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Isabel Maria Quesada

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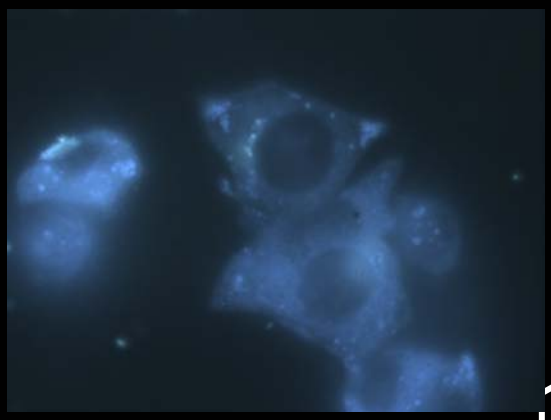

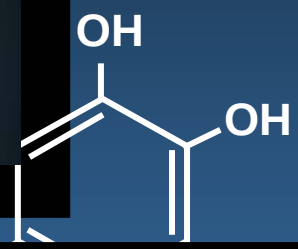

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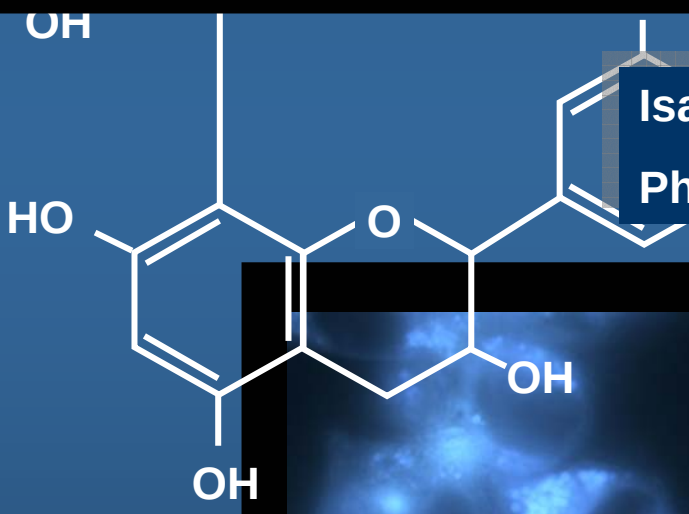

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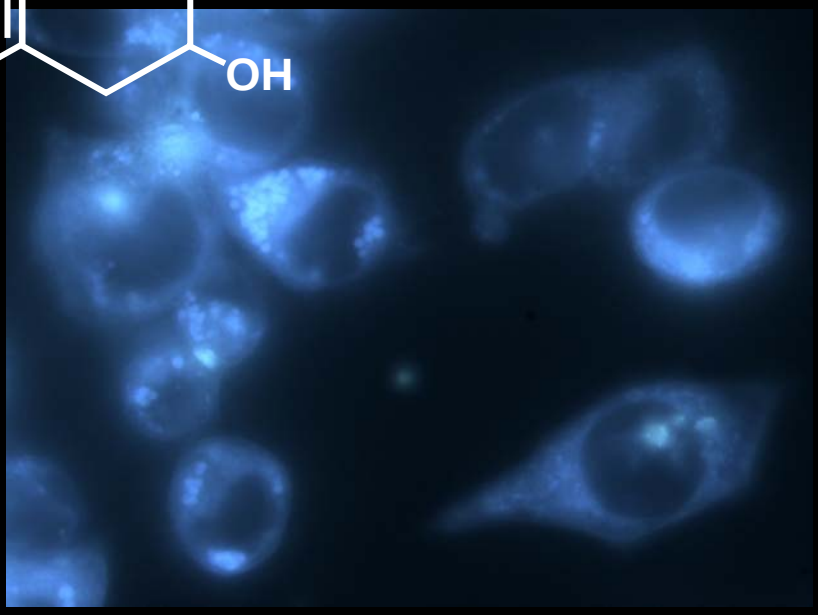

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Isabel María Quesada

Effects of dietary catechins and proanthocyanidins on zinc homeostasis in hepatic cells

Ph Doctoral Thesis

Directed by Dr. Juan Bautista Fernández Larrea
Department of Biochemistry and Biotecnology
Nutrigenomics Research Group
Universitat Rovira i Virgili
Tarragona

UNIVERSITAT ROVIRA I VIRGILI

Tarragona, October 2010

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Juan Bautista Fernández-Larrea, professor agregat del Departament de Bioquímica i Biotecnologia de la Universitat Rovira i Virgili,

CERTIFICO:

Que aquest treball, titulat "**Effects of dietary catechins and proanthocyanidins on zinc homeostasis in hepatic cells",** que presenta Isabel María Quesada per a l'obtenció del títol de Doctor, ha estat realitzat sota la meva direcció al Departament de Bioquímica i Biotecnologia d'aquesta universitat.

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A Mario y nuestro hijo Tomás. Sin su amor y apoyo nada de esto hubiese sido posible.

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"*El único modo de superar una prueba es realizarla.*

Es inevitable."

EL ANCIANO CISNE NEGRO REAL

En "Las voces del desierto"

De Marlo Morgan

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SUMMARY

Effects of dietary catechins and proanthocyanidins on zinc homeostasis in hepatic cells.

Catechins and their polymers procyanidins are health-promoting flavonoids found in edible vegetables and fruits. They act as antioxidants by scavenging reactive oxygen species and by chelating the redox-active metals iron and copper. They also behave as signaling molecules, modulating multiple cell signaling and metabolic pathways and gene expression, including that of antioxidant enzymes. Previous results of the Nutrigenomics Reseach Group showed that an oral acute dose of a grape-seed procyanidin extract (GSPE) represses the expression of the zinc-binding protein metallothionein (MT) genes in rat liver, and enhances the expression of the orfan nuclear receptor small heterodimer partner (SHP/Nr0b2) (Del Bas et al., 2005). In addition, it was shown that procyanidins act as transcriptional coactivators of the nuclear bile acid receptor Farnesoid X Receptor (FXR), which in turns upregulates SHP expression, thereby exerting an hypotrygliceridemic effect (Del Bas et al., 2008; Del Bas et al., 2009).

The objectives of this Ph.D. Thesis were to determine whether catechins and procyanidins interact with the redox-inactive metal zinc, to evaluate their effect on zinc homeostasis in hepatic cells -including the expression of MT genes, used here as a biomarkers of procyanidin activity in hepatic cells-, and to disect the mechanisms by which procyanidins affect cellular zinc homeostasis, in particular to asses whether MT genes are targets of SHP and FXR.

Our results show that GSPE, as well as individual catechins and procyanidins tested, including the green tea flavonoid (-)-epigallocatechin-3-gallate (EGCG), bind zinc cations in solution with higher affinity than the zinc-specific chelator Zinquin. In human hepatocarcinoma HepG2 cells, GSPE inhibits intracellular zinc accumulation and counteracts the toxic effects of excess zinc on cell viability. At the mRNA expression level, GSPE downregulates MTs and zinc-efflux transporters while upregulating zinc-influx transporters. Zinc importers of the Trans-Golgi network are upregulated by GSPE. In addition, GSPE blocks the induction of MTs expression by the proinflammatory cytokine IL-6, the ROS generator tBOOH, the glucocorticoid receptor agonist dexamethasone, and the metals copper and zinc.

EGCG reproduces the major effects of GSPE on zinc homeostasis in HepG2, downreguling the expression of MTs and zinc-efflux transporters, while upregulating the expression of zinc-influx transporters, concomitantly inhibiting intracellular zinc accumulation and the toxicity of

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high zinc doses. Procyanidin dimer B1 and trimer C1 behave opposite to GSPE and EGCG with regard to MT expression and intracellular zinc accumulation in HepG2 cells.

Concerning cytoplasmic labile zinc, the tiny fraction of total cellular zinc that modulates signaling and metabolic pathways, we found that GSPE, EGCG and trimeric procyanidin C1 greatly elevate Zinquin-detectable labile zinc in HepG2 cells.

Experiments with SHP-null and FXR-null mice demonstrate that GSPE downregulates postprandial expression of MT genes in the liver, in a SHP-independent but FXR-dependent manner. In addition, chenodeoxycholic acid, a physiological ligand and activator of FXR, represses the expression of MT genes in HepG2 cells. Thus, MT genes are targets of FXR and, consequently, FXR is revealed as a modulator of zinc homeostasis.

To explain these results, we postulate that catechins and procyanidis may act both as sequestrants of zinc -thereby impeding the entrance of zinc cations to the cell through plasma membrane zinc transporters-, and as zinc ionophores -thereby cotransporting zinc cations through the lipid bilayer and increasing the levels of cytoplasmic labile zinc. Repression of MT expression by procyanidin-activated FXR might also contribute to the increment of the labile pool of zinc, by hindering the sequestration of zinc-cations by *de novo* synthesized apo-thionein.

Given the role of labile zinc as modulator of multiple intracellular signaling and metabolic pathways, we forward the hypothesis that extracellular complexation of zinc cations and subsequent elevation of cytoplasmic labile zinc may be relevant mechanisms underlying the health-promoting activity of catechins and procyanidins and, therefore, that the signaling and metabolic pathways modulated by labile zinc will be aslo a target of these flavonoids.

Literature:

Del Bas, J.M., Fernandez-Larrea, J., Blay, M., Ardevol, A., Salvado, M.J., Arola, L. and Blade, C.: Grape seed procyanidins improve atherosclerotic risk index and induce liver CYP7A1 and SHP expression in healthy rats. Faseb J 19 (2005) 479-81.

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RESUM

Efectes de catequines i proantocianidines de la dieta sobre l'homeòstasi del zinc en cèl·lules hepàtiques.

Les catequines i els seus polímers, les procianidines, són flavonoids presents en hortalisses i fruits amb efectes beneficiosos sobre la salut. Actuen com a antioxidants segrestant espècies reactives d'oxigen (ROS) i quelant els metalls ferro i coure. També es comporten com a molècules senyalitzadores, modulant múltiples vies de senyalització i metabòliques i l'expressió gènica, incloent-hi la d'enzims antioxidants. Resultats previs del Grup de Recerca en Nutrigenòmica mostren que una dosi oral aguda d'un extracte de procianidines de llavor de raïm (GSPE) reprimeix l'expressió de les metal·lotioneïnes (MT), proteïnes lligadores de zinc, a fetge de rates, i tanmateix incrementa l'expressió del receptor nuclear orfe *small heterodimer partner*.(SHP/Nr0b2) (Del Bas et al., 2005). Igualment, es va demonstrar que les procianidines actuen com a coactivadors transcripcionals del receptor nuclear d'àcids biliars Farnesoid X Receptor (FXR), el qual es responsable de la sobre-expressió de SHP causada per GSPE a cèl·lules hepàtiques, i de l'efecte hipotrigliceridèmic de les prociandines effect (Del Bas et al., 2008; Del Bas et al., 2009).

Els objectius d'aquesta Tesi van ser determinar si les catequines i procianidines interaccionen amb el zinc, avaluar el seu efecte sobre l'homeòstasi del zinc en cèl·lules hepàtiques -incloent l'efecte sobre l'expressió de genes MT, utilitzats aquí com a biomarcadors de l'activitat de les procianidines a cèl·lules hepàtiques-, i diseccionar els mecanismes pels quals les procianidines afecten l'homeòstasi del zinc, en particular confirmar si els gens MT són dianes de SHP i FXR.

Els resultats obtinguts mostren que GSPE, així com diverses catequines i procianidines pures, incloent-hi el flavonoid del te verd (-)-epigallocatechin-3-gallate (EGCG), lliguen cations de zinc en solució amb una afinitat més gran que el quelant específic de zinc Zinquin. En cèl·lules d'hepatocarcinoma humanes HepG2, GSPE inhibeix l'acumulació intracel·lular de zinc i contraresta els efectes tòxics de dosis elevades de zinc sobre la viabilitat cel·lular. GSPE reprimeix l'expressió de gens de MTs i d'exportadors de zinc mentre que estimula l'expressió d'importadors de zinc. L'expressió dels importadors de zinc de la xarxa Trans-Golgi és estimulada per GSPE. A més a més, GSPE bloqueja la inducció de l'expressió de MTs per la citoquina proinflamatoria IL-6, pel generador de ROS tBOOH, per l'agonista de receptors de glucocorticoids dexametasona, i pels metalls coure i zinc.

EGCG reprodueix els efectes de GSPE sobre l'homeòstasi del zinc en HepG2, reprimint l'expressió de MTs i d'exportadors de zinc, estimulant l'expressió d'importadors de zinc, i

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inhibint l'acumulació de zinc intracel·lular i la toxicitat de dosis elevades de zinc. La procinidina dimèrica B1 i la trimèrica C1 es comporten tenen efectes contraris als de GSPE i EGCG pel que fa a l'expressió de MT i l'acumulació de zinc total en cèl·lules HepG2.

Pel que fa al zinc làbil citoplasmàtic, la minúscula fracció del total del zinc cel·lular que modula múltiples vies metabòliques i senyalitzadores, tant GSPE com EGCG i C1 eleven en gran manera els nivells de zinc làbil detectable per Zinquin a cèl·lules HepG2.

Experiments amb ratolins KO per SHP o per FXR han demonstrat que GSPE reprimeix l'expressió postprandrial de gens MT a fetge per una via que no depen de SHP però que és depenent de FXR. A més, l'àcid biliar CDCA, un lligand fisiològic i activador de FXR, reprimeix l'expressió de gens MT a cèl·lules HepG2. Per tant, els gens MT són diana de FXR i, conseqüentment, FXR apareix com un receptor nuclear que modula l'homeòstasi del zinc.

Per explicar aquests resultats, proposem que catequines i procianidines poden actuar tant com a segrestadors de zinc -evitant la seva entrada a la cèl·lula a través dels transportadors de zinc de membrana plasmàtica-, com d'ionòfors de zinc -cotransportant cations zinc a través de la bicapa lipídica i incrementant així els nivells de zinc làbil citoplasmàtic. La repressió de gens MT induïda per l'activació de FXR per GSPE podria també contribuir a l'increment de zinc làbil, en impedir que els cations zinc siguin segrestats per apo-tioneïna sintetitzada *de novo*.

Donat el paper del zinc làbil com a modulador de múltiples víes de senyalització i metabòlics, formulem la hipòtesi que la quelació extracel·lular de cations de zinc i l'elevació de zinc làbil citoplasmàtic són mecanismes subjacents a l'activitat biològica de catequines i procianidines i, per tant, que les vies metabòliques i de senyalització afectades pel zinc làbil, ho seràn també per aquests flavonoids.

Referències bibliogràfiques:

Del Bas, J.M., Fernandez-Larrea, J., Blay, M., Ardevol, A., Salvado, M.J., Arola, L. and Blade, C.: Grape seed procyanidins improve atherosclerotic risk index and induce liver CYP7A1 and SHP expression in healthy rats. Faseb J 19 (2005) 479-81.

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ABREVIATIONS

ARE: Antioxidant Response Element

Akt/PKB: Protein kinase B

B: Procyanidin Dimer B

C: (+)-catechin

C1: Procyanidin Trimer C1

CDCA: Chenodeoxycholic acid

C/EBP: CCAAT enhancer-binding protein

CREB: cAMP responsive element binding protein

Cu: Copper

CaMPK: Calcium-calmodulin activated protein kinase

Dex: Dexamethasone

Dnmt: DNA methyltransferase

EC: (-)-epicatechin

ECG: (-)-epicatechin gallate

EGCG: epigallocatechin gallate

ER: Endoplasmic Reticulum

ERK1/2: Extracellular signal Regulated protein Kinase

FXR: Farnesoid X receptor

GSPE: Grape Seed Procyanidin Extract

GCLC: Glutamate-Cysteine Ligase, Catalytic subunit

HDAC: Histone deacetylase

IL: Interleukin

iNOS: Inducible nitric oxide synthase

IR: Insulin receptor

IRS-1: Insulin receptor substrate

JAK: Cytokine receptor-mediated intracellular signal transduction Janus kinase

JNK: c-Jun N-terminal kinase

KO: Knock Out

LDL: Low Density Lipoprotein

MT: Metallothionein

MAPK: Mitogen-activated Protein Kinase

MTF-1: Metal responsive transcription factor 1

MRE: Metal regulatory Response Element

NF-kB: Nucear Factor kappa Beta

PI3K: Phosphatidylinositol-3-kinase

PDE: Cyclic nucleotide phosphodiesterase

PTP: Protein tyrosine phosphatases

PKC: Protein kinase C

RNS: Reactive nitrogen species

ROS: Reactive oxigen species

SHP: Small Heterodimer Partner (NR0B2)

SOD: Superoxide Dismutase

STAT: Signal transducers and activators of the transcription

TPEN: N,N,N',N'-tetrakis(2- phridylmethyl) ethylenediamine

TDPC: Tetramethylated dimeric procyanidins

TNF-α: Tumor Necrosis Factor-α

tBOOH: tertbutyl- hydroperoxide

WT: Wild Type

Zn: Zinc

ZnT/SLC30: Zinc Transporter / Soluted-linked carrier family 30

ZIP/SLC39: Zrt-and Irt-like proteins / soluted-linked carrier family 39

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I. INTRODUCTION

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1. CATECHINS AND PROANTHOCYANIDINS: BIOACTIVE COMPOUNDS

Catechins and proanthocyanidins are polyphenolic compounds of flavonoid type fairly abundant in numerous aliments and drinks of plant origin such as grapes, cocoa, different berries, apples, nuts, red wine, chocolate and tea, and are considered bioactive micronutrients that form an integral part of human diet (Scalbert et al., 2000; Havsteen, 2002; Beecher, 2003; Shi et al., 2003; Bhagwat, 2004; Cos et al., 2004; Gu et al., 2004; Manach et al., 2004; Laboratory, 2007). Also, proanthocyanidin-rich extracts from different sources, such as grape seeds, pomegranate and pine bark, are commercialized as food additives and nutritional supplements (Ariga, 2004; Weber et al., 2007). The interest of proanthocyanidins in nutrition arises from the demonstrated and potential benefits of regular consumption of proanthocyanidins for human health (Ross and Kasum, 2002; Scalbert et al., 2005). Thus, numerous population and intervention studies have demonstrated that proanthocyanidin-rich diets reduce the risk of cardiovascular diseases (Kuo, 1997; Bagchi et al., 2000; Reed, 2002; Bagchi et al., 2003; Steinberg et al., 2003; Keen et al., 2005; Manach et al., 2005a; Rasmussen et al., 2005; Engler and Engler, 2006; Kar et al., 2006; Robbins et al., 2006; Erdman, 2007; Stangl et al., 2007), and cancer (Kuo, 1997; Bagchi et al., 2000; Scalbert et al., 2005; Williamson and Manach, 2005; Yance and Sagar, 2006; Zaveri, 2006; Thomasset et al., 2007; Zhao et al., 2007) and many intervention studies and works with animal models have also shown the benefits proanthocyanidins and other flavonoids for prevention and amelioration of cardiovascular disease-associated pathologies such as diabetes, atherosclerosis, obesity, and hypertension (Ariga, 2004). Consumption of proanthocyanidins could also prevent and ameliorate diverse neurodegenerative diseases (Esposito et al., 2002; Li et al., 2004; Mandel et al., 2004a; Mandel et al., 2004b; Mandel and Youdim, 2004; Lau et al., 2005; Zhao, 2005; Kim et al., 2006; Sutherland et al., 2006). Since oxidative stress and concomitant inflammation is a common backstage to the etiology and progression of these diseases, and since dietary antioxidants, in general, may act as cancer chemopreventive and antiinflammatory agents and reduce risk of cardiovascular and cancer

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mortality, the beneficial effects of catechins and proanthocyanidins have been largely attributed to their well known antioxidant and associated antiinflammatory activity (Bagchi et al., 2000; Dragsted, 2003; Shi et al., 2003; Ariga, 2004; Cos et al., 2004). The protective properties of these polyphenolic compounds have placed them in the focus of nutrition research, in order to understand the processes and molecular mechanisms responsible for their effects.

CLASSIFICATION AND CHEMICAL STRUCTURE OF PROANTHOCYANIDINS

Polyphenols constitute one of the most numerous and ubiquitously distributed groups of plant secondary metabolites. They are characterized by having at least one aromatic ring with one or more hydroxyl groups attached. They can be classified by the number and arrangement of their carbon atoms into two categories: the flavonoids and the non-flavonoids (Figure I.1.1). The general chemical structure of flavonoids (Figure I.1.2) includes a C15 (C6-C3-C6) skeleton joined to a chroman ring (benzopyran moiety) that in turn bears an aromatic ring at C2, C3 or C4 (Crozier, 2009). Based on the heterocyclic ring (C-ring) saturation and degree of oxidation, dietary flavonoids further divide into six groups: anthocyanidins, flavonols, flavones, flavanones, isoflavones, and flavanols (also known as flavan-3-ols or catechins and proanthocyanidins) (Figure I.1.1), while those that are comparatively minor components of the diet are dihydroflavonols, flavan-3,4-diols, coumarins, chalcones, dihydrochalcones and aurones (Crozier, 2009).

Flavan-3-ols are the most structurally complex subclass of flavonoids, ranging from the simple monomers (+)-catechin and its isomer (-)-epicatechin, through to complex structures including the oligomeric and polymeric proanthocyanidins (also known as condensed tannins) (Aron and Kennedy, 2008). The two chiral centres at C2 and C3 of the flavan-3-ols produce four isomers for each level of B ring hydroxylation, two of which, (+)-catechin and (-)-epicatechin, are widespread in nature whereas (-)-catechin and (+)-epicatechin are comparatively rare. Proanthocyanidins that consist exclusively of (+)-catechin or (-)-epicatechin are called procyanidins, and are the most abundant type of proanthocyanidins.

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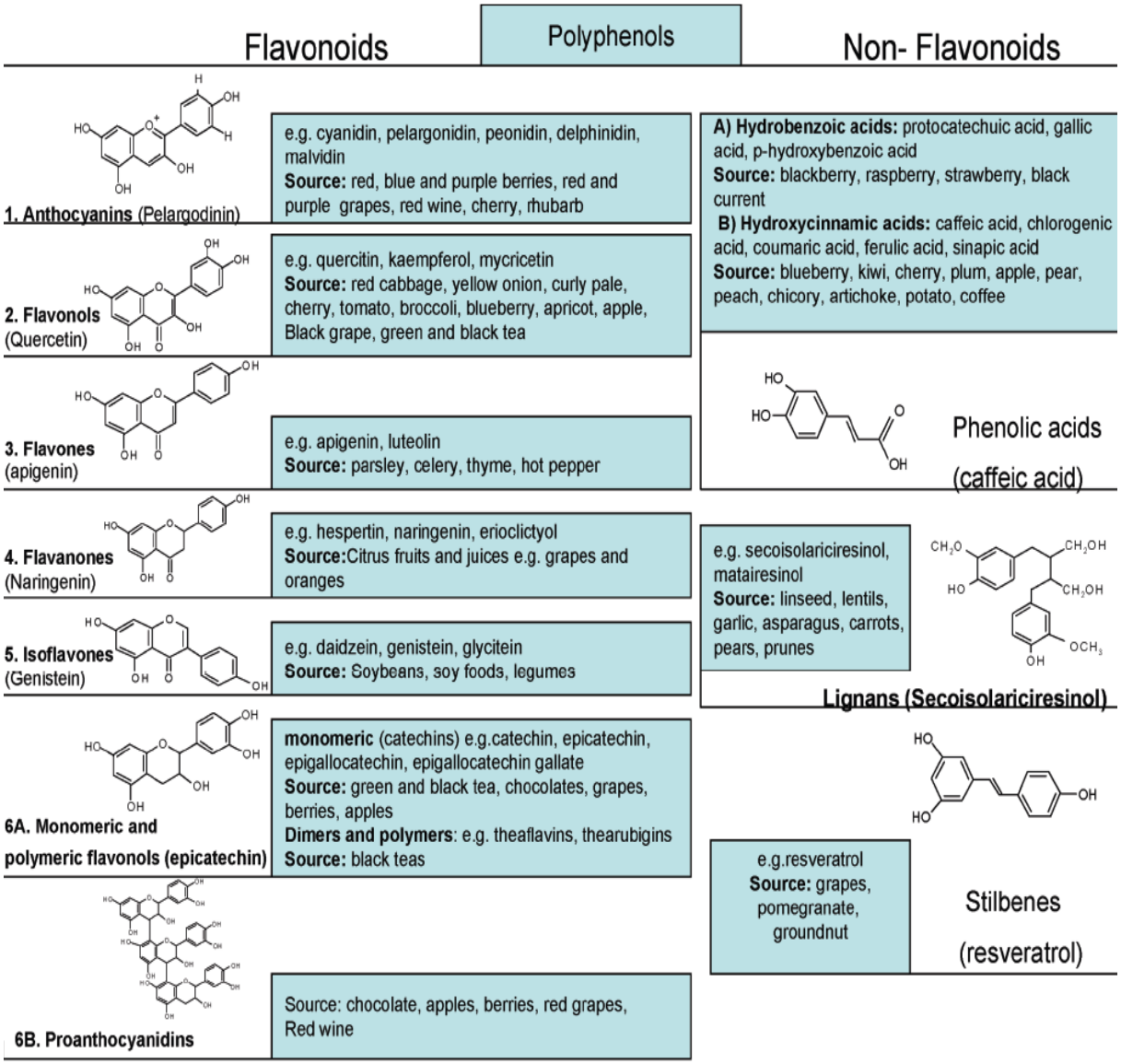
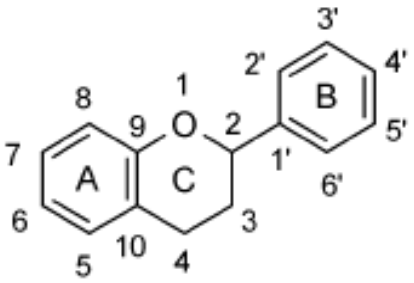


Figure I.1.1. Main groups of polyphenols with their individual compounds and food sources. Taken from (Singh et al., 2008).

Figure I.1.2. Basic structure of flavonoids.
From (Singh et al., 2008).



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INTAKE, BIOAVAILABILITY AND METABOLISM OF PROANTHOCYANIDINS

Daily proanthocyanidin intake can vary from 10 mg to 0.5 g/day, with dimers B1 and B2 most likely consumed (Aron and Kennedy, 2008) with the main sources being tea, chocolate, apples, pears, grapes, and red wine. Gu *et al*. estimated the daily average intake of proanthocyanidins in the USA and found that in total the daily intake of proanthocyanidins is about 53.6 mg/day excluding the monomers, and 57.7 mg/day including the monomers. An estimated 74% of ingested proanthocyanidins possess degree of polymerization >3 (Gu et al., 2004).

The process of bioavailability for polyphenols refers to their liberation and digestion in the stomach and the gastrointestinal tract, their transport across the intestinal membrane into the blood, their tissue distribution, their metabolism and efficacy (or biological effects) and, last, their elimination. Bioavailability and blood biokinetics depend on the type of polyphenolic compound, dietary source (food matrix and backgroung diet), form (galloylation of EGC markedly decreases absorption, and the aglycones (i.e., non-glycosilated forms) are generally the most readily absorbed forms), dose, gut transit time, and fecal degradation rate (Erdman et al., 2007; Galli, 2007). Digestion and absorption of polyphenols begin in the stomach, where the aglycones of many polyphenols may be absorbed. The intestinal epithelium, rich in drug-metabolizing enzymes, is important in the metabolism of the different flavonoids. Flavonoid aglycones, in general, and flavonoid glycosides (bound to glucose, but the attached glucose is removed during absorption, except for anthocyanins) are absorbed in the small intestine, where they are rapidly taken up and metabolized to form hydroxylated, methoxylated, sulphated and/or glycosylated metabolites (Mazza, 2002; Wu, 2002; Felgines et al., 2003; Kay, 2004; Manach et al., 2004; Kay, 2005; Manach et al., 2005b). The majority of flavonoids exist naturally as glycosides and the linked sugar is often glucose or rhamnose. The number of sugar moieties is commonly one, but could be two or three, and there are several positions of substitution on the polyphenol (Singh et al., 2008). Sugars and hydroxyl groups increase the water solubility of flavonoids, while other substituents, such as methyl

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groups and isopentyl units, make flavonoids more lipophilic. Also, flavan-3-ol subunits can carry an acyl substituent, being gallic acid the most common one. The molecular sites of metabolic modification include methylation on the B-ring catechol group, influencing both antioxidant and pro-oxidant properties, and glucuronidation/sulfatation on the A-ring (and C and B rings in certain structures). Glutathionylation can occur on the C ring and could be an important metabolic step in cell detoxification and export, but can potentially influence the homeostatic control of cell redox and thiol-dependent signaling (Galli, 2007). Flavonoids and flavonoid metabolites that reach the colon may be further metabolized by bacterial enzymes and absorbed. Thus, colonic degradation by the microflora is extensive for procyanidins, the flavonol quercetin, and flavan-3-ols. Polyphenols known to be particularly well absorbed in humans are isoflavones, followed by quercetin glycosides. Proanthocyanidins and the flavan-3-ol EGCG appear to be among the least well-absorbed polyphenols, but this may be a result of instability once absorbed and not of poor absorption (Manach, 1998; Galli, 2007) Peak concentrations of flavonoids typically occur approximately 2 h after ingestion of a test food, depending on the type of compound and site of absorption (Rein et al., 2000; Wang et al., 2000; Serra et al., 2009). There is, nevertheless, conflicting evidence on the absorption and metabolism of the oligomeric and polymeric flavan-3-ols in humans and animals. Tsang, C. *et al*. found that, after oral ingestion of procyanidins, rat plasma contained exclusively catechin glucuronides and methylated glucuronide metabolites which were also detected in the liver and kidneys (Tsang et al., 2005). However, Shoji, T. *et al*., established that procyanidins with polymerization degree >2 were found in plasma of rats feed procyanidin extracts from apple and from grape-seeds. This work showed that procyanidins from dimer to pentamer were detected in plasma and suggested that are directly involved in physiological functions (Shoji et al., 2006). Also, plasma concentrations of unmetabolized procyanidins reached, 2 hours after oral administration of an apple procyanidin extract, 0.15 μM for trimer C1, 0.4 μM for dimer B2, and 0.2 μM for dimer B1. When pure individual compounds were orally administered, they were found in plasma unmodified at high concentrations: 3 μg/mL for dimers, 7 μg/mL for trimers, 7 μg/mL for tetramers, and 7 μg/mL for pentamers (Shoji et

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al., 2006). Likewise, Serra, A. *et al*. recently established that, upon oral administration to rats of 1 g GSPE per kg of body weight, the plasma concentration of metabolized flavanols was even greater than that of unmodified, parent compounds (Serra et al., 2009). Tetrameric and pentameric procyanidins are also absorbed in rats and reach concentrations of 14 mg/g in liver and 15 mg/L in plasma, 2 hours after treatment (Garcia-Ramirez et al., 2006). Likewise, EGCG may reach concentrations of up to 1.5 μM in human plasma after oral intake of a single 800 mg dose of EGCG (Chow et al., 2001).

Available evidence suggests that there are no long-term stores of polyphenols in the body. However, some flavonoids have been shown to concentrate in different tissues at measurable concentrations (Hong, 2001; Garcia-Ramirez et al., 2006; Henning et al., 2006; Serra et al., 2009).

The hydrophilic nature of polyphenols makes them difficult to pass through cell membranes. Glucuronide and sulfate moieties, as mentioned above, contribute to their water solubility and therefore influence the chemical, physical and the biological properties of the polyphenol (Scalbert et al., 2000; Scalbert and Williamson, 2000; Tarahovsky et al., 2008). There are two possible relevant interactions that occur between flavonoids and lipid bilayers of cell membranes postulated by Oteiza *et al*. On one hand, the partitioning of the polyphenol in the non-polar core of the membrane, associated with the hydrophobic nature of the flavonoid (this interaction is higher for the flavones and flavanones). The more hydrophobic the flavonoid is, the more permeabilizating effect has and this fact depends on pH (Oteiza et al., 2005). On the other hand, the interaction of the hydrophilic flavonoids and oligomers with the polar headgroups of lipids at the lipid–water interface, mainly associated with the formation of hydrogen bonds (Oteiza et al., 2005). In addition, in a more recent study, Tarahovsky Y*. et al*. point out that flavonoid molecules preferably located in the hydrophobic region of the bilayer can initiate formation of raft-like domains (raft-making effect), while the molecules located in the polar interface region of the bilayer can fluidize membranes (raft-breaking effect), or initiate formation of interdigitated or micellar structures (Tarahovsky et al., 2008) (Figure I.1.3).

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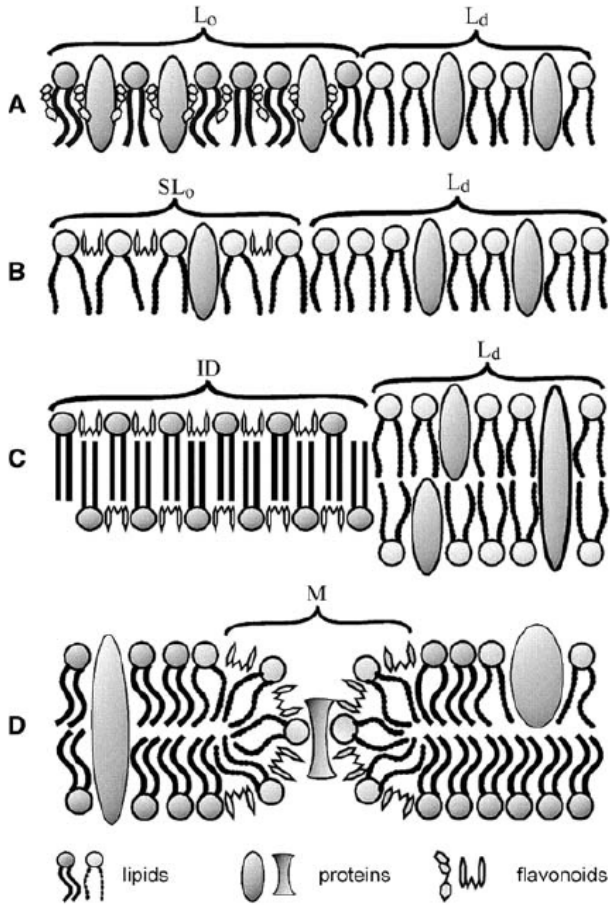

Figure I.1.3. Hypothetical process of lipid and protein lateral segregation in membranes, initiated by different flavonoids. For simplicity, only one lipid monolayer is shown on (a) and (b). (a) Flavonoid molecules (e.g., quercetin) intercalate between the lipid molecules in the periphery of the hydrophobic region and initiate formation of a liquid ordered phase (Lo). The rest of the lipid molecules is in a liquid disordered state (Ld). Hydrophobic proteins and their complexes with flavonoids are also presented. (b) Flavonoid molecules (e.g., phloretin) intercalate the polar heads of lipid. As a result, the distance between the hydrocarbon chains is increased and lipids form a superliquid ordered phase (SLo). (c) Interdigitated structure (ID) and (d) bilayer defects and micelles (M) caused by intercalation of phloretinlike flavonoids between the polar heads of lipids. After (Tarahovsky et al., 2008).

Once absorbed and metabolized, metabolites of polyphenols are ‘‘lost’’ from the body via urinary and biliary excretion. The quantities of polyphenols found intact in urine vary from one phenolic compound to another. They are particularly low for quercetin and rutin, but reach higher values for catechins in green tea, isoflavones in soy, flavanones in citrus fruits or anthocyanidins in red wine. A major part of the polyphenols ingested (75–99%) is not found in urine. This implies they have either not been absorbed through the gut barrier, absorbed and excreted in the bile or metabolized by the colonic microflora or by different tissues (Scalbert et al., 2000).

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INTERACTIONS OF FLAVONOIDS WITH INTRACELLULAR SIGNALING PATHWAYS

The beneficial effects of proanthocyanidis, and of flavonoids in general, have been largely attributed to their well known antioxidant activity (Bagchi et al., 2003; Cos et al., 2003; Dragsted, 2003; Shi et al., 2003; Ariga, 2004; Cos et al., 2004). It has been deeply studied that flavonoids exert their antioxidant activity by three mechanisms: (a) direct scavenging of reactive oxigen spicies (ROS) – such as superoxide radical anion, hydroperoxyl radical, hydrogen peroxide, and hydroxyl radical –, and reactive nitrogen species – such as peroxinitrite-; (b) by chelating redox-active transition metals – such as iron and copper–, that may act as powerful generators of ROS; and (c) by inhibition of ROS producing enzymes, in particular xanthine oxidase, NADPH oxidase and lipoxygenases (Premysl, 2010). By the first two mechanisms, flavonoids act as potent inhibitors of LDL peroxidation triggered by different pro-oxidant systems, both *in vitro* and *in vivo*, and hence are active against genesis and progression of inflammation and atherosclerosis (Plumb, 1998; Aviram and Fuhrman, 2002; da Silva Porto et al., 2003; Aviram et al., 2004).

Yet, the bioactivity of flavonoids is not limited to their direct antioxidant actions. Several flavonoids and proanthocyanidin species have been shown to interact with specific plasma membrane receptors, cytoplasmic signal transduction factors and nuclear receptors, serving themselves as signaling agents and eventually modulating gene expression at the transcriptional level. Actions of flavonoids on phosphoinositide 3-kinase (PI 3-kinase), Akt/protein kinase B (Akt/PKB), tyrosine kinases, protein kinase C (PKC), and mitogen activated protein kinase (MAP kinase) signaling cascades are well characterized (Williams et al., 2004b). Moreover, recent studies show that flavonoids can bind directly to some protein kinases, including Akt/PKB, Fyn, Janus kinase 1 (JAK1), mitogen-activated protein kinase kinase 1 (MEK1), PI3K, mitogen-activated protein (MAP) kinase kinase 4 (MKK4), Raf1, and ζ chain-associated 70-kDa protein (ZAP-70) kinase, and then alter their phosphorylation state to regulate multiple cell signalling pathways in carcinogenesis processes (Hou, 2010)(Figure I.1.4).

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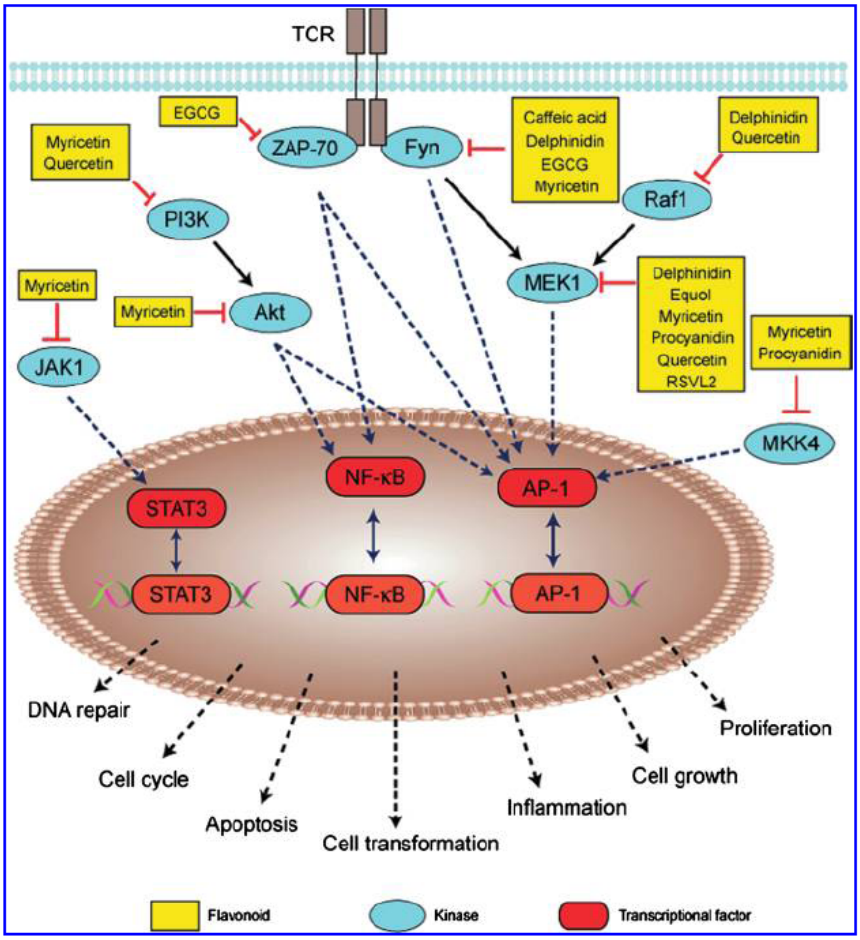

Figure I.1.4. Cellular signaling pathways regulated by the direct binding of protein kinases with flavonoids. Flavonoids are indicated within square mark. Kinases are indicated within ellipse mark. Transcript factors are indicated within ellipse–square mark. Dotted arrows represent the indirect downstream events. Solid arrows represent the direct binding of flavonoids to protein kinases. Taken from (Hou, 2010).

An additional level of antioxidant and also the antiinflammatory action of flavonoids is exerted by modulating the redox cellular state, thus controlling the activity of redox-sensitive signaling cascades and transcription factors, notably Nrf2 (nuclear redox factor 2 or NF-E2 related factor 2), AP-1 (activator protein 1) and NF-kB (nuclear factor kappa beta). Nrf2 target genes which are upregulated by flavonoids include genes involved in synthesis and utilization of glutathione, such us gamma-glutamyl cysteine ligase (GSC), glutathione S-transferase (GST) and glutathione peroxidase (GI-GPx), detoxification of ROS/RNS, such as Superoxide Dismutase – Cu,Zn-SOD, Mn SOD- and heme oxygenase-1 (HO-1), and detoxification of

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xenobiotics, such us NADP(H):quinone oxidoreductase-1 (NQO1) (Kuo, 2002; Rosenkranz et al., 2002; Frei and Higdon, 2003; Gohil, 2004; Mackenzie et al., 2004; Williams et al., 2004a; Puiggros et al., 2005; Surh et al., 2005; Moon et al., 2006; Rahman and Kilty, 2006; Kluth et al., 2007; Gohil, 2000). Special interest in the context of antioxidant and anti-inflammatory action of flavonoids is their documented modulatory effect on NF-kB dependent gene expression on a wide range of cellular types (Park, 2000; Kong, 2001.; Owuor and Kong, 2002; Rosenkranz et al., 2002; Rushmore and Kong, 2002; Balogun et al., 2003; Cos et al., 2004; Mackenzie et al., 2004; Feng et al., 2005; Shen et al., 2005; Surh et al., 2005; Xu et al., 2005; Andreadi et al., 2006; Kweon et al., 2006; Lee-Hilz et al., 2006; Nam, 2006; Narayanan, 2006; Rahman et al., 2006; Wu et al., 2006; Kluth et al., 2007; Liu et al., 2007; Terra et al., 2007). ROS enhance inflammation through the activation of NFkB and AP-1 transcription factors, and nuclear histone acetylation and deacetylation in various inflammatory diseases (Rahman et al., 2004; Rahman and Adcock, 2006; Rahman et al., 2006; Rahman and Kilty, 2006). NF-kB controls the expression of genes encoding pro-inflammatory cytokines (e. g., IL-1, IL-6, TNF-a), inducible pro-oxidant enzymes (cyclooxygenases COX-1, COX-2 and inducible nitric oxide synthase, iNOS) (Mutoh, 2000), some of the acute phase proteins, which play critical roles in controlling most inflammatory processes (Nam, 2006), lipoxygenases and xanthine oxidase. Catechins and proanthocyanidins have been shown to suppres the IkB/ NF-kB signaling pathway even in the presence of NF-kB stimulation, and trigger a reduced expression of many NF-kB target genes such as the survival factors Bcl2 (Roig et al., 1999; Roig et al., 2002; Stevens et al., 2002; Frei and Higdon, 2003; Noonan et al., 2007).

Flavonoids have also been reported to inhibit several isoforms of phosphodiesterase (PDE) (Peluso, 2006). Therapeutic effects of flavonoids on platelet aggregability and blood pressure have been attributed to competitive inhibition of cyclic nucleotide phosphodiesterase, an elevation in cAMP level, and subsequent activation of protein kinase A (cAMP-dependent protein kinase) (Squadrito F, 2002; Liu, 2005). In addition, flavonoids also suppress pathways of lipid biosynthesis and of very low-density lipoprotein production in cultured

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hepatocytes. Thus, the soy isoflavone genistein has been shown to inhibit several PDE isoforms and to enhance cellular cAMP accumulation in numerous cell types (Stringfield TM, 1997; Nichols MR, 1999; Nichols MR, 2000; Beecher, 2003). Furthermore, in rat hepatocytes, genistein has shown to augment the glucagon-mediated elevation in cAMP (Keppens, 1995). Flavonoids can also induce lipolysis in adipose tissue, likely through competitive inhibition of PDE and antagonism of cAMP degradation. For instance, luteolin, apigenin, quercetin, diosmetin, genistein, and other flavonoids have been shown to inhibit PDE3 and to induce lipolysis in isolated rat adipocytes (Kuppusamy UR, 1992). Augmentation of lipolysis by genistein in adipocytes was shown to depend on PKA (Szkudelska K, 2000), and grape seed procyanidins were shown to elevate cellular cAMP levels and to induce lipolysis through activation of PKA in 3T3-L1 adipocytes (Pinent et al., 2005). The inhibition of PDEs activity by flavonoids has been postulated to lay on the ability of the flavonoids to fit in the catalytic site of PDEs. The three-dimensional structure of many flavonoids, such as apigenin, genistein, daidzein, and quercetin, fit very well in the catalytic site of cAMP PDE3 and PDE4 binding pocket (Figure I.1.5).

To sum, the modulation of multiple signaling pathways exerted by flavonoids may explain their numerous benefitial effects on health.

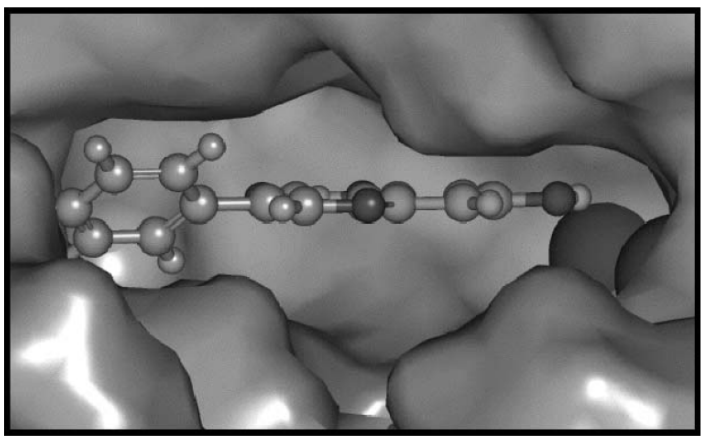

Figure I.1.5. Close-up view of genistein in the substrate binding cavity of human PDE4B2. Genistein was docked in the catalytic site of human PDE4B2. The electrostatic surface shading is not shown. The two zinc cations within the catalytic pocket are shown on the right as spheres. The graphical representation was rendered using PyMOL. Taken from (Peluso, 2006)

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METAL CHELATION BY POLYPHENOLS

Due to their chemical structure, polyphenols easily chelate metal ions and create polyphenol-metal complex compounds. The essential sites for metal chelation are hydroxyl groups. The peri positions of two fused rings may also provide a chelation center. Also, the oxidation products, semiquinone and quinone, are able to coordinate metal cations, but generally with reduced affinity. Thus, all types of flavonoids possess three domains able to react with metal ions: (a) between the 5-hydroxyl and 4-carbonyl group, (b) between the 3-hydroxyl and 4-carbonyl group, (c) between the 3′,4′-dihydroxyl group located on the B ring. The last chelation place (c) seems to play a principal role in chelating (Premysl, 2010) (Figure I.1.6).

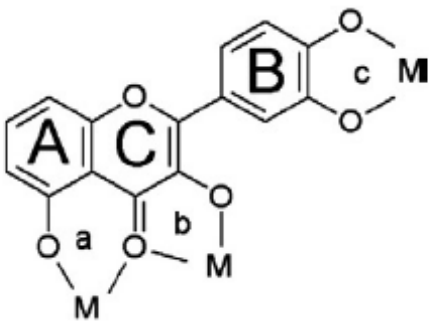

Figure I.1.6. The commonest possible metal (M) chelation sites of flavonoids. Capital letters indicate the prevailing designation of the flavonoid structure, and lower case letters mark the most common iron chelation sites. Taken from (Premysl, 2010).

Therefore, polyphenols that posses more than one chelation place are much more effective scavengers of metal cations than those that have only one. Thus, quercetin has three potential metal-binding sites, A, B, and C, with the affinity of site A being greater than sites B and C at pH 7.0. With kaempferol there are two potential binding sites, D and E, but only one or the other will be used for chelation, both cannot be used simultaneously (Hider et al., 2001) (Figure I.1.7A). Thus, these kind of polyphenols that have more than one metal-binding site might be capable of polymerization and oligomerization as indicated in Figure I.1.8. This aggregation effect could reduce the ability of polyphenols to partition in membranes and therefore to gain access to intracellular compartments (Hider et al., 2001). Hence, flavonoid complexes may play an important role in modulating flavonoid and or metal bioavailability in the cell.

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When polyphenols are metabolized a phenol group is conjugated with a glucoside, methyl or sulphate moiety loosing one metal-binding center. However, they still may have the capacity to bind metals. For instance, rutin (a glycosilated form of quercetin) still retain two metal-binding sites (Figure I.1.7A) (Hider et al., 2001). Catechin or epicatechin glucuronide still have a bidentate metal chelating site and a monodentate metal chelating site (Figure I.1.7B). Thus, this metabolization will also reduce the ability of the polyphenol to donate hydrogen and, therefore, they will be less effective scavengers of oxygen and nitrogen species relative to their parent aglycone form (Williams et al., 2004b). In addition, the gallate moiety of the gallocatechins is also capable to bind metals (Kumamoto et al., 2001) (see Figure I.1.7B). Thus, esterification of gallic acid to a glucose moiety in the gallotannin β-glucogallin increases the affinity for iron (Scalbert et al., 1999).

Figure I.1.7. A)

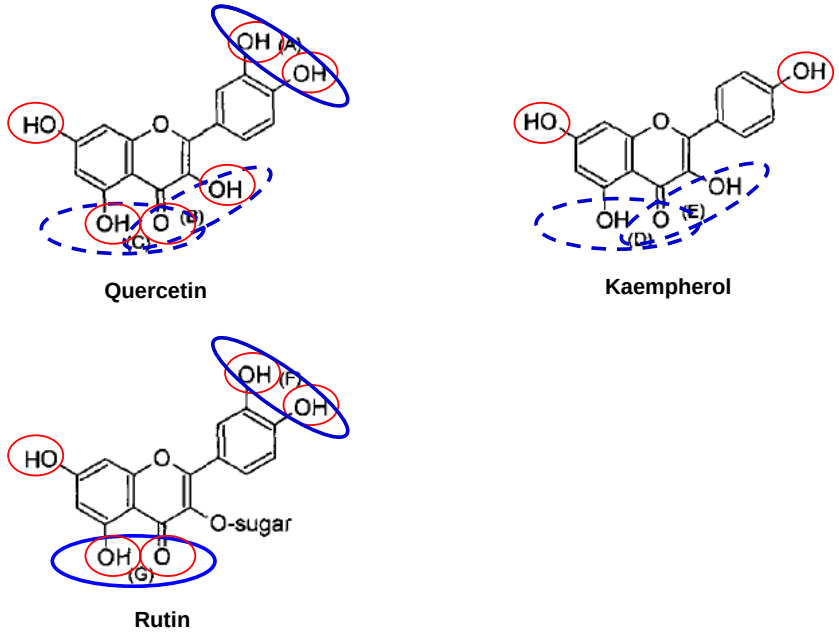

Figure I.1.7. Structures and potential metal-binding sites of different flavonoids. Based on (Hider et al., 2001). Red circles indicate monodentate metal binding sites. Blue ellipses indicate bidentate, strong metal binding sites. Blue punctuated ellipses indicate alternative bidentate metal binding sites. A) Quercetin and quercetin-derived flavonoids. B) Catechin and epicatechin isomers C) Dimeric and trimeric procyanidins. D) Glucuronidated and methylated metabolites of (-)-Epicatechin found in plasma of rats fed with proanthocyanidins. Note that these metabolites still have metal-binding sites.

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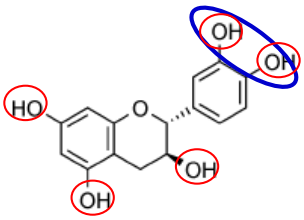
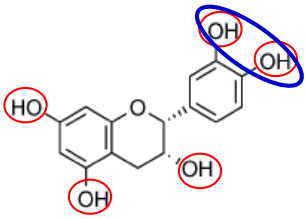
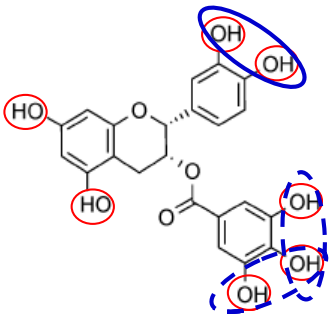
Figure I.1.7. B)



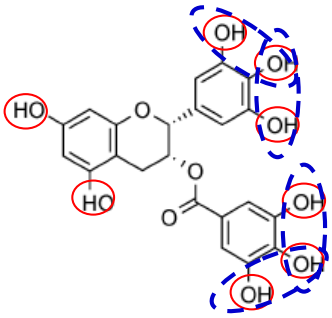
+(-)Catechin



-(-)Epicatechin

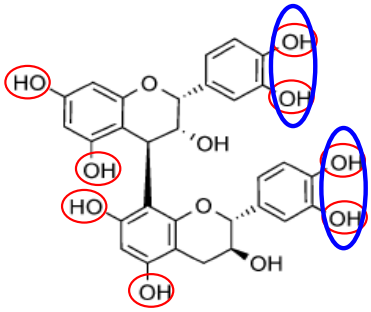


-(-)Epicatechin-3-*O*-gallate

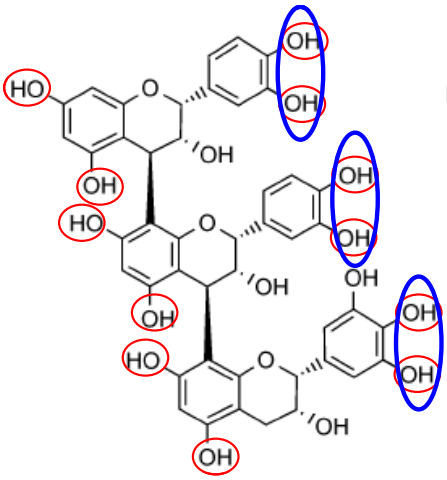


-(-)Epigallocatechin-3-*O*-gallate

Figure I.1.7. C)

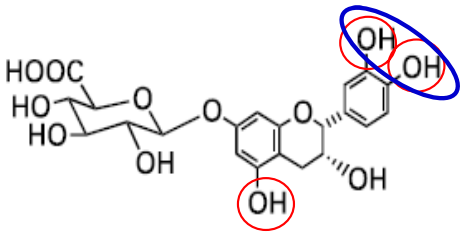


Procyanidin dimer B1

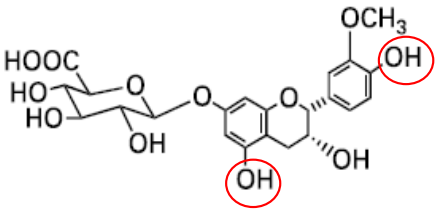


Procyanidin trimer C1

Figure I.1.7. D)



(-)-Epicatechin-7-*O*-glucuronide



3'-*O*-Methyl-(-)-epicatechin-7-*O*-glucuronide

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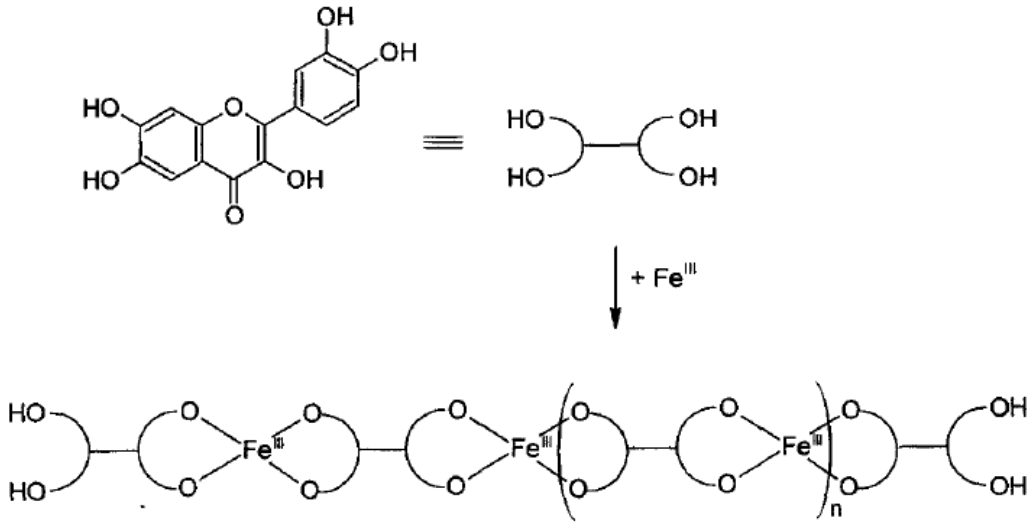

Figure I.1.8. Iron(III)-induced polymerization of a flavonoid that possesses two metal-chelating centers. Taken from (Hider et al., 2001)

The most suitable cations for polyphenol chelation are Fe(II), Fe(III), Cu(II) and Zn(II) because they have high charge density that favours the interaction with the phenoxide group that has a high negative charge density. In general polyphenols prefer tribasic cations to dibasic cations, so they also can bind Al(III). Polyphenols do not chelate alkali and alkaline earth cations, e.g., sodium, potassium, and calcium. There may be some weak interaction with magnesium, but a catechol function will lose magnesium ions in competition with phosphate (Hider et al., 2001).

Since flavonoids are weak polybasic acids that tend to protonate, pH has a considerable impact on complex formation. At pH below 3.0, flavonoids remain undissociated, which is unfavourable for complex formation. At high pH values flavonoids are deprotonated making them able to complexation. Although the pKa value of most phenols is in the region of 9.0-10.0, in the presence of suitable cations, such as iron(III) or copper(II), the proton is displaced at much lower pH values, e.g. 5.0-8.0. Due to this fact, metal chelation by polyphenols can occur at physiological pH values. Thus, at slightly acidic or neutral pH, flavonoid-metal complexes with the highest coordination number are typically formed, but rarely in basic media. For instance, Fe(III) is coordinated by a single ligand under acidic

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conditions, while in media close to neutrality it is tetracoordinated by two molecules of the gallotannin β-glucogallin (Scalbert et al., 1999) In general, the optimal pH for complex formation, although strongly dependent on the features of the metal ion, is around pH 6 (Hider et al., 2001; Malesev, 2007) (Kumamoto et al., 2001).

For steric reasons, the metal-flavonoid complexes usually include no more than two flavonoid molecules. However Zhou and co-workers using fluorescence spectroscopy found 3:1 complexes of quercetin with eight rare-earth metal ions. It is important to highlight that the relative proportion of each form largely depends on the concentration of both metal and ligand and the pH of the solution (Zhou et al., 2001).

Understanding the mechanisms of chelation of flavonoids by metal ions permits a better understanding of their multiple properties. As explained before, one of the mechanisms by which flavonoids exert their anioxidant activity is by chelating redox-active transition metals, primarily iron and copper which are known to catalyse many biological processes leading to the production of free radicals. The catechol B ring of flavonoids is known to be responsible for their antioxidant activity (Rice-Evans, 1999; Heim, 2002) and at the same time hydroxyl groups of the B ring are preferentially involved in metal chelation. However, flavonoids complexed with divalent cations does not render the flavonoid inactive as antioxidant, they still retain ROS scavenging activity (Arora A, 1998). Moreover, experimental data have shown that the chelates are considerably more effective free radical scavengers than the free flavonoids. For instance, Kostyuk *et al*. found that complexes of rutin, dihydroquercetin or epicatechin with Fe(II), Fe(III), Cu(II) or Zn(II) were more effective radical scavengers than the free flavonoids, due to the acquisition of additional superoxide dismutating centers (Kostyuk et al., 2001). Furthermore, Kumamoto *et al*. found that Cu(II) ion strongly increased the antioxidant activity of EGCG, while Fe(II) ion largely inhibited the antioxidative activity of EGCG (Kumamoto et al., 2001).

Although much less studied, it is known that flavonoids can also chelate Zn(II) (Figure I.1.9). In spite of Zn(II) being a redox-innocent cation, many reports have studied Zn complexes to

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evaluate the antioxidant activity of the flavonoid. For instance, Bodini *et al*. have reported that the complexation of catechin with Zn(II) ions favours the antioxidant activity of catechin decreasing the oxidation potentials (Bodini et al., 2001). Le Nest *et al*. have also studied the formation of complexes between quercetin and catechin with Zn(II) in two media at neutral pH in the absence of oxygen and its influence on their antioxidant properties (Le Nest, 2003). In addition, Sun Sh. *et al*. described that complexation of Zn(II) with EGCG influences the rate of absortion of EGCG by prostate cancer (PC-3) cells (Sun et al., 2008). Likewise, Kuo et al. found that diverse flavonoids may affect the expression of metallothionein in human intestanal CaCo cells (Kuo et al., 1998; Kuo and Leavitt, 1999; Kuo et al., 2001) an effect that was then atributed to the antioxidant action of the flavonoids, and not to a direct interaction of the flavonoids with the metals.

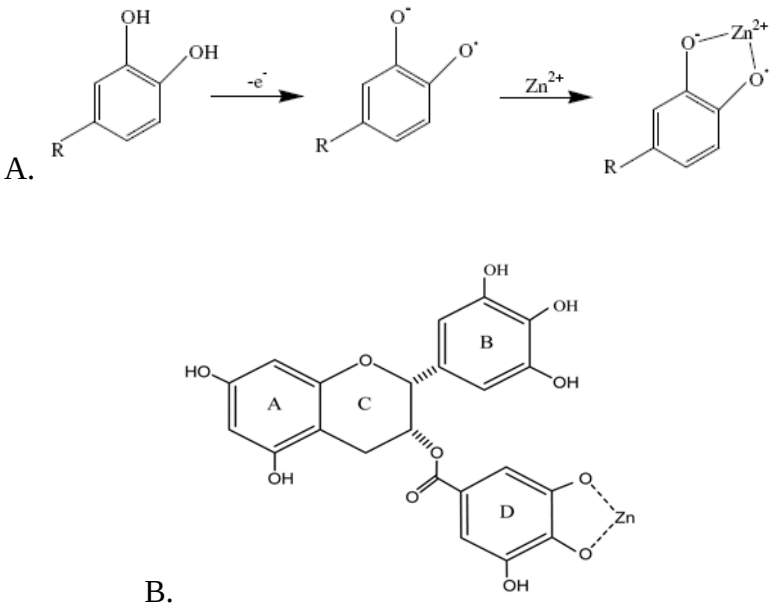

Figure I.1.9. Examples of flavonoid-Zn(II) complexes.
A. o-Semiquinone–Zn(II) acetate complex: only ring B is representated, R= Rings A and C. From (Le Nest, 2003)
B. A possible coordination scheme proposed for zinc-EGCG complex. The D-ring OH groups were occupied by Zn^{2+} at a molar ratio of 1:1. From (Sun et al., 2008).

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2. ZINC: AN ESSENTIAL METAL

Zinc (Zn) is a small ion with atomic number 30, atomic weight 65.41, and oxidation state +2. It has a highly concentrated charge and a small size. It is a strong Lewis acid and can bind strongly to donors such as thiolates and amines. Zinc is an ubiquitous and essential trace element in all organisms, i.e. it is indispensable for the growth and development of bacteria, fungi, plants, and animals. In mammals, it is found in all body tissues and fluids in relatively high concentrations. In vertebrates, relative large amount of zinc are found in bones and muscles, but these stores are not in equilibrium with the rest of the organism. Small amount of readily available zinc are stored in the liver, kidney and pancreas (McClain et al., 1995). The average amount of Zn in the adult human body is about 1.4–2.3 g Zn (Vallee and Falchuk, 1993). Average zinc intake in humans ranges from 107 to 231 micromol/day depending on the source, and human zinc requirement is estimated at 15 mg/day. In multicellular organisms, almost all zinc is found intracellularly, 40% is located in the cell nucleus, 50% in the cytoplasm, organelles and specialized vesicles and the remainder in the cell membrane (Tapiero and Tew, 2003). Zinc ions are hydrophilic and do not cross cell membranes by passive diffusion. In general, transport has been described as having both saturable and non-saturable components, depending on the extracellular Zn(II) concentrations involved (Tapiero and Tew, 2003). At the molecular level, zinc is almost completely bound to proteins or chelated by low molecular weight ligands, leading to a very low concentration (in the nanomolar range) of the free ionized species (Paski et al., 2003). Protein bound zinc is involved in a wide variety of metabolic processes including carbohydrate, lipid, nucleic acid and protein synthesis, and degradation. Recent studies estimate that zinc-binding proteins make up to 10% of the human proteome (Andreini et al., 2006; Andreini et al., 2009).

Zinc carries out its biochemical functions as a divalent cation primarily when bound to enzymes and other proteins. Under physiological conditions, zinc does not undergo reduction or oxidation. This lack of redox change renders it stable in a biological medium whose

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oxidoreductive potential is subject to continual flux. Another relevant physicochemical quality is the fact that it is amphoteric, existing both as the aquo and hydroxo metal complex at pH values near neutrality. It has a variable coordination sphere and a stereochemical adaptability to assume multiple coordination geometries, unusual features that contribute to its biochemical versatility. These different geometries result in coordination numbers varying from two to eight. However, four, five, and six coordinate complexes are the ones most frequently encountered in enzymatic, and other, biological functions of zinc, with geometries ranging from regular or distorted tetrahedral to trigonal bipyramidal, square pyramidal, and octahedral. This plethora of coordination numbers and geometries reflects the ability of zinc to cooperate with the demands of its ligands, allowing them to alter its intrinsic reactivity. Thus, protein structure affects the chemistry of bound zinc as much as zinc, in turn, affects the conformation and adaptability of these macromolecules. The magnitude of the stability constants of metal binding proteins varies quite widely and has served to differentiate operationally between two classes, metalloproteins and metal-protein complexes (Vallee and Falchuk, 1993). The metal is bound sufficiently firmly to metalloproteins. In contrast, in metal-protein complexes, the metal is bound loosely and, in most cases, the mechanism by which zinc binding affect protein function remains poorly characterized.

ZINC PROTEINS AND ZINC ENZYMES

The number and diversity of zinc-binding enzymes and the postulated roles of the metal in their function can be appreciated by examination of Table I.2.1. Four distinct types of zinc binding sites in enzymes have thus far been identified: **catalytic, cocatalytic, structural** and **protein interface.** Imidazole nitrogen atoms and cysteine thiol groups appear to be the predominant ligands in the catalytic and structural sites of enzymes, respectively, and the most frequent amino acid ligands are His, Glu, Asp, and Cys (Tables I.2.2 and I.2.3) (Vallee and Falchuk, 1993; Patel et al., 2007; Maret and Li, 2009). A summary of the ligand types to zinc proteins and enzymes and their net charges are shown in Tables I.2.3 and I.2.4.

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Name	Source	Role
Class I, Oxidoreductases		
Alcohol dehydrogenase	Yeast	c, s
Alcohol dehydrogenase	Vertebrates, plants	c, s
Sorbitol dehydrogenase	Vertebrates	c
D-Lactate dehydrogenase	Barnacle, bacteria	?
D-Lactate cytochrome reductase	Yeast	?
Superoxide dismutase	Vertebrates, plants, fungi, bacteria	ca
Class II, Transferases		
Transcarboxylase	*P. shermanii*	?
Aspartate transcarbamylase	*E. coli*	s
Phosphoglucomutase	Yeast	?
RNA polymerase	Wheat germ, bacteria, viruses	c
Reverse transcriptase	Oncogenic viruses	c
Nuclear poly(A) polymerase	Rat liver, virus	c
Terminal deoxyribonucleotidyl transferase	Calf thymus	?
Mercaptopyruvate sulfur transferase	*E. coli*	?
Class III, Hydrolases		
Leukotriene A_4 hydrolase	Human	c
Alkaline phosphatase	Mammals, bacteria	c, ca
5'-Nucleotidase	Bacteria, lymphoblast, plasma	?
Fructose-1,6-bisphosphatase	Mammals	ca
Phosphodiesterase (exonuclease)	Snake venom	c
Phospholipase C	*B. cereus*	c, ca
Cyclic nucleotide phosphodiesterase	Yeast	?
Nuclease	Microbes	?
α-Amylase	*B. subtilis*	s
α-D-Mannosidase	Mammals, plants	?
Aminopeptidase	Mammals, fungi, bacteria	c, ca
Aminotripeptidase	Rabbit intestine	c
Astacin	Crustacea	c
Meprin	Mammals	?
Enkephalinase	Mammals	?
Thermolysin	Bacteria	c
Dipeptidase	Mammals, bacteria	c
Class III, Hydrolases (*continued*)		
Angiotensin-converting enzyme	Mammals, bacteria	c
Carboxypeptidase A	Vertebrates, crustacea	c
Carboxypeptidase B	Mammals, crustacea	c
Carboxypeptidase (other)	Mammals, plants, bacteria	c
Carboxypeptidase DD	*S. albus*	c
Elastase	*P. aeruginosa*	c
Neutral protease	Vertebrates, fungi, bacteria	c
Collagenase	Mammals, bacteria	c
Protein kinase C	Mammals	s
Hemorrhagic protease	Snake venom	c
Aminoacylase	Pig kidney, microbes	?
Dihydropyrimidine aminohydrolase	Bovine liver	?
Dihydroorotase	*Clostridium oroticum*	?
β-Lactamase II	*B. cereus, P. maltophila*	c
Creatininase	*P. putida*	?
AMP deaminase	Rabbit muscle	?
Inorganic pyrophosphatase	Yeast	?
Nucleotide pyrophosphatase	Yeast	c
Adenosine deaminase	*E. coli*, mammals	?
Class IV, Lyases		
Fructose-bisphosphate aldolase	Yeast, bacteria	c
1-Rhamnulose-1-phosphate aldolase	*E. coli*	c
Carbonic anhydrase	Animals, plants	c
δ-Aminolevulinic acid dehydratase	Mammalian liver, erythrocytes	c
Glyoxalase I	Mammals, yeast	c
Class V, Isomerases		
Phosphomannose isomerase	Yeast	?
DNA topoisomerase I	*E. coli*	?
Class VI, Ligases		
tRNA synthetase	*E. coli, B. stearothermophilus*	c
Pyruvate carboxylase	Yeast, bacteria	?

c, catalytic role; s, structural; ca, coactive; ?, available information is insufficient to make an assignment.

Table I.2.1. Zinc enzymes comprise all 6 classes of enzymes established by the IUPAC. Taken from (Vallee and Falchuk, 1993).

A **catalytic role** specifies that the metal participates directly in enzyme catalysis. If the metal is removed by chelating or other agents, the enzyme becomes inactive. This abolition of activity is attributed primarily to the fact that zinc itself participates directly in the catalytic process; this does not exclude the possibility that there may also be a concomitant structural change (e.g., in local conformation and/or that of the ligands). **A coactive (or cocatalytic) zinc** atom enhances or diminishes catalytic function in conjunction with another active site zinc atom in the same enzyme, but is not indispensable of itself for either enzyme activity or stability. **Structural zinc** atoms are required solely for structural stability of the protein but do not directly participate in catalysis, and can help stabilize the quaternary structure of oligomeric holoenzymes. Alcohol dehydrogenases of vertebrates contain both a catalytic and a structural zinc atom. **Protein interface zinc** sites have an impact on the quaternary structure of a protein. They are composed of amino acid ligands that reside in the binding surface between two protein subunits or interacting proteins and generally have the

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coordination properties of catalytic or structural zinc binding sites (Maret and Li, 2009). (Figure I.2.1)

Enzyme	L_1	X	L_2	Y	L_3	Z	L_4
Class I							
Alcohol dehydrogenase	Cys	20	His	106	Cys (C)		H_2O
Alcohol dehydrogenase*	Cys	2	Cys	2	Cys (C)	7	Cys (C)
Class II							
Aspartate transcarbamylase*	Cys	4	Cys	22	Cys (C)	2	Cys (C)
Class III							
Carboxypeptidase A	His	2	Glu	123	His (C)		H_2O
Carboxypeptidase B	His	2	Glu	123	His (C)		H_2O
Thermolysin	His	3	His	19	Glu (C)		H_2O
B. cereus neutral protease	His	3	His	19	Glu (C)		H_2O
Carboxypeptidase DD	His	2	His	40	His (N)		H_2O
β-Lactamase	His	1	His	121	His (C)		H_2O
Phospholipase C	His	3	Glu	13	His (N)		H_2O
Alkaline phosphatase	Asp	3	His	80	His (C)		H_2O
P. aeruginosa elastase	His	3	His	19	Glu		H_2O
Class IV							
Carbonic anhydrase I	His	1	His	22	His (C)		H_2O
Carbonic anhydrase II	His	1	His	22	His (C)		H_2O

Table I.2.2. Zinc ligands and their spacing for the catalytic and structural zinc. X, number of amino acids between ligand 1 (L_1) and L_2; Y, number of amino acids between L_2 and its nearest zinc ligand neighbor; Z, number of amino acids between L_3 and L_4. L_3 is contributed by either the amino (N) or the carboxy (C) portion of the protein. * Structural zinc site. All others are in catalytic zinc sites. From (Vallee and Falchuk, 1993).

Catalytic	$(L)_3^*Zn\text{-}H_2O$	Zn_2 cluster	Zn_2Cys_6
Coactive	$(L)_3^*Zn\text{-}H_2O$ Zn-Asp-Mg	Zn_3 cluster	Zn_3Cys_9
Structural	$ZnCys_4$	Zn_4 cluster	$Zn_4\ Cys_{11}$
	$ZnCys_3His$	Zn_n finger	$(ZnCys_2His_2)_n$
		Zn_2 twist	$(ZnCys_4)_2$

Table I.2.3. Zinc binding sites in proteins. $(L)_3$*, 3 amino acid ligands of zinc. From (Vallee and Falchuk, 1993)

Example	Ligands	Charge
Catalytic		
Carbonic anhydrase	$(His)_3(H_2O)$	+2
Thermolysin	$(His)_2Glu(H_2O)$	+1
Alcohol dehydrogenase	$(Cys)_2His(H_2O)$	0
Structural		
TFIIIA	$(Cys)_2(His)_2$	0
Protein kinase C	$(Cys)_3His$	−1
Alcohol dehydrogenase	$(Cys)_4$	−2
Glucocorticoid receptor	$(Cys)_4$	−2
Clusters		
GAL4	$(Cys)_6(Zn)_2$	−2
Metallothionein	$(Cys)_9(Zn)_3$	−3
	$(Cys)_{11}(Zn)_4$	−3

Table I.2.4. Net charge of zinc binding sites in some zinc proteins. No account is taken of any partial charge from the water molecule. From (Vallee and Falchuk, 1993).

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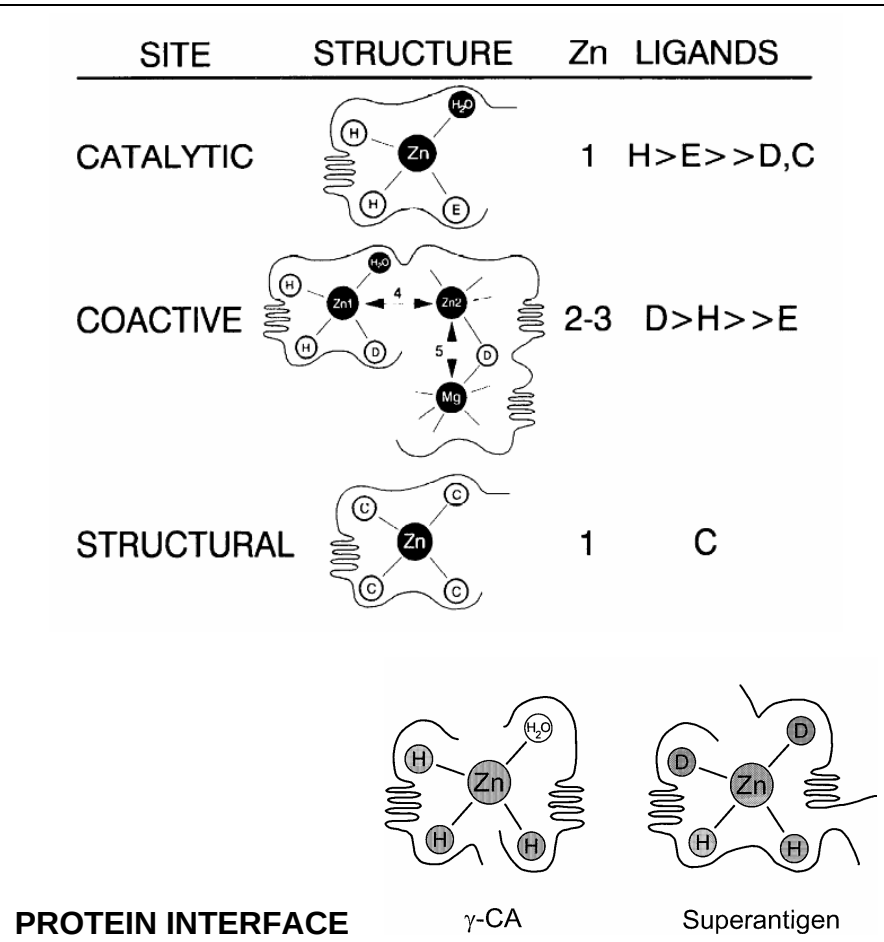

Figure I.2.1. Zinc binding sites in zinc proteins. Three amino acids (1 letter code) and one water molecule are the zinc ligands in the **catalytic zinc** site. Enzymes with a **coactive zinc** site exhibit both a catalytic zinc site, Zn1, and a separate coactive site, Zn2. In such multizinc enzymes, both sites act as a unit for catalytic activity. Zn2 sites modulate but are not essential for catalysis. These 2 Zn atoms can bind to 1 amino acid, usually aspartic acid, sharing it as a bridge. Furthermore, one of the amino acid ligands of a Zn2 site can additionally form a bridge to another, i.e., 3rd metal, in this case, a magnesium atom. In **structural zinc** site shown, metal is coordinated tetrahedrally to 4 Cys residues that prevent access of other ligands to the coordination sphere. In protein kinase C, Zn is coordinated to 3 Cys and 1 His residues. Taken from (Vallee and Falchuk, 1993). **Protein interface zinc** binding sites generally have the coordination properties of catalytic or structural zinc binding sites. *γ*-CA, *γ*-carbonic anhydrase; Superantigen, staphyloccus enterotoxin C2. From (Auld, 2001).

1. Catalytic zinc

Catalytic zinc sites are generally composed of a bound water molecule and three aminoacids, two of which come from a short amino acid spacer, with His being the most frequent, followed by Glu, Asp, and Cys residues that coordinate the zinc by imidazole and

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carboxyl groups (Table I.2.2, Figure I.2.1). The zinc dissociation constant for these protein sites is in the nM to pM range. One catalytic zinc atom per subunit of enzyme is the rule. Zinc is bound to three His in human carbonic anhydrases I and II (Figure I.2.2.). It is bound to two His and one Glu in carboxypeptidases A and B. The sole exception to the above thus far is alcohol dehydrogenase, the catalytic zinc site which uniquely contains just one His as well as two Cys. However, in this case, too, the third zinc ligand at the active site appears to be variable and has been deduced to be Cys, Glu, or Asp in different enzymes within the alcohol dehydrogenase superfamily. A water molecule is the fourth ligand at all catalytic sites. Mechanistically, the water molecule can be ionized, polarized, or displaced. Ionization or polarization provides hydroxide ions at neutral pH, while the displacement of the water leads to Lewis acid catalysis.

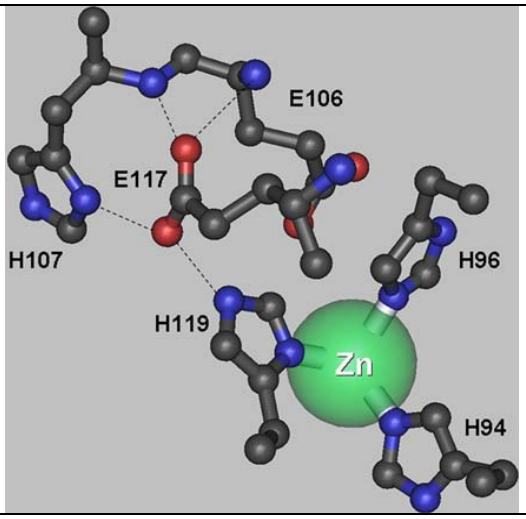

Figure I.2.2. The catalytic zinc atom in the human carbonic anhydrase II. Carboxyl oxygens of E117 hydrogen bond the imidazole NH of H107, the non-zinc binding NH of H119 and amide NH's of H106 and E106. From (Auld, 2009).

2. Coactive (cocatalytic) zinc

Cocatalytic zinc sites are found in enzymes containing two or more zinc and/or other transition metals in close proximity to each other that operate in concert as a catalytic unit. The additional zinc (or other metal) site has been named coactive (or "cocatalytic"). The distance between the metals is determined by type of amino acid (Asp, Glu, His, or a carboxylated Lys) that bridges the two metals (Figure I.2.1). Sometimes a water molecule forms a bridge between the metal atoms in a cocatalytic site. Asp and His are the most

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frequent ligands in this type of site. The ligands to these sites often come from nearly the whole length of the protein and the metals are important not only to catalytic function but to protein folding. There are two bridged metal atoms, zinc and magnesium, in alkaline phosphatase, two zinc atoms in phospholipase C, zinc and magnesium in bovine lens leucine aminopeptidase, zinc and copper in bovine erythrocyte superoxide dismutase, as one as zinc and magnesium in zinc atoms in human PDE4 cAMP phosphodiesterases (Figure I.2.3).

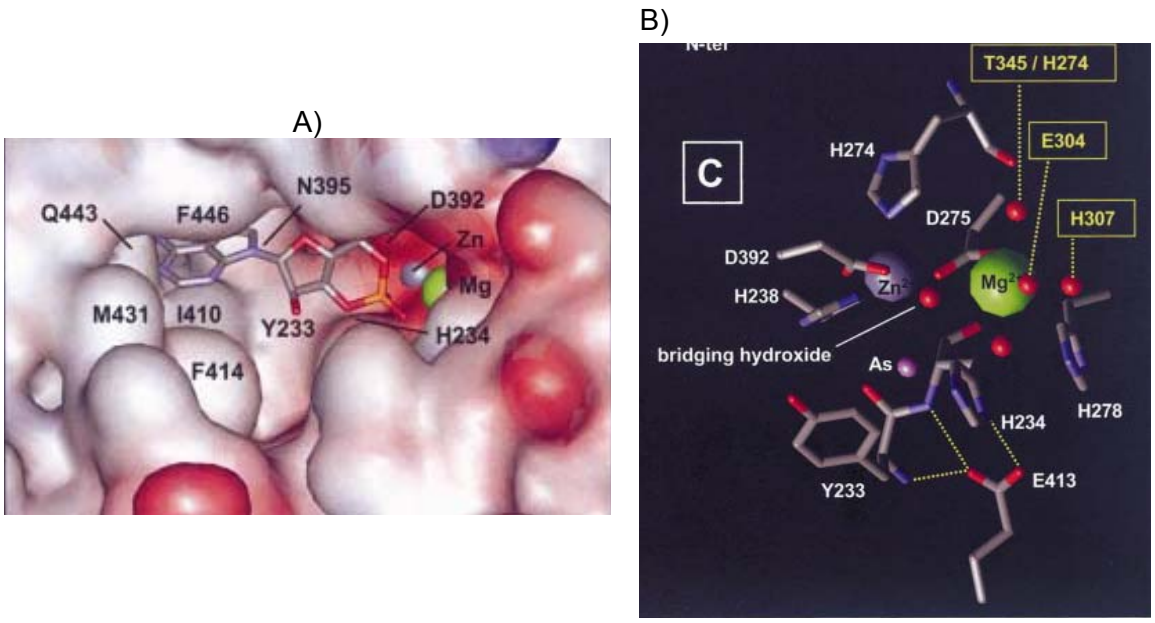

Figure I.2.3. X-ray crystal structure of cAMP phosphodiesterase PDE4B2 core catalytic unit illustrating metal-ion environment and probable substrate-binding mode. (A) Detail of proposed binding mode for cAMP substrate. (B) Detail of metal-ion environment, including solvent ligands. Taken from (Houslay and Adams, 2003)

3. Protein interface zinc

Protein interface zinc sites have an impact on the quaternary structure of a protein. They are composed of amino acid ligands that reside in the binding surface between two protein subunits or interacting proteins and generally have the coordination properties of catalytic or structural zinc binding sites. *γ*-carbonic anhydrase, Superantigen, and insulin are examples

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of proteins that use zinc to form quaternary structures (Figure 1.2.1 and I.2.4). (Auld, 2009; Maret and Li, 2009).

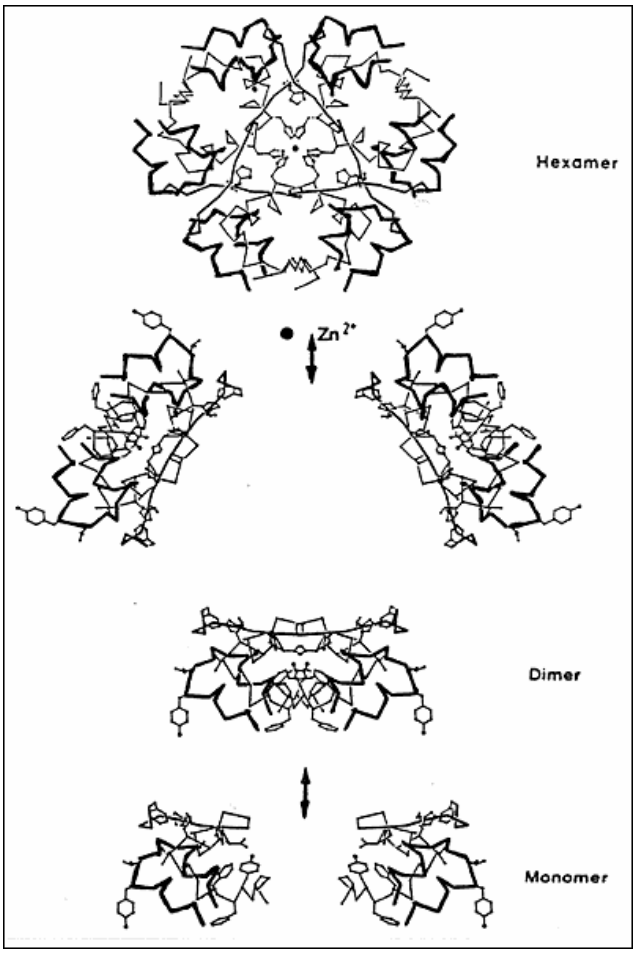

Figure I.2.4. Assembly and disassembly of the 2-Zn insulin hexamer. The A-chain of insulin is illustrated as a thickened black line, and the B-chain as a thinner line. As explained in the text, monomers assemble into dimers as the concentration of protein increases, and into hexamers in the presence of divalent cations such as zinc. The side chains of the six HisB10 residues are shown coordinating with the zinc ions in the hexamer. The pathway is reversed when the hexamer is secreted from β-cells and diffuses down its concentration gradient into the blood. Taken from (Pittman et al., 2004).

4. Structural zinc

Structural zinc sites contain four protein ligands and no metal bound water. While Cys is the most frequent ligand of these sites, any combination of four Cys, His, Glu, and Asp residues in principle can form this type of zinc site. Twelve combinations of the 22 permutations of these four ligands have been observed so far. The role of the structural zinc site is to maintain the localized structure of the protein which could in turn influence protein folding or function by supplying residues involved in catalysis that arise from within the spacer arms. Structural zinc atoms have been best characterized in three enzymes, **protein kinase C**, **aspartate transcarbamylase**, and **alcohol dehydrogenase** (Vallee and Falchuk, 1993).

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The structural motif in protein kinase C contains 4 g·atoms zinc/mol protein located in the noncatalytic domain of the enzyme. Each zinc atom is fully coordinated tetrahedrally to three cysteines and one histidine. This motif has previously been observed in DNA binding protein g32p from bacteriophage T4. In aspartate transcarbamylase and alcohol dehydrogenase the structural zinc is fully coordinated tetrahedrally to four cysteines (Figure I.2.5.) (Auld and Bergman, 2008).

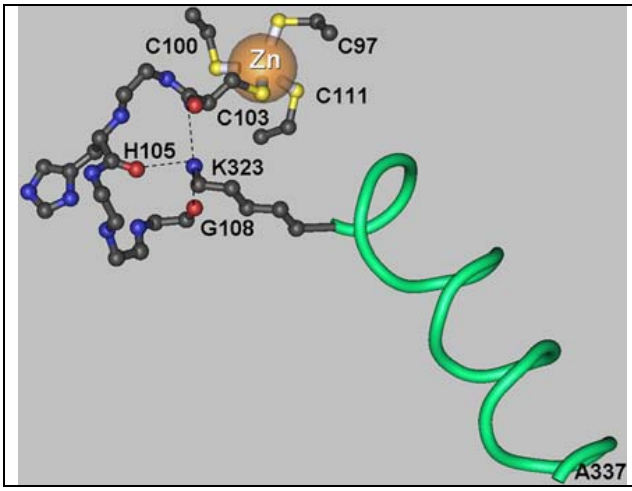

Figure I.2.5. Three-dimensional structure of the structural zinc site of alcohol dehydrogenase (PDB# 2JHG). The helix ending with Ala337 positions the ε-amino group of Lys323 within H bonding distance of the backbone carbonyls of amino acid residues Cys103, His105, and Gly108. From (Auld, 2009).

Zymogen activation (transformation of a structural to a catalytic zinc atom in matrix metalloproteases).

Matrix metalloproteases are expressed in an inactive pro-form. Their propeptides contain a cysteine residue in a highly conserved short sequence of amino acids. The dissociation or displacement of the cysteine from the catalytic zinc atom in the latent form leds to its activation mechanism since the dissociation of the cysteine from the zinc in the latent enzyme switches the role of the zinc from a non-catalytic to a catalytic one. The amino acids surrounding the propeptide cysteine act like Velcro in stabilizing the tetracoordinate structural like zinc site in the latent form. This represented a new zymogen activation mechanism based on zinc coordination properties.

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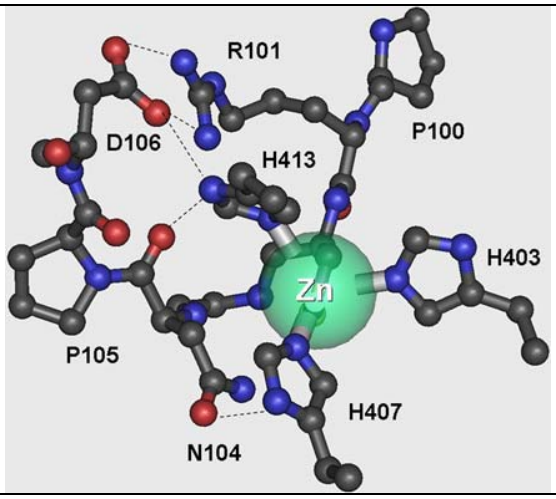

Figure I.2.6. Stabilization by Zn of the latent form of human proMMP-2 by the interactions of the amino acids (PRCGNPD) on each side of C102 bound to the catalytic zinc from beneath. From (Auld, 2009).

The collagenases and gelatinases are matrix metalloproteinases that hydrolyze the major components of the extracellular matrix. On the basis of their substrate preferences, the matrix metalloproteinases have been grouped into three classes: the interstitial collagenases, the type IV collagenases (gelatinases), and the stromelysins. The enzymes are synthesized as zymogens, i.e., inactive precursors. Matrix metalloproteinases can be activated by surfactants such as sodium dodecyl sulfate, disulfides such as glutathione, and oxidants such as NaOCl in addition to the well established activating agent for these enzymes, trypsin (Auld, 2009). The single zinc atom in the inactive precursors of this family of enzymes is coordinated tetrahedrally to four amino acid residues as found in structural sites (Figure 1.2.6). The coordination sites of the metals in these proenzymes, therefore, are filled; neither water nor substrate has ready access to the metal. One of the ligands to the zinc is a highly conserved cysteine in the “activation” peptide (PRCGN(V)PD) that forms a mercaptide with the metal atom and is removed in the activation process. The cysteine appears to act like Velcro by “sticking” to the zinc atom through its SH-group and blocks it from participating in the catalytic process. Dissociation and/or displacement of that cysteine from the metal atom transforms the zinc from tetradentate, i.e., structural, to tridentate with respect to protein ligands, with water becoming the fourth ligand and rendering it “catalytic”. The displacement of the zinc bound cysteine by water converts a structural-like zinc site into a catalytic zinc site (Figure 1.2.7). This activation is called the ‘‘Cysteine Switch’’ (Auld, 2009).

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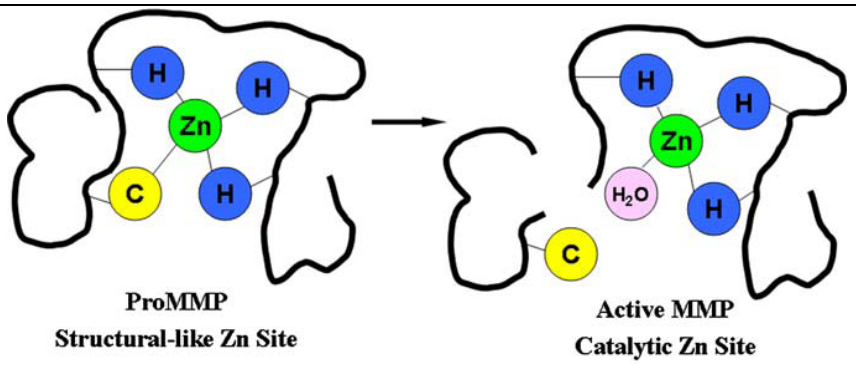

Figure 1.2.7. Schematic of mechanism of activation of promatrix metalloproteinases. The displacement of the propeptide cysteine by water is induced by either proteolytic cleavage and/or conformational changes of the propeptide. From (Auld, 2009).

Gene regulatory proteins

A promitent role of structural zinc is found in the conformation of certain zinc-dependent protein domains, such as zinc fingers, zinc clusters, and RING fingers, which are commonly found in transcriptional regulatory proteins. While the majority of the zinc-finger proteins are transcription factors, others may participate in protein-protein interactions that provide signalling via kinase-binding domains (Cousins, 1998; Krishna et al., 2003).

Since 1985, more than 1000 nucleoproteins containing functionally important zinc atoms and directly involved with replication and transcription of DNA in viruses, bacteria, yeast, and mammals, among other organisms, have been shown to contain amino acid sequences in DNA binding domains including combinations of four or more conserved Cys and/or His residues that could serve as metal binding sites. DNA binding proteins can be categorized into different structurally distinct groups (Figure I.2.8), constituted by **zinc fingers (TFIIIA), "zinc twists" [glucocorticoid (GR) and estrogen receptors (ER)], and "zinc clusters" (GAL4 protein).** There are important differences in the mode of binding of the zinc to these protein types. The finger types require only one zinc atom for each site, whereas the others require at least two. The ligands to the metal of five activator proteins critical to transcription and one DNA-binding protein (g32p) are shown in Table I.2.5.

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Protein	Zinc/mole	Ligands/zinc
TFIIIA	2	2 Cys, 2 His
TFIIIA	7-11	2 Cys, 2 His
Glucocorticoid receptor	2	4 Cys
Estrogen receptor	2	4 Cys
GAL4	2	4 Cys*
g32p	1	3 Cys, 1 His

Table I.2.5. Zinc in replication and transcription regulatory proteins. * In this zinc cluster, a total of 6 Cys coordinate with 2 zinc atoms such that 2 of the Cys are shared by both zinc atoms. Formally, 4 Cys are involved in the coordination of each zinc, 2 of the Cys with both metals. From (Vallee and Falchuk, 1993).

TFIIIA, a transcription factor protein from *Xenopus laevis* oocytes, interacts with the 50-bp internal control region of the 5S RNA gene, thereby activating transcription by RNA polymerase III. It also interacts with 5S RNA itself, forming a 7S complex that is readily obtainable from the eggs of Xenopus laevis. TFIIIA was the first transcription factor to be identified as a zinc protein. The initial publication reported the presence of 2 g·atoms zinc/mol purified protein, whereas the 7S intact protein-RNA particles were shown to bind from 7 to 11 zinc atoms. The variation in in zinc stoichiometry could be due to physiological differences that might be brought about by the action of thionein, which can remove zinc from TFIIIA, thereby rendering the transcription factor inactive (Zeng et al., 1991). Such chemical monitoring of TFIIIA action could be postulated to modulate the number of zinc atoms bound to the protein, thereby controlling the number of zinc fingers actively engaged in the transcription process at any given time. The primary structure of TFIIIA contains highly conserved sequences comprised of 2 Cys and 2 His residues separated by variable numbers of amino acids in 9 repeat units of -30 amino acids. The Cys and His residues in each of the conserved repeat units of TFIIIA form tetrahedral coordination complexes with one zinc atom and generate a loop structure containing the DNA binding domain of the protein in the sequence intervening between the pairs of Cys and His residues, resulting in the “zinc finger” DNA binding motif. Zinc is required for DNA binding of TFIIIA.The protein will not bind to DNA if zinc is removed by chelating agents. The molecule binds in the major groove of B-

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DNA. The primary contacts are in a 3-bp subsite in a guanine-rich strand of the DNA. The contacts with the bases are made by conserved arginines that are located in the β-sheet region that immediately precedes the α-helix.

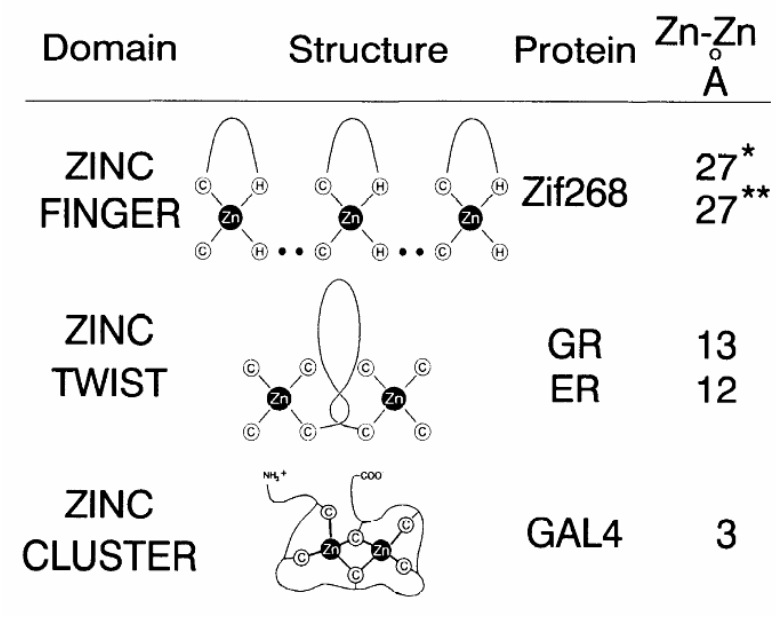

Figure I.2.8. Estructural zinc binding sites in gene regulatory proteins. In the zinc finger type, metal is coordinated to 2 Cys and 2 His residues. Structure has been defined crystallographically using a 3-finger construct. * Zn-Zn distance from 1st zinc finger to 2nd finger; ** Zn-Zn distance from 2nd to 3rd finger. In both the zinc twist type, found in the glucocorticoid receptor (GR) and estrogen receptor (ER), and the zinc cluster type of the GAL4 protein, metal is coordinated to 4 Cys residues. Structure of resultant DNA binding domain differs for each type. From (Vallee and Falchuk, 1993).

The **glucocorticoid** and **estrogen receptors** are members of a multigene family that includes receptors for thyroid hormone, retinoic acid, and vitamin D, which incorporate three polypeptide domains, each interacting with a different ligand. The first domain binds to a specific hormone, e.g., cortisol or estrogen; the second interacts with enhancerlike DNA segments; and the third binds to RNA polymerase. The enhancer regions are usually present in a dyad symmetry, suggesting that the interaction with the receptor involves dimerization of the protein. The steroid itself or the hormone binding domain is not required for DNA binding,

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although the hormone-receptor complex facilitates the interaction between the DNA binding domain and the enhancer region. The metallo-DNA binding domain interacts specifically with the glucocorticoid response element sequence. Removal of zinc by chelating agents at low pH yields an apoprotein that does not bind to specific DNA fragments. Binding is fully restored by reconstitution with either zinc or cadmium, but less so with cobalt, and not with other metals. The primary structure of the DNA binding domain of the glucocorticoid receptor encompasses one His and nine Cys residues. Each metal is coordinated to four isolated sulfur ligands. There are no bridging sulfur ligands. The DNA binding motif is helical and does not have a finger structure. Other members of the hormone receptor family of proteins exhibit sequences in the DNA binding domain that contain potential zinc-binding ligands, and the estrogen receptor has been shown to contain 2 g·atoms zinc/mol.

The **GAL4** protein from yeast activates the genes utilized for galactose metabolism. It consists of 881 amino acids, but only the NH_2-terminal 74 amino acid residues are involved in binding to the upstream activation sequence. Zinc is required for GAL4 function.The two bound metals are coordinated by six cysteines, two of which form bridging ligands between the Zn ions. The resultant binuclear metal clusters are similar in principle to those observed in metallothionein. The structure of the DNA binding domain composed of such a cluster is clearly distinct from those of the TFIIIA and glucocorticoid receptor proteins.

The ligands that bind zinc and the resultant DNA binding motifs generated when the metal binds to the known zinc transcription/replication proteins are quite varied in the regulatory proteins that have been examined (Tables I.2.3 and I.2.5, Fig. I.2.8). The first suggestion that zinc could bind to a transcription activator in a manner that differs from that observed with TFIIIA arose from work with the transactivating tat protein from human immunodeficiency virus. This protein has been shown by optical absorption spectroscopy to bind 2 zinc atoms/monomer and form a metal-linked dimer, not a zinc finger structure.

UNIVERSITAT ROVIRA I VIRGILI
EFFECTS OF DIETARY CATECHINS AND PROANTHOCYANIDINS ON ZINC HOMEOSTASIS IN HEPATIC CELLS
Isabel Maria Quesada
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Metallothioneins

MTs are low-molecular weight metal-binding proteins. In mammals, MT is composed of 61-62 amino acids; among them, 20 are cysteines which are critical to the protein structure and function. Metallothionein-like proteins and peptides have been found in numerous unicellular and multicellular organisms and grouped together as a family composed of three classes. **Class I** metallothioneins typically occur in mammalian organisms and their primary structures are highly conserved. **Class II** metallothioneins are found in unicellular eukaryotes, such as yeast, and their primary structures bear little or no resemblance to those of the class I metallothioneins. **Class III** is present in plants, composed of chains varying from 2 to 11 γ-glutamylcysteinyl units collectively designated phytochelatins when glycine is the COOH-terminal residue or homophytochelatins if alanine is at that site. Phytochelatins are believed to be synthesized from glutathione through the action of phytochelatin synthase.

Class I MTs can bind a variety of metals, but in mammalian cells and tissues that have not been exposed to excess metal ions for which MTs have a higher affinity (Ag, Hg, Cd) they are bound primarily by zinc and secondarily by copper. The metals in MT are organized into two clusters (α and β) with differential capacity to associate to metals. The C-terminal α domain contains 11 cysteines Zn_4Cys_{11} (residues 33-60) and the N-terminal β domain contains nine cysteines Zn_3Cys_9 (residues 5-29), with five and three cysteine residues acting as bridging ligands between two metal ions in each cluster, respectively (Figure I.2.9).

The 20 Cys residues in MTs allow it to bind up to a total of seven zinc or cadmium ions, or up to 12 copper ions (Haq et al., 2003). In vitro studies have shown that MT can interchange Zn ions with other metalloproteins, notably zinc finger transcription factors such as ER (Cano-Gauci, 1996), TFIIIA (Zeng, 1991.), Sp1 (Zeng, 1991.), and the direction of this transfer is dependent upon redox cellular state and is facilitated by the GSSG/GSH system (Jacob et al., 1998).

In mammals, MT exists in 4 highly homologous isoforms, namely MT-1, MT-2, MT-3 and MT-4 which are encoded by different genes and differ in their expression pattern. MT-1 and MT-2

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isoforms are expressed in all tissues, with prevalence, but not restricted to, liver, pancreas and muscle. MT-3 (also known as neural tumor repression factor 3) and MT-4 are expressed specifically in the nervious system and epidermal and scamous cells respectively. Only one of each isoform, encoded by 4 different genes, are found in rodents, whereas in humans MT-1 isoforms are encoded by at least 10 different MT genes, placed in tandem in chromosome 16 together with the unique genes which code for the MT-2, MT-3 and MT-4 isoforms (Haq et al., 2003).

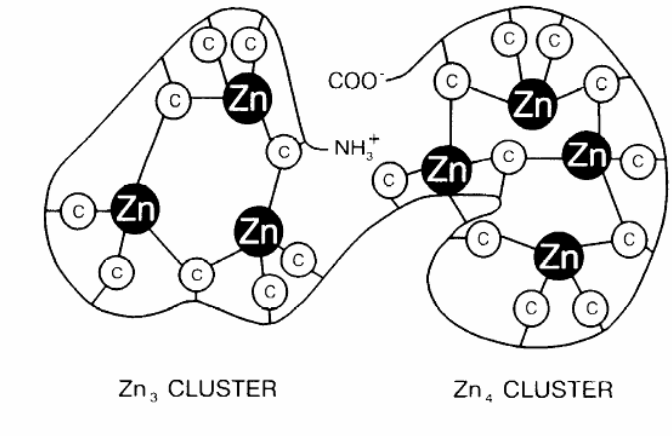

Figure I.2.9. Zinc thiolate clusters of mammalian metallothionein. Each molecule is composed of 2 clusters, the NH_2- and COOH-terminal regions containing 3 (Zn_3) and 4 (Zn_4) zinc atoms, respectively, in each cluster. Each metal is tetrahedrally coordinated to 4 thiolate bonds with some of the thiolate ligands sharing the zinc ion. From (Vallee and Falchuk, 1993).

MTs play an important regulatory role in Zn uptake, distribution, storage and release. It is well known that MTs bind Zn more tightly than most other Zn proteins and Zn bound to MTs represents 5–10% of the total Zn in human hepatocytes, serving as a reservoir of cellular zinc (Bühler RH, 1974; Maret, 2000). The roles of MT include the detoxification of heavy metals, homeostatic regulation of essential metals, and protection of tissues against various forms of oxidative injury (Haq et al., 2003). These biochemical functions of MT protein are functionally related with the diverse stress signals that upregulate MT gene transcription acting through distinct signal transduction pathways.

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Activation of MT expression.

A large number of physiological and pathological agents induce thionein (the metal-free protein or apo-protein) synthesis *in vivo* (Figure I.2.10). These include the heavy metal atoms Zn, Cd, Hg, Cu and Bi, to which MT bind, and Ni and Co, to which MT does not bind. Transcriptional regulation of MT-1/MT-2 genes by heavy metals is conferred by MREs (Metal Response Elements) (Figure 1.2.11), to which MTF-1 (Metal response element-binding Transcription Factor-1), binds (Figures 1.2.MTF1 and **I.2.MTF12**). MTF-1 is a zinc-dependent transcription factor which sense intracellular available zinc ions amd induces transcription of its target genes, including metallothioneins, in response to cellular zinc. MTF-1 contains six finger structures of which the first binds zinc with low affinity. After complexing zinc, MTF-1 is translocated from the cytoplasm to the nucleus (Smirnova IV, 2000) where it binds to metal response elements (MRE) of the target genes promoters (Langmade et al., 2000).

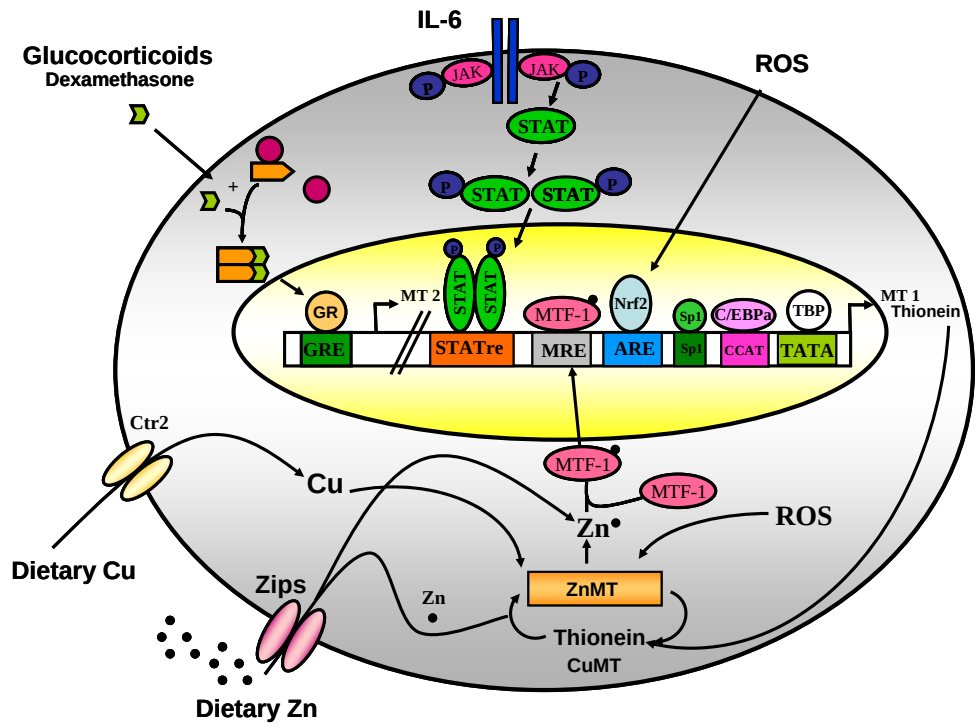

Figure I.2.10. Induction of mammalian MT gene expression through diverse signal transduction pathways. See text for details. Based on (Coyle et al., 2002; Haq et al., 2003; Cousins et al., 2006).

MTF-1 activates other important genes besides those encoding MTs, and that is why the double knockout of MT-I and -II genes was viable, while the knockout of the control protein

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MTF-1 was lethal. Gamma glutamyl-cysteine synthetase heavy chain (γGCShc), a key enzyme for glutathione synthesis, is one of them (Gunes et al., 1998). Also, C/EBPα another transcription factor, that is required to maintain the differentiated, non-proliferating state of hepatocytes and α-foetoprotein, that is responsible for maintenance of embryonic colloid-osmotic pressure and that also acts as a scavenger for heavy metals and reactive oxygen intermediates (ROI), are *in vivo* target genes of MTF-1 (Mizejewski, 1997; Gunes et al., 1998) The zinc transporter ZnT1, the main exporter of zinc out of the cell, was shown to be yet another *in vivo* target gene of MTF-1 (Cuajungco and Lees, 1997) (Figure I.2.MTF12). In sum, MTF-1 is a crucial transcriptional regulator for basal expression of at least three important genes (MT-I, MT-II an ZnT1) involved in zinc metabolism.

Optimal induction of metallothionein gene transcription by the redox-active species cadmium and H_2O_2 appears to require at least a MRE and an ARE (Antioxidant Response Element), which is bound by Nrf2, in the mouse MT-I gene promoter, while induction with tBHQ or zinc (a redox-inactive metal) depends only on MREs. The induction of MT with transition metals (both Cd and Zn) requires phosphorylation of MTF-1 by a kinases pathway that includes PI3K (Phosphoinositol-3 kinase), PKC (Protein kinase C) and JNK (c-jun N-terminal kinase) (LaRochelle et al., 2001; Saydam et al., 2002; Rutherford and Bird, 2004) (Figures 1.2.12 and 1.2.13).

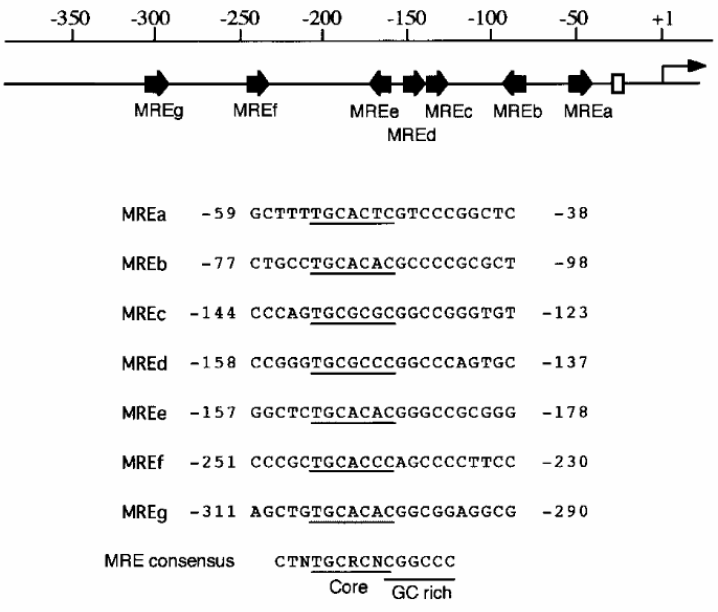

Figure 1.2.11. Arrangement of Metal Responsive Elements (MRE) in the promoter region of human MT2A gene. The TATA box is idicated by and open box and the MREs by arrows. The sequence of each MRE is shown bellow the figure, and the core sequence is underlined. Taken from (Otsuka, 2001).

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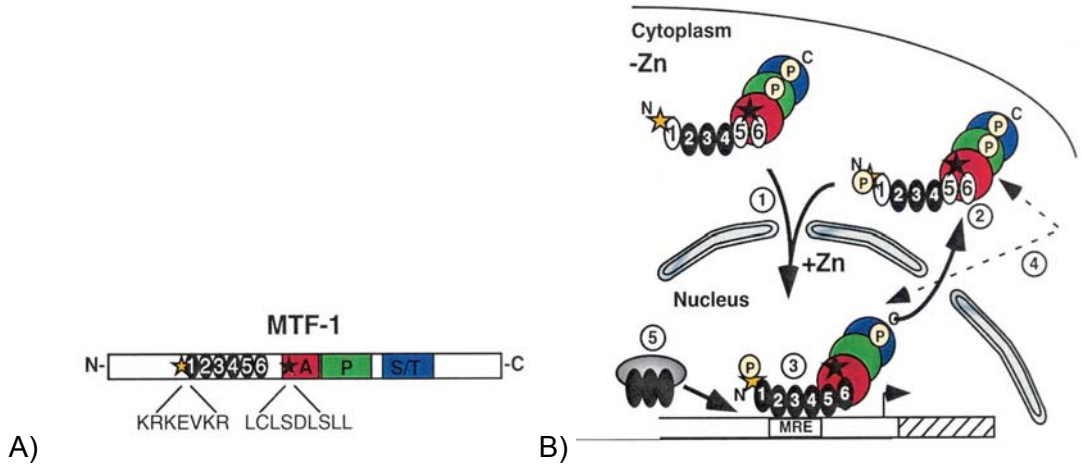

Figure I.2.12. MTF-1 structure and regulation. A) Scheme of mouse MTF. Shown are: zinc finger domains (numbered black ovals), MTF-1 nuclear localization signal (gold star), MTF-1 NES (black star), and MTF-1 acid-, proline-, and serine/threonine-rich activation domains (red, green, and blue boxes labeled A, P, and S/T, respectively). (B) The multiple levels of MTF-1 regulation: 1, nuclear translocation; 2, nucleocytoplasmic shuttling; 3, DNA-binding control; 4, posttranslational modification by phosphorylation; 5, interactions and binding inhibition by other factors. The absence of zinc in a metalloregulatory finger is indicated by a white oval. Taken from (Rutherford and Bird, 2004).

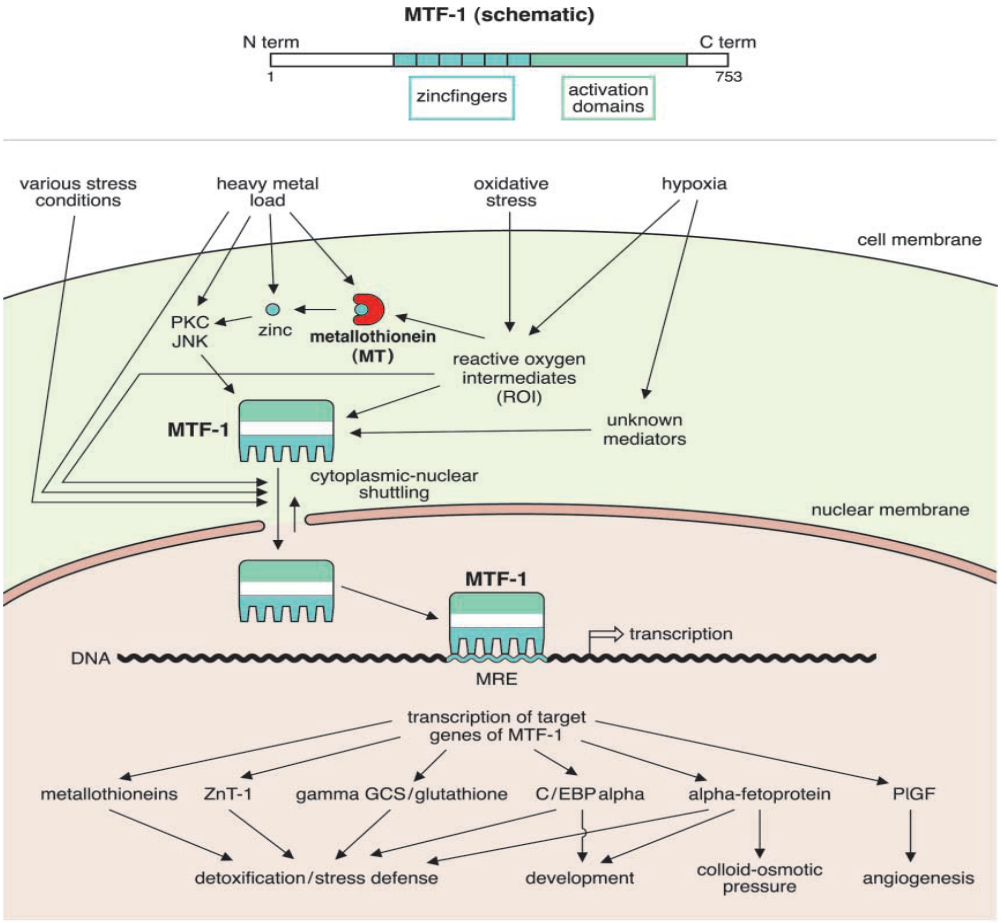

Figure I.2.13. Overview of activation pathways and downstream functions of MTF-1. Taken from (Lichtlen and Schaffner, 2001).

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Similar to induction of acute phase proteins (such as C-reactive protein and fibrinogen) inflammation induces the expression of hepatic MT. This induction is mediated by **glucocorticoid** hormones, acting through the GR (Glucocorticoid Receptor) and GREs (GR-Response Elements) in the promoter region of MT genes, and also by **pro-inflammatory cytokines**, such as IL-6, that activates MT expression through the JAK (Janus kinases) and STAT (Signal Transducer and Activator of Transcription) kinases, which bind to STAT-response elements in the regulatory regions of MT genes (Haq et al., 2003). IL-6 also acts through MTF-1 to fully activate MT transcription by upregulating the expression of the plasma membrane zinc importers ZIP6 and ZIP14 that, in turn, will promote the entrance of zinc cations into the cell thus activating MTF-1 (Figure I.2.14). This mechanism is responsible for the hypozincemia and zinc accumulation in hepatic cells that occurs after inflammation (Liuzzi et al., 2005; Cousins et al., 2006).

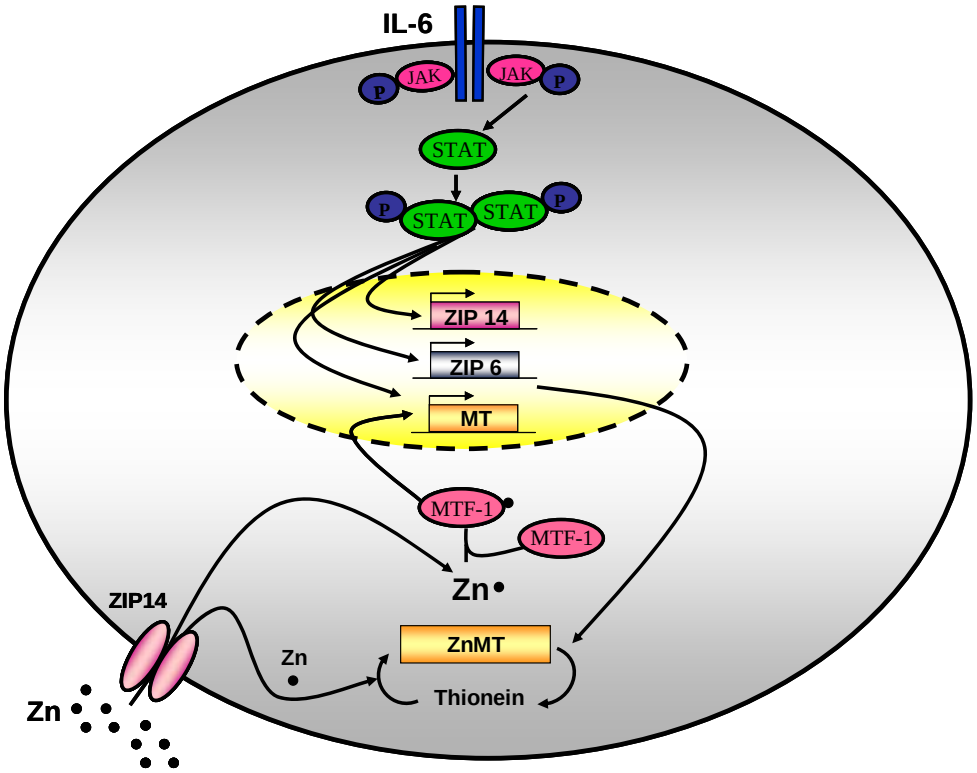

Figure I.2.14. Regulation of intracellular zinc homeostasis by proinflammatory cytokines. Proinflammatory cytokines, including the interleukins IL1 and IL6, stimulate STAT-mediated signaling, up-regulation of Zip14 and Zip6, and cellular zinc influx. Adapted from (Cousins et al., 2006).

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Epigenetic repression of MT genes.

MT genes can be repressed by epigenetic changes in their promoter region, such as methylation of cysteine residues within short stretches of CpG dinucleotides and CpG islands, and histone methylation and deacetylation (Majumder et al., 1999a; Majumder et al., 1999b; Ghoshal et al., 2000; Ghoshal and Jacob, 2001; Ghoshal et al., 2002; Majumder et al., 2002; Datta et al., 2005; Majumder et al., 2006).

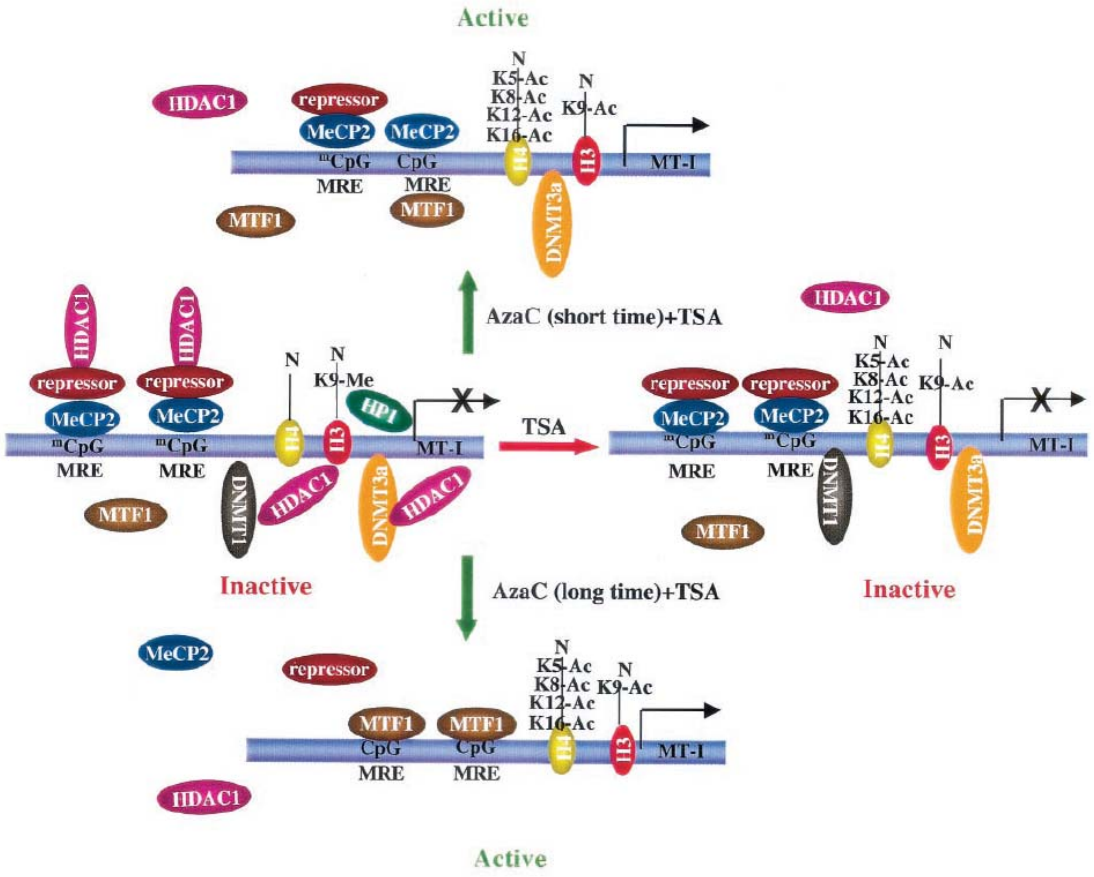

Figure I.2.15. Model of epigenetic silencing of MT-I in lymphosarcoma cells. The model depicts the involvement of different components of the methylation machinery in silencing the MT-I gene in lymphosarcoma cells and their dissociation after treatment with inhibitors of Dnmts and HDACs. Untreated cells, cells treated 12 h with TSA, cells treated 24 h with 5-AzaC and then 12 h with TSA, and cells treated with 5-AzaC for 120 h are shown. Taken from (Ghoshal et al., 2002).

Analysis of regulatory regions of rodent MT genes has revealed that they contain CpG islands with CpG content much higher than the minimum required to define an island (i.e.,

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CpG/GpC > 0.6). Thus, MT gene promoter regions in rodent tumor cell lines derived from liver and thymus have been shown to be highly methylated at CpG sites, in good correlation with low MT transcription from those genes. DNA methylation can repress gene transcription either by inhibiting binding of positive factors to the promoter or by recruiting transcriptional co-repressors. An example of the latter is that DNA methylation can lead to recruitment of HDAC (Histone deacetylases) that deacetylate histones in chromatin and promote an alteration of chromatin structure that inhibits transactivator access. In fact, histone hypoacetylation has been shown to be responsible for MT-I and MT-II silencing in mouse lymphosarcoma cells. These genes could be activated in these tumors by inhibition of Dnmts (DNA methyl-transferases) using 5-AzaC, and by HDACs, using TSA (Ghoshal and Jacob, 2001; Ghoshal et al., 2002) (Figure I.2.15).

LABILE ZINC, REGULATORY ROLE OF ZINC AND ZINC SIGNALING.

The multiples functions of zinc in cells, including structural, catalitic and regulatory functions of enzymes, requires a strict regulation of the cellular zinc content and subcellular distribution. To regulate the availability of Zn dynamically, eukaryotes have first compartmentalized Zn and at the same time they have the metallothionein/thionein pair, which controls the pico- to nanomolar concentrations of the pool of “labile” intracellular zinc (Maret, 2003; Krezel et al., 2007; Maret, 2009). This pool, which has also been called metabolically active, mobile, or available zinc, can reversibly bind to regulatory sites in signaling proteins. Consequently, changes of the lable oncentration can affect cell signaling patways and might even act as zinc signals. Estimations of “free” zinc concentrations have varied over many orders of magnitude, ranging from femtomolar concentrations in bacterial cells (Outten and O'Halloran, 2001) to micromolar concentration in hepatocytes (intracellular free zinc in isolated hepatocytes was estimated to be 1.26 +/- 0.27μM) (Brand and Kleineke, 1996). However, free zinc concentrations that trigger transcription of zinc uptake or efflux machinery are femtomolar, or six orders of magnitude less than one atom per cell. This is not

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consistent with a cytosolic pool of free zinc and suggests an extraordinary intracellular zinc-binding capacity. Several techniques have been used to determine the free zinc concentration experimentally. Measurements with radioactive ^{65}Zn yielded a concentration of 24 pM (Simons, 1991), and ^{19}F-NMR spectroscopy with 5-F-BAPTA gave a concentration of 0.5 nM free intracellular zinc (Benters J, 1997). With fluorescent probes traditionally employed for Ca(II) analysis, intracellular free Zn(II) concentrations were estimated to be 1 nM with FURA-2 (Atar D, 1995) and 2 nM with Mag-Fura-2 (Sensi SL, 1997). Whereas these probes are not specific for Zn(II) and thus their signals are difficult to separate from those for Ca(II), the zinc-specific fluorescent probe Zinquin allows a specific detection of intracellular zinc. It has been shown that Zinquin does not only detect free zinc, but also some of the zinc atoms that are bound to metallothionein, and probably also to other proteins (Coyle et al., 1994). Zinquin cannot be used for a quantification of free cytosolic zinc ions but it has been shown to be an excellent tool to investigate the amount and intracellular distribution of loosely bound, or labile, zinc, i.e. the pool of zinc cations that can be easily exchanged between proteins and therefore should be considered as the mediator of a signaling function for zinc. Other fluorescent probe usually used to estimate labile zinc is Zinpyr-1, which has been developed with an apparent dissociation constant of 2nM. (Krezel and Maret, 2006).

How much of (the total) zinc is in the labile pool depends on the zinc buffering capacity i.e. the ratio between zinc-bound and unbound cellular ligands. About 30% of this zinc buffering capacity is due to sulfur donors (thiols), and thus, serves at the same time as redox (oxidant) buffering capacity (Maret, 1994; Krezel et al., 2007; Maret and Krezel, 2007; Maret, 2009) (Figure I.2.16).

Zinc ions released intracellularly from proteins with zinc/thiolate coordination environments, such as metallothionein, become messengers in a fundamental molecular pathway, in which a redox signal is converted into a zinc signal (Figures 1.2.17 and 1.2.18 and 1.2.19):

$$\text{Redox signal} \rightarrow \text{proteins with zinc/thiolate coordination} \rightarrow Zn^{2+} \rightarrow \text{target(s)}$$

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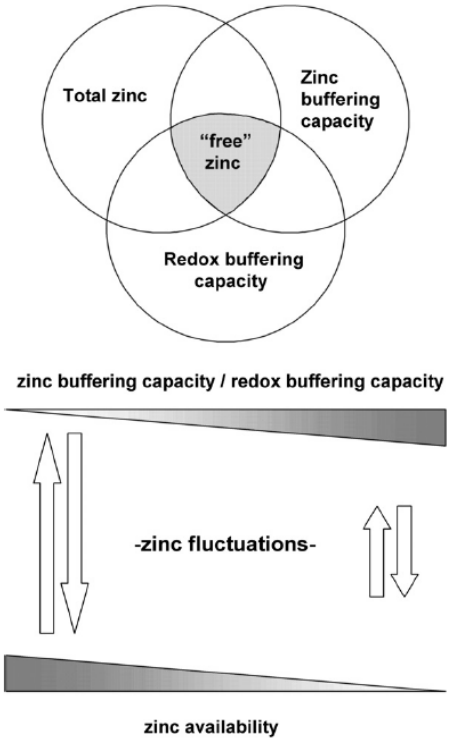

Figure I.2.16. Factors affecting the cellular concentrations of "free" (labile) zinc. Venn diagram illustrating how the total zinc concentration, zinc buffering capacity, and the redox buffering capacity determine the amount of "free" zinc. Increases of zinc or redox buffering capacity decrease zinc availability and limit the amplitudes of "free" zinc fuctuations**.** (Krezel et al., 2007)

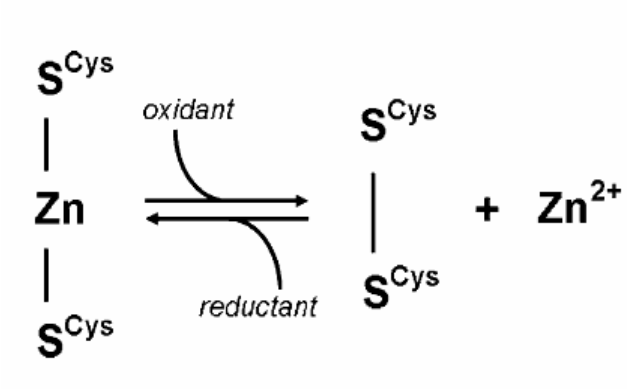

Figure I.2.17. A redox zinc switch. Cysteine/cystine interconversion can control zinc release and binding, a molecular mechanism that links zinc and redox metabolism. From (Maret, 2009)

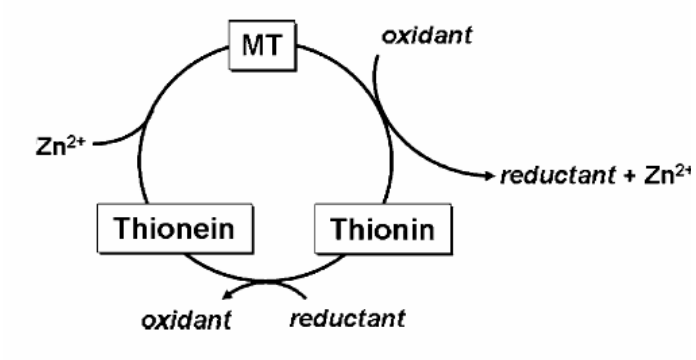

Figure I.2.18. Metallothionein redox cycle. In this cycle, one redox couple controls zinc release from MT, the zinc-loaded state, and generates a partially oxidized and zinc-loaded state (thionin). The same or another redox couple restores the reduced protein (thionein) for subsequent loading with zinc ions. From (Maret, 2009)

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Factors that interfere with zinc buffering will change the zinc potential, either making not enough “free” zinc available for vital functions or raising “free” zinc to pathological or cytotoxic levels. Interference can stem from chemicals with metal-binding capacity, which increase zinc buffering and make less zinc available. It can also stem from chemicals that react with the buffering ligands or from metal ions such as cadmium or mercury that bind more tightly than zinc to the corresponding ligands, decreasing the buffering capacity and increasing “free” zinc and inducing MT (Figure I.2.20) (Maret and Krezel, 2007). Other classes of agents that increase free zinc ion concentrations are reducible selenium compounds and endogenous reactive carbonyls formed during oxidative stress, lipid peroxidation, hyperglycemia-induced glycations and environmental exposures (Jacob et al., 1999; Hao and Maret, 2006; Maret and Krezel, 2007).

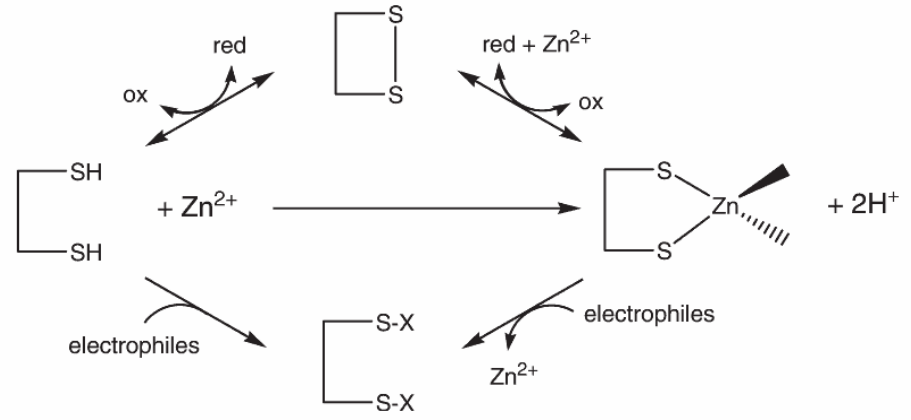

Figure I.2.19. Availability of “free” zinc ions. Competition among oxidation or covalent modification of thiols, zinc ion binding, and oxidation or covalent modification of zinc/thiolates determines the availability of “free” zinc ions. From (Maret and Krezel, 2007)

Fluctuations of labile zinc

In order for a metal ion to transduce signals, its intracellular concentration has to fluctuate in response to stimulation. Many examples can be found in the literature, some of which are listed in the Table I.2.7. In many reports cultured cells were exposed to zinc supplementation to the culture medium, resulting in an influx and a rise in labile zinc. This is a convenient way

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to increase labile intracellular zinc to investigate its effects on signal transduction, but probably not a source of zinc fluctuations *in vivo.* The physiological level of extracellular zinc is only between 12 and 16 μM, and plasma contains high amounts of zinc buffering proteins like albumin, and α2-macroglobulin. For albumin alone, the concentration in human serum has been estimated to be 0.6 mM (Peters, 1995) and its dissociation constant for zinc is $2.95 \cdot 10^{-8}$ (Masouka, 1993). Therefore, a maximum of nanomolar concentration of extracellular labile zinc can be expected, and is not clear if changes in the overall extracellular zinc concentration that could induce significant intracellular fluctuations do occur *in vivo*, or if these would be buffered (Haase, 2007).

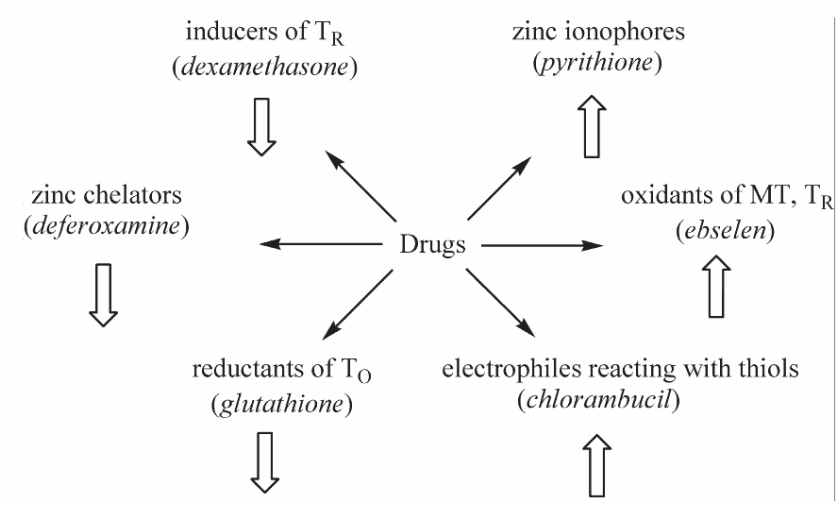

Figure I.2.20. Drugs change the availability of "free" zinc by several mechanisms. Zinc chelation, induction of TR or generation of TR (reduced thionein) by reduction of T0 (Thionein) decrease zinc (left). Oxidation or other covalent modification of thiols and compounds that serve as carriers of zinc into the cell increase zinc (right). From (Maret and Krezel, 2007).

To investigate the impact that zinc fluctuations have on zinc signaling, several tools have been developed that can be used to modulate labile zinc. On the one hand, ionophores are used to increase labile zinc, including pyrithione, PDTC (pyrrolidine dithiocarbamate), and A23187. On the other hand, a reduction of the concentration of cellular labile zinc can be achived by chelators. This can be done by either depleting the culture medium of zinc, or by direct intracellular chelation of labile zinc. Depletion can be achived by chelex treatment, or

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addition of DTPA (Diehylenetriaminepentaacetic acid), EGTA (Ethylene glicol-bis(2-aminoethylesther)-N,N,N',N' tetraacetic acid), EDTA (Ethylenediamineteraacetic acid), BAPTA (1,2-bis(2-aminophenoxy) ethane N,N N',N' tertaacetic acid). For the intracellular chelation of zinc, membrane permeable chelators like oPA (1,10-orthophenanthroline) and the frequently used TPEN (N,N,N',N'-Tetrakis(2-pyridylmethyl)ethylenediamine) can be used (Haase, 2007).

STIMULUS	FLUCTUATION	DETECTION METHOD	CELL TYPE
electrical stimulation	<0.1nM resting level to 1 nM after stimulation	Fura-2	rat cardiomyocytes
glucagon-cAMP	decrease in intracellular zinc	Zinquin	rat hepatocytes
nitric monoxide	increase in nuclear and cytosolic fluorescence	Zinquin, fluorescence microscopy, flow cytometry	L929 cell line, mouse splenocytes, rat endothelial cells
hypochlorus acid, selenite	from 0.2nM resting level to 7nM after stimulation	Fura-2	rabbit ventricular myocytes
nitric monoxide	vesicular fluorescence, total zinc-dependent fluorescence doubled	TSQ, microscopy, fluorescence spectrometry	C6 rat glioma cell line
IGF-1 serum withdrawal	increase in nuclear fluorescence	Zinquin	myoblast cell lines
2,2' dilhiodipyridine	intracellular zinc release	Newport Green, fluorescence imaging	rat neurons
PMA, H2O2	increase in cytoplasmatic fluorescence	TSQ, confocal microscopy	NIH 3T3
VP-16	increase in intracellular zinc	Zinquin	human lymphoma cell lines
cytokines	increase in nuclear fluorescence	Zinquin	murine aortic endothelial cells
NMDA, mitochandrial depolarization	1-3nM increase in labile zinc	FluoZin-3	rat cortical neurons
2,2' dilhiodipyridine	intracellular zinc release from MT	Mag-Fura-2	rat astocytes
PDGF, EGF, IGF-1	increase due to higher retention	^{65}Zn	3T3
PMA	intracellular labile zinc doubled	FluoZin-3, flow cytometry	primary human monocytes

Table I.2.6. Fluctuation of labile zinc in mammalian cells. From (Haase, 2007)

Given that zinc ion fluctuations occur at such low concentrations and zinc interacts strongly with proteins, released zinc ions are potent intracellular signals. Two models of zinc signaling have been proposed: 'late Zn signalling' and 'early Zn signalling' (Hirano et al., 2008). The

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latter is a rapid change in free cytoplasmic Zn levels, occurring in minutes, directly induced by an extracellular stimulus, as described in mast cells stimulated via the high affinity immunoglobin E receptor (Yamasaki et al., 2007). It is important to highlight that the source of zinc for this zinc wave was thought to originate from the ER, consistent with Hogstrand *et al*. (Hogstrand et al., 2009a) observation that siRNA to ZIP7, located on the ER, was able to prevent the zinc wave. Late Zn signaling is also triggered by extracellular stimuli but occurs in hours and involves transcriptional changes in plasma membrane zinc transporters, as described in dendritic cells stimulated with lipopolysaccharides (LPSs) (Kitamura et al., 2006; Murakami and Hirano, 2008) (Figure I.2.21). To sum, Zn acts as a conventional intracellular secondary messenger capable of transducing an extracellular stimulus into intracellular events.

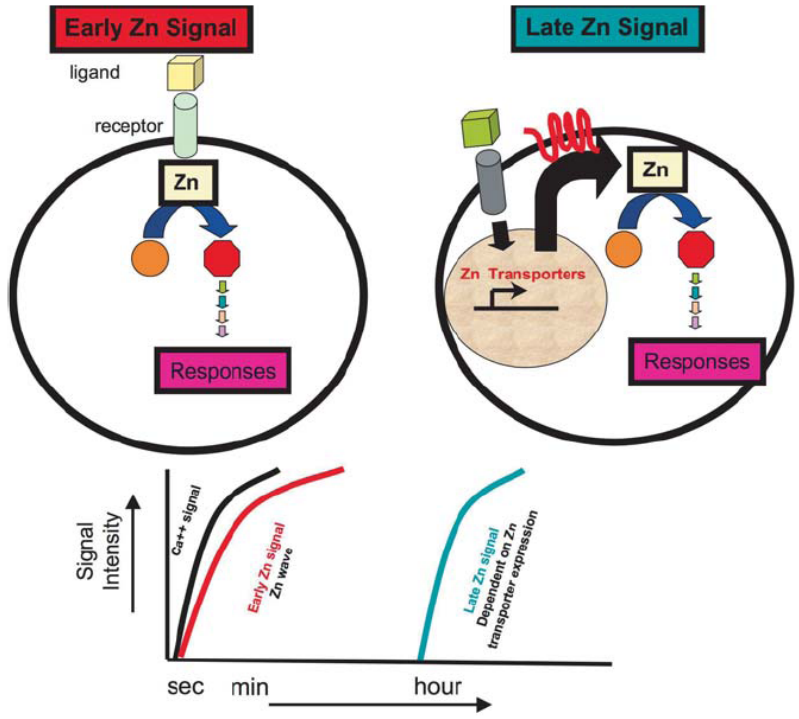

Figure I.2.21. Early and late zinc signalling. Intracellular zinc can be classified as early signalling, such as the Zn wave, which is directly induced by extracellular stimuli, and late signalling, which is dependent on transcriptional regulation of Zn transporter expression. From (Murakami and Hirano, 2008)

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Effects of zinc on major cellular signaling mechanisms:

Protein Kinases:

A common feature in the transduction of signals along signaling pathways is reversible protein phosphoryation, especially on tyrosine, serine and threonine residues. Their phosphorylation is governed by the balance of activities of kinases, enzymes that transfer the terminal phosphate group of the ATP to these amino acids, and phosphatases, which cleave it.

Zinc imitates the effects of insulin, the so-colled insulinomimmetic effect, activating the same signaling pathways, including tyrosine phosphorylation of the insulin receptor (IR) β subunit, PI3K and Akt (Tang and Shay, 2001; Jansen et al., 2009) Zinc may come from the extracellular milieu or be released from zinc-loaded MT upon oxidation (Maret, 2009) (Figure I.2.18) (Figure I.2.22). Hereby, zinc can induce tyrosine phosphorylation of the Irs activating tyrosine residues 1158, 1162, and 1163 and the adaptor protein IRS-1, and induces downstream activation of p21-activated kinase 1 and 2 phosphorylation. Zinc has also a dual effect on extracellular regulated kinase (ERK)-1/2 phosphorylation, activating at low concentrations, but inhibiting at higher ones. The IR, IRS-1 and both kinases were only affected when zinc was added together with pyrithione, indicating an intracellular site of action of zinc (Haase and Maret, 2003; Haase and Maret, 2005b; Haase and Maret, 2005a). After stimulation with insulin, membrane permeable zinc chelators were able to abrogate insulin induced signals, an indication that cellular zinc is involved in these pathways. So did addition of TPEN inhibit the activating phosphorylation of the IR (Haase and Maret, 2003) and zinc was also shown to be required for insulin stimulated phosphorylation of p70S6 kinase, since this was abrogated in the presence of oPA. The authors ascribed this effects to a direct activation of mTOR by zinc (Lynch CJ, 2001).

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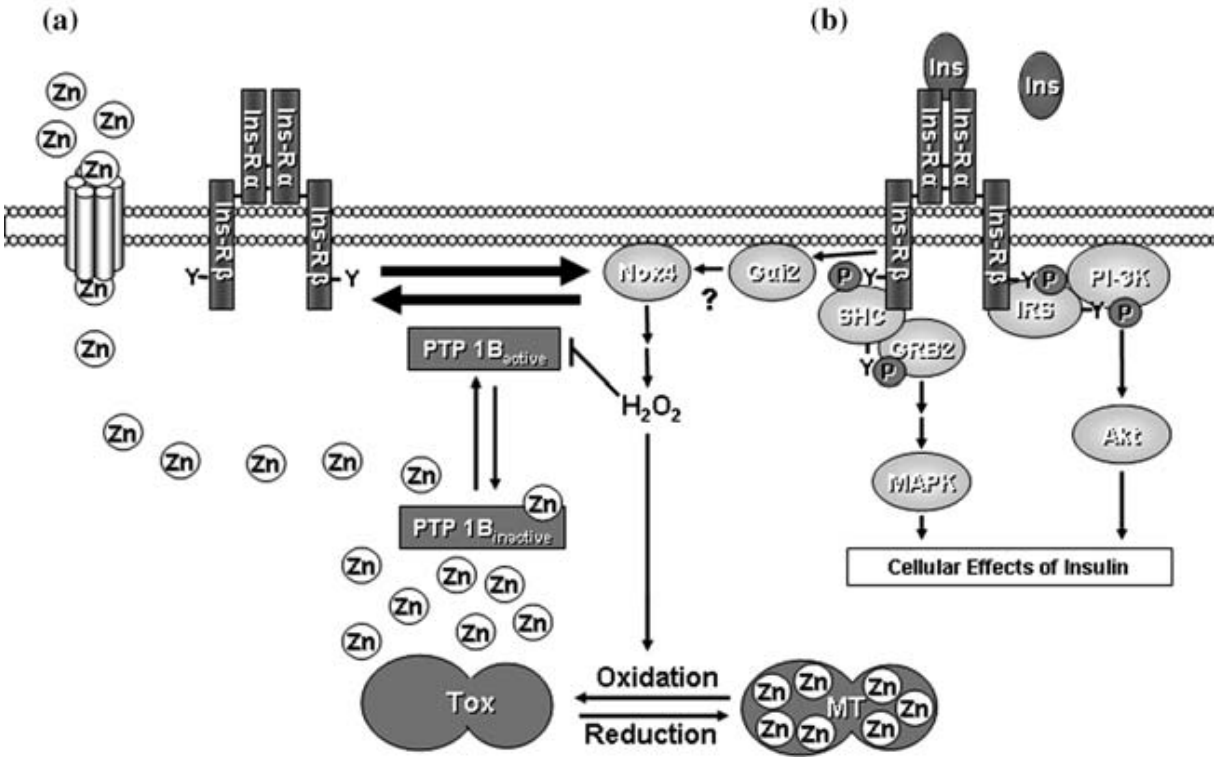

Figure I.2.22 Zinc and redox modulation of insulin phosphorylation signaling. (a) Extracellular zinc enters the cell and inhibits PTP 1B, thus increasing net phosphorylation of the insulin receptor and enhancing activation of downstream pathways such as MAP, PI-3 and Akt kinases. (b) Insulin binding to its receptor induces autophosphorylation, assembly of multiprotein complexes with adaptor proteins such as IRS and SHC/GRB2, and activation of insulin signaling cascades. Through a pathway involving a G-protein and the oxidase Nox4, insulin induces the production of hydrogen peroxide. The hydrogen peroxide can oxidize PTP 1B or release proteinbound zinc from proteins such as metallothionein (MT). In this process oxidized thionein (Tox) is formed. Both pathways inactivate PTPs and attenuate the insulin signal. Taken from (Haase and Maret, 2005b; Haase and Maret, 2005a).

Many reports describe an activation of kinases after zinc incubation, frequently involving the activation of MAP kinases, but also numerous of other kinases. Thus, Hansson *et al.* indicate that an incubation with zinc leads to tyrosine phosphorylation and ERK activation (Hansson, 1996). In mouse cortical cells, zinc activated ERK and hereby the tranacription factor Erg-1, an effect which was inhibited by an inhibitor of the mitogen-activated/extracellular signal-regulated kinase kinases (MEK), which act upstream of ERK (Park JA, 1999). Extracts of zinc- stimulated cells showed that zinc had induced kinase activity of PKC, and the MAPKs ERK, p38, and c-Jun N- termianl kinase (JNK) (LaRochelle et al., 2001). When

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oligodendrocytes were treated with peroxynitrite, activation of ERK-1/2, 12 lipooxygenase, and reactive oxygen species were aconsequence of zinc release, since all these effects were blocked by TPEN (Zhang et al., 2004). In neuroblastoma cells, zinc treatment also induce phopphorylation of p70S6 kinase, GSK-3β, and the MAP kinses ERK-1/2, JNK, and p38 via mTOR (An et al., 2005). Treatment of cultured cells with zinc resulted in a phosphorylation and activation of p70S6 kinase, which was mediated by PI3K, but was independent of calcium or PKC (Kim et al., 2000).

When periphereal blood mononuclear cells are incubated with zinc, this stimulates secretion of cytokines like IL-1β and TNFα. Studies with low molecular weight inhibitors showed that the zinc induced activation involved in protein tyrosine kinases and cyclic nucleotide dependent kinases (Wellinghausen et al., 1996). However, the effect of zinc on kinases is not only an activating one, zinc ions have also been shown to specifically inhibit IL-1 receptor associated kinase 1 (IRAK-1), abrogating subsequent signaling events, a mechanism by which zinc can inhibit the stimulation of T-cells by IL-1 (Wellinghausen et al., 1997). (Figure I.2.23)

Protein kinase C structure and activity. The PKC family of serine/threonine kinases consists of several different isoforms. The amino acid sequences of most isoforms of PKC contain two identical zinc-binding motifs at the N-terminus, the regulatory region of this enzyme (Parker PJ, 1986).

A regulatory function of zinc for PKC is inferred from the observation that nanomolar concentrations of zinc can activate PKC and cause a translocation to the plasma membrane, a central event in the activation of PKC (Csermely P, 1988a; Csermely P, 1988b). Zinc also seems to regulate the translocation of PKC to the cytoskeleton (Forbes IJ, 1990). Furthermore, it was shown that a chelatable pool of intracellular zinc increases the binding of the PKC activator phorbol dibutyrate (Forbes IJ, 1990) and that the zinc-binding cysteines are essential for phorbol ester binding (Ono Y, 1989).

It has been suggested that the above-mentioned four zinc-binding sites mediate the regulatory effects of zinc on PKC. But on the other hand, the zinc finger bound zinc could not

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even be removed by high affinity heavy metal ion chelators, making regulation by free zinc at these sites unlikely (Hubbard SR, 1991). Nevertheless, the chelators TPEN (Csermely P, 1988b) and 1,10-phenanatroline (Forbes IJ, 1990) were able to inhibit PKC activation. So the activation of PKC by zinc is mediated by a chelatable pool of zinc that is not identical with the metal ions bound to the zinc finger structures. Furthermore, zinc was found to modulate the autonomous activity of PKC, i.e., the activity in the absence of activating cofactors. The oxidation of the zinc-binding cysteine residues led to a release of zinc and to an increase of the autonomous PKC activity, but to a loss of sensitivity to regulating cofactors (Knapp LT, 2000). This indicates a possible involvement of the cellular redox state in PKC signaling mediated by the zinc finger structures. (Figure I.2.23)

Protein Phosphatases:

Zinc has been reported to be an inhibitor of protein tyrosine phosphatases (PTP). For instance, it is an inhibitor of specific phosphatases, like PTP 1B. It is a very potent inhibitor of PTP, with nanomolar inhibition constants for T cell PTP and PTP 1B (Haase and Maret, 2003; Haase and Maret, 2005b; Haase and Maret, 2005a). These constants indicate that labile intracellular zinc constitutively inhibits a part of PTP activity. This is supported by the finding that chelation of labile zinc interrupts insulin and IGF-1-induced tyrosine phosphorylation of the receptor and downstream signals (Haase and Maret, 2003; Haase and Maret, 2005a) indicating that cellular zinc really does inhibit a part of PTP under normal conditions, and that this inhibition is essential for signal transduction.

Expression of PTP is also regulated by zinc. Culture of osteoblastic cells in the presence of zinc increased PTP activity, an effect that was sensitive to protein synthesis inhibition, and zinc enhanced an IGF-1-induced rise in PTP activity (Yamaguchi and Fukagawa, 2005). The mechanism by which inhibits PTP enzymatic activity is still unresolved. Since the truncated form of ΔSH2/SHP-1, which consist only of the catalytic domain, was still inhibit by nanomolar concentrations of zinc, the inhibitory binding site is somewhere on this domain (Haase and Maret, 2003). The catalytic domain is highly conserved between the PTPs,

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indicating that zinc may affect a considerable number of these enzymes (Haase and Maret, 2005b; Haase and Maret, 2005a) (Figure I.2..22)(Figure I.2.23).

***Calcium signaling*:**

Zinc interferes with diferent aspects of calcium regulation. In some cells types, elevation of extracellular zinc evoked intracellular Ca^{+2} mobiliaztion. For instance, in primary hepatocyte cultures, 100 μM Zn(II) caused and increase in the intracellular free calcium concentration by stimulation of hormone sensitive intracellular zinc stores (McNulty TJ, 1999). For the calcium/calmodulin-dependent protein kinase-2 (CaMPK-2), opposite effects of low and elevates zinc concentrations were observed. Whereas low zinc concentrations resulted in an increase of calmodulin-independent activity, high levels of zinc inhibites the binding of Ca^{+2}-calmodulin and the activity of the kinase (Lengyel I, 2000). (Figure I.2.23)

***Cyclic nuclotide metabolism*:**

The second messengers, cyclic adenosine monophosphate (cAMP) and its guanosine analogue (cGMP), are synthesized by adenylate cyclases (ACs) or guanylate cyclases (GCs), respectively. Their degradation is mediated by cyclic nucleotide phosphodiesterases (PDEs). Zinc has been shown to affect these signal transduction pathways (cAMP, cGMP) by modulating PDE activities. Several isoforms of PDEs are activated at low zinc concentrations because they require a catalytic zinc ion, bound to two tandem histidine-containing amino acid sequences in the catalytic domain (Conti M, 2007). Conversely, slightly higher concentrations of zinc than those necessary for activation inhibit PDEs *in vitro* (Francis SH, 1994; Percival MD, 1997; Haase H, 2009) (Figure I.2.23).

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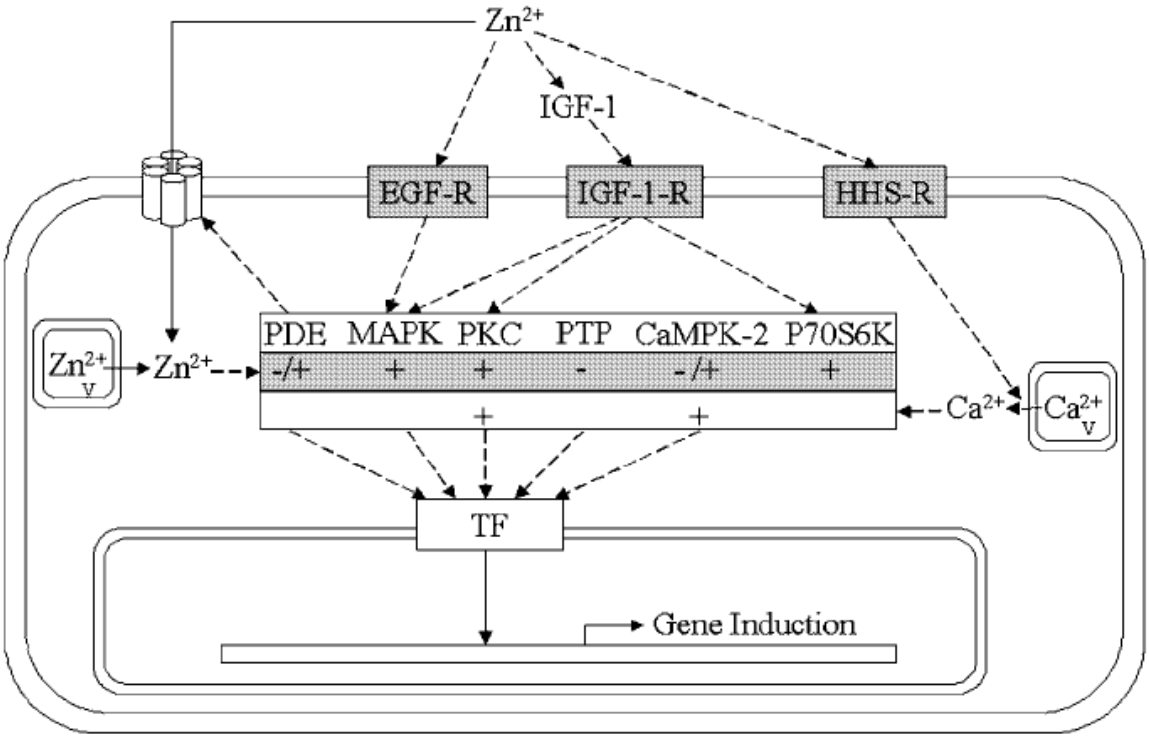

Figure I.2.23. Effects of zinc on signal transduction pathways. Extracellular zinc can increase the formation of insulin-like growth factor (IGF), and stimulate the epidermal growth factor-receptor (EGR-R). The activation of a hepatic heavy metal ion stimulated-receptor (HHS-R) causes the intracellular release of Ca^{2+} in hepatocytes. At the level of protein phosphorylation, Zn^{2+} taken up and/or Zn^{2+} released from zincosomes can modulate the activity of cyclic nucleotide phosphodiesterase (PDE), mitogen-activated protein kinase (MAPK), protein kinase C (PKC), protein tyrosine phosphatases (PTP), Ca2+-calmodulin activated protein kinase-2 (CaMPK-2), and P70S6 kinase (P70S6K). Activation of protein kinases or phosphatases leads to changes in the phosphorylation state of transcription factors (TF) and gene activities. Activating and inhibitory interactions are represented by + and −, respectively. Index V: vesicular localization. From (Beyersmann and Haase, 2001).

Zinc in cell proliferation, differentiation and apoptosis:

Biochemical mechanisms for the function of zinc in **cell proliferation** were detected when zinc was shown to be a structural element in enzymes involved in DNA synthesis (Springgate CF, 1973; Chesters JK, 1989), transcription (Wu W, 1999), aminoacyltRNA synthesis (Hicks SE, 1987) and ribosomal function (Härd T, 2000). Furthermore, zinc is present in the zinc finger structures of transcription factors that control the activity of genes responding to growth factors (Berg JM, 1996). Zinc is not only a structural element but is also involved in regulatory mechanisms of cell proliferation. Based on observations that serum addition to

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mammalian cell cultures enhanced the cellular uptake of zinc, and that zinc deprivation by metal chelators caused decreased growth and DNA synthesis, zinc was proposed to be a second messenger of mitogenic signaling (Grummt F, 1986).

A novel role for zinc mediated by MT was found in the process of **differentiation** of 3T3L1 preadipocytes (Schmidt and Beyersmann, 1999). After stimulation of differentiation by insulin and dexamethasone, these cells enter into a phase of rapid proliferation with a concomitant rise in cellular zinc and MT contents. Simultaneously MT is translocated from the cytoplasm into the nucleus. Upon entry of the cells into the subsequent actual differentiation, the elevated levels of zinc and MT return to the initial amounts, and a redistribution of MT to the cytoplasm occurs. Similar changes in subcellular localization of zinc and MT were also observed in the course of differentiation of two myoblast cell lines to myotubes (Apostolova et al., 1999; Apostolova et al., 2000).

Zinc regulates the activity of enzymes in the **apoptotic cascade**. The calcium-dependent endonuclease that mediates DNA fragmentation is inhibited by zinc (Duke RC, 1983). However, this target is beyond the point of no return for programmed cell death, and an inhibition could explain a suppression of DNA fragmentation during apoptosis, but not the effect on cellular survival. Another important group of enzymes in apoptosis are cysteine-aspartic acid proteases (caspases), which form a cascade to transduce initial apoptotic signals to the effector enzymes that mediate the organized destruction of cells characteristic for programmed cell death. In this process, inactive procaspases are activated by proteolytic cleavage. An additional regulatory mechanism is indicated by inhibition of caspases-3, -6, -7, and -8 by low micromolar zinc concentrations (Perry DK, 1997).

While in general zinc depletion is regarded as an inducing factor for apoptosis, excessive zinc supplementation, leading to excess of intracellular labile zinc, has also been shown to induce apoptosis (Figure I.2.24), in some instances even in the same experimental system and in the absence of any additional apoptotic stimuli (Haase et al., 2001; Watjen et al., 2002; Chimienti et al., 2003; Mann and Fraker, 2005; Makhov et al., 2008) and zinc quelators producing either sequestration of the metal or acting as ionofors, and thereby decreasing or

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increasing, respectively the pool of intracellular labil zinc, are now recognized as potential anticancer agents (Ding and Lind, 2009).

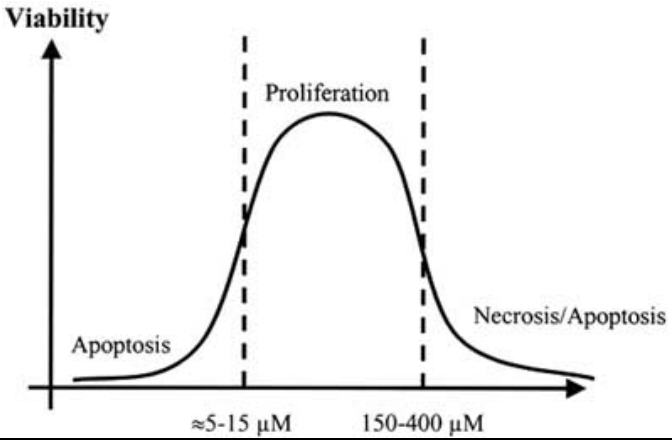

Figure I.2.24. Relationship between extracellular zinc concentration and cell fate. From (Chimienti et al., 2003)

CELLULAR ZINC HOMEOSTASIS: TRAFFICKING, STORAGE AND BUFFERING

The complexity and importance of zinc homeostasis is reflected by the large number of proteins that are dedicated to zinc transport, storage and buffering. At least several dozen human genes code for cellular proteins involved in this control (see Table I.2.7 and Figures I.2.27, 1.2.28). They include membrane transporters (exporters and importers from the ZnT (SLC30) and ZIP (SLC39) families) that transport Zn through the cytoplasmic and intracellular membranes (Kambe et al., 2004; Liuzzi and Cousins, 2004; Cousins et al., 2006; Eide, 2006; Sekler et al., 2007; Mocchegiani et al., 2008; Hogstrand et al., 2009b; Lichten and Cousins, 2009), zinc sensors, such as metal response element (MRE)-binding transcription factor-1 (MTF-1) (Andrews, 2001; Lichtlen and Schaffner, 2001; Laity and Andrews, 2007), glutathione, and a family of at least 10 functional human metallothionein proteins (see Table 1.2.7).

Zinc transporters

Two protein families of mammalian zinc transporters exist, the ZnT (SLC30) and the ZIP (SLC39) families (Figures I.2.25, I.2.26, I.2.27 and I.2.28) (Table 1.2.7).

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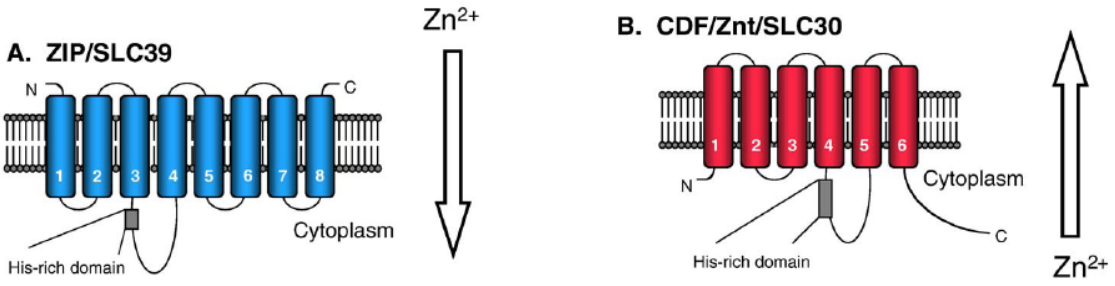

Figure I.2.25. Predicted membrane topologies of the ZIP/SLC39 and the CDF/ZnT/SLC30 families of mammalian Zn2+ transporters. The transmembrane domains are numbered 1, 2, etc. Figure taken from (Eide, 2006).

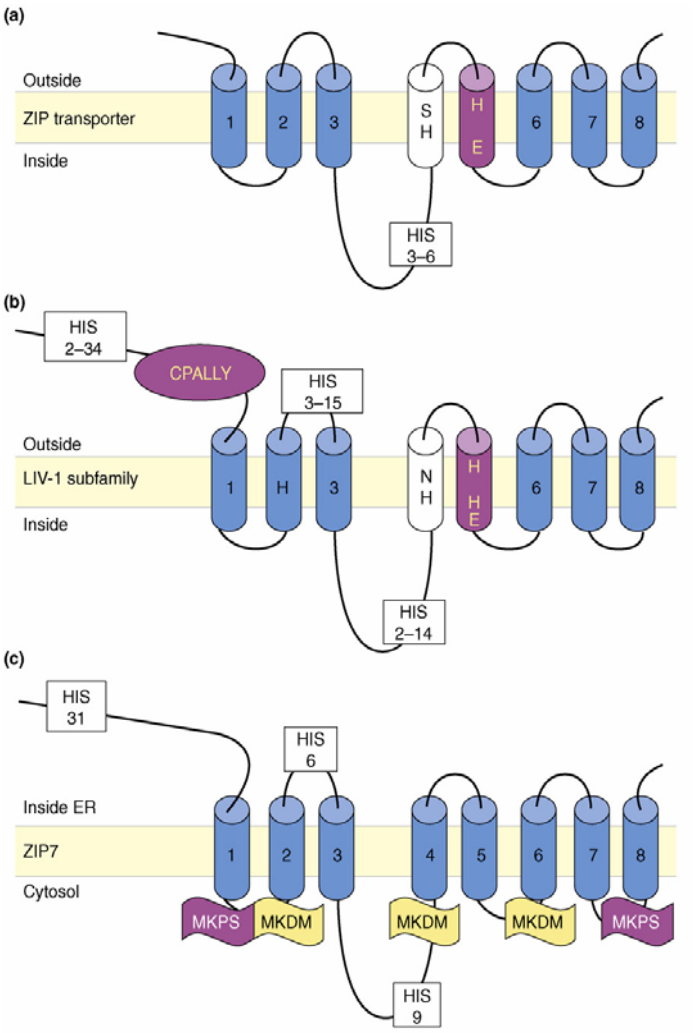

Figure I.2.26. Schematic of ZIP transporters. (a) The 8 transmembrane (TM) structure of the ZIP family of transporters containing a ZIP consensus sequence in TM4 (white barrel) and conserved His residues in TM4 and TM5 (pink barrel). The histidine-rich motif (white box) contains between 3 and 6 histidines. **(b)** The LIV-1 subfamily contains additional motifs such as the conserved HEXPHE motif unique to the LIV-1 family in TM5, with the initial histidine matching the conserved ZIP histidine. They also have 3 areas of histidine-rich motifs with more histidine residues positioned on both sides of the membrane, as well as an N-terminal CPALLY motif. **(c)** ZIP7 is present on the endoplasmic reticulum (ER) membrane with histidine residues (white box) and no CPALLY motif. ZIP7 contains 2 cytosolic MAPK phosphorylation sites (MKPSs) and 3 cytosolic MAPK docking motifs (MKDMs), suggesting MAPK binding. From (Hogstrand et al., 2009b).

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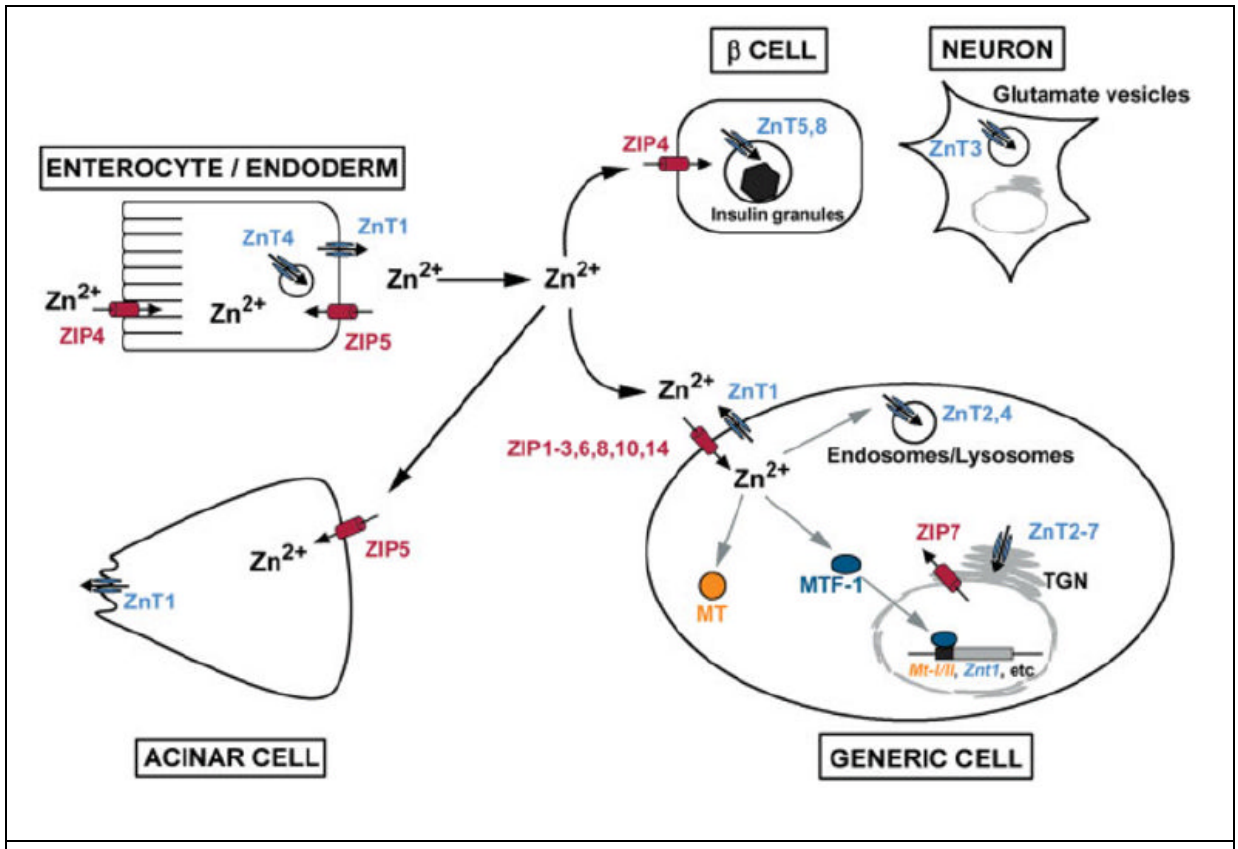

Figure I.2.27. Overview of zinc homeostasis. Twenty four different genes encode proteins that are involved in the uptake (*Zip* genes) or efflux (*ZnT* genes) of this metal in a cell-specific, developmentally regulated, and zinc-regulated manner. The functions of many of these genes remain to be determined. ZIP4 plays a critical role in the absorption of dietary zinc by ENTEROCYTE/ ENDODERM cells when zinc is limiting, but other transporters must also play important roles. Zinc is thought to be exported into portal blood or into the conceptus by ZnT1 localized on the basolateral membrane. Other ZnT proteins (i.e. ZnT4) also likely play a role in this process. ZIP5 is localized to the basolateral membranes of enterocytes, endoderm cells and pancreatic acinar cells where it may serve to remove zinc from the blood when zinc is replete. In peripheral tissues (GENERIC CELL), zinc is probably taken up by various ZIP transporters localized on the plasma membrane. To date ZIPs1, 2, 3, 6, 8, 10, and 14 have each been shown to have zinc transport activity in transfection or oocyte injection studies, and most show tissue-specific patterns of expression. Inside the cell, free zinc levels are kept low and zinc can be bound to MT or transported into secretory vesicles, endosomes/lysosomes or zincosomes by ZnT2 and ZnT4. Zinc activates the zinc-sensing transcription factor MTF-1 which regulates transcription of the mouse *Mt-I/II* and *Znt1* genes and represses expression of *Zip10* in an effort to control excess zinc. ZnTs2–7 participate in the delivery of zinc into the secretory pathway, whereas ZIP7 may transport zinc out of the Golgi apparatus into the cytoplasm. ZnT3 transports zinc into glutamate containing vesicles in the brain whereas ZnT8 transports zinc into pancreatic β-cell insulin secretory granules. ZIP4 is expressed in β-cells, but its localization in those cells has not been reported. Taken from: (Kambe et al., 2008).

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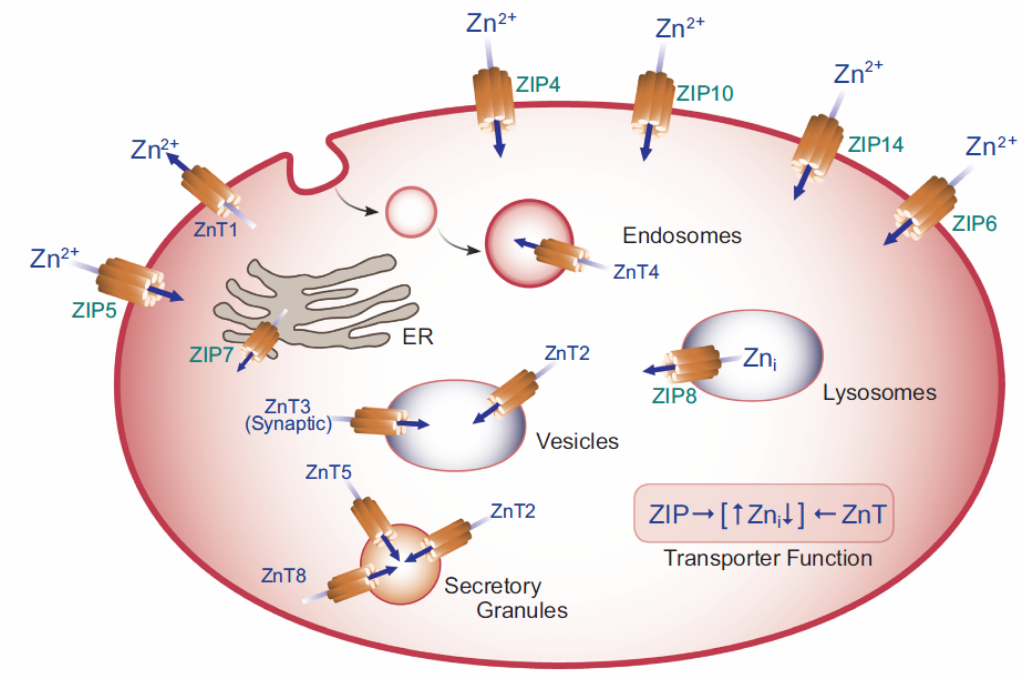

Figure I.2.28. Generalized cell showing locations of some key zinc transporter proteins. The six and eight trans-membrane domains for the majority of ZnT and ZIP proteins, respectively, are shown. Restricted localization specific to the plasma membrane or specific intracellular organelles has yet to be established for other members of both protein families. As shown, the function of the Znt and ZIP transporter families is to reduce and increase the cytoplasmic zinc concentrations, respectively. Such diverse distribution of these proteins suggests individual roles in executing the catalytic, structural, and regulatory roles of zinc. ER, endoplasmic reticulum. From (Lichten and Cousins, 2009).

The **ZnT family** of transporters acts to decrease cytoplasmic zinc levels by transporting zinc from the cytoplasm to the lumen of organelles or the extracellular space. Most members of this family have six predicted transmembrane domains (Figure I.2.25). Notable exceptions to this rule are the Msc2 and Znt5 proteins that form heteromeric complexes to transport zinc into compartments of the secretory pathway. Plasma membrane efflux transporters mediate the efflux of intracellular zinc to prevent the cellular over accumulation of the metal ion thereby saving the cell from the toxic consequences of zinc overload.

Zinc transporters of the **ZIP (Zrt-, Irt-like protein) family (**Figure I.2.25 and I.2.26), are responsible for increasing cytoplasmic zinc levels by transporting the metal either from the extracellular space or from organellar lumen into the cytoplasm. Thus, ZIP proteins work in

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opposition to the ZnT transporters (Liuzzi and Cousins, 2004; Cousins et al., 2006; Eide, 2006).

Zinc entering the cell was associated in the cytoplasm with a ‘muffler’ with a high affinity for zinc, and this muffler was perhaps composed of sulfur-rich molecules, such as metallothionein, that allowed zinc to be strongly buffered. Subsequently, the buffer-associated zinc would be transferred into a deep cellular store, such as the ER compartment, before release to the cytosol. Because of its location in the ER and ability to raise the cytosolic concentration of labile zinc (Taylor et al., 2004), ZIP7 is a strong candidate for being the entity that mediates this zinc release. The lumen of the ER is the site for the initial folding and post-translational modification of proteins destined for secretion or residency within secretory pathway, and many of these processes are zinc-dependent. For instance, glycosylphosphatidylinositol phosphoethanolamine transferases (GPIPETs) in the endoplasmic reticulum (Galperin MY, 2001). The Golgi also contains zinc-dependent resident proteins. For example, cleavage of the Alzheimer's disease Aβ protein from the amyloid precursor protein in the Golgi depends on resident zinc metalloproteases in that compartment (Mok SS, 1997). The packaging of insulin in secretory granules relies on zinc. Insulin is packaged in a crystalline structure within these vesicles with a 2:1 Zn:insulin stoichiometry (Dodson G, 1988). The hexamerization of proinsulin that occurs in the Golgi, an early step in forming the Zn:insulin crystal, is zinc-dependent as well (Huang XF, 1995). Zinc may also be exported from the Golgi. Also, ZIP7 protein localizes to the Golgi and appears to mediate transport of secretory pathway zinc back to the cytoplasm (Huang L, 2005). This may be a means to recover unused zinc in the secretory pathway prior to its loss by secretion. In addition, many mammalian cell types also contain membrane-bound vesicular structures, called zincosomes. These vesicles sequester high amounts of zinc and release it upon stimulation, e.g., with growth factors (Eide, 2006; Taylor et al., 2008)(Figure I.2.29).

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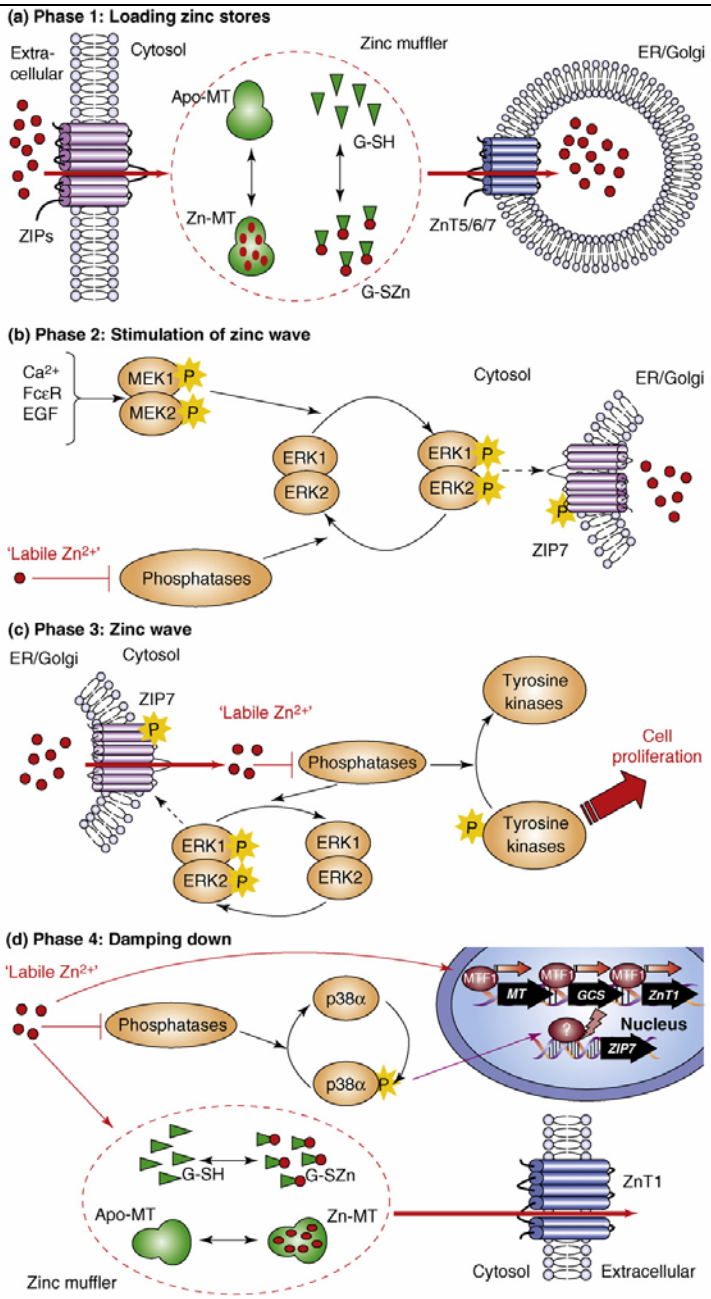

Figure I.2.29. Model for ZIP7-mediated zinc signalling in tyrosine kinase pathways. Extracellular zinc is transported into the cytosol through plasma membrane ZIP zinc transporters. Zinc is transferred directly to a 'muffler', where it is bound to metal free apo-metallothionein (apo-MT) and protonated glutathione (G-SH) to generate Zn-MT and G-SZn, respectively. From the 'muffler' it is compartmentalized into an intracellular zinc store, the ER and/or the Golgi via various ZnT zinc transporters **(a)**. Release of zinc from the ER and/or Golgi back into the cytosol is mediated by ZIP7, resulting in a cytosolic Zn^{2+} wave. ZIP7 might be stimulated to release zinc through phosphorylation by MAPKs, such as ERK1/2, but is flooded when the ER and/or Golgi is overloaded with zinc, as for example when cellular zinc influx is high. Release of zinc can be stimulated by extracellular triggers, such as epidermal growth factor (EGF), or in mast cells via FceR, and involves an intracellular Ca^{2+} transient, leading to phosphorylation of mitogen-activated protein kinase kinase 1 and 2 (MEK1/2), which in turn phorphorylates ERK1/2. Labile Zn2+ might lead to increased phosphorylation of ERK1/2 and result in an early positive feedback on ZIP7-mediated zinc release **(b)**. The cytosolic Zn^{2+} wave causes inhibition of phosphatases, resulting in a shift of MAPKs and tyrosine kinases to their phosphorylated and active states **(c)**. Taken from (Hogstrand et al., 2009b).

UNIVERSITAT ROVIRA I VIRGILI
EFFECTS OF DIETARY CATECHINS AND PROANTHOCYANIDINS ON ZINC HOMEOSTASIS IN HEPATIC CELLS
Isabel Maria Quesada
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Table I.2.7. The intracellular Zn homeostatic machinery. Data compiled from the following reviews: (Kambe et al., 2004; Liuzzi and Cousins, 2004; Cousins et al., 2006; Eide, 2006; Devirgiliis et al., 2007; Sekler et al., 2007; Kambe et al., 2008; Mocchegiani et al., 2008; Murakami and Hirano, 2008; Hogstrand et al., 2009b; Lichten and Cousins, 2009). (Continued on next pages).

Protein name (HUGO name)	Tissue Expression Pattern	Cellular Distribution	Molecular Function	Disease by Mutation	Response to Dietary/Extracellular Zinc Deficiency (ZnD) or Excess (ZnE)	Hormonal and Cytokine regulation of expressioN	Phosphorylation residue
Zinc transporters							
ZnT family							
ZnT-1 (SLC30A1)	Ubiquitous	Plasma membrane	Removal of cytoplasmic Zn	Alzheimer's disease, lung tumours KO: embryonic lethal	ZnD (−) Ubiquitously ZnE (+) Ubiquitously Activated by MTF-1		S506
ZnT-2 (SLC30A2)	Small intestine, kidney, pancreas, testis, seminal accumulation vesicles, mammary gland, epithelial cells	Vesicles, lysosomes	Enhance Zn accumulation in lysosomal and vesicular compartments	Self-limiting acrodermatitis enteropathica: produce zinc-deficient milk in women. KO cells: lower zinc tolerance	ZnD (−) in small intestine, liver, pancreatic acinar cells, and kidney ZnE (+) in Mammary gland	Glucorticoid hormone (+) in Pancreatic acinar cells Prolactin (+) in Mammary gland and Prostate	
ZnT-3 (SLC30A3)	Brain, testis	Synaptic and other vesicles	Accumulation of Zn in synaptic vesicles; possibly involved in reproductive activities, in testis	Alzheimer's disease, responsible for ZEN (zinc-enriched neurons) in Cornu Ammonis 1 area of the brain. KO: Prone to seizures elicited by kainic acid treatment			S38
ZnT-4 (SLC30A4)	Mammary gland, brain, small intestine, mast cells, placenta, blood, epithelial cells	Endosomes, Golgi	Transport of Zn into milk and in the vesicles of mast cells	Alzheimer's disease, lung tumours *lethal milk* syndrome locus (mouse) produce zinc-deficient milk	ZnD (−) in Small intestine	Immune activation (+) in T cells Cell differentiation (+) in Intestinal epithelium	
ZnT-5 (SLC30A5)	Pancreatic β-cells, intestine, heart, brain, liver, kidney, blood, epithelial cells	Insulin secretory vesicles, Golgi. Variants are expressed in plasma membrane complexed with ZnT-6	Transport of Zn into the lumens of the Golgi and the vesicular compartments. Znt-5 variant b is a bidirectional Zn transporter and mediates Zn uptake.	Activates tissue specific alkaline phosphatases. KO: Poor growth; osteopenia; low bodyfat; muscle weakness; male-specific cardiac death	ZnD (−)? in Small intestine ZnD (+) in HeLa cells (Devergnas et al., 2004)	IL-6 (+) in Liver cells	

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Table I.2.7 . The intracellular Zn homeostatic machinery (continued)

ZnT-6 (SLC30A6)	Small intestine, brain, liver, blood, adipose tissue	Golgi. Expressed in plasma membrana complexed with ZnT5 variant b	Transport of Zn into the lumens of the Golgi and the vesicular compartments.	Alzheimer's disease	ZnD (−) Small intestine ZnE (+) Small intestine		S382
ZnT-7 (SLC30A7)	Small intestine, liver, retina, spleen, blood, epithelial cells	Golgi	Facilitates Zn transport from the cytoplasm into the Golgi apparatus	Activates tissue specific alkaline phosphatase	ZnD (extracellular) (+) in HeLa cells (Devergnas et al., 2004)		
ZnT-8 (SLC30A8)	Pancreatic beta-cells	Insulin secretory vesicles (insulin granules)	Transports Zn within insulin secretory vesicles	Diabetes mellitus			
ZnT-9 (SLC30A9)	Ubiquitous	Cytoplasm and nucleus	Unknown				
ZIP family							
ZIP1 (SLC39A1) Subfamily II	Ubiquitous	Plasma membrane	Uptake of Zn from the outside to the cytosol of the cell	Reduced expression in prostate cancer KO: Abnormal morphogenesis of the embryo in zinc-limiting condition		Cell differentiation (+) in Osteoblasts Prolactin (+) in Prostate cells Testosterone (+) in Prostate cells IL-6 (+) in Liver cells	
ZIP2 (SLC39A2) Subfamily II	Subsets of immature dendritic cells, pericentral hepatocytes, developing keratinocytes, prostate glands, blood, uterus, cervical epithelium, optic nerve, monocytes	Plasma membrane	Uptake of Zn from the outside to the cytosol of the cell	Reduced expression in prostate cancer KO: Abnormal morphogenesis of the embryo in zinc-limiting condition			
ZIP3 (SLC39A3) Subfamily II	Widespread; more expressed in mammary epithelial cells, prostate epithelial cells, blood	Surface plasma membrane, transiently relocalized to intracellular compartments	Uptake of Zn from the outside to the cytosol of the cell	Reduced expression in prostate cancer KO: Abnormal morphogenesis of the embryo and depletion of thymic pre-T cells in zinc-limiting condition	ZnE (-) Blood cells	Prolactin (+) in Mammary cells	S125, S129
ZIP4 (SLC39A4) LIV-1 subfamily	Small intestine, stomach, colon, cecum, kidney, pancreatic beta-cells.	Apical plasma membranes	Uptake of Zn from the outside to the cytosol of the cells	Acrodermatitis Enteropathica; severe zinc deficiency. KO: embryonically lethal.	ZnD (+) Small intestine, colon		
ZIP5 (SLC39A5) LIV-1 subfamily	Kidney, liver, spleen, colon, stomach, pancreatic acinar-cells	Basolateral membranes of polarized cells	Cellular Zn uptake and Zn removal from the body (serosal-to-mucosal Zn transport) (Dufner-Beattie et al., 2004)			IL-6(+) in Liver cells	

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Table I.2.7 . The intracellular Zn homeostatic machinery (continued).

ZIP6 (SLC39A6) LIV-1 LIV-1 subfamily	Ubiquitous	Plasma membrane	Zn uptake	Breast cancer.		IL-6/IL-1 (+) in Liver cells Lipopolysaccharide (+) in Dendritic cells	S472, Y522
ZIP7 (SLC39A7) LIV-1 subfamily	Ubiquitous	Endoplasmic reticulum and Golgi	Zn into cytosol	Tamoxifen resistance in Breast cancer. Migration, gastrulation			S275, S276
ZIP8 (SLC39A8) LIV-1 subfamily	Ubiquitous	Vesicles, plasma membrane, mitochondria	Zinc, cadmium and manganese uptake/influx	Faslodex resistance in Breast cancer. Cd testicular susceptibility locus.		Lipopolysaccharide (+) in Monocytes Immune activation (+) in T cells TNFα (+) in Lung epithelial cells	T424
ZIP9 (SLC39A9) Subfamily I	Ubiquitous	Unknown	Unknown				
ZIP10 (SLC39A10) LIV-1 subfamily	Ubiquitous	Plasma membrane	Zink uptake	Breast cancer	ZnD (+) Brain, liver, erythroid progenitor cells Repressed by MTF-1	Thyroid hormone (+) Intestine and Kidney cells	S546, Thr553, S574, S591
ZIP11 (SLC39A11) GufA	Ubiquitous	Unknown	Unknown				
ZIP12 (SLC39A12) LIV-1 subfamily	Brain, lung, testis, retina	Plasma membrane	Zink uptake	Asthma. SNP linked to schizophrenia			
ZIP13 (SLC39A13) LIV-1 subfamily	Ubiquitous	Golgi	Zn into cytosol	Ehlers-Danlos syndrome			
ZIP14 (SLC39A14) LIV-1 subfamily	Ubiquitous	Plasma membrane	Zn influx, non-transferrin-bound iron uptake	Asthma. IL-6-mediated inflammation.		IL-6/IL-1 (+) in Liver cells Nitric oxide (+) in Liver cells Expressed during adipocyte differentiation	

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Table I.2.7 . The intracellular Zn homeostatic machinery (continued).

Intracellular Zinc Storage and Distribution							
Metallothionein-1 (MT-1)	Ubiquitous	Cytoplasm, nucleus, intermembrane space of mitochondria	Buffering of cytoplasmic Zn, distribution of Zn to other proteins, transfer of Zn from cytoplasm to mitochondria	MT-1, -2 KO: sensitive to heavy metal overload. Obese in adult live. (REF) Dysfunction of macrophages (Sugiura et al., 2004)	ZnD (−) Ubiquitously ZnE (+) Ubiquitously Activated by MTF-1		
Metallothionein-2 (MT-2)	Ubiquitous	Cytoplasm, nucleus, intermembrane space of mitochondria	Buffering of cytoplasmic Zn, distribution of Zn to other proteins, transfer of Zn from cytoplasm to mitochondria	MT-1, -2 KO: sensitive to heavy metal overload. Obese in adult live. (REF) Dysfunction of macrophages (Sugiura et al., 2004) MT-2 SNP (promoter):	ZnD (−) Ubiquitously ZnE (+) Ubiquitously Activated by MTF-1		
Metallothionein-3 (MT-3)	Brain, testis	Cytoplasm, nucleus	Buffering of cytoplasmic Zn, distribution of Zn to other proteins, vesicular trafficking				
Metallothionein-4 (MT-4)	Squamous epithelia	Cytoplasm, nucleus	Buffering of cytoplasmic Zn, distribution of Zn to other proteins, regulation of Zn and copper metabolism				
Glutathione	Ubiquitous	Cytoplasm	Detoxification Zinc buffering				
Glutathione synthase	Ubiquitous		Glutathione biosynthesis		Activated by MTF-1		
Gamma glutaril cytsteine ligase	Ubiquitous		Glutathione biosynthesis		Activated by MTF-1		
Zinc Homeostasis Regulatory Transcriptions Factors							
Metal-responsive Transcription Factor 1 (MTF-1)	Ubiquitous	Cycles between cytoplasm and nucleus	Zinc Binding; Transcriptional activity; Binding to Metal Response Elements	KO: embryonic lethal			PI3K and PKC dependent phosphorilation of S residues.
Extracellular Zinc Distribution							
Albumin	Liver	Plasma	Reversible Zinc Binding				
A2-macroglobuiin	Liver	Plasma	Estructural zinc				

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ZINC METABOLISM

Zinc absorption and body distribution

The trace element zinc is an essential micronutrient and a novel dietary supplement, and serves a wide range of biological functions in human and animal health. Zinc is released from food as free ions during digestion and bound to secreted ligands before transporting into the enterocytes in the duodenum and jejunum (Blanchard RK, 1996). The absorbed zinc will then be carried directly to the circulation system for delivery to the respective tissues. The total zinc content in plasma is usually 100 μg zinc/100 mL varying as a function of age, sex, pregnancy, and time of day. Specific transport proteins facilitate the passage of zinc across the cell membrane into the portal circulation: 30-40% of plasma zinc is a tightly linked component of α2-macroglobulin, and most of the remainder is loosely bound to serum albumin. A small fraction is chelated to serum free amino acids or small peptides (Vallee and Falchuk, 1993). In α2-macroglobulin fraction, the metal is tightly bound to this protein and does not exchange with ^{65}Zn. This zinc is necesary to enable the protein to mantain the esterolytic activity of trypsin in the presence of soybean trypsin inhibitor. A large portion of zinc is initially taken up by liver, with ligands such as metallothionein, followed by bone marrow, bone, skin, kidney, and thymus, respectively (Dunn MA, 1987; Huber KL, 1988). This suggest that the liver may be central to zinc transfer and distribution and that liver metallothioneins provide the mechanism of homeostatic control which governs the amount of zinc that can reach the bloodstream (Vallee and Falchuk, 1993). At the hepatic level, glucocorticoids, insulin and glucagon produce transient dysregulation of zinc metabolism, which produces a decrease in plasma zinc concentrations. Similarly, immune-regulatory peptides, including interleukins 1 and 6, produce tissue-specific changes in zinc metabolism (Vallee and Falchuk, 1993; Blanchard and Cousins, 1996). All of these hormone effects are believed to be associated with an increase in zinc uptake by liver, concomitant with the uptake of aminoacids, iron and other metal ions (Vallee and Falchuk, 1993).

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Half of all zinc is eliminated from the body through the gastrointestinal tract. Considerable amount of zinc is secreted through the biliary and intestinal secretions, but most of it is reabsorbed and this process is an important point of regulation of zinc balance. Other routes of zinc excretion include the urine and surface losses through skin, hair and sweat. The amount required to replenish these losses and mantain the system in balance is obtained by dietary intake (Cousins and McMahon, 2000).

Recommended dietary allowance (RDA) for zinc

	RDA (mg/day)
Infants	5
Children 1–10 years old	10
Males > 10 years old	15
Females > 10 years old	12
Pregnant Woman	15
Lactating Women in 1st Trimester	19
Lactating Women in 2nd Trimester	16

Table I.2.8. Recommended dietary allowance (RDA) for zinc. From (Chien et al., 2006).

The absorption of zinc ions from the intestinal lumen into mucosal cells is controlled by a solubility, zinc-binding ligands and dietary interactions. The dietary components have a substantial impact on the absorption of zinc. Substances that influence intestinal permeability to zinc affect bioavailability. Also, other dietary substances can influence the bioavailability of zinc, either by chelating or binding zinc or by competitively inhibiting its absorption. Thus, phytate and calcium inhibit zinc absorption (Vallee and Falchuk, 1993; Oberleas, 1996; Lonnerdal, 2000). Phytate is a strong chelator of minerals and can not be digested or absorbed. Minerals, including zinc, that are bound to phytate also pass through the intestines unabsorbed. Calcium may form an insoluble calcium-zinc-phytate complex in the intestinal tract which inhibits zinc absorption (Oberleas, 1996). Also, calcium and dietary non-heme iron are two inorganic elements that interfere with zinc uptake by direct competition. Polyphenolic substances (tannins) in tea and coffee and oxalic acid of green leafy herbs have been show to inhibit zinc absortion (Sreenivasulu et al., 2008). On the other hand, a series of compounds have been suggested as enhancers or promoters of zinc absorption, including amino acids, sugars, picolinic acid, citric acid, and prostaglandins. Only the first two

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classes of compound are likely to be of physiological significance (Vallee and Falchuk, 1993; Oberleas, 1996; Lonnerdal, 2000).

Zinc metabolism and diseases.

Whereas at the cellular level zinc it is critically involved in proliferation, differentiation, apoptosis, and signal transduction (see above), at the organism level, zinc functions include immunity, control of glucose and lipid metabolisms, reproduction, vision, taste, cognition behaviour, neurogenesis, synaptogenesis, neuronal growth, and neurotransmission (Figure I.2.30) (Chien et al., 2006).

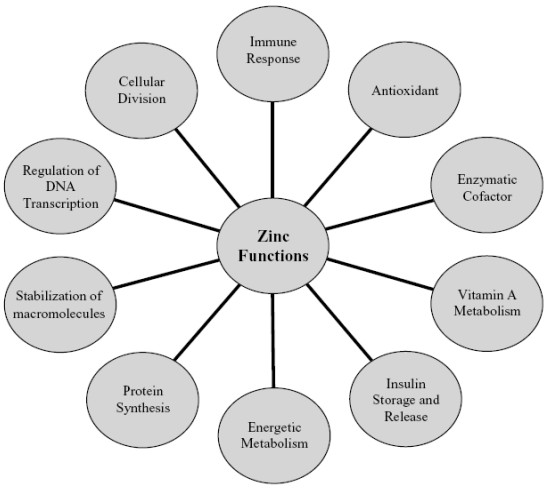

Figure I.2.30. Role of zinc in cellular and biochemical events. From (Chien et al., 2006)

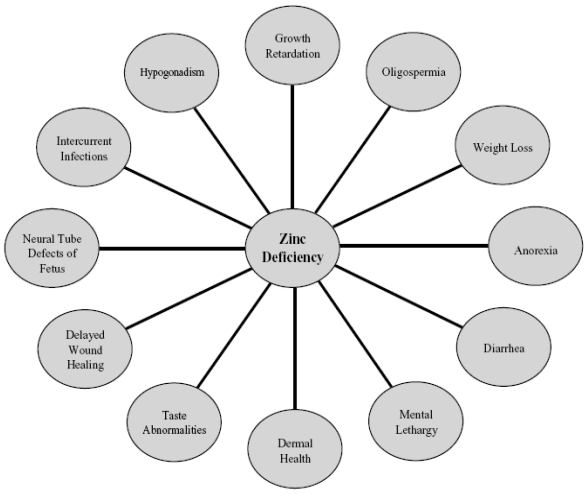

Figure I.2.31. Consequences of dietary zinc deficiency. From (Chien et al., 2006)

Ddisturbances on zinc homeostasis are associated with several kinds of diseases, such as diabetes, atherosclerosis, Alzheimer's dementia, asthma, obesity, cancer and age related immune dysfunctions (Costello and Franklin, 2006; Devirgiliis et al., 2007; Smidt et al., 2007; Anzellotti AI, 2008; Mocchegiani et al., 2008; Taylor, 2008; Ding and Lind, 2009). Several conditions can produce a pathophysiological metabolism of zinc. When the supply of dietary zinc is insufficient to support their functions, biochemical abnormalities and clinical signs may develop. Thus, **zinc deficiency** leads to impairments of growth, dermal, gastrointestinal, neurologic and immunologic systems (Tapiero and Tew, 2003; Chien et al., 2006; Stefanidou

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et al., 2006) (Figure I.2.31). In addition, the lack of this essential nutrient causing a sustained deficiency syndrome can also secondarily alter hormone secretion and action and thereby affect gene expression directly and indirectly in the most complex ways. The zinc status in mammals is known to affect growth and this is mediated at least in part through the somatotropic axis with alterations in the level of circulating insulin-like growth factor I (IGF-I) (McNall AD, 1995; Ninh NX, 1995; Ninh NX, 1998). Correspondingly, zinc deficient rats show reduced expression of hepatic growth hormone receptor (GHR), the GHR binding protein (GHRBP), IGF-I, the IGF binding proteins IGFBP1 and IGFBP2 and a subunit of IGFPB (Daniel H., 2004). Thyroid hormones also affect growth, and it appears that zinc also influences the biological function of the thyroid hormones and their receptors, but the mechanism by which zinc acts on the thyroid axis is not yet clear (Freake HC, 2001) (Figure 1.2.32). In the same zinc deficient rats, a variety of transcripts coding for proteins of hepatic fat and glucose metabolism are found (Daniel H., 2004) (Figure I.2.33).

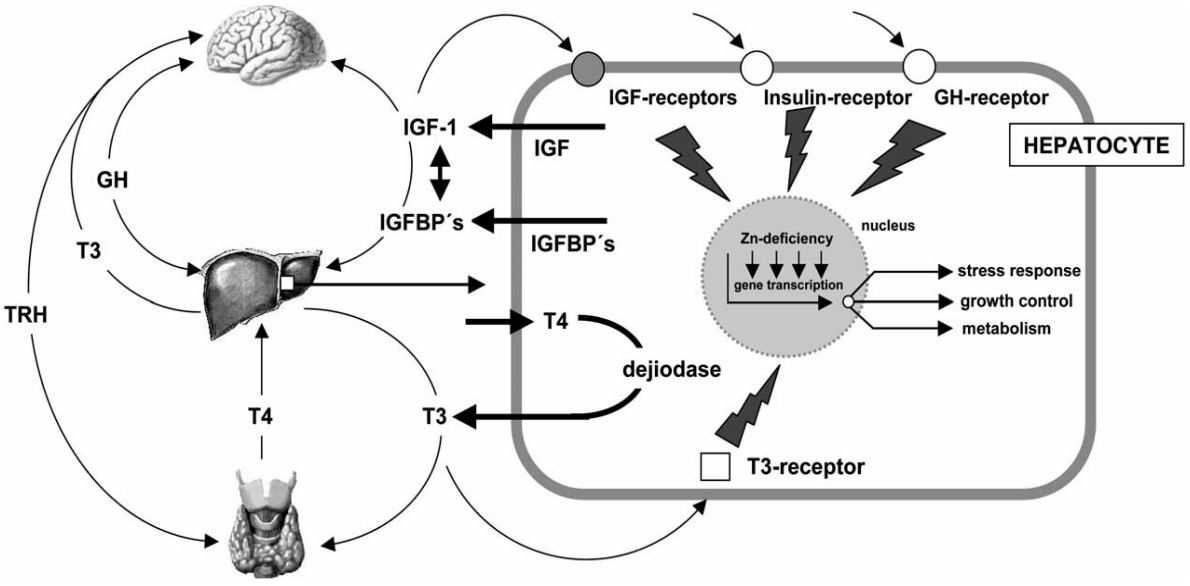

Figure I.2.32. Zinc-dependent hormone pathways in liver. A simplified model for the effects of Zn deficiency on hepatic processes that in turn affect circulating hormone levels of the GH, IGF, insulin and thyroid axis and with the liver as a target organ for the action of these hormones. Postulated impairments in signaling cascades that secondarily affect hepatic gene transcription are indicated. Taken from (Daniel and tom Dieck, 2004).

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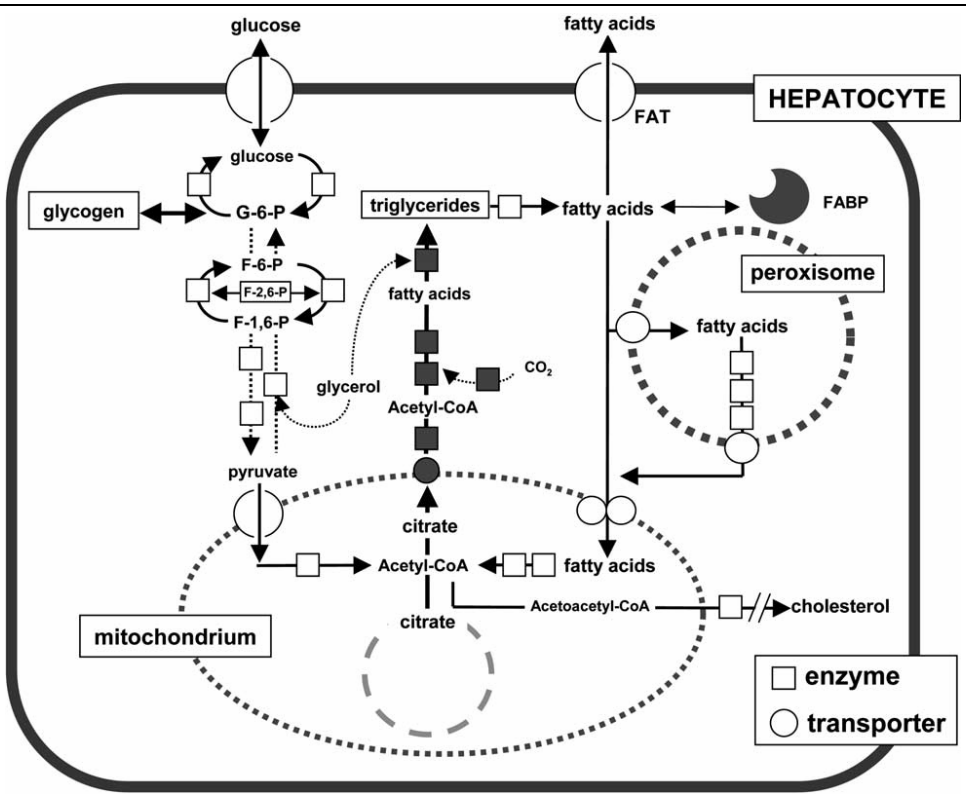

Figure I.2.33. Alterations of hepatic metabolism in zinc deficiency. Metabolic processes and individual enzymatic reactions in hepatic carbohydrate and fat metabolism that responded with significant changes (□2-fold) in transcript/protein levels to experimental Zn deficiency in the rat model. Open boxes indicate proteins (enzymes) with lowered transcript/protein levels, filled boxes those with increased levels, circles indicate the relevant transport proteins. From (Daniel and tom Dieck, 2004).

Another relevant consequence of zinc defficient status is diabetes. The predominant effect on zinc homeostasis in diabetes is the hypozincemia which may be the result of hyperzincuria or decreased gastrointestinal absorption of Zn or both. Hyperglycemia from either Type 1 or Type 2 diabetes is the most probably responsible for the increased urinary loss and decreases in total body zinc. Furthermore, zinc might play a role in the development of diabetes, since genetic polymorphisms in the gene of zinc transporter 8 and in metallothionein-encoding Zn plays a clear role in the synthesis, storage and secretion of insulin as well as conformational integrity of insulin in the hexameric form. Also, Zn deficiency affects the ability of the islet cell to produce and secrete insulin, particularly important in Type 2 diabetes (Devirgiliis et al., 2007; Mocchegiani et al., 2008; Jansen et al., 2009).

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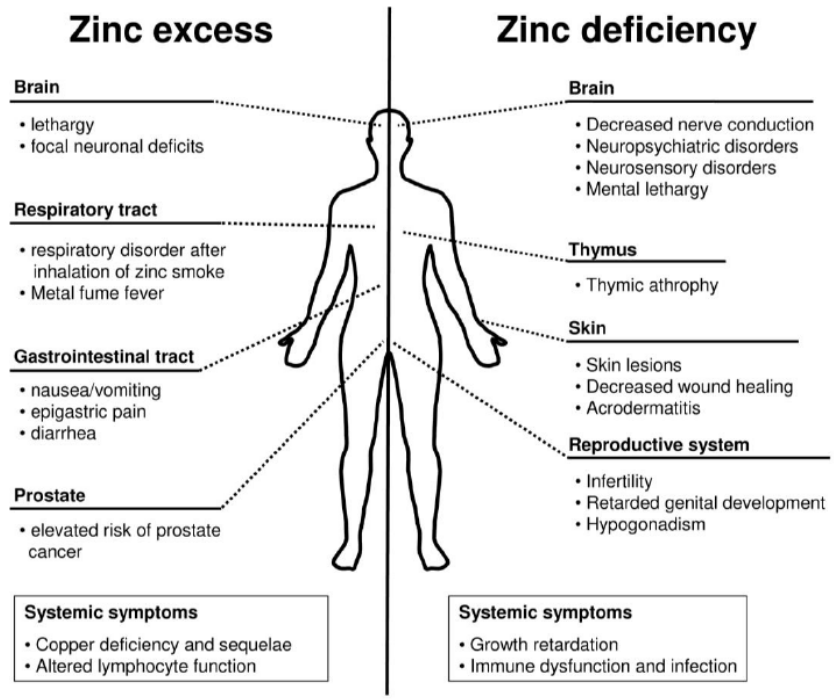

Figure I.2.34. Comparison of the effects of zinc intoxication versus deficiency. Intoxication by excessive exposure to, or intake of, zinc (left hand side), and deprivation of zinc by malnutrition or medical conditions (right hand side), have detrimental effects on different organ systems. Effects that could not be attributed to a certain organ system or affect several organs are classified as systemic symptoms. After (Plum et al., 2010).

On the other hand, toxic damage produces **zinc accumulation** in diseases such as stroke, head trauma and epileptic brain injury. In addition, Taneja *et al*. have shown that zinc in excess in the diet for a long period of time induces metabolic syndrome-X in rats (Taneja et al., 1996). Dysregulation of zinc transporters and of free zinc has also been implicated in the formation of β-amyloid plaques associated with Alzheimer's disease (Smith et al., 2006; Smith et al., 2007; Talmard et al., 2009; Miller et al., 2010). In these cases, zinc chelators are promising therapies (Devirgiliis et al., 2007) (Figures I.2.34 and 1.2.35).

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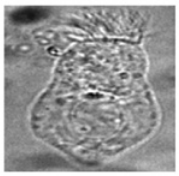
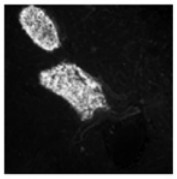
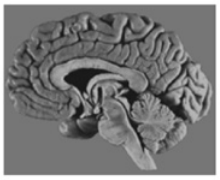
	Lung Epithelium 	**Endocrine Pancreas** 	**Brain*** 
Zn imbalance in the associated pathologies	***Asthma*** (Zn deficiency)	***Diabetes*** (Zn deficiency)	***Alzheimer's disease*** (Zn accumulation)
Zn localization	Apical cytoplasm [31,32]	Secretory vesicles [42]	Synaptic vesicles [66]
Zn function	Antioxidant?	Insulin/glucagone secretion [43,52]	Synaptic transmission [69]
Expressed ZIPs	ZIP1, 6, 7, 8, 14 [37]	ZIP3, 4, 6, 7, 8, 14 [51]	ZIP6, 7* [74]
Expressed ZnTs	ZnT1, 2, 4, 5, 7 [37]	ZnT1, 4, 5, 6, 7, 8 [51]	ZnT1, 3, 4, 5, 6, 7 [74]
Human genes containing SNPs associated with disease	ADAM33 (Zn-dependent metalloprotease)[37]	MT2A promoter [10]	?
Effects of Zn deficiency	↑ disease risk ↑ apoptosis [33]	↑ disease risk ↑ Islet cell damage [39,40]	
Effects of Zn supplementation	↓ inflammation [37]	Protection from chemically-induced diabetes ↑ MT gene expression [61]	
Effects of Zn chelation			↓ Aβ plaque deposition [64]

Figure I.2.35. Features of Zn fluxes in tissues displaying alterations associated with disease. *Expression of all known members of ZIP and ZnT families has not been investigated in the brain. From (Devirgiliis et al., 2007). Numbers within brackets refer to references given in (Devirgiliis et al., 2007).

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UNIVERSITAT ROVIRA I VIRGILI
EFFECTS OF DIETARY CATECHINS AND PROANTHOCYANIDINS ON ZINC HOMEOSTASIS IN HEPATIC CELLS
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3. COPPER: OVERVIEW OF COPPER HOMEOSTASIS

Copper is an integral part of many important enzymes such as cytochrome c oxidase (CCO), lysyl oxidase, tyrosinase, copper/zinc superoxide dismutase (SOD1) and ferroxidases, involved in a number of vital biological processes (Peña MM, 1999; Shim H, 2003; Prohaska JR, 2004).

Free copper rarely exists *in vivo* and it is normally bound to proteins, however Cu ions may be released and therefore turned toxic because of its ability to redox cycle and support Fenton chemistry leading to the production of free radicals (Halliwell B, 1984). Thus, copper homeostasis is very tightly regulated and several proteins play key roles in the uptake, distribution and export of copper from the cells. These include a high-affinity transporter Ctr1, a low-affinity transporter Ctr2, copper chaperones (CCS, Atox1, Cox17, SCO1 and SCO2), the copper efflux transporters ATP7A and ATP7B and MTs (Gupta, 2009).

Estimates of copper in humans are 2 mg/d approximately and it is readily absorbed from the diet across the small intestine (Turnlund, 1998), but the mechanisms of uptake of dietary copper are not well understood (Maryon EB, 2007b). Ctr1 (Slc31a1), a high affinity, trimeric copper transport protein, is essential for acquisition of dietary copper (Nose Y, 2006). The subcellular localization and abundance of Ctr1 are cell-type specific (Kuo YM, 2006). Once absorbed in the enterocyte, copper is exported into the portal blood stream via the ATP7A P-type ATPase transporter which is localized to the basolateral membrane and secretory pathway (Hamza I, 2003; Nyasae L, 2007) (Figure I.3.1). Recessive mutations in the gene encoding ATP7A result in Menkes disease, a fatal X-linked neurodevelopmental disorder. ATP7A deficiency results in a lack of copper transport through the placenta and the intestinal epithelium, leading to systemic copper deficiency and the failure to provide essential cuproenzymes with the metal (Shim H, 2003).

Then, copper is taken up by the liver, which represents the predominant storage organ for copper. In the hepatocyte, copper is imported via Ctr1. Once intracellular, copper has one of

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four different possible fates: a) joining the copper/metallothionein pool, b) trafficking to the mitochondria for cytochrome c oxidase incorporation via the copper chaperone cox17, c) binding to CCS (copper chaperone for SOD) for delivery to nascent Cu, Zn-SOD, or d) trafficking to the Wilson disease P-type ATPase, which resides in the trans-Golgi network (TGN) by HAH1 (human atox-1 homologue) for subsequent copper incorporation into the cuproprotein ceruloplasmin, a ferroxidase which is the primary copper-binding protein in serum. Excess copper is excreted into the bile and eliminated in feces (Figure I.3.2) (Turnlund, 1998; Shim H, 2003; Lutsenko S, 2007). This proces is also regulated by ATP7B. Patients with Wilson's disease have mutations in ATP7B resulting in the hyperaccumulation of copper in the liver because of impaired excretion of copper into the bile and secretory pathway (where copper is loaded into ceruloplasmin) (Shim H, 2003).

Ctr1

The best characterized yeast and human Ctrs differ in size and show only moderate sequence similarity. Nevertheless, they share an overall homotrimer architecture, in which each monomer has four key domains: an extracellular N-terminus; three transmembrane (TM) helices; an intracellular loop of variable length, connecting the first and second TM helices; an intracellular C-terminal tail. Human Ctr1 monomer consists of 190 amino acid residues (Gupta et al., 1999) (Figure I.3.3). hCtr1 is N- and O-glycosylated (Maryon EB, 2007a) with the glycosylation sites located at the extracellular N-terminal domain (residues Asn-15 and Thr-27, respectively). The extracellular N-terminus, which is rich in Met and His residues, has been implicated in copper binding (De Feo CJ, 2007). There is some discrepancy on the intracellular localization of this transporter (the apical, basolateral and intracellular localization were reported). However, in cultured cells, Ctr1 is typically observed in two locations: at the plasma membrane and intracellularly in vesicles. In enterocytes and hepatocytes, as mentioned above, Ctr1 is responsible for making copper available in the cytosol for utilization by various cellular proteins and further efflux.

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Ctr2

Ctr2 has sequence homology with Ctr1. The residues in the TM portion that are known to be essential for the transport function of Ctr1 are conserved in Ctr2 (Klomp AE, 2002). There are also notable differences between hCTR2 and hCTR1. Interestingly, Ctr2 lacks the extended N-terminal domain characteristic of Ctr1 that is important for high-affinity copper transport, and does not contain the appropriate consensus sites for N-glycosylation. These observations support the hypothesis that hCtr2 does transport copper, but suggest that this occurs with a lower affinity than that of hCtr1. However, the function of hCtr2 is currently unknown.

The vast majority of this protein is localized in late endosomes and lysosomes and facilitates cellular copper uptake (van den Berghe PV, 2007; Bertinato J, 2008). Less than 5% of Ctr2 is located at the plasma membrane (Bertinato J, 2008). Given its predominantly intracellular location, Ctr2 might uptake copper at the plasma membrane. It could be that the predominantly intracellular localization of Ctr2 simply reflects a pool of inactive transporters that could be recruited to the plasma membrane under certain physiological conditions. In addition, the intracellular Ctr2 may facilitate copper release from intracellular copper stores, as it does in yeast (Bertinato J, 2008).

Van den Berghe *et al.* postulated that Ctr2 mediates an alternative low-affinity copper import pathway in human cells, since Ctr1-null mice still have some residual cellular copper uptake (van den Berghe PV, 2007).

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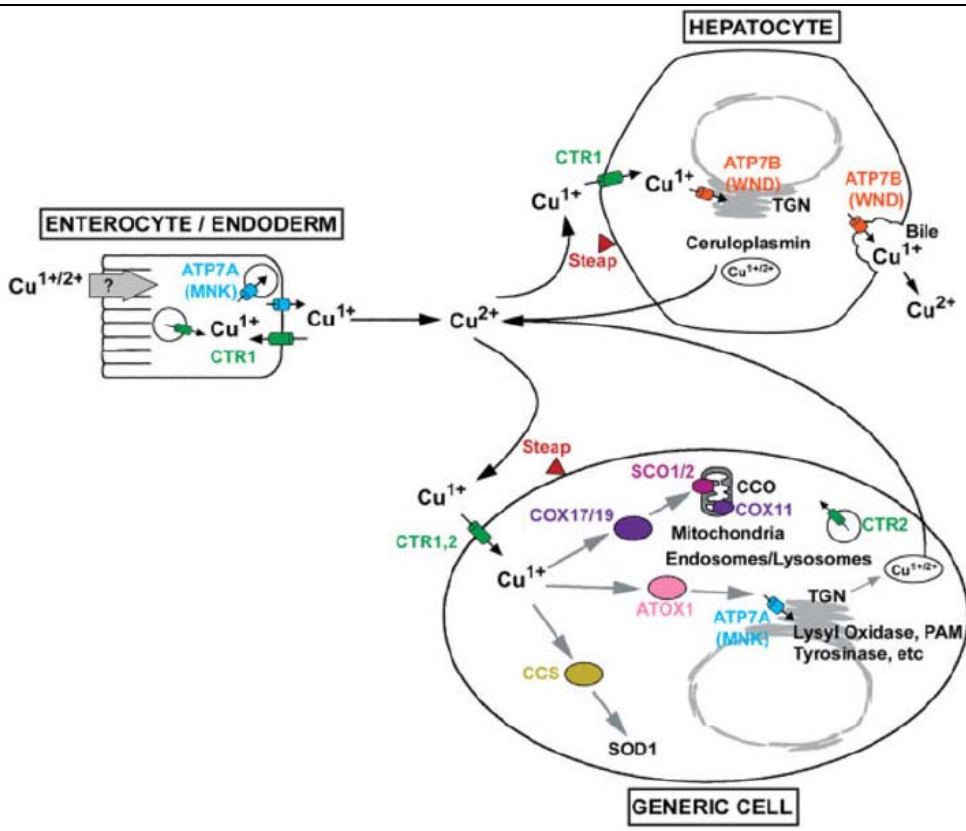

Figure I.3.1. Copper homeostasis. Copper is oxidized (Cu2+) in the intestinal lumen and in the serum, but is reduced to Cu1+ before transport into cells. The Steap ferric/cupric reductases (Steaps 2, 3, and 4) are localized to endosomes/lysosomes and the plasma membrane and are essential for the reduction of copper, as well as the reduction of iron (Ohgami et al., 2006). Copper is transported into ENTEROCYTE/ENDODERM cells by unknown mechanisms (perhaps endocytosis). CTR1 is essential for the acquisition of dietary copper but its function in the enterocyte is unknown. In many other cell types (GENERIC CELL), copper is taken up by CTR1 localized to the plasma membrane and perhaps also by CTR2 (Slc31a2). Copper taken up by ENTEROCYTE/ENDODERM is exported into portal blood/conceptus, respectively, by ATP7A (Menkes disease protein, MNK) which is localized to vesicles trafficking toward the basolateral membrane and to the basolateral membrane. Copper exported to portal blood is taken up into the liver, the primary organ that regulates copper homeostasis. In the HEPATOCYTE, ATP7B (Wilson's disease protein, WND) effluxes excess copper into the bile and puts copper into the trans-Golgi network (TGN) where it is loaded into ceruloplasmin, a ferroxidase that is the primary copper binding protein in serum. Inside the cell (GENERIC CELL), copper is distributed to cytoplasmic copper chaperones (COX17/19, ATOX1, CCS) which, in turn, deliver copper to mitochondrial inner membrane and ultimately cytochrome C oxidase (CCO) or to ATP7A in the TGN, and cytoplasmic SOD1, respectively. It is thought that copper in COX17/19 (and probably COX23/MTCP1) is first transferred to both COX11 and SCO1/2 and ultimately to CCO. ATP7A transports copper into the TGN and activates copper containing secretory and membrane-bound proteins [lysyl oxidase, tyrosinase, peptidylglycine α- amidating monooxygenase (PAM)]. The cellular localization and abundance of CTR1, ATP7A, and ATP7B are dynamically regulated by copper availability which is not reflected in this static cartoon. Taken from (Kambe, 2008)

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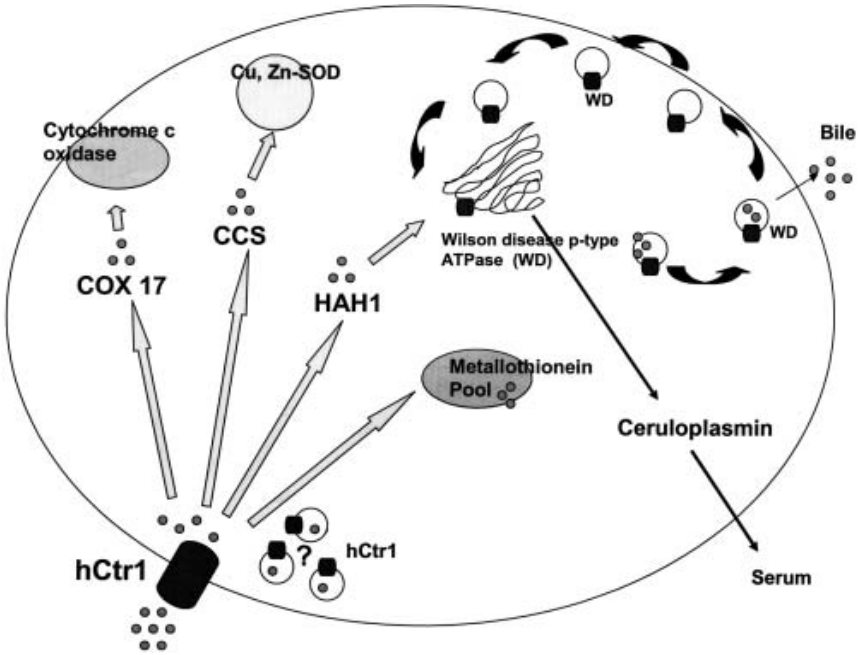

Figure I.3.2. Model of copper trafficking in a hepatocyte. Copper enters the hepatocyte bound to either albumin or histidine and traverses the cell via the copper transport protein, hCtr1. Characterization of hCtr1 confirms localization on the plasma membrane and also suggests the presence of hCtr1 on a separate intracellular perinuclear vesicular compartment. Copper, once inside the hepatocyte, has one of four possible fates: a) joining the copper/metallothionein pool, b) trafficking to the mitochondria for cytochrome c oxidase incorporation via the copper chaperone cox17, c) binding to CCS for delivery to Cu, Zn-SOD, or d) trafficking to the Wilson disease P-type ATPase, which resides in the trans-golgi network by HAH1 for subsequent copper incorporation into the cuproprotein ceruloplasmin. Localization studies of the Wilson P-type ATPase reveal redistribution of the ATPase from the trans-golgi network to a vesicular compartment that moves out toward the biliary epithelium under conditions of high copper concentration providing a mechanism for copper excretion in bile. From (Shim H, 2003).

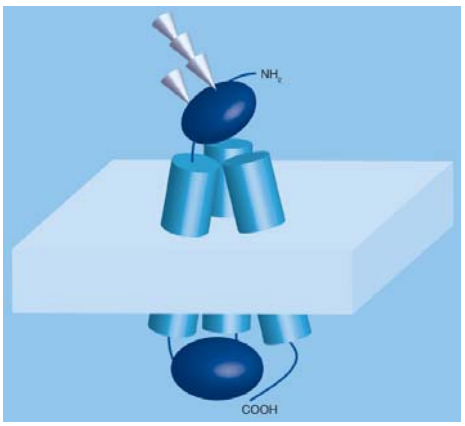

Figure I.3.3. Overall architecture of the Ctr1 monomer. Ctr1 has a glycosylated N-terminal domain exposed to the outside milieu; three transmembrane (TM) segments, a cytosolic loop connecting TM1 and TM2 and the cytosolic C-terminus. In the fully assembled functional Ctr1, the three monomers associate into a stable complex. Taken from (Gupta et al., 1999).

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4. ANTECEDENTS

The research work carried out in this Ph. D. Thesis is part of a more general research project developed by the Nutrigenomics Research Group of the Rovira i Virgili University, which deals with the mechanisms of action and potential beneficial effects of dietary procyanidins in preventing and ameliorating the metabolic disorders associated with Metabolic Syndrome. This syndrome is characterized by metabolic disorders such as insulin resistance, obesity, atherogenic dyslipidemia, high blood pressure and proinflammatory or prothrombotic that are associated with a higher risk of cardiovascular diseases (CVD) and Type-2 Diabetes Mellitus (Grundy et al., 2004; Grundy et al., 2005; Huang, 2009; Roberts and Sindhu, 2009).

Previous studies of our research group (Del Bas et al., 2005) showed that oral administration of a grape seed procyanidin extract (GSPE) to healthy rats lowers plasma triglyceride levels and ApoB-containing VLDL particles in the postprandial state. Analysis of liver gene expression profile associated to GSPE consumption identified numerous target genes of procyanidin action in hepatic cells. Among these genes, mRNA levels of the orphan nuclear receptor small heterodimerpartner (SHP, Nr0b2) were increased threefold in the liver of GSPE-treated animals compared with control, untreated animals. SHP is known to be involved in cholesterol, TG, and glucose homeostasis and mediates the hypotriglyceridemic effect of bile acids. Bile acids -such as CA (cholic acid) and CDCA (chenodeoxycholic acid)- exert their hypotriglyceridemic effect by binding to, and thereby activating the transcriptional activity of, the bile acid receptor Farnesoid X Feceptor (FXR), which then binds to the promoter of the SHP gene activating its transcription. SHP then represses the transcription of diverse genes involved in the biosynthesis and secretion of tryglicerides in hepatic cells, notably the Steroid Response Element Binding Protein-1 and -2 (SREBP1 and SREBP2), ultimately leading to an .improvement of the plasma lipid profile and the atherosclerotic risk index in the postprandial state in GSPE-treated animals (Watanabe et al., 2004) (Figure I.4.1.) It was later on demonstrated that procyanidins exert their hypotrygliceridemic effects in

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a SHP- and FXR-dependent way, by acting as coactivators of bile acid-activated FXR (Del Bas et al., 2008) thus enhancing the hypotrygliceridemic effects of bile acids (Del Bas et al., 2009). Activators of FXR activity are considered promising therapeutical agents to treat Metabolic Syndrome (Cariou and Staels, 2007; Zhang and Edwards, 2008; Prawitt et al., 2009).

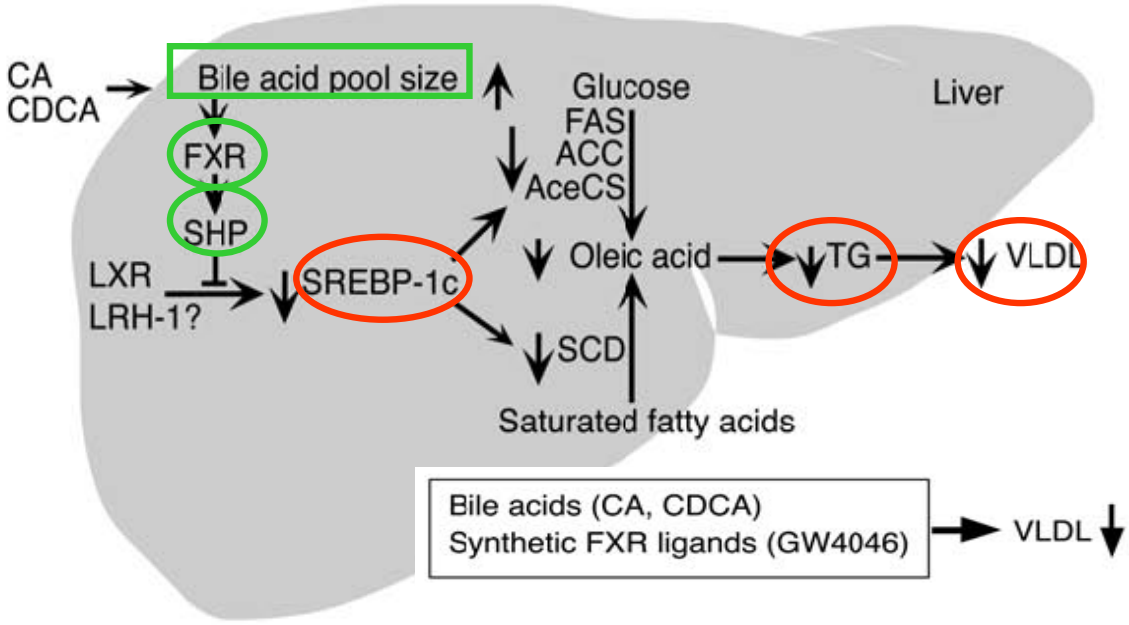

Figure I.4.1. The FXR-SHP-SREBP-1c regulatory cascade. Schematic representation of the proposed role of SHP in mediating the effects of FXR agonists on SREBP-1c expression and lipogenesis. Alter (Watanabe et al., 2004).

Small heterodimer partner (SHP; NR0B2), an excepcional member of the mammalian nuclear receptor family, directly modulates the activities of conventional nuclear receptors by acting as an inducible and tissue-specific corepressor. SHP is a pleitropic regulator of diverse metabolic pathways in the liver, including cholesterol, bile acids, fatty acids and trygliceride synthesis and gluconeogenesis (Yamagata et al., 2004; Bavner et al., 2005; Boulias et al., 2005; Yamagata et al., 2007). Among other mechanisms, SHP is known to act as a repressor of transcription by different mechanisms including recritment of Histone Deacetylases (HADC), Histone Methyl Transferases (HMT) and DNA Methyl Transferases

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(Dnmt) to the promoter region of its targets genes, ultimately producing rearrangements in chromatin structure and epigenetic silencing of gene expression (Bavner et al., 2005; Gobinet et al., 2005; Fang et al., 2007) (Figure I.4.2).

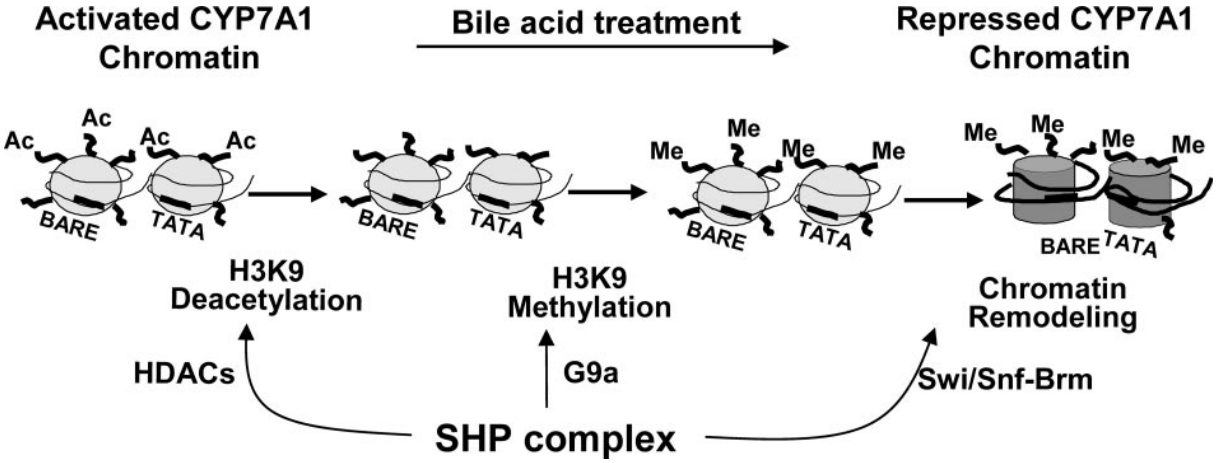

Figure I.4.2. Coordinated sequential recruitment of HDACs, G9a, and Swi/Snf-Brm in the SHP-mediated suppression of CYP7A1 in the hepatic bile acid metabolism. Bile acid-induced SHP may compete with coactivator HATs, including p300, CBP, and SRC-1, at the CYP7A1 promoter, which contributes to a decrease in histone acetylation. SHP directly interacts with HDACs and G9a through its C and N terminus, respectively, recruiting these cofactors to the promoter, which results in deacetylation and subsequent dimethylation at the H3K9. H3K9 methylation and deacetylation create a binding surface for the recruitment of the Swi/Snf-Brm complex, which results in ATP-dependent chromatin remodeling and subsequent gene silencing of CYP7A1. From (Fang et al., 2007).

Yet other targets genes of GSPE in the liver of the rats fed GSPE were found to be the metallothionein (MT) genes MT-I and MT-II. mRNA levels of MT genes were 3 fold less abundant in the liver of GSPE-treated rats than in control animals. As described above, metallothioneins are metal binding proteins crucially involved in heavy metal metabolism, especially in zinc and copper homeostasis, in mammalian cells. Notably, as described above, silencing of MT expression in diverse hepatocarcinoma cell lines has been adscribed to epigenetic silencing mediated by HADCs, HMTs and Dnmts (Majumder et al., 1999a; Majumder et al., 1999b; Ghoshal et al., 2000; Ghoshal and Jacob, 2001; Ghoshal et al., 2002; Majumder et al., 2002; Datta et al., 2005; Majumder et al., 2006), a mechanism strikingly similar to that described for SHP-mediated gene silencing.

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Albait the complexation of polyphenols with the essential transitions metals iron, copper and zinc is well recognized, the vast majority of studies on polyphenol-metals interactions have foucused of the interaction of polyphenols with iron and copper and the consequences of this interaction on the antioxidant action of polyphenols, given the potency of copper and iron to generate reactive oxygen species through Fenton chemistry (see section I.1). Very few studies have previously dealed with the complexation of polyphenols with zinc cations, although a few studies had stablished that zinc cations may alter the biological effects of flavonoids. Thus, zinc yields (−)-epigallocatechin-3-gallate (EGCG) effective in protecting cultured rat hepatocytes against hepatotoxin-induced cell injury (Kagaya et al., 2002) and enhances its antiproliferative effects on prostate cancer cells (Sun et al., 2008). Zinc also stimulates the apoptotic effect of genistein in osteoclastic cells (Uchiyama and Yamaguchi, 2007). Therefore, bioactivity of flavonoids and zinc metabolism might be interconnected. No studies, however, have previously studied the consequences of zinc complexation by polyphenols on zinc metabolism and homeostasis. The strong downregulation of MT genes revealed in the microarray study and the scarce information about the relationship between MT and proanthocyanidins, led us to focus, first, on the mechanisms by which these flavonoids trigger the down-regulation of MT genes in hepatic cells, using the expression of MT as a biomarker of procyanidin action, and second, on the study of the effects of proanthocyanidins on different parameters of cellular zinc homeostasis.

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II. OBJETIVES

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The experimental evidence previously obtained in the Nutrigenomics Research Group had shown that the expression of MT genes is repressed in the liver of GSPE-treated rats concomitantly with the upregulation of the orfan nuclear receptor SHP. It was also known that procyanidins act as enhancers of the transcriptional activity of bile acid-activated FXR (see section I.4) Given the central role of MT in the control of cellular zinc homeostasis and zinc signaling, the purpose of this thesis has been to characterize and understand how dietary catechins and proanthocyanidins modulate MT gene expression and zinc homeostasis in hepatic cells. With this aim, six objectives were sequentially proposed:

1. **To determine the effect of GSPE on modulation of metallothionein genes in hepatic human cells (HepG2) cultured in vitro**, with the aim of stablishing whether MT genes are bona fide target of procyanidins in hepatic cells, excluding the effects that procyanidins exert on extrahepatic tissues in *in vivo* models.
2. **To asses the effect of GSPE on the expression of metallothioneins in HepG2 under conditions that mimic the postprandial situation**, i.e., when MT expression is induced with: zinc overload, copper overload, the proinflammatory cytokine IL-6, the ROS generator tBOOH and the glucocorticoid receptor agonist dexamethasone. This approach is intended to identify which of the different pathways that control MT expression at the transcriptional level are targets of procyanidins action.
3. **To establish the molecular mechanisms by which GSPE downregulate metallothionein genes *in vitro* in both basal and induced conditions.** Considering the different mechanisms known to regulate metallothionein expression, and the resuls obtained in the experiments performed under the previous objective, two different mechanisms of MT regulation by catechins and procyanidins have been propossed:

3.1. Epigenetic silencing of MT expression: to asses this hypothesis the objective was to elucidate the possible implication of SHP and FXR in the silencing of MT gene elicited by

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GSPE, monitoring the changes in hepatic MT expression elicited by oral administration of GSPE in SHP-null and FXR-null mice.

3.2. Diminished zinc bioavailability: to asses this hypothesis, total intracellular zinc content in HepG2 cells we measured both in basal concentrations of zinc in the culture medium and under different conditions that stimulate hepatic cells to accumulate zinc.

4. **To asses whether different catechins and procyanidins interact with zinc cations in solution.** This objective is intended to know if the flavonoids under study react with zinc ions to form complexes in solutions, and if they interact with enough affinity to interfere with different parameters of cellular zinc homeostasis.

5. **To characterize the modulation of parameters of cellular zinc homeostasis by catechins and procyanidins in HepG2 cells.** To gain deeper knowledge of the effects of catechins and procyanidins on cellular zinc homeostasis, different parameters will be analyzed:

5.1. **Effect of catechins and procyanidins on expression of plasma membrane and organelles zinc transporters in HepG2 cells,** cultured in standard conditions and treated with substances that increase de expression of MT genes and stimulate cellular uptake of zinc. Changes on the expression of zinc transporters should provide information on the availability of extracellular zinc and of intracellular distribution of zinc in HepG2 cells treated with the flavonoids under study.

5.2. **Effect of catechins and procyanidins on the cytoplasmatic labile pool of zinc in HepG2 cells.** Because this pool of zinc is endowed with a modulatory and signaling function, changes triggered in the amount of labile zinc may relevant to explain the effect of the flavonoids on diverse metabolic and signaling pathways which are targets of labile zinc.

6. **To evaluate the effect of GSPE on copper homeostasis in HepG2 cells.** Given that flavonoids can chelate copper ions and that MT expression, which is profoundly affected by GSPE, is regulated principally by zinc but also by copper, we aimed to analyze the

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effects of GSPE on copper homeostasis evaluating the expression of copper transporters and copper chaperones and measuring total intracellular copper content.

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III. MATERIALS AND METHODS

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***Chemicals**:*

GSPE was from Les Dérives Résiniques et Térpeniques (Dax, France). This extract consist of monomeric catechins (16.55%), dimeric (18.77%), trimeric (16%), tetrameric (9.3%) and oligomeric (5-13 units) (35.7%) procyanidins, and phenolic acids (4.22%). Pure procyanidin C1 was provided by Prof. Jean-Michel Mérillon and Dr. Xavier Vitrac (Polyphénols Biotech, Bordeaux, France). All other procyanidins, catechins, TPEN (N,N,N',N'-tetrakis(2-phridylmethyl) ethylenediamine), $ZnCl_2$, $CuCl_2$ dexamethasone, Zinquin (ethyl (2-methyl-8 ptoluenesulfonamido- 6-quinolyloxy), CDCA, DMSO and Non-essential aminoacids were from Sigma and IL-6 from Roche. Dulbecco's Modified Eagl's Medium (DMEM), fetal bovine serum (FBS), penicillin/streptomicyn and L-Glutamine were from BioWittaker.

***Interaction of catechins and procyanidins with zinc in solution**:*

UV-Vis absorption spectra of flavonoids (10 μM) in PBS at pH 7.4, was recorded before and after addition of 5 μM $ZnCl_2$, as previously reported [38], using an Hitachi U-1900 Spectrophotometer, with a 4 nm slit width.

Fluorescence spectra of flavonoids in 50 mM Tris-HCl buffer, 0.1M NaCl, pH 7.450mM, was recorded before and after addition of $ZnCl_2$, using a Perkin Elmer LS 50 spectrofluorimeter with excitations set at 230 and 260nm (5nm slit width) and emmisions from 240 to 600nm.

Zinc-dependent fluorescent emission of Zinquin (485-490 nm) dissolved in 50 mM Tris-HCl buffer, 0.1M NaCl, pH 7.4 (Devergnas et al., 2004) was recorded in a Perkin Elmer LS 50 spectrofluorimeter, with excitation set at 365-370 nm, at 25ºC. Quenching of zinc-dependent Zinquin fluorescence by flavonoids was monitored 5 minutes after addition of different amounts of the flavonoids to the solution containing 10 μM Zinquin and 1 μM zinc, or 25μM Zinquin and 100 μM zinc.

***Cell Cultures and treatments**:*

Human hepatoblastoma HepG2 cells (American Type Culture Collection, ATCC) were cultured in Dulbecco's Modified Eagl's Medium (DMEM) suplemented with 10% fetal bovine

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serum, 1% penicillin/streptomicyn, 2mM L-Glutamine, 1% Non-essential aminoacids. This medium contains 4.9±0.2 μM zinc as determined by flame atomic absorption spectrometry (FAAS). Cells were incubated at 37ºC in a humidified, 5% CO2-enriched atmosphere and routinely splitted at a 1:5 ratio when reached 80% confluence.

For treatments, cultures at 80% confluence were trypsinized, resuspended at a density of $0.5x10^6$ cells/mL and 1 mL aliquots were seeded per well in 12-well plates (Orange Scientific). 24 hours later medium was replaced with 1 mL of fresh medium containing the different test substances or vehicle (final 0.2% ethanol). Samples were collected at different times.

For induction of MT expression, Inducers were added by supplementing the growth medium with either 100μM Zinc Chloride (Sigma) for 12 hours, 50μM Copper chloride (Sigma) for 15 hours, 200μM tert-butylhydroperoxide (Sigma) for 7 hours, 10μM Water-soluble Dexamethasone (Sigma) for 12 hours or 1000 U/ml Interleukin 6 (ROCHE) for 15 hours. Control cells of zinc and copper inductions were grown in growth medium with 0.2% ethanol. Control cells of tBOOH and Dexamethasone inductions were grown in the same growth medium as zinc and copper plus 5μM zinc and control cells of IL-6 induction was the same as tBOOH and Dex plus 1μM dexamethasone.

Cell viability assays:

Cytotoxicity of GSPE, EGCG, C1 and $ZnCl_2$ were assessed by measuring lactate dehydrogenase (LDH) leakage in cells treated 24 h with different concentrations of the test substances. It was determined spectrophotometrically by the rate of NADH utilization in the enzyme-catalyzed back reaction of pyruvate conversion to lactate using the LDH Kit (QCA, Barcelona, Spain).

Animals*:*

Male Wistar rats, 2 months old and weighing 250 g, were purchased from Charles River (Barcelona, Spain). The Animal Ethics Committee of University Rovira i Virgili approved all

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procedures. The animals were housed in animal quarters at 22ºC with 12 h light/dark cycle (light from 8 a.m. to 8 p.m.) and were fed ad libitum. At 11 a.m. on experimental day, the rats (6 animals/group) were fed an oral dose gavage of GSPE in aqueous solution (250 mg/Kg body wt.; GSPE group) or were fed an oral dose gavage with vehicle (tap water; Control group). The used procyanidin dose is one-fifth of the no-observed-adverse effect level (NOAEL) described for GSPE and male rats. Five hours after treatment, the rats were killed by beheading. Liver was excised, frozen immediately in liquid nitrogen, and store at -80ºC until RNA extraction as described (Del Bas et al., 2005).

Materials and methods of expriments done in SHP-null and FXR-null mice are detailed in (Del Bas et al., 2008) (Del Bas et al., 2009).

Gene expression analysis

RNA purification*:* Total RNA was purified from frozen livers or HepG2 cells using the NucleoSpin RNA II (Machery-Nagel, Düren, Germany) following the instructions of manufacturers.

Reverse Transcription*:* cDNA was generated from total RNA using TaqMan Reverse Transcription Reagents (Applied Biosystems).

Quantitative PCR amplification and detection were performed in cDNA samples using the Applied Biosystems Real Time 7000 PCR System termocycler and software. Specific TaqMan Assay-on-Demand Probes (Applied Biosystems) were used in HepG2 experiments to quantify changes in expression of MT1A, MT1X, MT2A, MT1E, MT1G, MT1F, MT1H, MT1B, ZnT1, ZIP1, ZIP4, ZIP14, GCLC and SHP. Cyclophilin was used as reference gene. In rat and mouse experiments the relative levels of mRNA of the tested genes were assesed using Syber Green Master Mix (Applied Biosystems). The forward and reverse primers for MT-1 were: 5'- ATGGACCCCAACTGCTCCTGCT-3' and 5'-CACAGCCCTGGGCACATTTGGAG-3' respectivly. The forward and reverse primers for MT-2 were: 5'- ATGGACCCCAACTGCTCCTGTG-3' and 5'-AGCCCTGGGAGCACTTCGCA-3'

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respectivly. GAPDH was used as reference gene. For **microarray hybridizations**, RNAs from individual HepG2 samples (GSPE-treated or control cells, 3 per group) were pooled. The integrity of the pooled RNA was assessed using the Agilent 2100 Bioanalyzer. For microarray hybridization, Cy3- or Cy5-labelled cDNA was obtained from each RNA pool by using the Agilent Low RNA Input Fluorescent Linear Amplification Kit as described in the Agilent manual (Part Number 5185-5818). Labelled cRNAs were hybridized against Agilent 40K Human Oligo Microarrays (Part Number G4122A) following the Agilent 60-mer oligo microarray processing protocol. Images of hybridized microarrays were acquired with the Agilent G2565BA scanner, and data from the microarray images were obtained and analyzed with the Agilent Feature Extraction software. For each pair of RNA samples being compared, duplicate hybridizations with a dye-swap labelling was performed. Hybridization, image acquisition and data extraction of microarrays were performed in the in microarray facility of Centre de Regulació Genòmica de Barcelona. Microarray data were validated by analyzing gene expression of MT1A, MT1X, MT2A, MT1B, MT1G, MT1E, ZnT1, ZIP1, ZIP4, ZIP14, GCLC and SHP by RTqPCR, using Cyclophilin A as the endogenous control.

Measurements of total intracellular zinc and copper:

To quantify total intracellular zinc and copper, cells were thoroughly washed with PBS and lysated with 0.01 M NaOH, 0.01% SDS. Aliquots of the cell lysates were used to quantify zinc or copper by FAAS as previously described (Reaves et al., 2000), using an Hitachi Z-8200 Polarized Zeeman AA Spectrophotometer. Total zinc and copper conetent were normalized per total protein conetnt of the cells and determined by Bradford method.

Measurements of cytoplasmatic labile zinc:

To measure changes in intracellular labile zinc, cells were washed with PBS after treatments, incubated 30 minutes at 37ºC in 25 μM Zinquin ethyl ester in PBS, washed again and finally suspended in PBS; protein content and Zinquin fluorescence was determined in cell aliquots. Background fluorescence of Zinquin unloaded cells was substracted from readings to derive Zinquin-dependent fluorescence as described (Coyle et al., 1994).

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For microscopy visualization, cells were attached to glass coverslips, the medium was discarded after treatments and the cells were washed (three times) with PBS. Coverslips were immersed in a solution of Zinquin (25 μM) in PBS and incubated for 30 min at 37 ºC. Coverslips were inverted on to microscope slides, and images were captured at 1000x magnification with a Leica DM 4000B microscope using UV light illumination (λex = 340-380 nm) and a blue emission filer (λem ≥ 425nm).

Statistical analysis:

For statistical analysis in cytotoxicity assays, zinc and protein quantification, quantitative RT-PCRs, and fluorescence measurements, t-test and one way ANOVA analyses were performed using SPSS software. Except for microarray analysis, all data are the result of at least 3 independent experiments. Differences were considered significant for P values ≤ 0.05.

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IV. RESULTS

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1. GSPE

Modulation of hepatic MT and SHP expression by GSPE in the postprandial phase of healthy rats.

In this work we first confirmed the previous data obtained in by microarray hybridization experiments done by our research group to monitor the changes in gene expression in the liver of healthy rats in the postprandial phase after oral ingestion of an aqute dose of GSPE (Del Bas et al., 2005), using quantitative PCR, and specific primers to discern between the MT-I and the MT-II genes. The results confirm that oral administration of a non-toxic dose of GSPE results in downregulation of MT-I and MT-II mRNA levels to 45% and 50%, respectively, and in twofold upregulation of SHP of control values in the liver of rats (Figure IV.1.1). Thus, the expression of both MT and SHP genes are targets of GSPE action in hepatic cells *in vivo*. But this behaviour of MT and SHP genes in hepatic cells *in vivo* does not, however, allow us to conclude whether procyanidins themselves directly trigger this transcriptional response in hepatic cells or, either, this response is caused by procyanidins metabolites produced during digestion in the intestinal tract, or even whether it is an indirect response of the liver to the action of procyanidins or procyanidins metabolites on other tissues, such as the intestinal or the adipose tissue.

Modulation of basal MT and SHP expression by GSPE in cultured human hepatocytes.

In order to asses whether procyanidins may act directly on hepatic MT and SHP expression, excluding the intervention of other tissues, we next tested the effect of GSPE administration on human cultured hepatic cells of the HepG2 cell line. GSPE were added to a standard culture medium (5μM zinc) at different concentrations and cells were collected for analysis at different time intervals. The relative changes in mRNA levels of metallothionein genes MT1A, MT1B, MT1E, MT1F, MT1G, MT1H, MT1X and MT2A and SHP were analyzed by quantitative PCR. The results show (Figure IV.1.2), that the basal expression of metallothionein genes in HepG2 cells is inhibited by the addition of GSPE to the culture

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medium in a dose and time dependent manner. In a first set of experiments (Figure IV.1.2A) we determined the effect of three different concentrations of GSPE in the culture medium, namely 15 mg/L (low dose), 75 mg/L (moderate) and 150 mg/L (high) on the basal expression of MT1A, MT1X, and MT2A, at 9 and 12 hours after GSPE addition. These doses of GSPE have been previously shown in our laboratory to be non toxic for HepG2 as measured by LDH leakage and Alamar Blue quantification (Puiggros et al., 2005; Del Bas et al., 2008). Whereas the low GSPE dose has no detectable effect, the medium and high GSPE doses markedly inhibit basal expression of these MT genes. At each time point tested, the effect of the high GSPE dose is greater than the effect of the medium dose. The higher GSPE dose repressed the expression of MT1X, MT1A and MT2A to 25%, 30% and 63% after 12 hours of treatment. These results indicate that the mechanism/s that mediate inhibition of basal MT expression by GSPE are not saturated at the medium (75 mg/L) dose.

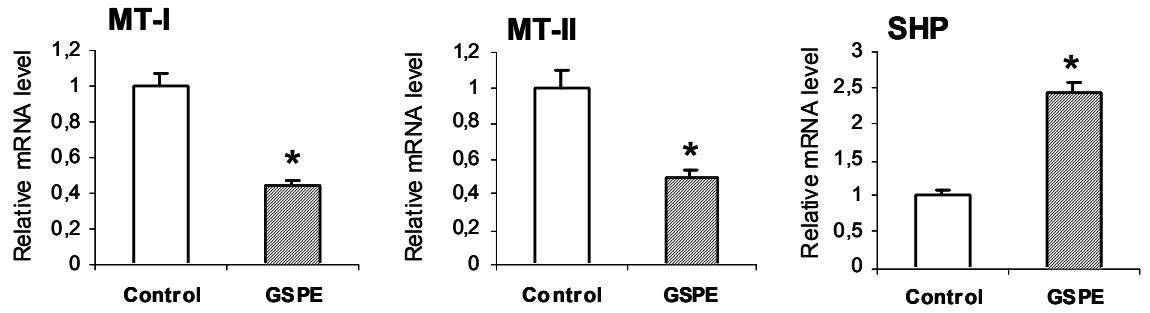

Figure IV.1.1. Modulation of MT and SHP expression by GSPE in the liver of healthy rats in postprandial phase. A single oral dose of GSPE (250 mg/Kg body weight) was administered to healthy rats. Control rats were administered only vehicle (distilled water). 5 hours after GSPE administration, animals were sacrificed; immediately afterwards slices of liver were frozen in liquid nitrogen. Total liver RNA was extracted and changes in MT and SHP gene expression were assessed by RT-PCR. GAPDH was used as reference gene. Values are mean ± SEM of three different biological samples. *Significant differences (P< 0.01) versus control value were determined using a Student's *t* test.

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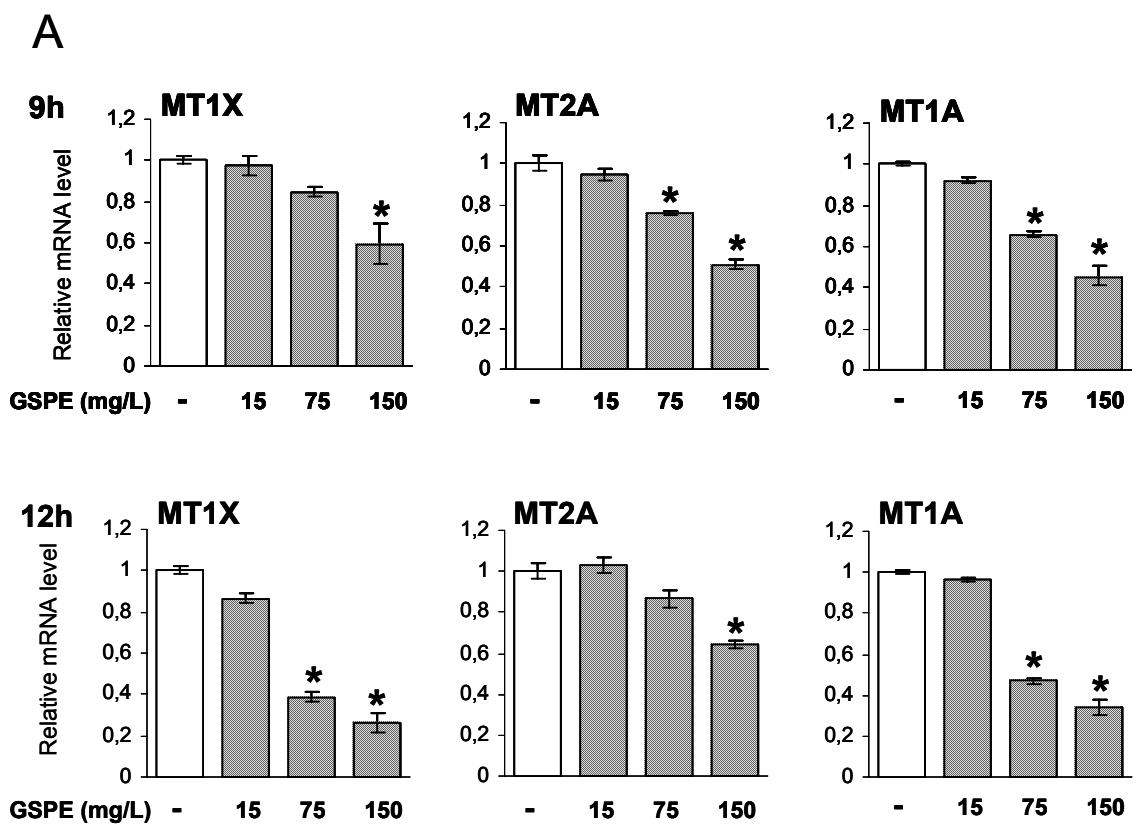

Figure IV.1.2. Time and dose dependent inhibition of MT genes expression by GSPE in HepG2 cells (A). Effect of different doses of GSPE on MT1A, MT1X and MT2A mRNA levels in HepG2 cells. HepG2 cells were treated with either 0, 15, 75 or 150 mg/L GSPE. Samples were collected at indicated periods of time. Total RNA was purified from the cells and changes in MT1A, MT1X and MT2A gene expression were assessed by RT-PCR. Cyclophilin was used as reference gene. Experimental values are the means ±SEM of three different experiments in triplicate. *Significant differences ($P<0.05$) by the Student's t test.

Next, we used the higher non-toxic dose of GSPE to monitor the time course of inhibition of MT expression over longer time periods (from 3 to 24 hours); five additional MT genes (MT1B, MT1E, MT1F, MT1G and MT1H) were included in this experiment (Figure IV.1.2B). The relative levels of mRNA of the different MT genes, at the beginning of the experiments, taking the mRNA of cyclophilin A (PPIA) as control, were as follows: PPIA >>MT2A> MT1G >MT1X >MT1F >>MT1A> MT1E>>> MT1B and MT1H. The mRNA levels of MT1B and MT1H were too low in HepG2 cells to quantify their levels with confidence. All genes were strongly downregulated between 9 and 12 hours of GSPE treatment. Thereafter, 24 hours

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after GSPE addition, only MT1A, MT1X and MT1G remain downregulated, whereas, in contrast, downregulation of mRNA levels of MT1E, MT1F and MT2A was transient and turned back to control levels after 24 hours of GSPE treatment. Conversely, SHP expression was increased, reaching the maximum values 12 hours after GSPE treatment (Figure IV.1.3).

These experiments clearly show that, in cultured human hepatocytes, basal expression of all 6 human MT genes tested, expressed as their levels of mRNA, is inhibited by procyanidins in a time and dose dependent manner. On the contrary, a time dependent upregulation of SHP expression was observed. Thus, there is not necessary the intervention of extrahepatic tissues to explain the observed modulation of MT and SHP expression *in vivo* in the liver of rats. Therefore, we may consider metallothionein and SHP genes as bona fide procyanidin target genes in hepatic cells, both *in vivo* and *in vitro*.

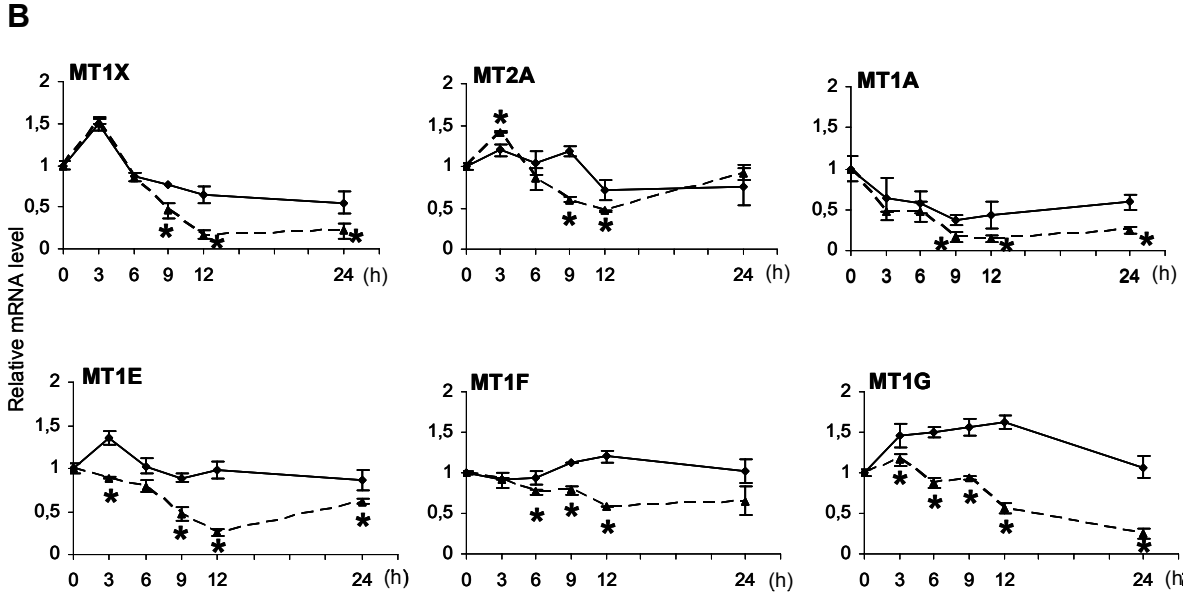

Figure IV.1.2. Time and dose dependent inhibition of MT genes expression by GSPE in HepG2 cells (B). Time course effect of GSPE administration on expression of MT1X, MT2A, MT1A, MT1E, MT1F, MT1G in HepG2 cells. HepG2 cells were treated with either vehicle (control cells) or 150 mg/L GSPE, and collected at the indicated times. Total RNA was extracted from the cells and changes in mRNA levels of MT1X, MT2A, MT1A, MT1E, MT1F, MT1G were determined as in Fig. IV.1.2A. Results are shown as means ±SEM of 3 different experiments in triplicate. *Significant differences ($P<0.05$) by the Student's t test

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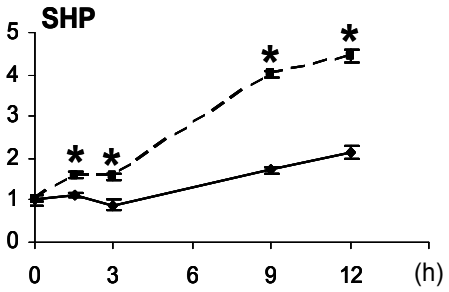

Figure IV.1.3. Time dependent upregulation of SHP expression by GSPE in HepG2 cells. Time course effect of GSPE administration on SHP expression in HepG2 cells. HepG2 cells were treated with either vehicle (control cells) or 150 mg/L GSPE, and collected at the indicated times. Total RNA was extracted from the cells and changes in mRNA levels of SHP were determined as in Fig. IV.1.2A. Results are shown as means ±SEM of 3 different experiments in triplicate. *Significant differences ($P<0.05$) by the Student's t test.

Inhibition of induced MT expression by GSPE in cultured human hepatocytes.

Next, we tested whether GSPE could inhibit the activation of MT transcription when induced by different agents. The aim of these set of experiments was to identify which of the different pathways that control MT expression at the transcriptional level are the target of GSPE. We tested five known inducers of MT transcription, which act through different although interconnected signaling pathways, namely zinc(II) cations (Zn), copper(II) cations (Cu), the glucocorticoid analogue dexamethasone (Dex), the alkoxyl and peroxyl radical generator tertbutyl-hydroperoxide (tBOOH), and the pro-inflammatory cytokine interleukin 6 (IL-6). As expected, all five inducers triggered a marked increase in MT1X and MT2A mRNA levels in the absence of procyanidins, with the only exception of the dexamethasone treatment which did not significantly induce the expression of MT1X (Figure IV.1.4), which has not a glucocorticoid receptor element in its promoter. GSPE added to the cultures blocked the induction by all 5 inducers to various extents, ranging from a 22 % inhibition of the induction triggered by tBOOH on MT2A to a complete inhibition of the induction elicited by IL-6 on MT1X. Except for Dex, inducers were more effective in inducing MT1X than MT2A, and so it was the repressive effect of GSPE.

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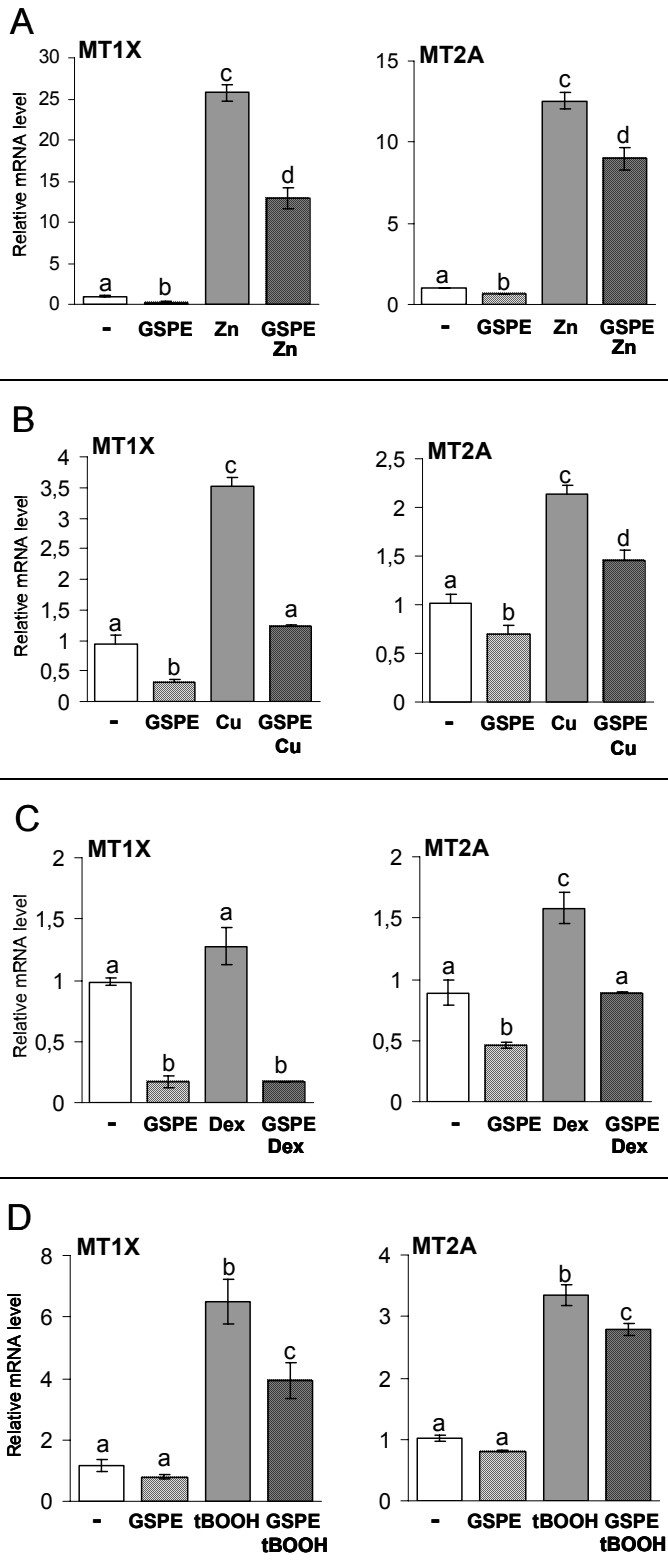

Figure IV.1.4. Inhibition by GSPE of induced-MT expression in HepG2 cells. See legend in next page.

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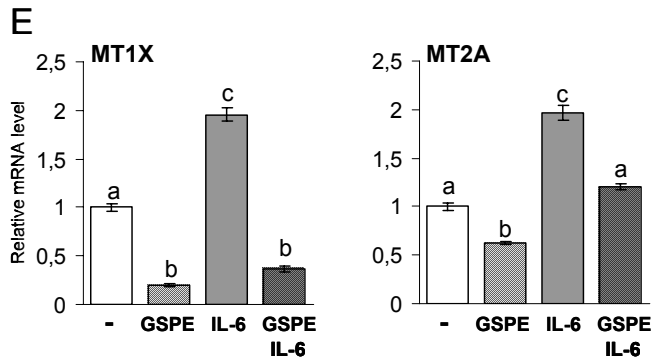

Figure IV.1.4. Inhibition by GSPE of induced-MT expression in HepG2 cells. Either vehicle (control), GSPE (150 mg/L) or inducers were added to HepG2 cells. Samples were collected for analysis at indicated times, as described in *Materials and Methods*. Inducers and time of treatment were: (A) 12 hours with 100 μMCl_2Zn; (B) 15 hours with 50μM Cl_2Cu; (C) 12 hours with 10μM Dex; (D) 7 hours with 200μM tBOOH; (E) 15 hours with 1000 U/ml IL-6. Total RNA was extracted from the cells and mRNA levels of MT1X and MT2A, relative to mRNA levels of Ciclophylin, were determined as described in Fig. IV.1.2. Experimental values are the means ± SEM of three different biological samples in triplicate. Significant differences ($P<0.05$) were obtained with ANOVA

Elucidation of the mechanisms by which MT is repressed by GSPE both *in vivo* and *in vitro.*

The lack of specificity of procyanidins in repressing the induced expression of MT genes in HepG2, points out to a common underlying mechanism which interferes with the different signal transduction pathways used by inducers. This basic mechanism used by GSPE to repress MT expression is also operative in basal conditions, i.e., in the absence of added inducers (supplementary zinc, copper, IL-6, dexamethasone and tBOOH), as evidence by the repression of basal MT expression in HepG2. Low amounts of zinc in the culture medium, and endogenously generated physiological levels of ROS are, however, always present in basal conditions.

Current knowledge about the control of MT genes expression allows us to postulate different mechanisms that could bring about repression of MT by GSPE in basal conditions (see Introduction, section I.2: 1. Repression through chromatin modification (epigenetic changes) and 2) diminished zinc availability.

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1) Epigenetic changes

The aim of this set of experiments was to know weather the repression of MT genes by GSPE could be due to chromatin modifications performed by the intervention of SHP. In support of this possibility, we have previously shown that GSPE induces, in the postprandial phase, liver expression of the orphan nuclear receptor Small Heterodimer Partner (SHP/NR0B2) (Del Bas et al., 2005; Del Bas et al., 2008) a promiscuous transcriptional repressor that have been shown to recruit histone deacetylases, histone methyltranferases and DNA methyltranferases to the promoters of several of their target genes (Boulias and Talianidis, 2004; Bavner et al., 2005; Gobinet et al., 2005; Fang et al., 2007a; Fang et al., 2007b), a mechanism that is coincident with the known silencing of MT expression in diverse hepatocarcinoma cell lines (Majumder et al., 1999a; Majumder et al., 1999b; Ghoshal et al., 2000; Ghoshal and Jacob, 2001; Ghoshal et al., 2002; Majumder et al., 2002; Datta et al., 2005; Majumder et al., 2006). Also, as shown above, GSPE upregulates SHP concomitantly with the downregulation of MT genes in HepG2 cells. SHP expression in HepG2 cells treated with GSPE reached a 2 fold overexpression compared to control cells (see Figure IV.1.3) after 12 hours of treatment, at a moment in which all MT genes are strongly downregulated (Figure IV.1.5).

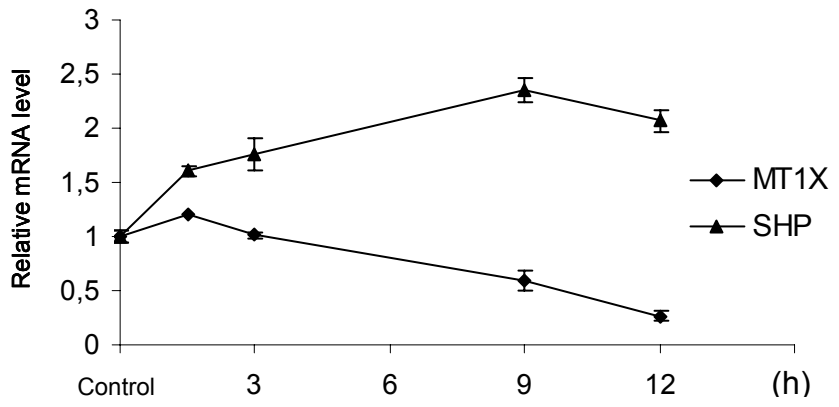

Figure IV.1.5. GSPE downregulates MT1X and upregulates SHP expression in HepG2 cells. HepG2 cells were treated with either vehicle (Control) or GSPE (150 mg/L) during 12 hours. Samples were collected at indicated periods of time. Total RNA was extracted from the cells and changes in mRNA levels of MT1X and SHP were determined by RT-PCR. Cyclophilin was taken as the control gene.

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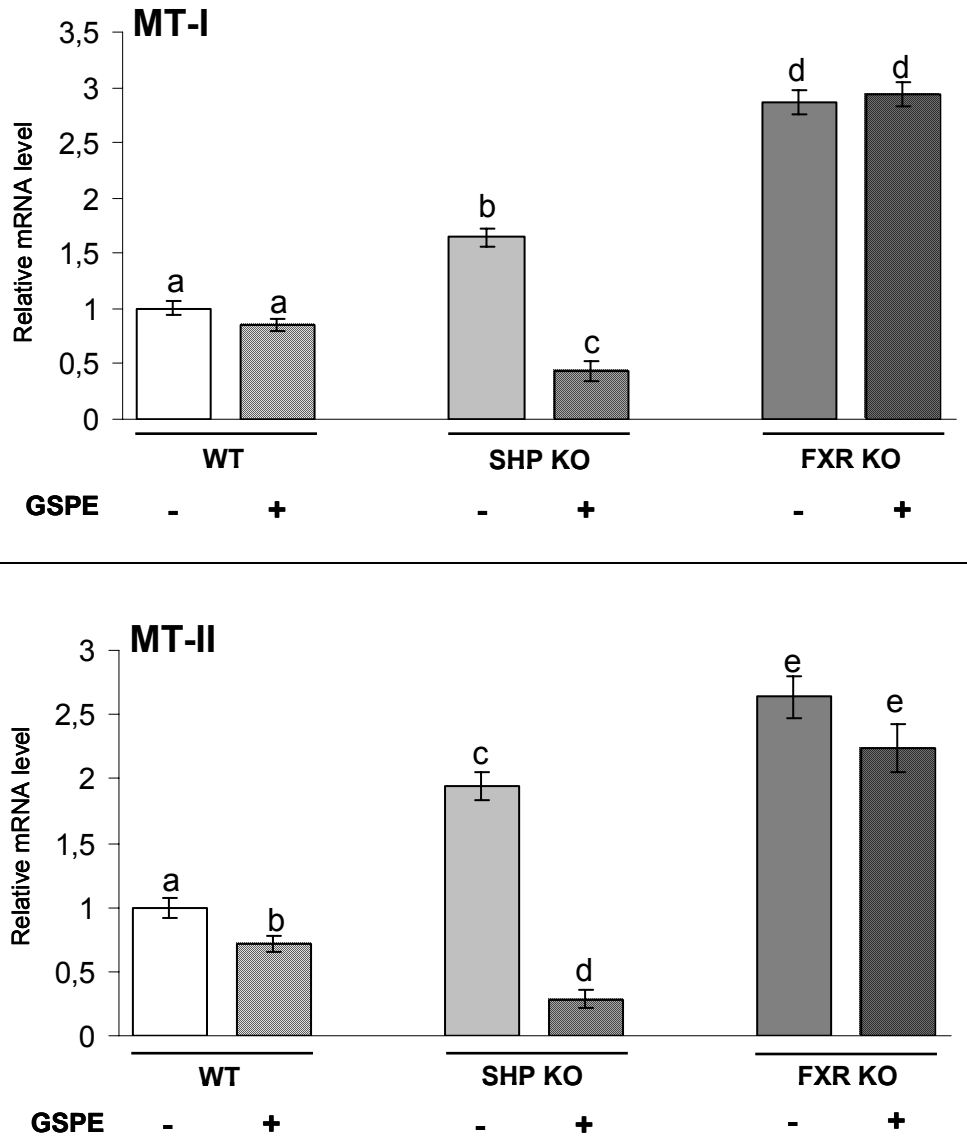

Figure IV.1.6. Effect of GSPE on hepatic MT-I and MT-II expression in wild-type, SHP and FXR -/- mice. Wild type (WT), SHP and FXR KO mice were fed with vehicle (control) or GSPE (250 mg/Kg) via oral gavage (n=5 in each group, age 8-10 week). Total liver RNA was extracted and changes in MT-I and MT-II gene expression were assessed by RT-PCR. GAPDH was used as reference gene. Significant differences ($P<0.05$) were obtained with ANOVA.

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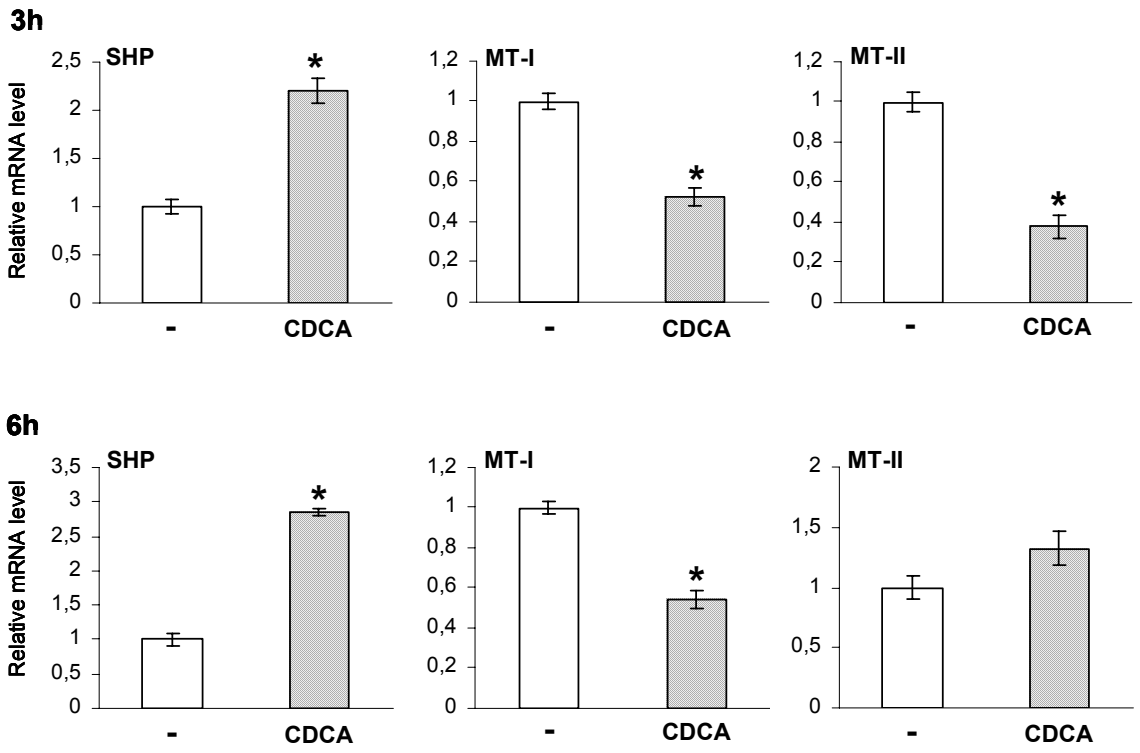

Figure IV.1.7. Effect of CDCA on SHP and MT expression in HepG2 cells. HepG2 cells were incubated with 100 μM CDCA. After 3 and 6 hours of treatment, total RNA was obtained and expression levels of SHP, MT-I and MT-II were determined as in figure IV.1.2. Actib B was taken as the control gene. Values are the mean +/- SEM of three independent experiments. *Significant differences (P<0.05) by the Student's t test.

Effects of GSPE on liver MT-I and MT-II expression in SHP-null and FXR-null mice.

So far, there are no reporters about the relationship between MT and SHP and FXR. Given the potentiential of SHP to act as transcriptional correpresor of its taget genes, and given that FXR controls the expression of SHP and is co-activated by procyanidins, we aim to asses whether MT repression by GSPE in hepatic cells might be mediated by SHP and/or FXR. To adress this point, we took advantage of the experiments performed in our group by Dr. J.M. Del Bas with SHP-null and FXR-null mice (Del Bas et al., 2008; Del Bas et al., 2009). First, we analyzed hepatic MT-I and MT-II expression after oral gavage of GSPE in WT mice (Figure IV.1.6). In wild type mice, oral GSPE gavage downregulates MT-II expression, while it does not affect MT-I gene. In SHP-null and FXR-null mice, MT-I and MT-II were highly

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overexpressed compared with wild type mice, indicationg that, in basal conditions, both SHP and FXR are implicated, directly or indirectly in the control of basal levels of MT expression. When SHP KO mice were treated with GSPE, both MT genes were downregulated even more strongly than in WT mice suggesting that repression of MT expression by GSPE is independent of SHP. However, administration of GSPE to FXR KO animals produced no change in MTs expression, indicating that the repression of MT expression by GSPE in the liver of healthy mice follows an FXR-dependent pathway.

The bile acid CDCA downregulates SHP and upregulates MTs gene expression in HepG2 cells.

In addition, chenodeoxycholic acid (CDCA), which induces the expression of SHP by activating the transcriptional activity of the bile acid receptor Farnesoid X Receptor (FXR), repressed the expression of MT genes concomitantly with the induction of SHP in HepG2 cells (Figure IV.1.7). This result supports the concept that FXR acts, directly or indirecty, as a transcriptional repressor of MT genes in hepatic cells.

To conclude, these results indicate that 1, expression of MT genes is kept to basal levels in WT hepatic cells with the intervention of both FXR and SHP; 2, GSPE represses the expression of MT genes in hepatic cells through a mechanism which is SHP-independent, and hence probably independent of recruitment of hitone deacetylases, histone methyltranferases or DNA methyltranferases to the promoter of MTs genes; and 3, GSPE represses the expression of MT genes in hepatic cells in using a pathway that depends on the transcriptional activity of FXR. Hence MT genes are revealed as novel target genes of FXX and FXR-ligands and, consequently, FXR is revealed as a nuclear receptor involved in the control MT expression and thereby in the control of cellular zinc homeostasis.

However, these results are do not discard the option that diminished zinc availability contributes to downregulation of MT expression by GSPE.

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2) Diminished zinc availability

An alternative, though not excludent, explanation for the repression of MT expression in HepG2 in basal conditions is that GSPE diminish the availability of zinc ions to the cells. Flavonoids have been widely reported to chelate metals (Hider et al., 2001), especially redox active metals such as iron and copper (Hider et al., 2001; de Souza and De Giovani, 2005; Scalbert et al., 2005) and make them less bioavailable to the cell. But little is known about the biological effects of chelation of flavonoids with redox inactive metals such as zinc (Chen et al., 2007; Sun et al., 2008). To address this issue, we first analyzed the interaction in buffered solutions of several catechins and procyanidins with zinc cations using several techniques.

UV-ViS Spectra of Flavonoid-Zn(II) complexes

If flavonoids indeed react with metals, the absorption spectrum of flavonoids would change after the addition of metal ions in the solution. Thus, we monitored the modification of the UV-Vis absorption spectrum of (+)-catechin (C), (-)-epicatechin (EC), (-)-epicatechin gallate (ECG), EGCG, dimeric procyanidins B1 and B3, the procyanidin trimer C1 and GSPE immediately after and 20 hours after the addition of Zn(II) to a solution containing the flavonoids. Based on the previously described 2:1 stoichiometry in flavonoid-Fe(II) complexes (Kuo et al., 1998), the concentration of flavonoids was set at 10 μM and that of metal was 5 μM (Figure IV.1.8). We first monitored the changes in UV-ViS spectra of the flavonoids immediatly after addition of Zn(II) to the solution (Figure IV.1.8A). Among the flavonoids tested, (+)-catechin, dimers B1, B3 and trimer C1 did not change their spectral properties. On the other hand, EGCG and GSPE showed the major changes upon addition of Zn(II) to the solution. We also performed the UV-ViS spectra of these flavonoids in the absence and presence of Zn(II) after overnight incubation in the dark (Figure IV.1.8B). Only EC and dimer B3 did not show any changes. On prolonged incubation of flavonoids in the buffer all of them changed their spectra but EGCG and GSPE exhibited the most important changes. EC, ECG, B1, B3 and C1 were found to be more stable compared with EGCG and

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GSPE, since their spectra showed little changes after overnight incubation in the absence of metal. Table IV.1.1 summarize the changes in the UV-ViS spectra of the flavonoids tested.

To conclude, the observed changes in the absorption spectrum of flavonodis upon addition of Zn(II), implies that the flavonoids interact and form estable complexes with zinc cations when they are free in solution, in the conditions of neutral pH and the concentrations tested.

Fluorescence Spectra of Flavonoid- Zn(II) interaction.

Many metal–flavonoid complexes have been synthesized and characterized in the past several years. Elemental and thermal analyses, conductivity and cyclic voltammetry, as well as IR, Raman, 1H-NMR, 13C-NMR, UV–Vis and fluorescence spectroscopy have been used to asses relevant interactions of flavonoids and metal ions (Malesev, 2007). If flavonoids could change the absorption spectra by addition of zinc cations, they could also be able to affect their fluorescence spectra. So we performed a fluorescence spectra of monomer EGCG, dimer B1, trimer C1 and GSPE before and after addition 100 μM of Zn(II) at excitation wavelenghts of 230 and 260nm (Figure IV.1.9). The emmision was recorded from 240 to 600nm. The concentrations used for GSPE was 75 mg/L and for EGCG, B1 and C1 were 1 μM. For all flavonoids tested, their fluorescence spectra changed after addition of Zn(II). Thus, we can confirm, by another spectroscopic analysis, the interaction of these flavonoids with zinc ions in solution.

	no Zn(II)	Zn(II) added	
Compound	**Spectrum change 20h later**	**Spectrum change at 0h**	**Spectrum change 20h later**
(+)-Catechin	**+**	**-**	**-**
(-)-Epicatechin	**+**	**+**	**-?**
(-)-Epicatechin Gallate	**+**	**+**	**+**
Epigallocatechin Gallate	**+**	**+**	**+**
B1	**+**	**-**	**+**
B3	**+**	**-**	**-**
C1	**+**	**-**	**+**
GSPE	**+**	**+**	**+?**

Table IV.1.1. Summary of changes in UV-ViS Spectra of flavonoid and flavonoid-metal solutions in PBS.

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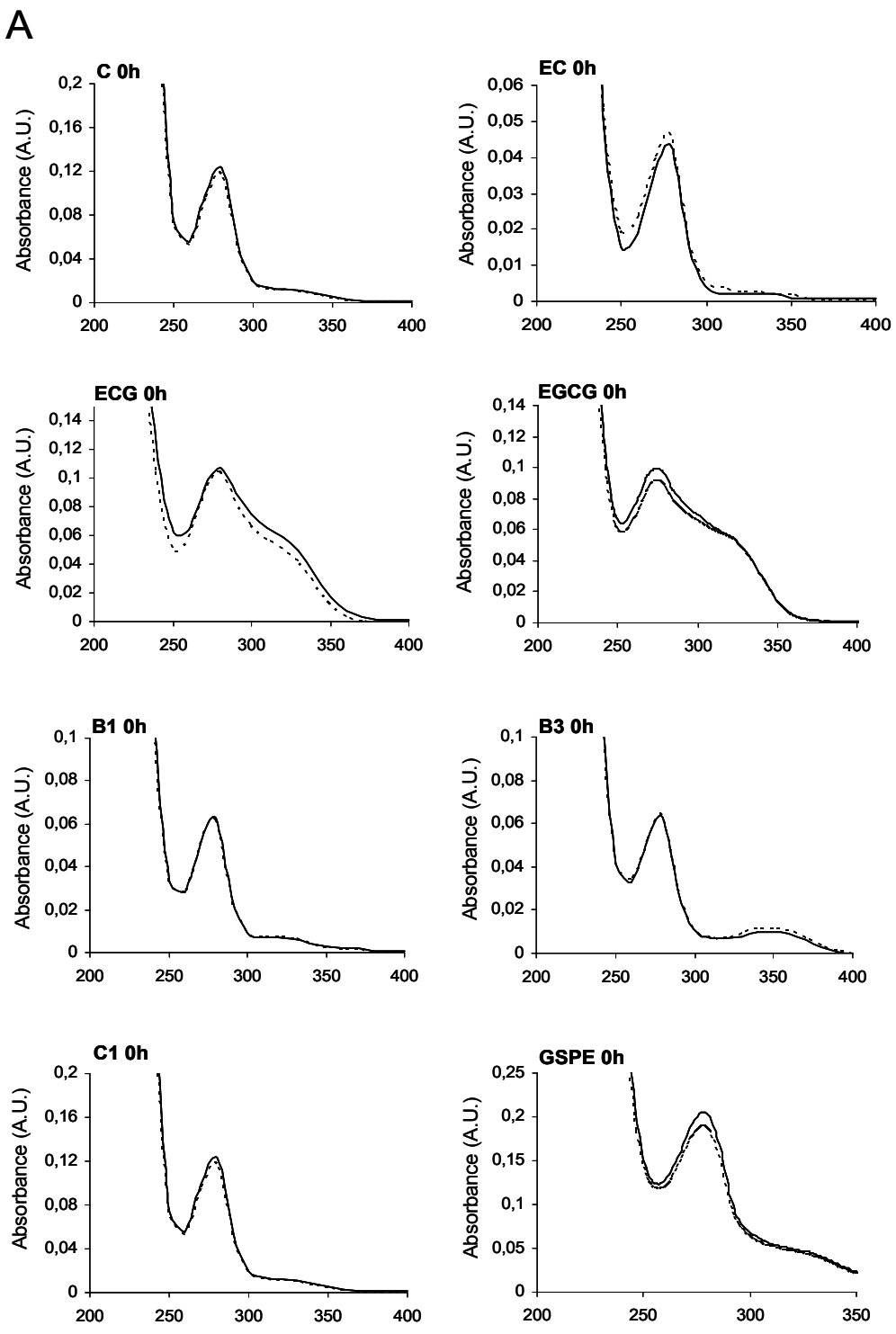

Figure IV.1.8. UV-Vis absorption spectra of different flavonoids coexisted in solution with Zn(II). A. After short time of incubation. Continuous lines represent the UV-Vis spectra of 10 μM flavonoid dissolved in PBS, recorded 5 min after the substances have been added to the buffer. Dashed lines represent the corresponding spectra when the substances were dissolved in PBS containing 5 μM Zn(II). AU, arbitrary units.

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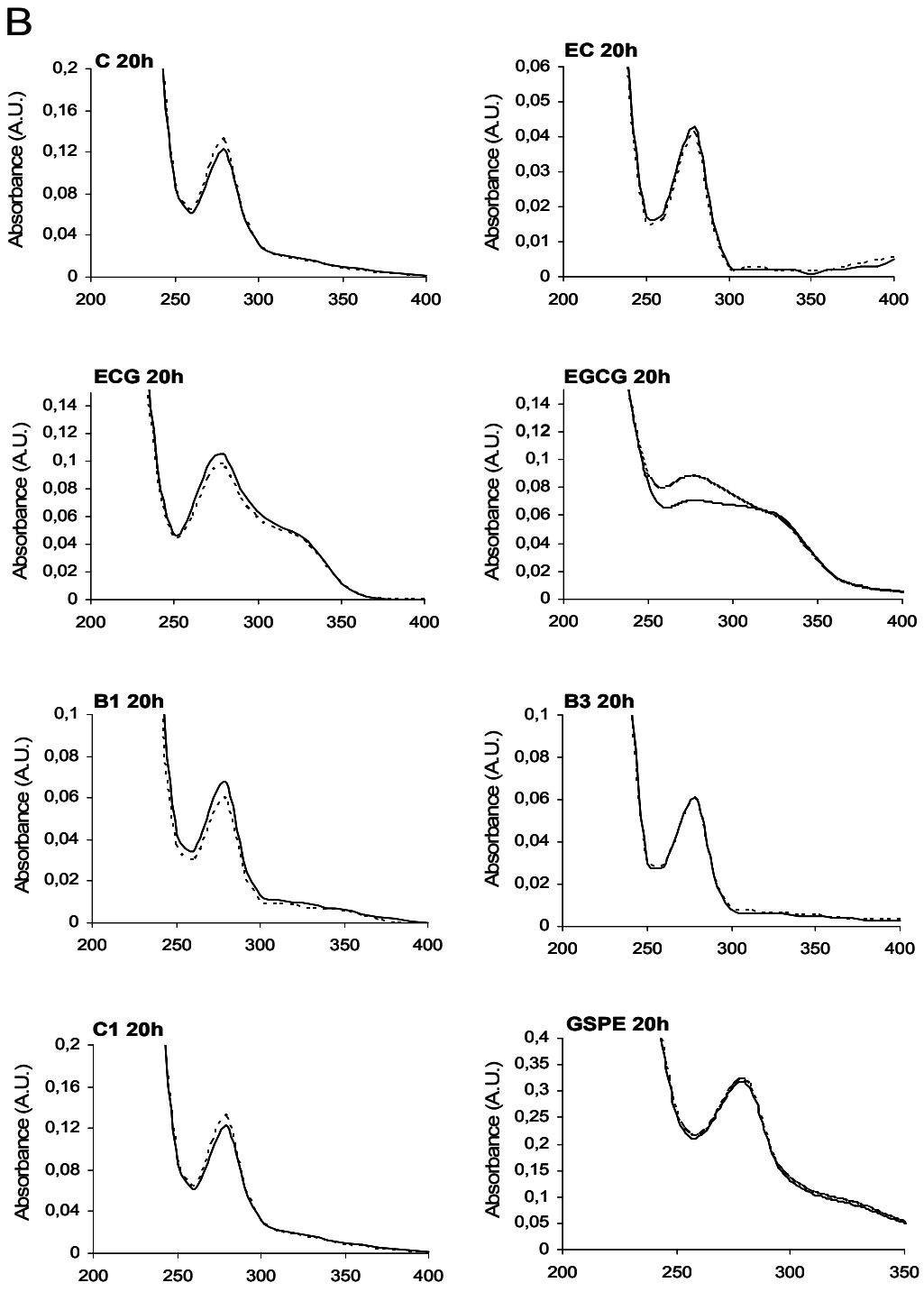

Figure IV.1.8. UV-Vis absorption spectra of different flavonoids coexisted in solution with Zn(II). B. After overnight incubation. Continuous lines represent the UV-Vis spectra of 10 μM flavonoid dissolved in PBS, recorded 20 hours after the substances have been added to the buffer. Dashed lines represent the corresponding spectra when the substances were dissolved in PBS containing 5 μM Zn(II). AU, arbitrary units.

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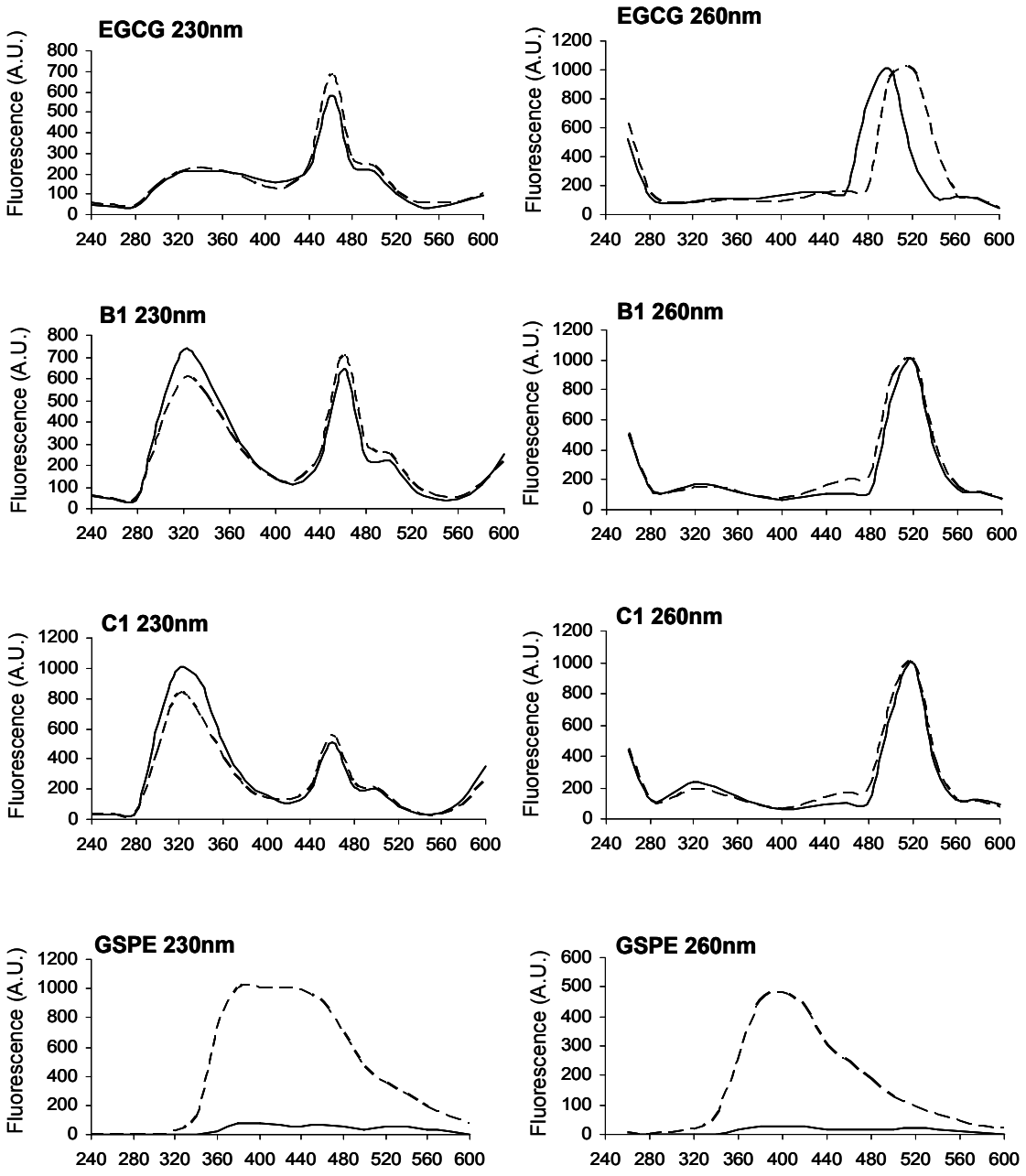

Figure IV.1.9. Fluorescence spectra of different flavonoids coexisted in solution with Zn(II). Continuous lines represent the fluorescence spectra of flavonoid (75mg/L GSPE and 1 μM for EGCG, B1 and C1) dissolved in Tris 50mM NaOH 0.1M pH 7.4, recorded 5 min after the substances have been added to the buffer. Dashed lines represent the corresponding spectra when the substances were dissolved in Tris 50mM NaOH 0.1M pH 7.4 containing 100 μM Zn(II). AU, arbitrary units.

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Ability of catechins and procyanidins to compete with Zinquin ethyl ester

If flavonoids can chelate metals as indicated above, they should be able to prevent the interaction of metal with other organic compounds. Zinquin ethyl ester, a zinc-specific fluorescent chelator widely employed to measure concentrations of labile (free plus loosely bound) zinc within cells and in biological fluids (Coyle et al., 1994; Devergnas et al., 2004; Zalewski et al., 2006) was used to demonstrate this hypothesis. We used this assay as an alternative method to test the ability of flavonoids to chelate Zn(II) in solution. First, we performed a titriation curve to asses the range of Zn(II) concentrations in which Zinquin fluorescence is proportional to Zn(II) concentration, i.e, is not saturated by zinc cations. In Figure IV.1.10, fluorescence of a 10 μM solution of Zinquin in buffer Tris 50mM NaOH 0.1M pH 7.4 increased linearly from 0 nM free Zn(II) (added in the form of $ZnCl_2$) up to 3 μM Zn(II), and was insensitive to Zn(II) concentrations □ 3 μM. Fluorescence was enhanced 180-fold by de addition of 1 μM Zn(II) to the buffer containing 10 μM Zinquin. This results indicates that Zinquin ethyl ester forms 3:1 Zinquin-zinc complexes in the conditions tested.

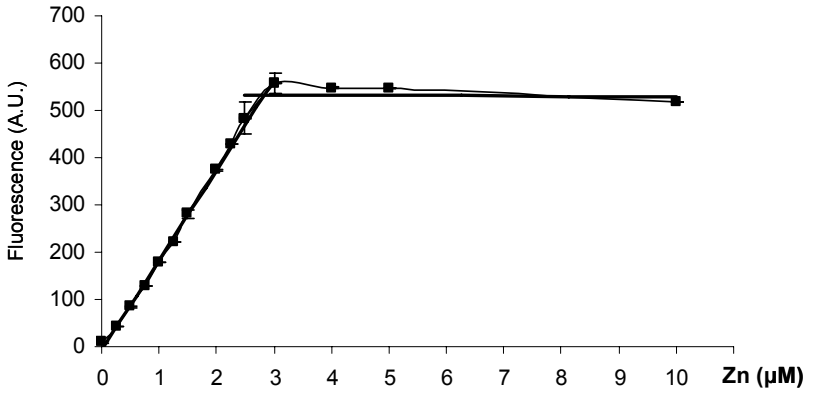

Figure IV.1.10. Calibration of zinc-dependent Zinquin fluorescence in buffered solution. 10 μM Zinquin ethyl ester solution in Tris 50mM NaOH 0.1M pH 7.4 buffer was tritated with different concentrations of Zn(II). Fluorescence signal was quantified at 365-370nm excitation and 485-490 emission at 25ºC. Zinc-dependent Zinquin fluorescence was monitored 5 minutes after addition of Zn(II). Values are direct readings of fluorescence signal. Values are the mean +/- SEM of three independent experiments.

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Next, we tested the ability of GSPE, catechin (C), epicatechin (EC), epicatechin gallate (ECG), EGCG, dimeric procyanidins B1, B2, B3 and B4 and the procyanidin trimer C1 to quench the zinc-dependent fluorescence of Zinquin. GSPE and the individual flavonoids quenched zinc-dependent Zinquin fluorescence rapidly and dose-dependently, with higher efficacy than the zinc chelator TPEN. Fluorescence quenching was evident at flavonoid to Zinquin ratios of 10:25 and 100 μM zinc and even at 0.1:10 flavonoid to Zinquin ratio in the presence of 1 μM zinc, for both GSPE and EGCG (Fig IV.1.11). These results imply that these flavonoids bind zinc cations in solution with enough affinity to cause its displacement from Zinquin-zinc complexes.

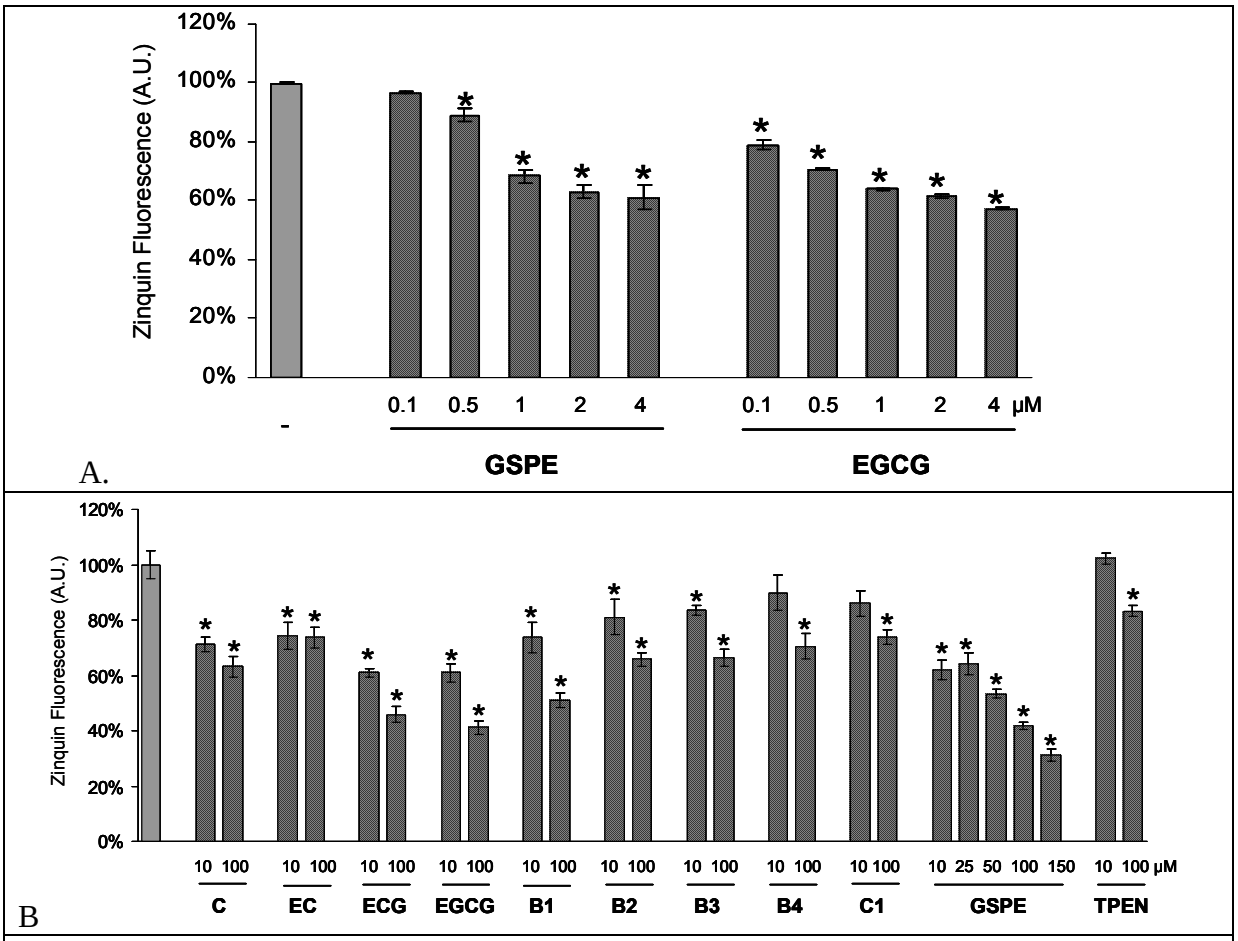

Figure IV.1.11. Quenching of zinc-dependent Zinquin fluorescence by catechins and procyanidins in solution. Fluorescent emission of zinc-Zinquin complexes formed in 50 mM Tris-HCl buffer, pH 7.4, 25 ºC, at 10 μM Zinquin and 1μM $ZnCl_2$ (A) or 25 μM Zinquin and 100 μM $ZnCl_2$ (B) was measured before (control values, 100 arbitrary units of fluorescence) and 5 minutes after addition of different catechins and procyanidins at the indicated concentrations. Asterisks indicate significant difference ($P<0.05$) versus control sample (left gray bar) using independent samples t test.

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Subsequently, in order to discern which of the fractions contained in GSPE was more effective in interacting with zinc ions, we tested different fractions of GSPE obtained by HPLC (Terra et al., 2007). The results showed that all fractions tested were able to remove zinc ions to Zinquin, being trimers and oligomers longer than trimers the most effective zinc chelators. The effectivness of GSPE at the same concentrations used in fractions (25mg/L) was between fraction II and VI (monomers and dimers) (Figure IV.1.12A). When we used the theorical concentrations of fractions that are contained in 150mg/L GSPE (Figure IV.1.12B), also trimers and oligomers were more effective and GSPE 150mg/L had the same effectiveness as trimers and oligomers implying that, in GSPE, trimeric procyanidins have the major effects in quenching zinc-dependent Zinquin fluorescence in the conditions tested.

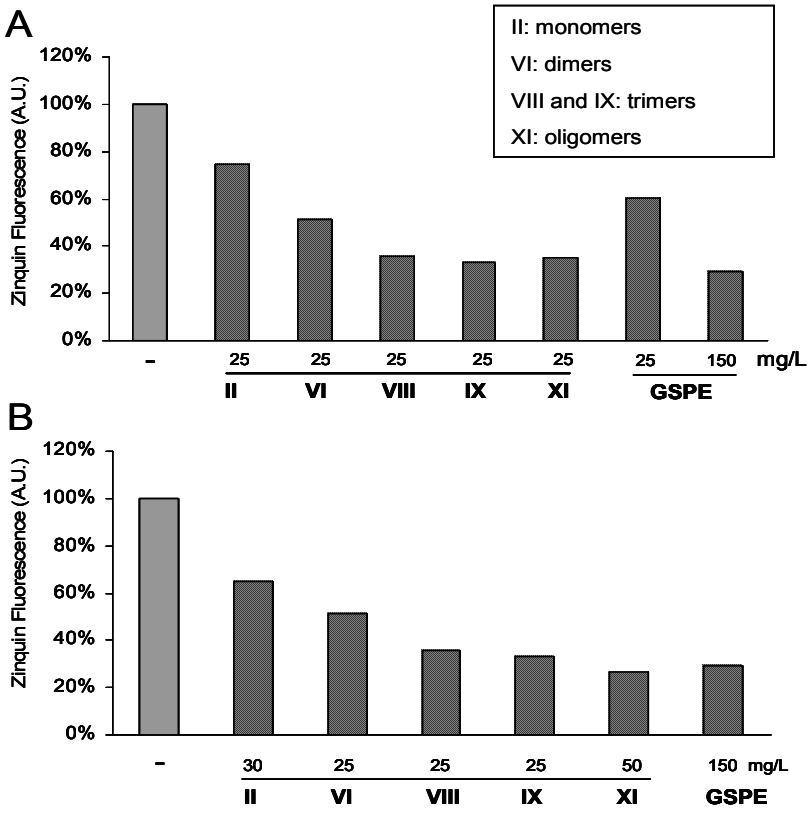

Figure IV.1.12. Quenching by fractions contained in GSPE.of zinc-dependent Zinquin fluorescence in solution. Fluorescent emission of zinc-Zinquin ethyl ester complexes formed in 50 mM Tris-HCl buffer, pH 7.4, 25 ºC, at 25 µM Zinquin and 100 µM $ZnCl_2$ was measured before (control values, 100 arbitrary units of fluorescence) and 5 minutes after addition of different fractions and GSPE at the indicated molar concentrations.

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Knowing that GSPE has enough affinity to remove zinc ions to Zinquin-Zn(II) complexes we wanted to know if GSPE was also able to remove zinc ions bound to TPEN, a specific zinc chelator with a dissociation constant of 0.26 fM for zinc. As shown in figure IV.1.13A, 100μM TPEN was able to completly remove zinc ions to Zinquin-Zn(II) complexes (10:1 ratio). However, after addition of GSPE to that mixture, it was capable to remove some zinc ions chelated by TPEN and dose dependently. (Figure IV.1.13B)

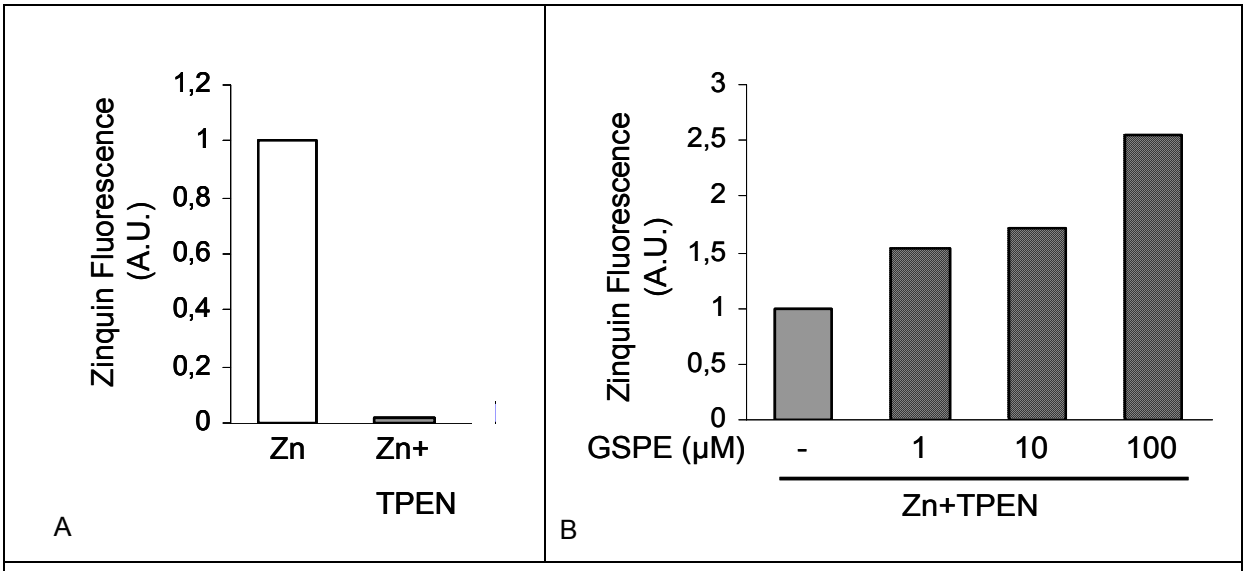

Figure IV.1.13. Competition between GSPE and TPEN for the quenching of zinc-dependent fluorescence of Zinquin in solution. Reaction conditions were as in Figure IV.1.12.

Taken together, these results demonstrate the interaction between different flavonoids and zinc metal ion using different techinques. The flavonoids tested, at concentration which are physiologically relevant, display eneogh affinity for Zn cations to displace them from Zinquin-Zn and TPEN-Zn complexes, implicating that chelation of Zn(II) by these flavonoids may be relevant *in vivo*, and in cultured cells. The metal-flavonoid interaction could affect the bioavailability of both flavonoid and zinc ions, either preventing or enhancing their entrance into the cells, and thereby affect zinc homeostasis.

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Modulation of basal expression of zinc and copper transporters by GSPE in HepG2 cells.

In order to asses whether the ability of catechins and procyanidins to complex with zinc may affect cellular zinc homeostasis, we first monitored changes in the expression profile of genes involved in zinc transport and storage in HepG2 cells grown 12h in standard culture medium (5 μM zinc) supplemented with 150mg/L GSPE, using oligonucleotide microarray hybridization. Table IV.1.2 shows the fold change in mRNA levels triggered by GSPE treatment on 24 known human genes encoding zinc transporters, 10 members of the ZnT/SLC30/CDF family and 14 members of the ZIP/SLC39A family. There are at least 10 ZnT and 15 ZIP transporters in humans cells (Cousins et al., 2006; Lichten and Cousins, 2009). Probes for ZIP15 are absent from the microarray. Some genes are represented by more than one probe in the microarrays. Each mean fold change is the mean of the two dye-swap hybridization for all probes and has been considered valid only when the two hybridizations gave consistent results, i.e., the values were up or down regulated in both assays. Among the zinc transporters of the ZnT family (efflux of zinc from the cytoplasm) only ZnT1 and ZnT10 (extrusion of zinc out of the cell) were clearly downregulated, whereas the expression of ZnT5 and ZnT7 (uptake of zinc into the Golgi network) were upregulated. Regarding the ZIP family of zinc transporters (import of zinc into the cytoplasm), mRNAs levels of ZIP1, ZIP4, ZIP6, ZIP10, and ZIP13 (located in the plasma membrane) became elevated upon GSPE treatment whereas ZIP3, ZIP5, ZIP7 (which extrude zinc form the Golgi apparatus) and ZIP11 (unknown location) were downregulated. GCLC and GCLM, which encode the catalytic and regulatory subunit, respectively, of the rate-limiting enzyme in glutathione biosynthesis, were strongly upregulated by GSPE treatment. Also albumin and alpha-2-macrogobulin, the main carriers of zinc in plasma, became upregulated by GSPE. Therefore, the expression of the main genes involved in zinc homeostasis is modulated by GSPE.

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Genebank ID	Gene symbol/s; name	Mean Fold Change GSPE *vs.* Control	
		µArray	RT-PCR
Metallothioneins			
NM_005946	MT1A	0.54	0.34
NM_005947	MT1B	0.61	0.26
NM_175617	MT1E	0.45	0.48
NM_005949	MT1F	0.64	
NM_005950	MT1G	0.69	0.34
NM_005951	MT1H	0.74	
NM_175622	MT1J	0.65	
NM_176870	MT1K	0.46	
NR_001447	MT1L	0.77	
NM_005952	MT1X	0.54	0.26
NM_005953	MT2A	0.70	0.64
SLC30A family of zinc transporters (cytoplasmic zinc export)			
NM_021194	SLC30A1 / ZnT1	0.46	0.68
NM_001004434	SLC30A2 / ZnT2	-	
NM_003459	SLC30A3 / ZnT3	1.23	
NM_013309	SLC30A4 / ZnT4	-	
NM_022902	SLC30A5 / ZnT5	1.80	
NM_017964	SLC30A6 / ZnT6	2.09	
NM_133496	SLC30A7 / ZnT7	4.19	
NM_173851	SLC30A8 / ZnT8	-	
NM_006345	SLC30A9 / ZnT9	0.93	
NM_018713	SLC30A10 / ZnT10	0.51	
SLC39A family of zinc transporters (cytoplasmic zinc import)			
NM_014437	SLC39A1 / ZIP1	1.77	2.25
NM_014579	SLC39A2 / ZIP2	-	
NM_144564	SLC39A3 / ZIP3	0.51	
NM_130849	SLC39A4 / ZIP4	1.45	1.60
NM_173596	SLC39A5 / ZIP5	0.61	
NM_012319	SLC39A6 / ZIP6	2.04	
NM_006979	SLC39A7 / ZIP7	0.65	
NM_022154	SLC39A8 / ZIP8	-	
NM_018375	SLC39A9 / ZIP9	1.57	
NM_020342	SLC39A10 / ZIP10	4.97	
NM_139177	SLC39A11 / ZIP11	0.51	
NM_152725	SLC39A12 / ZIP12	-	
NM_152264	SLC39A13 / ZIP13	1.80	
NM_015359	SLC39A14 / ZIP14	1.10	0.90
Glutathione biosynthesis			
NM_001498	GCLC; glutamate-cysteine ligase, catalytic subunit	3.73	3.35
NM_002061	GCLM; glutamate-cysteine ligase, modifier subunit	2.73	
Plasma zinc carriers			
NM_000477	ALB; albumin	2.62	
NM_000014	A2M; alpha-2-macroglobulin	1.34	
Transcription factors			
NM_005955	MTF1; metal-regulatory transcription factor 1	0.98	
Antioxidant enzymes			
NM_000454	SOD1; Cu/Zn-SOD; superoxide dismutase 1, soluble	0.89	
NM_000636	SOD2; Mn-SOD; superoxide dismutase 2, mitochondrial	7.07	
NM_001752	CAT; catalase	0.67	

Table IV.1.2. Effect of GSPE on mRNA levels of genes involved in zinc homeostasis in HepG2 cells. HepG2 cells cultivated in standard culture medium (5 M zinc) were incubated 12 h with 150 mg/L GSPE. Control cells where given only vehicle (final 0.1% ethanol). Total RNA from each group of cells (4 samples per group) were pooled and processed to obtain microarray hybridization data. Mean fold change (MFC) refers to the mRNA levels of each gene in GSPE-treated cells relative to that in untreated cells, and are the mean of two independent hybridizations with dye-swap labeling of RNA samples. - indicates that signal is similar to background fluorescence. MFC in bold characters were obtained by quantitative RT-PCR performed with non-pooled RNA samples.

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Given the inhibition of copper-induced MT transcription by GSPE in HepG2 cells (Figure IV.1.4B), we also monitored the expression of copper-transport and copper chaperones in the same microarray hybridization experiment to get insight into the effect of GSPE into copper status in HepG2. As shown in Table IV.1.3, the expression of the plasma membrane copper transporter gene Ctr2/SLC31A2, is highly upregulated by GSPE. Ctr2 transports copper ions from the extracellular space to the cytoplasm. The transcriptional upregulation of this gene by GSPE strongly suggests that procyanidins diminish copper availability to the cultured cells. To adress this point, we measured intracellular copper content both in basal and copper conditions. In HepG2 cells, Cu treatment increase intracellular copper levels 20-fold compared with untreated cells. In contrast, when GSPE was added to the cells, intracellular copper accumulation was diminished both in basal and copper-stimulated condition (Figure IV.1.14).

SLC31 transporters: copper influx into cytoplasm		Hybridization microarray 1			Hybridization microarray 2 (dye swap)		
Gene Symbol	Gene Bank	**Fold Change GSPE vs. Control**	GSPE Sample Signal	Control Sample Signal	**Fold Change GSPE vs. Control**	GSPE Sample Signal	Control Sample Signal
SLC31A1	**NM_001859**	**0.65**	753	1136	**0.80**	709	895
SLC31A1	**NM_001859**	**0.70**	463	650	**0.99**	567	582
SLC31A1	**NM_001859**	**0.90**	779	858	**0.83**	770	935
SLC31A1	**NM_001859**	**1.04**	1306	1241	**0.72**	932	1312
SLC31A2	**NM_001860**	**2.26**	1649	721	**4.06**	2308	576

Table IV.1.3. Modulation of expression of copper transporters by GSPE in HepG2 cells, assesed by mycroarray hybridization. Same conditions as in Table IV.1.2.

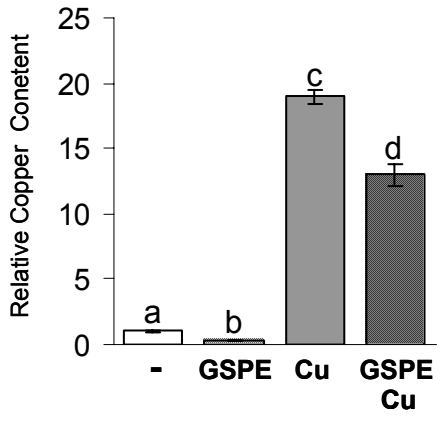

Figure IV.1.14. Effect of GSPE on total intracellular copper content in HepG2 cells. Vehicle (control), GSPE (150 mg/L), Cu (50μM) or both (GSPE Cu) were added to HepG2 cells. Samples were collected for analysis 15 hours after treatment. The total amount of copper within cells was determined by FAAS, and values normalized per total protein content of the cells. Values are the means ± SEM of 3 different biological samples, and 3 experiments. Significant differences ($P<0.05$) were obtained with ANOVA.

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Next, in order to confirm microarray data and asses time and dose dependency of the response of zinc-related genes to GSPE, we cultured HepG2 cells in the presence of 150 mg/L GSPE, and monitored changes in gene expression of 4 plasma membrane zinc transporters and GCLC at various times, using quantitative RT-PCR. The results show (Figure IV.1.15) that time-dependent expression of ZnT1 closely paralleled that of MT2A (see figure IV.1.2), suggesting a common regulatory mechanism for the downregulation by GSPE, whereas the levels of ZIP1 and ZIP4 mRNAs, which where upregulated by GSPE, progressed with an inverse tendency to that of MT2A and ZnT1. The expression of ZIP14 resulted unaffected by GSPE, and mRNA levels of GCLC increased steadily in GSPE-treated cells. Dose dependency was shown for changes in the expression of ZnT1, ZIP1, ZIP4 and GCLC in HepG2 cells treated 9 and 12 hours with 15, 75 or 150 mg/L GSPE (Figure IV.1.16).

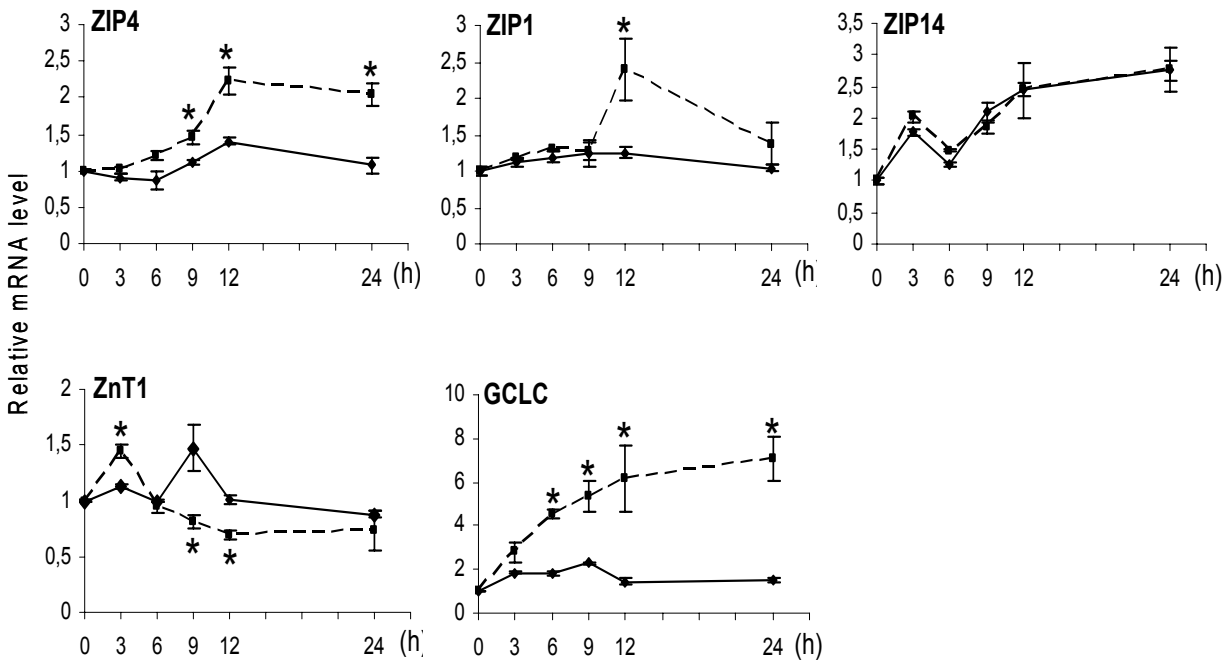

Figure IV.1.15. Kinetics of mRNA levels plasma membrane zinc transporters and GCLC in GSPE-treated cells. Black lines represent the evolution of the mRNA level of each gene in control cells, relative to the mRNA level of that gene in untreated cells just at the beginning of the treatment with 150 mg/L GSPE (dashed lines), determined by quantitative RT-PCR. Asterisks indicates significant difference ($P<0.05$) in treated cells versus control cells using independent samples t test.

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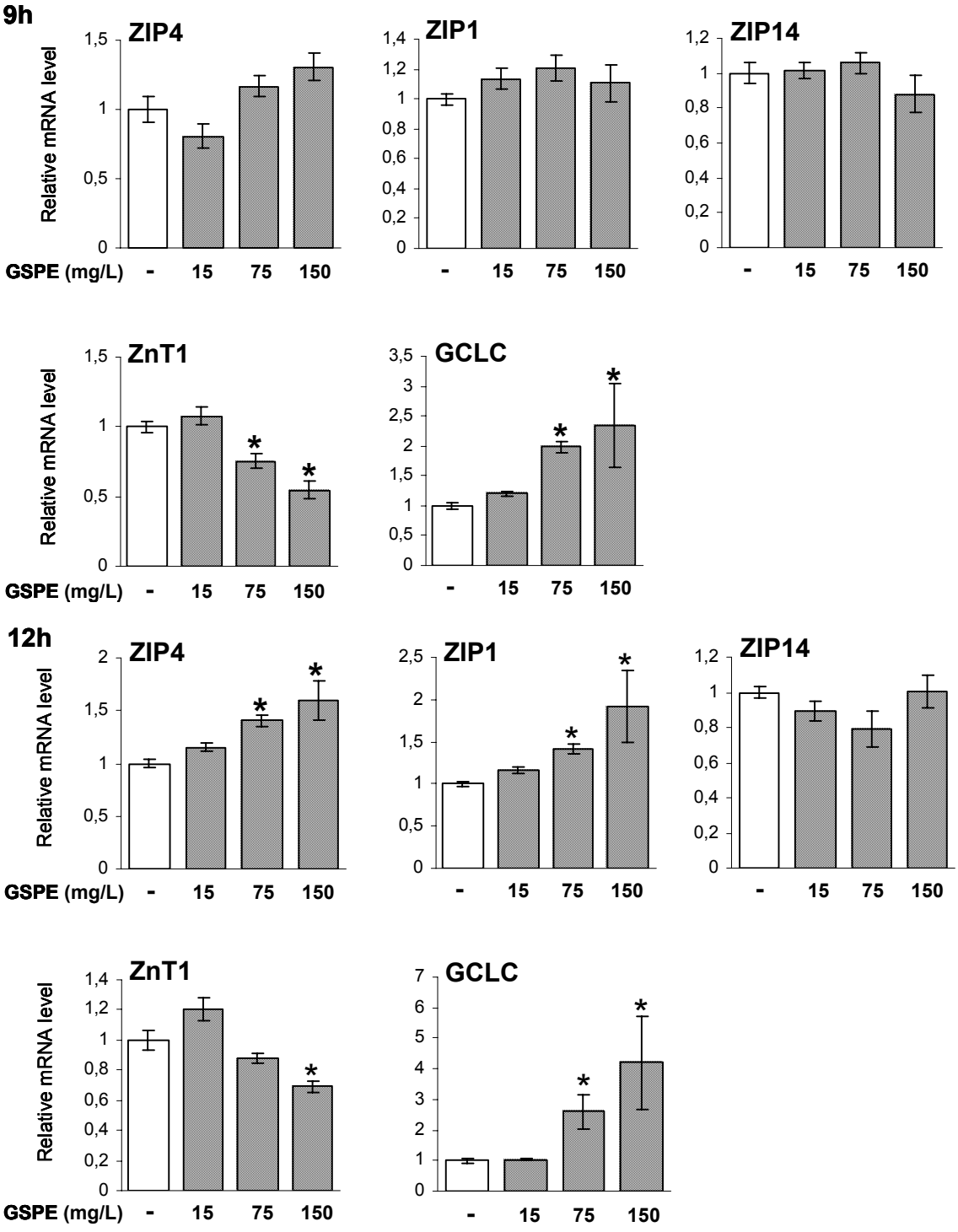

Figure IV.1.16. Dose dependency of the effects of GSPE on expression of plasma membrane zinc transporters and GCLC in HepG2 cells in basal conditions. HepG2 cells were treated with either vehicle (-), 15, 75 or 150 mg/L GSPE for 9 and 12 h, and relative mRNA levels of the indicated genes determined by RT-PCR. Asterisks indicates significant difference ($P<0.05$) in treated cells versus control cells using independent samples t test.

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Modulation of induced expression of zinc transporters by GSPE in HepG2 cells.

Zinc overload conditions.

As shown in Figure IV.1.17, addition of 100 μM zinc to the culture media, a concentration that is not toxic to HepG2 cells (Figure IV.1.21, see below), resulted in a 2 fold induction of ZnT1, whereas ZIP4 was downregulated to 60% of control value. Expression of ZIP1 and ZIP14 was unaffected by addition of zinc. In the presence of excess zinc, GSPE still upregulated ZIP1 and ZIP4 as in control cells, although to a lesser extend.

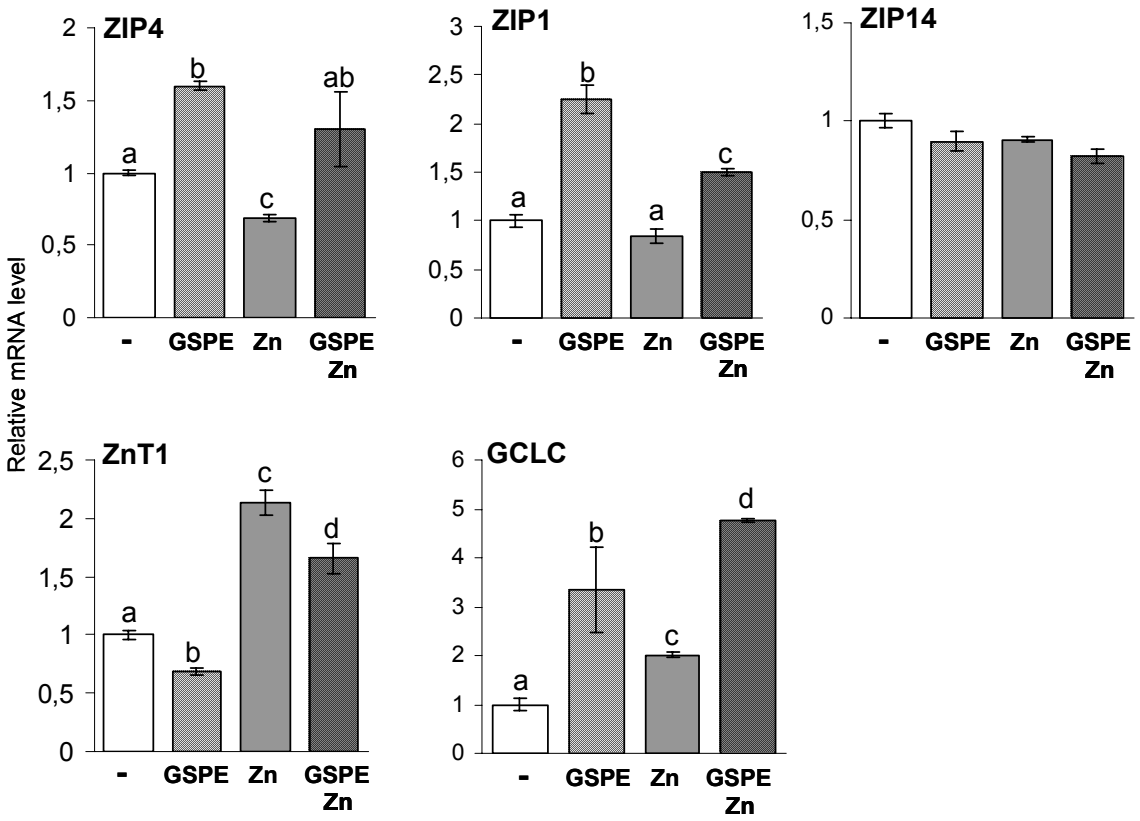

Figure IV.1.17. Effects of GSPE on plasma membrane zinc transporters and GCLC in HepG2 cells treated with excess zinc. mRNA levels of zinc transporter and GCLC genes upon 12 hours incubation with 150 mg/L GSPE (GSPE), 100 M ZnCl2 (Zn), or both (GSPE Zn), relative to the levels in untreated cells (-). Different letters denote different values after one way ANOVA test (P<0.05).

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Treatment with IL-6

Next, we tested whether GSPE may affect de expression of zinc transporters when cells are stimulated to take up zinc by the proinflammatory cytokine IL-6. In murine hepatocytes, IL-6 induces the expression of MT and ZIP14 signaling through the signal transducer and activator of transcription (STAT) pathway; subsequently, uptake of zinc mediated by ZIP14 further increases the activation of MT expression via MTF1; this response has been associated to the hypozincemia that accompanies the acute-phase response in infectious and inflammatory processes (Liuzzi et al., 2005; Cousins et al., 2006). In HepG2 cells, IL-6 induced a marked increase in the expression of ZIP14, and ZIP1 (Figure IV.18), ZnT1 and ZIP4 mRNA levels were not affected by IL-6 at this time. As in standard zinc conditions, ZnT1 was downregulated and ZIP4 was upregulated by GSPE independently of IL-6. Effect of GSPE on ZIP1 was not evident at this time.

In conclusion, GSPE treatment alters the expression of MT and zinc transporter genes in a time and dose-dependent manner in HepG2 cultured in basal zinc conditions, as well as in codition of zinc excess and IL-6 treatment. The pattern of changes elecitided by GSPE in the expression of MT and zinc-transporters is remarkably similar to that described for the zinc chelator TPEN in different cell types (see below), suggesting that extracellular, and perheaps intracellular, chelation of zinc cations by GSPE is a relevant mechanism underlying the observed modification in the expression of MT and zinc transporters elicited by GSPE in HepG2 cells.

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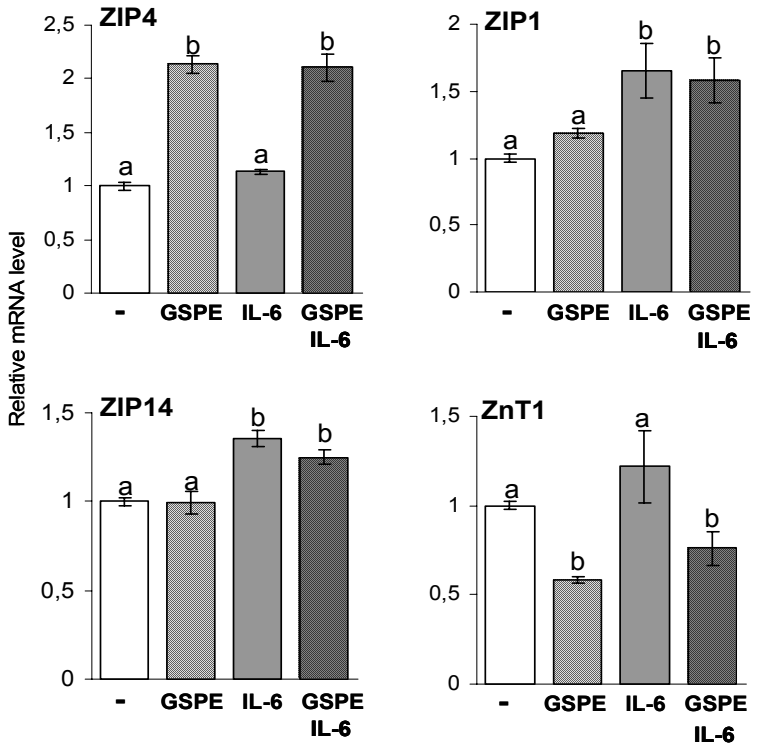

Figure IV.1.18. Effects of GSPE on plasma membrane zinc transporters in HepG2 cells treated with IL-6. mRNA levels of zinc transporter genes after 15 hours incubation with 150 mg/L GSPE, 1000 U/mL IL-6, or both treatments simultaneously, relative to control cells. 1 μM dexamethasone was added to all cultures. Different letters denote different values after one way ANOVA test (P<0.05).

GSPE diminishes total intracellular zinc accumulation in basal conditions (5μM zinc) in HepG2 cells.

The modification elicited by GSPE in the expression of MTs, ZnT1, ZIP1 and ZIP4, as well as ZnT5 and ZnT7, in HepG2 cells is remarkable similar to that described in other cell lines grown in conditions of reduced zinc availability, i.e., treated with the zinc chelator TPEN (Cao et al., 2001; Cousins et al., 2003; Devergnas et al., 2004; Shen et al., 2008) or in zinc-depleted medium (Langmade et al., 2000), that result in a reduction of total intracellular zinc. To address this point, we measured the total zinc content of control and GSPE-treated cells at different times (Figure IV.1.19). In control cells, total intracellular zinc increased steadily from 3.355 (SD 0.125) to 5.511 (SD 0.373) nmol zinc per mg of protein 15 h after the addition of fresh medium to the cells. In contrast, in cells treated with 150 mg/L GSPE, total intracellular zinc remained roughly constant from the time of GSPE administration until the end of cultivation. Therefore, GSPE hinders the normal entrance of zinc cations into HepG2 cells.

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GSPE diminishes total intracellular zinc accumulation in conditions of excess zinc, excces copper, and IL-6 treatment in HepG2 cells.

Zinc overload condition. GSPE counteracts the effect of excess zinc on intracellular zinc accumulation and on cell viability.

We first tested the capacity of GSPE to inhibit intracellular zinc accumulation in conditions of zinc overload (100μM). As shown in Figure IV.1.20, total intracellular zinc content normalized per protein content, was 4 times higher in zinc-treated cells than in control cells. Addition of GSPE (150 mg/L) to the media significantly hindered the zinc stimulated accumulation of intracellular zinc. These results suggested that GSPE should be able to counteract toxics effects of zinc. To test this, we performed LDH tests to evaluate the effect of increasing amounts of zinc on cell viability (Figure IV.1.21). Zinc concentrations above 200 μM resulted in significant LDH leakage, reaching 80% upon 24 h incubation of cells with 300 μM zinc. Addition of 150 mg/L GSPE to the cells prevented the noxious effects of 300 μM zinc by 50% after the LDH test. Thus, even if inhibiting MT expression, nontoxic amounts of GSPE are able to counteract the toxic effects of excessive zinc concomitantly reducing intracellular accumulation of zinc, strongly suggesting that GSPE renders zinc in the culture medium unavailable, and hence non toxic, to cells.

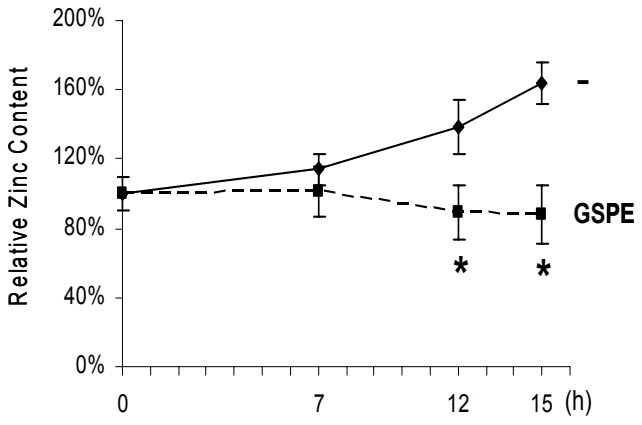

Figure IV.1.19. Progression of total intracellular zinc content in HepG2 in basal conditions. The total amount of zinc within cells was determined by FAAS, and values normalized per total protein content of the cells. The zinc to protein ratio in cells just at the beginning of the treatment with 150 mg/L GSPE was 3.355 (SD 0.125) nmol zinc per mg protein and is assigned the arbitrary value of 100. Asterisks indicates significant difference ($P<0.05$) in treated cells versus control cells using independent samples t test.

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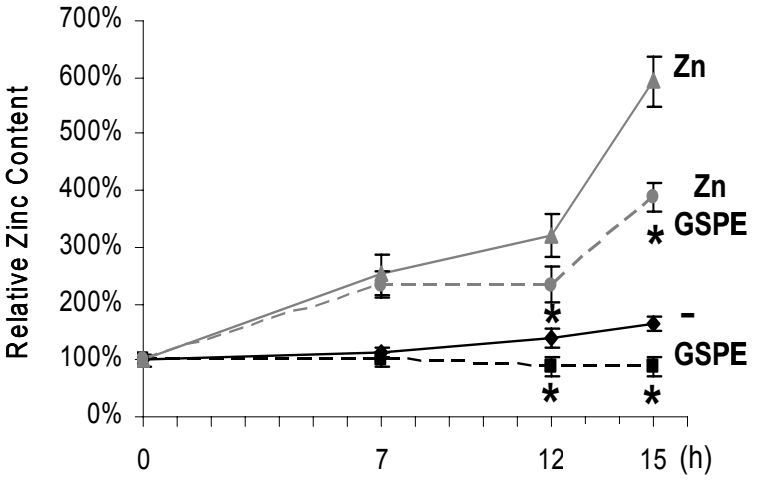

Figure IV.1.20. Progression of total intracellular zinc content in HepG2 cells in zinc overload conditions. Same conditions as in Figure IV.1.19.

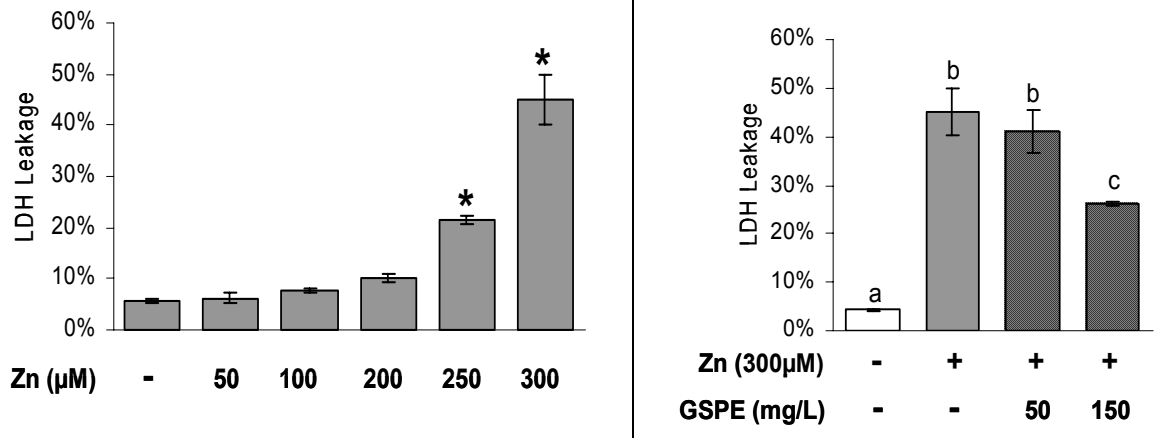

Figure IV.1.21. Effects of GSPE on cell viability in HepG2 cells treated with excess zinc. Dose-dependent toxicity of zinc on HepG2 cells and prevention of toxicity of 300 μM zinc in the culture medium by the addition of GSPE, assessed by the LDH test after 24 hours of treatment. Numbers indicate the percentage of total LDH activity of a cell culture which is present in the culture medium. Asterisks ndicates significant difference ($P<0.05$) from control cells. Different letters denote different values after one way ANOVA test ($P<0.05$).

Copper overload condition. GSPE counteracts the effect of excess copper on intracellular zinc accumulation.

Next, we tested the capacity of GSPE to diminish total intracellular zinc accumulation in copper overload condition (50μM). As zinc, copper is another phyisiological cation that induces MT synthesis. Thus, cells treated with 50 μM copper rised intracellular zinc content

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after 12 hours of treatment. Addition of GSPE (150 mg/L) to the media significantly hindered the copper stimulated accumulation of intracellular zinc. (Figure IV.1.22)

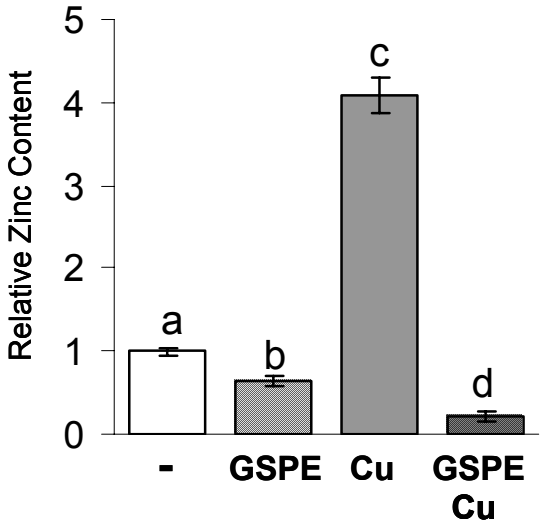

Figure IV.1.22. Effect of GSPE treatment on total intracellular zinc content in HepG2 cells in copper overload condition. Either vehicle (control), GSPE (150 mg/L), Copper (50μM) or both (GSPE Cu) were added to HepG2 cells. Samples were collected for analysis 15 hours after treatment. The total amount of zinc within cells was determined by FAAS, and values normalized per total protein content of the cells. Treatments were the same used in gene expression. Experimental values are the means ± SEM of three different biological samples in triplicate. Significant differences (P<0.05) were obtained with ANOVA.

IL-6 treatment. GSPE hinders the intracellular zinc accumulation elicited by IL-6.

Subsequently, we tested whether GSPE may affect zinc accumulation when cells are stimulated to take up zinc by stimuli different from zinc itself or other metal cations. In HepG2 cells, IL-6 increased intracellular zinc levels to 150% of untreated cells. When GSPE was added to the cells together with IL-6, intracellular zinc accumulation was completely blocked (Figure IV.1.23)

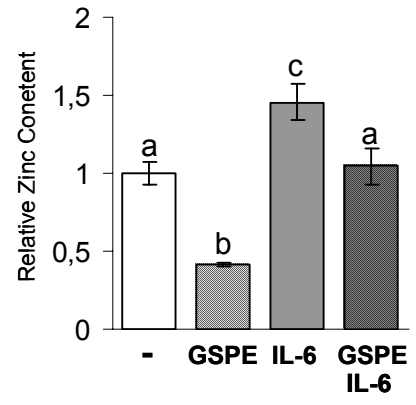

Figure IV.1.23. Total intracellular zinc content in IL-6 condition. Either vehicle (control), GSPE (150 mg/L), IL-6 (1000 U/mL) or both (GSPE IL-6) were added to HepG2 cells. Samples were collected for analysis 15 hours after treatment. The total amount of zinc within cells was determined by FAAS, and values normalized per total protein content of the cells. Treatments were the same used in gene expression. Experimental values are the means ± SEM of three different biological samples in triplicate. Significant differences (P<0.05) were obtained with ANOVA.

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These set of experiments show that the inhibition of zinc uptake and zinc accumulation by GSPE is relevant in standard as well as in zinc and copper-overload conditions and also in the cellular response to extracellular signals, such as those that mediate the acute-phase response in inflammatory processes (IL-6 condition).

GSPE elevates intracellular labile zinc in HepG2 cells

So far, we have shown that GSPE is able to profoundly modulate expression of MTs, cellular zinc transporters, extracellular zinc carriers, and total intracellular zinc accumulation, key factors in zinc homeostasis. However, there is a pool of cellular zinc, called “labile zinc”, which represents only a tiny fraction of the total amount of zinc in the cell, amounting nanomolar concentrations in the cytoplasm of hepatic cells, but is criticall to cell function, since fluctuations in the level of labile zinc consitute an intracellular zinc signal that modulate multiple metabolic and signalling pathways (see Introduction section). For that reason, we next monitored the effect of GSPE treatment on cytoplasmic levels of labile zinc, measured as zinc-dependent Zinquin fluorescence. As shown in Figure IV.1.24A, GSPE increased the cytoplasmic levels of Zinquin-detectable zinc in HepG2 cells in all conditions tested. After 12 hours of GSPE treatment, Zinquin fluorescence was enhanced by 12 fold compared with cells cultured in standard conditions. Addition of 100 μM zinc resulted in a 10 fold increase in Zinquin fluorescence, and co-incubation with 100 μM zinc and 150 mg/L GSPE produced a further increase of up to 120 fold compared to untreated cells. IL-6 treatment of HepG2 cells cultured in standard zinc conditions enhanced the labile pool of zinc by 1.4 fold, and addition of GSPE further increased it up to 18 fold. Microscopic observations of Zinquin-loaded HepG2 cells were done to visualize this effect of GSPE on cytoplasmic labile zinc in HepG2 cells grown in basal zinc concentrations as well as in conditions of zinc excess (Figure IV.1.24B). Thus, in spite of hindering intracellular zinc accumulation, GSPE produced an increase in the cytoplasmic pool of labile zinc in HepG2 cells, in standard growth conditions as well as when cells are stimulated to accumulate zinc.

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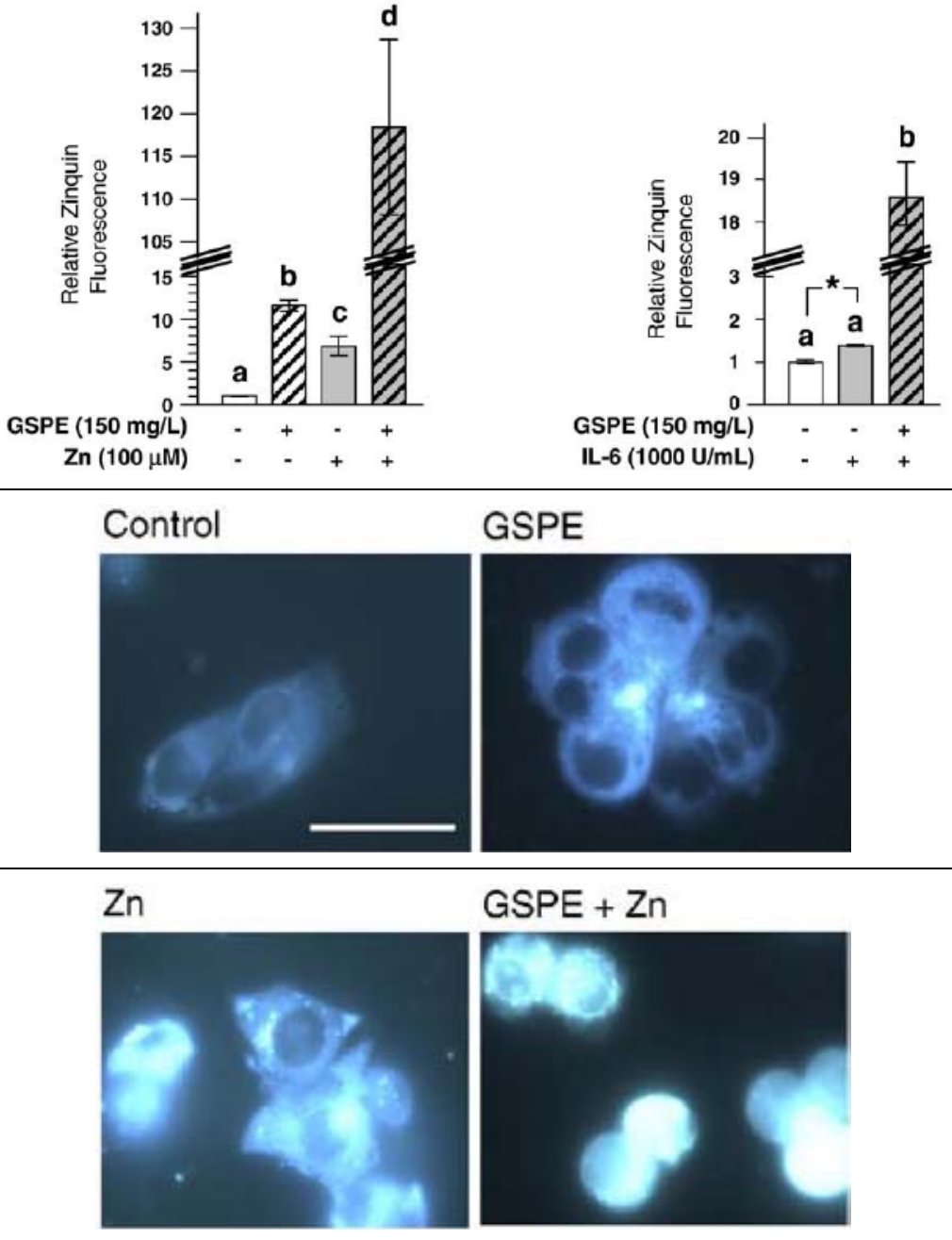

Figure IV.1.24. Effect of GSPE on cytoplasmic labile zinc in HepG2 cells. A. Fluorescence (arbitrary units) of Zinquin-loaded cells was normalized to protein content in each sample, and untreated cells were assigned the arbitrary value of 1. Treatments with GSPE, zinc and IL-6 were as in Fig IV.1.4. **B.** Images of Zinquin-loaded HepG2 cells. To visualize intracellular Zinquin fluorescence, cells were seeded in wells containing glass coverslips, treated as above, and loaded with Zinquin ester as described (Coyle et al., 1994); images at 1000x magnification were acquired with a Leica DM 4000B microscope using UV light illumination (λex = 340-380 nm) and a blue emission filer (λem ≥ 425nm). Barr indicates 50 μm.

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GSPE consist of a mixture of dozens of different catechins and procyanidins which, expectedly, will display different affinities for zinc. For the convenience of characterizing the effect of pure individual flavonoids on zinc homeostasis, we tested the effect of the monomer EGCG; the dimmeric procyanidin B1 and the trimeric procianidin C1 on different parameters of zinc homeostasis previously tested with GSPE in HepG2 cells.

2. EGCG

EGCG modulates the expression of genes involved in zinc homeostasis in HepG2 cells cultured in standard conditions.

In order to asses weather EGCG affects zinc homeostasis, we first performed gene expression analysis of MTs and zinc transporters. Thus, in basal zinc conditions (Figure IV.2.1), 100μM EGCG repressed MT1X, MT2A and ZnT1 expression 12 hours after treatment. Thereafter, 24 hours after EGCG addition, only MT1X and MT2A remain downregulated, whereas, in contrast, mRNA levels of ZnT1 were upregulated after 24 hours of EGCG treatment. All ZIP transporters tested were upregulated 24 hours after EGCG treatment, showing a later response compared with GSPE treatment. Only ZIP4 was also enhanced 12 hours after addition of EGCG. We also monitored the time course of GCLC expression (Figure IV.2.2). The results show an upregulation of GCLC after 24 hours of treatment.

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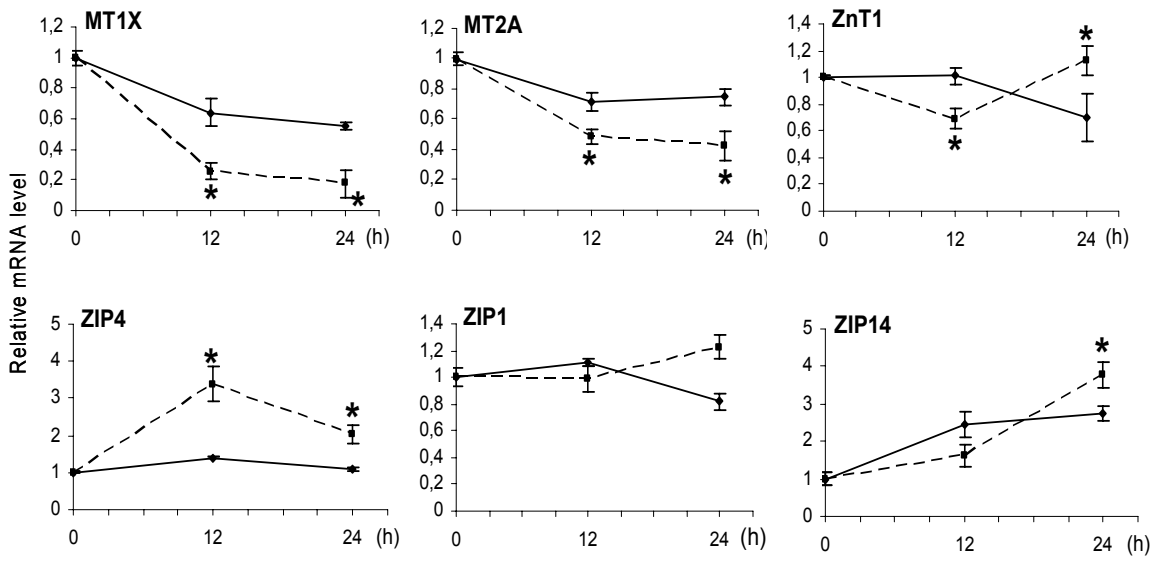

Figure IV.2.1. Modulation of basal MTs and zinc transporters by EGCG in HepG2 cells. Time course effect of EGCG administration on expression of MT1X, MT2A, ZnT1, ZIP4, ZIP1 and ZIP14 in HepG2 cells. Cells were treated with either vehicle (control cells) or 100 μM EGCG, and collected at the indicated times. Results are shown as means ±SEM of 3 different experiments in triplicate. *Significant differences (P<0.05) by the Student's t test.

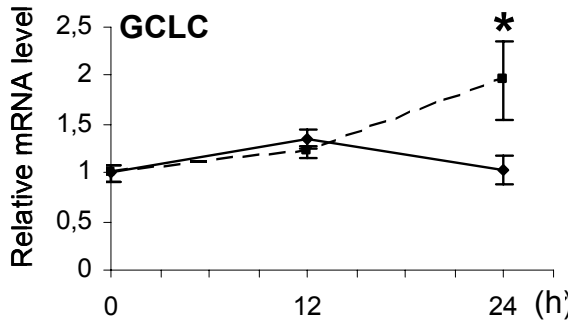

Figure IV.2.2. Upregulation of GCLC by EGCG in HepG2 cells. Time course effect of EGCG administration on expression of GCLC in HepG2 cells. Experiment was performed as described in Figure IV.2.1.

EGCG diminishes total intracellular zinc accumulation in basal conditions in HepG2 cells.

We tested the capacity of EGCG to inhibit intracellular zinc accumulation in standard cultured condition (5μM zinc) in HepG2 cells. As shown in Figure IV.2.3, EGCG 100μM was able to diminish total intracellular zinc 12 hours after treatment.

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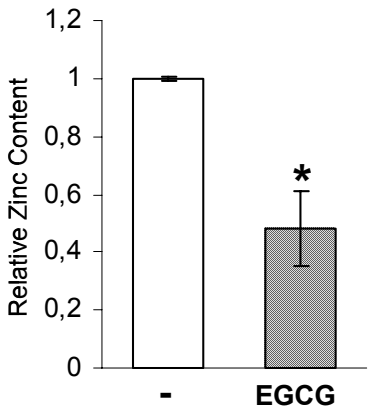

Figure IV.2.3. Total intracellular zinc content in basal condition. Either vehicle (control) or EGCG (100 μM) were added to HepG2 cells. Samples were collected for analysis 12 hours after treatment. The total amount of zinc within cells was determined by FAAS, and values normalized per total protein content of the cells. Experimental values are the means ± SEM of three different biological samples in triplicate. *Significant differences (P<0.05) by the Student's t test.

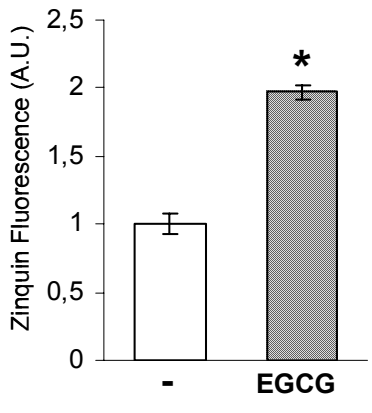

Figure IV.2.4. Effect of EGCG on cytoplasmic labile zinc in HepG2 cells. Fluorescence (arbitrary units) of Zinquin-loaded cells was normalized to protein content in each sample, and untreated cells were assigned the arbitrary value of 1. Treatments with EGCG were as in Fig. IV.2.1. Experimental values are the means ± SEM of three different biological samples in triplicate. *Significant differences (P<0.05) by the Student's t test.

EGCG elevates intracellular labile zinc in basal conditions in HepG2 cells.

We next monitored the effect of EGCG treatment on cytoplasmic levels of labile zinc. As shown in Figure IV.2.4, EGCG increased the cytoplasmic levels of Zinquin-detectable zinc 2-fold in basal condition in HepG2 cells after 12 hours of EGCG treatment.

EGCG modulates the expression of genes involved in zinc homeostasis in HepG2 cells cultured in zinc overload condition.

In order to determine weather EGCG also affects zinc homeostasis in zinc overload condition, we first performed gene expression analysis of MTs and zinc transporters. As shown in Figure IV.2.5, addition of 100 μM zinc to the culture media, resulted in an

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upregulation of MT1X, MT2A and ZnT1, whereas ZIP4, ZIP1 and ZIP14 were unaffected. Addition of EGCG reverted the zinc-induced upregulation of MTs and ZnT1 genes. Conversely, ZIP1 and ZIP4 resulted upregulated after addition of EGCG to the zinc supplemented medium.

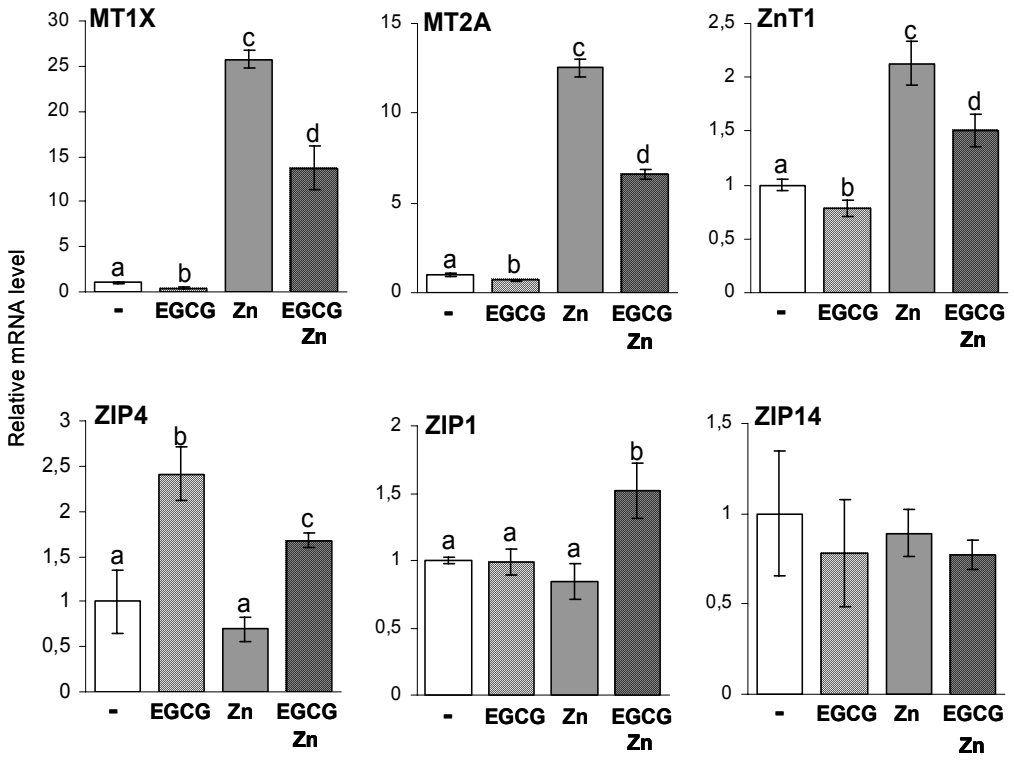

Figure IV.2.5. Effects of EGCG on MT and plasma membrane zinc transporters in HepG2 cells treated with excess zinc. mRNA levels of MT1X, MT2A and zinc transporters ZnT1, ZIP4, ZIP1 and ZIP14 genes upon 12 hours incubation with 100 μM EGCG (EGCG), 100 μM ZnCl2 (Zn), or both (EGCG Zn), relative to the levels in untreated cells (-). Different letters denote different values after one way ANOVA test ($P<0.05$).

EGCG counteracts the effect of excess zinc on intracellular zinc accumulation and on cell viability.

Next, we tested the capacity of EGCG to inhibit intracellular zinc accumulation in conditions of zinc overload (100 μM). As shown in Figure IV.2.6A, total intracellular zinc was 2 times

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higher in zinc-treated cells than in control cells. Addition of EGCG (100 μM) to the media significantly hindered the zinc stimulated accumulation of intracellular zinc. As with GSPE, these results also suggest that EGCG should be able to counteract toxic effects of zinc. To test this, we performed LDH tests, as done in GSPE experiments (Figure IV.2.6B). Addition of different concentrations of EGCG to the cells prevented the noxious effects of 300 μM zinc dose-depently after the LDH test.

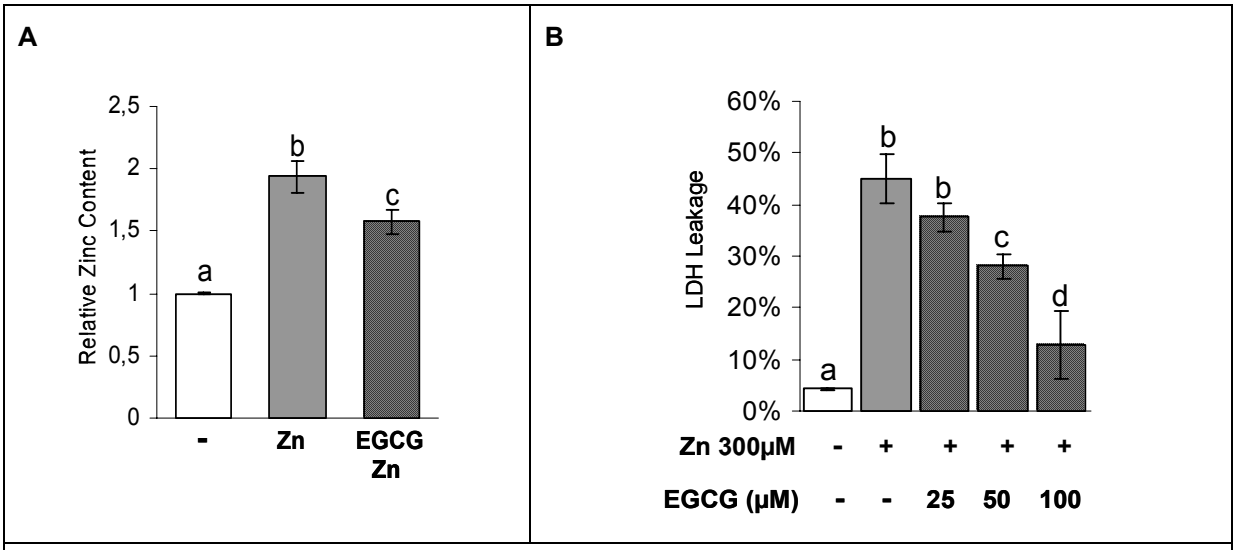

Figure IV.2.6. Effects of EGCG on total zinc accumulation and zinc toxicity in HepG2 cells treated with excess zinc. A. Total intracellular zinc content in zinc overload condition. Either vehicle (control), EGCG (100μM) or Zn (100μM) were added to HepG2 cells. Samples were collected for analysis 12 hours after treatment. The total amount of zinc within cells was determined by FAAS, and values normalized per total protein content of the cells. Treatments were the same used in gene expression. Experimental values are the means ± SEM of three different biological samples in triplicate. Significant differences ($P<0.05$) were obtained with ANOVA. **B. Effects of EGCG on cell viability in HepG2 cells treated with excess zinc.** Prevention of toxicity of 300 μM zinc in the culture medium by the addition of different doses of EGCG, assessed by the LDH test after 24 hours of treatment. Numbers indicate the percentage of total LDH activity of a cell culture which is present in the culture medium. Different letters denote different values after one way ANOVA test ($P<0.05$).

EGCG elevates intracellular labile zinc in zinc overload conditions in HepG2 cells.

We next monitored the effect of EGCG treatment on cytoplasmic levels of labile zinc in excess zinc condition. As shown in Figure IV.2.7, EGCG (100μM) markedly increased the

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cytoplasmic levels of Zinquin-detectable labile zinc in HepG2 cells treated with 100μM zinc after 12 hours of EGCG and Zn(II) administration to the cell culture.

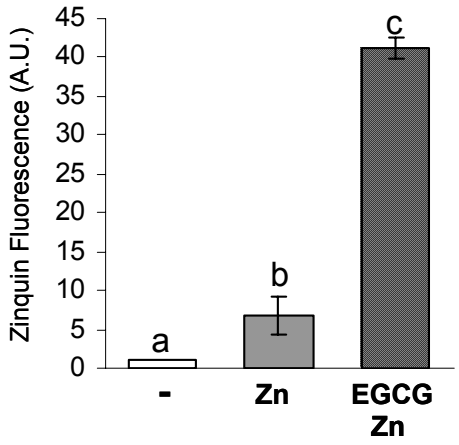

Figure IV.2.7. Effect of EGCG on cytoplasmic labile zinc in HepG2 cells in cell overload conditions. Fluorescence (arbitrary units) of Zinquin-loaded cells was normalized to protein content in each sample, and untreated cells were assigned the arbitrary value of 1. Cells were treated 12 hours with vehicle (-), 100μM Zn(II) (Zn) or with 100μM EGCG plus 100μM Zn(II) (EGCG Zn). Experimental values are the means ± SEM of three different biological samples in triplicate. *Significant differences ($P<0.05$) by the Student's t test.

EGCG modulates the expression of genes involved in zinc homeostasis in HepG2 cells cultured in IL-6 condition.

Next, we tested whether EGCG may affect de expression of MT and zinc transporters when cells are stimulated with IL-6. In HepG2 cells, IL-6 induced a marked increase in the expression of MT1X, MT2A, ZnT1 and ZIP14 (Figure IV.2.8), ZIP4 and ZIP1 mRNA levels were not affected by IL-6 at this time. Addition of EGCG to IL-6 treated medium elicited a downregulation of MT1X and MT2A. Effect of EGCG was not evident on ZIP14 and ZnT1.

EGCG hinders the intracellular zinc accumulation elicited by IL-6.

In HepG2 cells, IL-6 slightly increased intracellular zinc levels compared to untreated cells. This less augmentation of total intracellular zinc by IL-6 compared to the high levels observed with Zn treatment, might be due because IL-6 activates transcription of ZIP6 and ZIP14 via STAT and therefore, would lead to enter the small amount of zinc in the medium (a maximum of 10μM Zn: 5μM of the standard medium plus 5μM of supplemented, see

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materials and methods). This amount is 10 times lesser than 100μM Zn in Zn treatment. However, when EGCG was added to the IL-6 supplemented medium, it was able to hamper the accumulation of total intracellular zinc elicited by IL-6 (Figure IV.2.9)

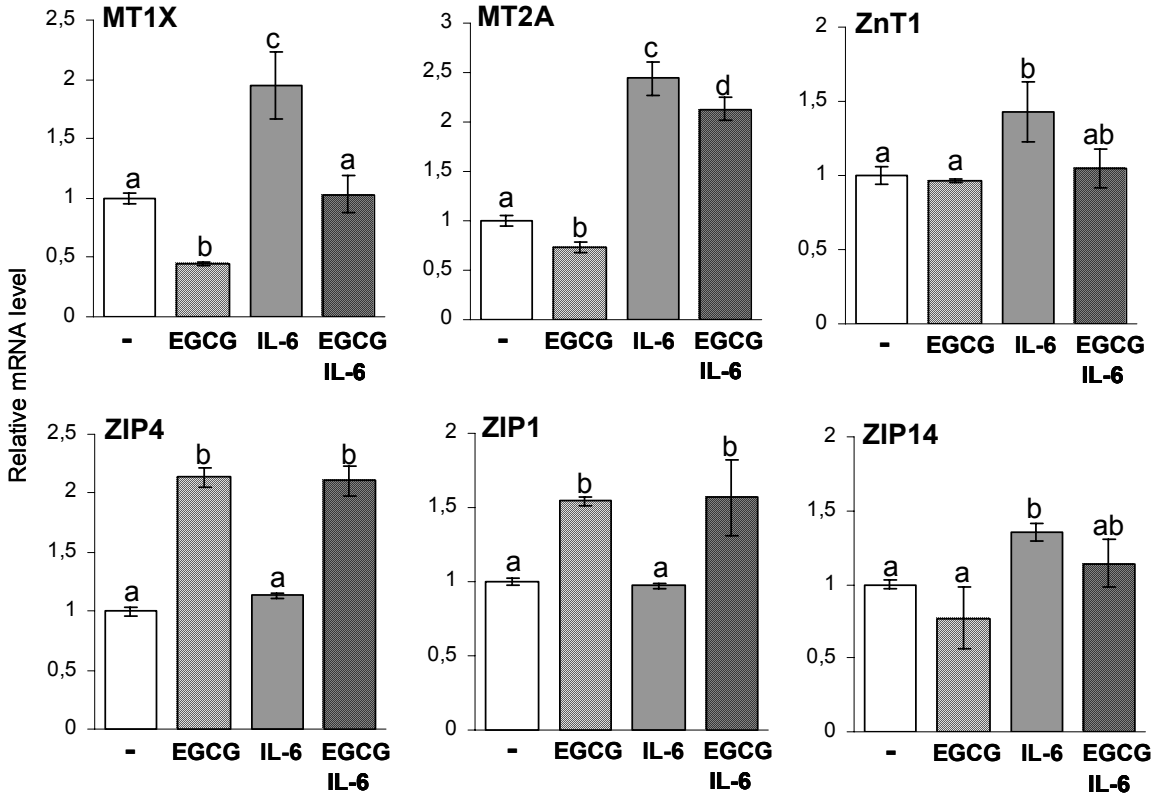

Figure IV.2.8. Effects of EGCG on MTs and plasma membrane zinc transporters in HepG2 cells treated with IL-6. mRNA levels of zinc transporter genes after 15 hours incubation with 100μM GSPE, 1000 U/mL IL-6, or both treatments simultaneously, relative to control cells. 1 μM dexamethasone was added to all cultures. Different letters denote different values after one way ANOVA test ($P<0.05$).

EGCG elevates intracellular labile zinc in HepG2 cells treated with IL-6.

Next, we monitored the effect of EGCG treatment on cytoplasmic levels of labile zinc in IL-6 condition. As shown in Figure IV.2.10, IL-6 slightly, but not significantly, increase this labile pool of zinc. Following the same criteria as in total intracellular zinc plus the fact that this pool is rapidly buffered, there is no visible agumentation in this pool of zinc. However, when the

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cells were co-incubated with EGCG and IL-6, cytoplasmic levels of Zinquin-detectable zinc were highly increased after 15 hours of treatment.

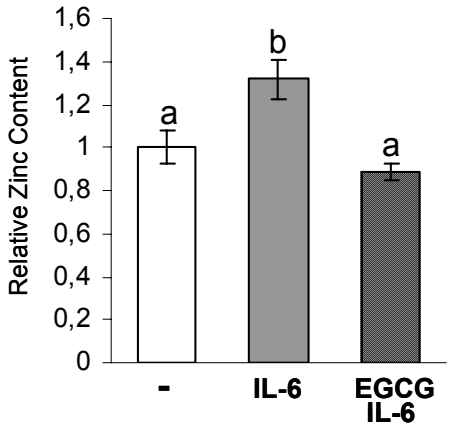

Figure IV.2.9. Effects of EGCG on total intracellular zinc content of HepG2 cells treated with IL-6. Either vehicle (control), EGCG (100μM) or IL-6 (1000 U/mL) were added to HepG2 cells. Samples were collected for analysis 15 hours after treatment. The total amount of zinc within cells was determined by FAAS, and values normalized per total protein content of the cells. Experimental values are the means ± SEM of three different biological samples in triplicate. Significant differences (P<0.05) were obtained with ANOVA.

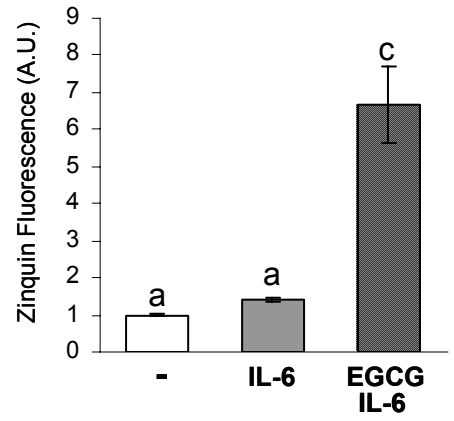

Figure IV.2.10. Effect of EGCG on cytoplasmic labile zinc in HepG2 cells in IL-6 condition. Fluorescence (arbitrary units) of Zinquin-loaded cells was normalized to protein content in each sample, and untreated cells were assigned the arbitrary value of 1. Treatments with EGCG and IL-6 were as in Figures IV.2.8 and IV.2.9. Experimental values are the means ± SEM of three different biological samples in triplicate. Significant differences (P<0.05) were obtained with ANOVA

3. TRIMER C1

Trimer C1 upregulates the expression of MT genes, increases total intracellular and cytoplasmatic labile zinc in basal conditions

First, we tested the capacity of trimeric procyanidin C1 to modulate zinc homeostasis in standard cultured medium conditions. As shown in figure IV.3.1, 100μM C1 was able to

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upregulate MT1X and MT2A expression 12 hours after treatment. ZnT1, ZIP1 and ZIP4 were unaffected. On the other hand, ZIP14 was downregulated. Strikingly, this results show that trimer C1 has opposite effects to GSPE and EGCG on MT expression. These results could lead us to believe that total intracellular zinc amounts would also be increased. To adress this issue, we measured total intracellular zinc in the same conditions. Trimer C1, was able to increase total intracellular zinc accumulation 2-fold compared with untreated cells. When we measured the cytoplasmatic labile zinc, we also found a marked increase in this pool of zinc.

Trimer C1 upregulates the expression of MT genes, increases total intracellular and cytoplasmatic labile zinc in zinc overload condition (100μM).

Next, we tested whether trimer C1 could also modulate zinc homeostasis in excess zinc condition. As expected, zinc treatment upregulate MT1X, MT2A and ZnT1 and increased total intracellular zinc accumulation. ZIP1, ZIP4 and ZIP14 were unaffected by zinc. Addition of 100μM trimer C1 could only downregulate ZIP14 and slightly (but not significantly) increase intracellular zinc content (Figure IV.3.2A and B). These results suggest that trimer C1 might not be able to counteract toxic effects of zinc. To adress this hypothesis, we performed LDH tests to evaluate the effect of trimer C1 when it is co-incubated with a toxic zinc concentration (300 μM) in HepG2. Addition of 100 μM trimer C1 to the cells, not only does not prevented the noxious effects of 300 μM zinc, but also increased zinc toxicity after the LDH test (figure IV.3.2C).

Thus, trimer C1 does not reproduce the major effects of GSPE and EGCG on zinc homeostasis. On the contrary, acted in opposite inducing MTs and increasing total zinc accumulation in basal conditions. On the other hand, trimer C1 behaved equal as GSPE and EGCG increasing cytoplasmic labile zinc in basal conditions.

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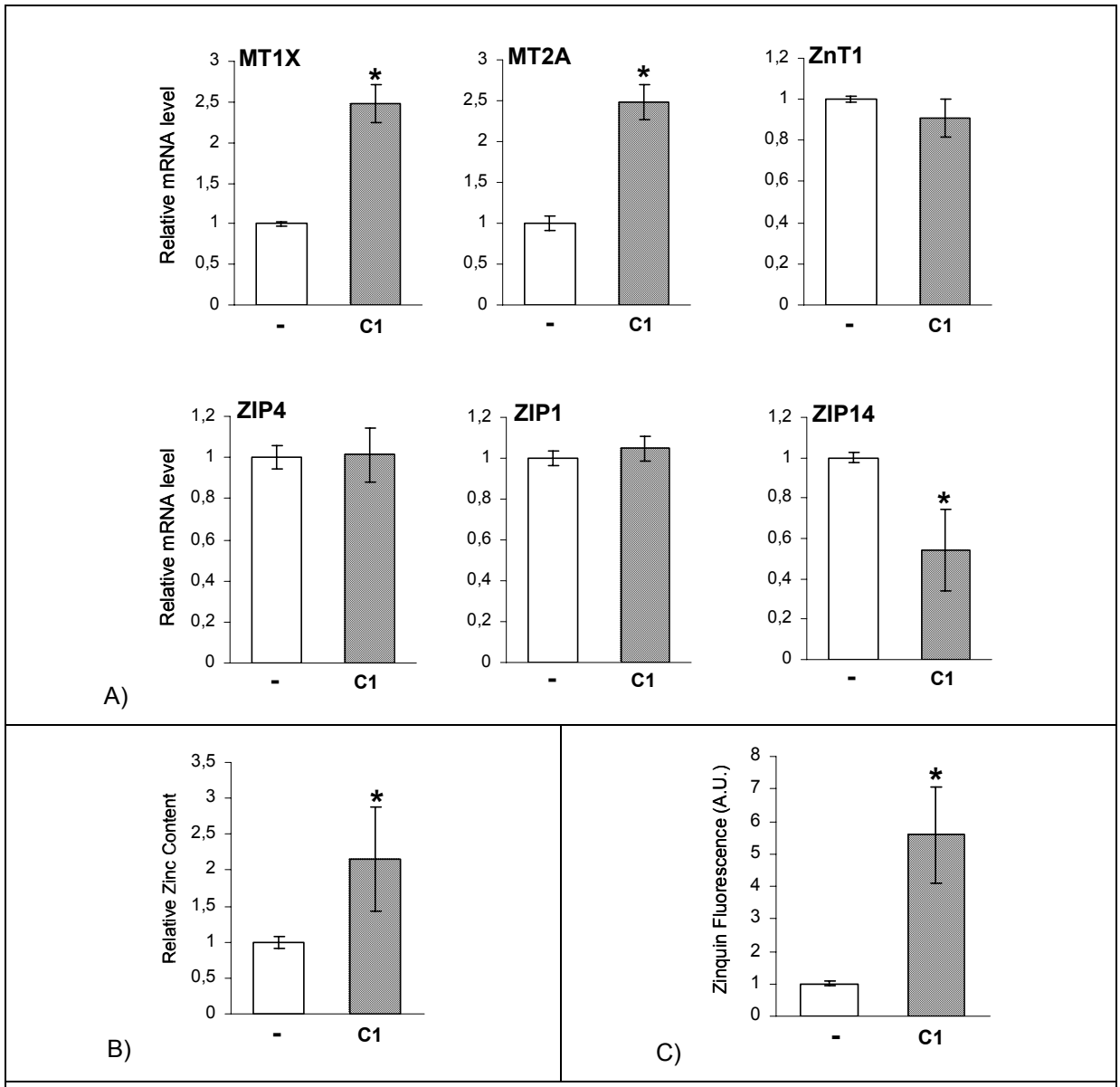

Figure IV.3.1. Effects of trimer C1 on zinc homeostasis in basal conditions in HepG2 cells.

A. Modulation of MTs and zinc transporters expression by trimer C1 in HepG2 cells cultured in standard conditions. Effect of 100μM trimer C1 administration on expression of MT1X, MT2A, ZnT1, ZIP4, ZIP1 and ZIP14 in HepG2 cells. Cells were treated with either vehicle (control cells) or 100 μM trimer C1, and collected 12 hours after treatment. **B. Effect of trimer C1 on total intracellular zinc accumulation in HepG2 cells in basal conditions.** The total amount of zinc within cells was determined by FAAS, and values normalized per total protein content of the cells. Treatments with C1 were as in Fig. IV.3.1A. **C. Effect of trimer C1 on cytoplasmic labile zinc in HepG2 cells in basal conditions.** Fluorescence (arbitrary units) of Zinquin-loaded cells was normalized to protein content in each sample, and untreated cells were assigned the arbitrary value of 1. Treatments with C1 were as in Fig. IV.3.1A. Experimental values are the means ± SEM of three different biological samples in triplicate. *Significant differences ($P<0.05$) by the Student's t test.

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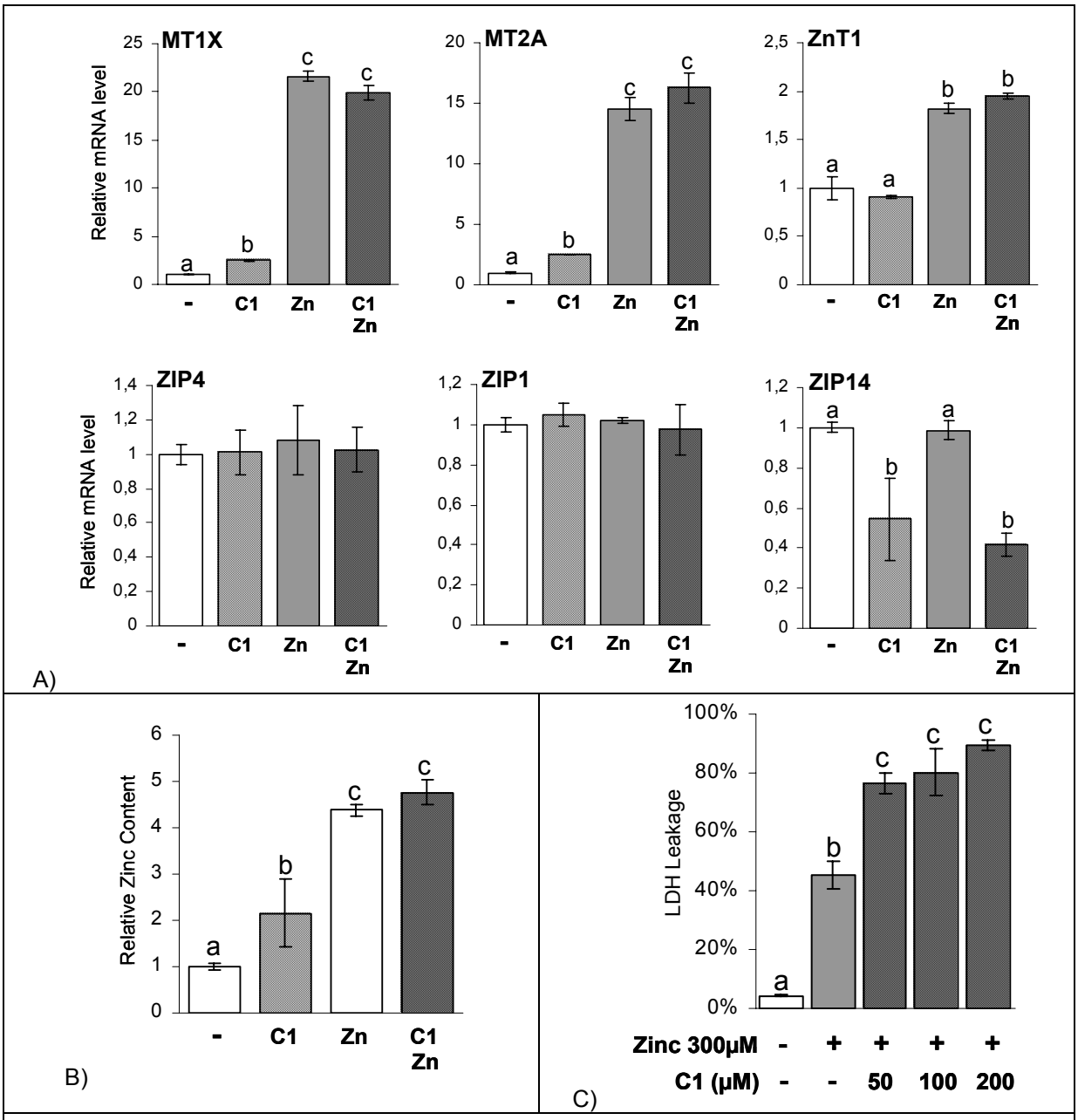

Figure IV.3.2. Effects of trimer C1 on zinc homeostasis in zinc overload condition in HepG2 cells. A. Modulation of MTs and zinc transporters by trimer C1 in HepG2 cells. mRNA levels of MT1X, MT2A and zinc transporters ZnT1, ZIP4, ZIP1 and ZIP14 genes upon 12 hours incubation with 100 µM trimer C1 (C1), 100 µM ZnCl2 (Zn), or both (C1 Zn), relative to the levels in untreated cells (-). Different letters denote different values after one way ANOVA test ($P<0.05$). **B. Effect of trimer C1 on total intracellular zinc accumulation in HepG2.** The total amount of zinc within cells was determined by FAAS, and values normalized per total protein content of the cells. Treatments were as in Fig. IV.2.6A. Different letters denote different values after one way ANOVA test ($P<0.05$). **C. Effect of trimer C1 on cell viability in HepG2 cells.** Toxicity of 300 µM zinc in the culture medium and co-incubation with different doses of C1, assessed by the LDH test after 24 hours of treatment. Numbers indicate the percentage of total LDH activity of a cell culture which is present in the culture medium. Different letters denote different values after one way ANOVA test ($P<0.05$).

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4. DIMER B1

Dimer B1 shows little effect of MT and zinc trasnporter expression and on total intracellular zinc accumulation in HepG2 cells cultured in basal conditions.

In order to asses the ability of dimeric procyanidin B1 to modulate zinc homeostasis, we monitored changes in the expression profiles of MT1X and MT2A and zinc transporters ZnT1, ZIP1, ZIP4 and ZIP14 in HepG2 cells grown 12 hours in standard culture media supplemented with 100 μM B1. In this conditions, this dimeric procyanidin B1 upregulated MT1X expression significantly and tended to upregulate MT2A. Of the zinc transporters tested, only ZIP14 was downregulated, whereas ZIP1, ZIP4 and ZnT1 were unaffected by B1 (Figure IV.4.1A). This results show a poor modulation of MT and zinc transporters by dimer B1 in these conditions. Next, we measured the total zinc content in the same conditions tested. Although there was a tendency to increase intracellular zinc accumulation, no significant statistical change was observed by the treatment of HepG2 cells with dimer B1 (Figure IV.4.1B).

Dimer B1 modulates MT and zinc trasnporter expression and increases total intracellular zinc accumulation in zinc overload conditions.

Next, we tested the capacity of dimer B1 to modulate genes involved in zinc homeostasis in zinc excess condition (Figure IV.4.2A). Addition of 100μM zinc to the culture medium, elicited a marked upregulation of MT1X, MT2A and ZnT1, while ZIP4 resulted downregulated. ZIP1 and ZIP4 showed no changes in their expression. In the presence of excess zinc, dimer B1 treatment still upregulated MT1X and MT2A, but had no effect on ZnT1, ZIP4 and ZIP1. Zinc stimulated accumulation of intracellular zinc was increased when dimer B1 was incubated in zinc overload condition (Figure IV.4.2B). This results suggest that the effect of dimer B1 is

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greater in upregulating MT and increasing intracellular zinc accumulation in zinc overload condition than in basal condition.

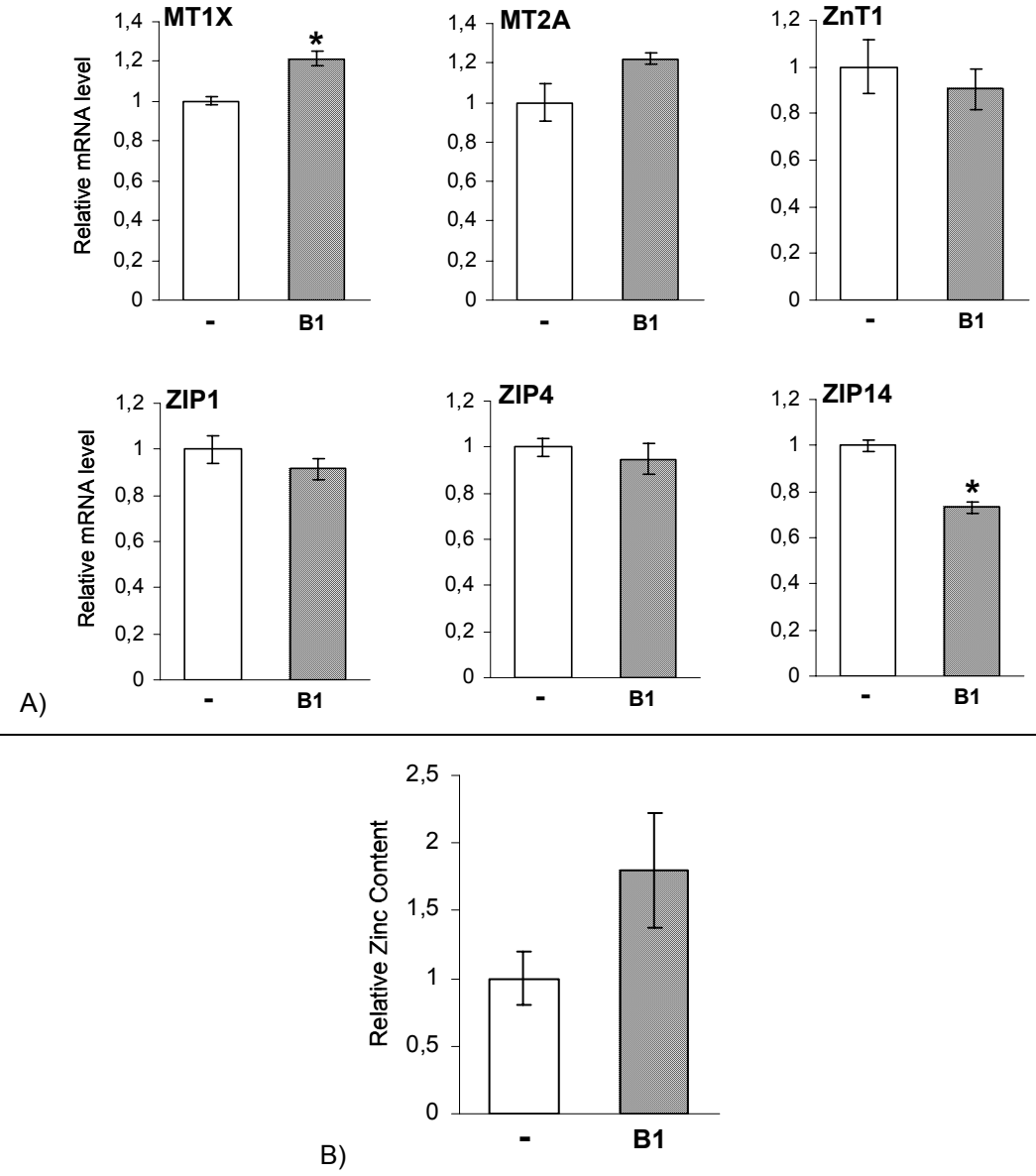

Figure IV.4.1. Effects of dimer B1 on zinc homeostasis in basal conditions in HepG2 cells.

A. Modulation of MTs and zinc transporters expression by dimer B1 in HepG2 cells in basal conditions. Effect of 100μM dimer B1 administration on expression of MT1X, MT2A, ZnT1, ZIP4, ZIP1 and ZIP14 in HepG2 cells. Cells were treated with either vehicle (control cells) or 100 μM dimmer B1, and collected 12 hours after treatment. **B. Effect of dimer B1 on total intracellular zinc accumulation in HepG2 cells in basal condition.** The total amount of zinc within cells was determined by FAAS, and values normalized per total protein content of the cells. Treatments with C1 were as in Fig. IV.2.6A. *Significant differences ($P<0.05$) by the Student's t test.

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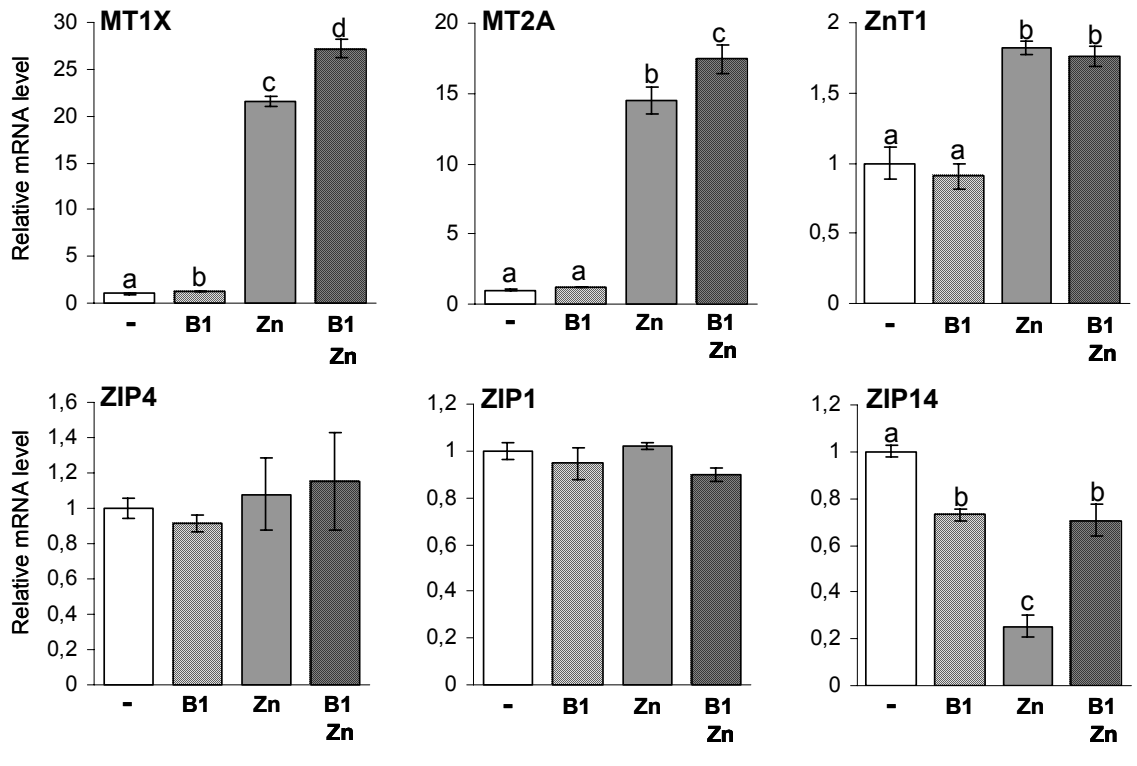

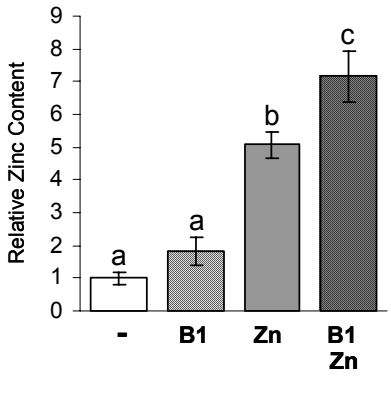

Figure IV.4.2. Effects of dimer B1 on zinc homeostasis in zinc overload condition in HepG2 cells. A. Modulation of MTs and zinc transporters by dimer B1 in HepG2 cells. mRNA levels of MT1X, MT2A and zinc transporters ZnT1, ZIP4, ZIP1 and ZIP14 genes upon 12 hours incubation with 100 μM dimer B1 (B1), 100 μM ZnCl2 (Zn), or both (B1 Zn), relative to the levels in untreated cells (-). Different letters denote different values after one way ANOVA test (P<0.05). **B. Effect of dimer B1 on total intracellular zinc accumulation in HepG2.** The total amount of zinc within cells was determined by FAAS, and values normalized per total protein content of the cells. Treatments were as in Fig. IV.2.7A. Different letters denote different values after one way ANOVA test (P<0.05).

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V. GENERAL DISCUSSION

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Previous results of our research group have shown that oral administration of a single GSPE dose to healthy rats in the postprandial phase triggers a marked reduction of MT-I, MT-II and an increased expression of SHP in the liver, detected 5 hours after treatment by microarray hybridization (Del Bas et al., 2005). Although this approach identifies metallothionein and SHP genes as targets of procyanidin action in the liver, it does not allow, however, to discern whether this effect is exerted by procyanidins acting directly on hepatic cells or, either, is an indirect effect, i.e., it results from a primary action of procyanidins on enterocytes, adipose tissue, vascular cells, and/or other extrahepatic tissues, which, in turn, affect hepatic metabolism and gene expression through the action of hormones and cytokines or by metabolic changes with indirectly affect liver functions.

We have here shown that GSPE directly represses MT and upregulates SHP expression in cultured human hepatocarcinoma HepG2 cells and, therefore, it is not necessary to invoke the intervention of non-hepatic tissues to explain the effects of GSPE on hepatic MT and SHP expression *in vivo*. In support of this hypothesis, it has bee previously shown (Garcia-Ramirez et al., 2006) that orally administered synthetic oligomeric procyanidins are rapidly absorbed and internally methylated in rats, and tetramethylated dimeric procyanidins (TDPC) are detectable in plasma and liver of treated rats as soon as 1 hour after intake, reaching concentrations of 14 mg/g in liver and 15 mg/L in plasma, 2 hours after treatment. The concentrations of TDPC in plasma and liver are in the same range of hormones and cytokines and, therefore, should be adequate to exert cytokine and hormone-like biological activities in the liver. Consistent with this view, it has been previously demonstrated insulin-like effects of orally administered GSPE on the adipose tissue of streptozotocin-induced diabetic rats and in vitro in 3T3-L1 adipocytes (Pinent, 2004; Pinent et al., 2005). In conclusion, MT and SHP genes are genuine targets of procyanidins in hepatic cells, both *in vivo* and *in vitro*, in human and rat hepatocytes.

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The observed diminished hepatic expression of MT genes triggered by ingestion of GSPE in rats in the postprandial phase, could in principle result from a repression of basal MT expression or, alternatively, from an inhibition of an induction of MT expression which could happen in postprandial phase. Although there is no information concerning the regulation of hepatic MT by food consumption, most, if not all, known inducers of hepatic metallothionein expression are elevated in plasma soon after a food intake. Thus, immediately after a meal there is a transitory increment of plasma levels of zinc that is followed by increased zinc uptake by the liver and other tissues; plasma zinc levels rapidly decline afterwards and drop to levels which are lower than those characteristics of the fasted state (postprandial hypozincemia) (Blalock TL, 1988; Cousins and Lee-Ambrose, 1992; King et al., 2000; Szczurek et al., 2001). On the other hand, the postprandial state displays some characteristics of an inflammatory response: several proinflammatory cytokines (IL-1, IL-6 and IL-10, TNF-α) are elevated in plasma and trigger an increased liver synthesis and secretion of several acute-phase proteins (Maret, 2003; Wautier JL, 2006; Lundman, 2007). Also, the production of reactive oxygen species from macrophages and endothelial cells is elevated in the postprandial period, due to the increased glycaemia and triglyceridemia and the action of the pro-inflammatory cytokines (Nappo et al., 2002; Natella et al., 2002; Ursini and Sevanian, 2002; Covas et al., 2003; Ceriello et al., 2004; Sies and Stahl, 2004; van Oostrom et al., 2004; Yano et al., 2004; Manuel-y-Keenoy et al., 2005; Neri et al., 2005; Saxena et al., 2005; Sies et al., 2005a; Sies et al., 2005b; Takeda et al., 2005; Bavenholm and Efendic, 2006; Fuhrman et al., 2006; Wautier JL, 2006; Wright et al., 2006; Alipour et al., 2007; Yamagishi et al., 2007). Plasma hypozincemia, inflammation and oxidative stress associated to the postprandial situation have been postulated to represent a defense mechanism against pathogens ingested with food (Sies and Stahl, 2004; Yano et al., 2004; Sies et al., 2005a; Wright et al., 2006). However, postprandial oxidative stress and inflammation are tightly linked to the development of atherosclerosis and diabetes (Nappo et al., 2002; Yano et al., 2004; Bavenholm and Efendic, 2006; Wautier et al., 2006; Wright et al., 2006; Alipour et al., 2007). Dietary flavonoids and other antioxidants have been proposed

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as preventive agents of postprandial oxidative stress. Also cortisol levels increase after a meal, and food-induced stimulation of cortisol secretion promote the expression of leptin by the adipose tissue, a mechanism that serves to produce the sensation of satiety (Rosmond et al., 2000; Sies and Stahl, 2004; Duclos et al., 2005). Excessive cortisol levels, however, have been linked to the development of several metabolic disorders associated with the metabolic syndrome and treatments with dexamethasone, a synthetic agonist of the glucocorticoid receptor, triggers an acute inflammatory response with the concomitant overproduction of acute phase proteins. To test the hypothesis that GSPE could hinder the induction of hepatic MT expression brought about by different inducers which peak in the postabsorptive and postprandial phases, we have tested the effect of GSPE in HepG2 cells under conditions that mimic different aspects of these phases, namely zinc and copper overload, addition of the proinflammatory cytokine IL-6, generation of oxidative stress by tBOOH, and treatment with the cortisol analogue dexamethasone, all of them inducers of liver metallothionein expression (Coyle et al., 2002a; Haq et al., 2003). The aim of these set of experiments was to discern which of the different MT activation pathways might be intercepted by procyanidins and responsible for the postprandial repression of liver MT expression in vivo. The results have shown that, in HepG2 cells, GSPE blocks the induction of metallothionein expression by all the inducers tested. Therefore, procyanidins exhibit the ability to inhibit the activation of MT transcription brought about by a wide array of inducers which act through different signal transduction pathways. This fact strongly suggest that repression of liver MT expression by procyanidins *in vivo* may be due to the simultaneous repression of the different activation pathways operating in the postprandial phase. On the other hand, this unspecificity of procyanidins in repressing the induced expression of MT in HepG2, points out to a common underlying mechanism which interferes with the different signal transduction pathways used by inducers. With the purpose to explain the mechanisms by which procyanidins repress MT expression, two different hypotheses were initially proposed: 1. epigenetic silencing through chromatin modifications in the promoter region of MT genes, mediated by SHP; 2. diminished zinc availability caused by zinc chelation.

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Following the finding that GSPE, EGCG and trimeric procyanidin C1 enhance the levels of cytoplasmic labile zinc in HepG2 cells, a third mechanism for silencing of MT expression by these flavonoids may be proposed: enhancement of Akt phosphorylation elicited by the increment in labile zinc (Figures I.2.22 and V.2) and subsequent inhibition of MTF1 activity by phosphorylated CAAT/Enhancer binding protein (C/EBP) α (Figure V.3).

1) The first mechanism proposed was the intervention of GSPE in **chromatin modification**. Many works have described the silencing of MT expression triggered by epigenetic changes in the promoter region of metallothionein genes, such as methylation of cysteine residues within short stretches of CpG dinucleotides and CpG islands, and histone methylation and deacetylation (Majumder et al., 1999a; Majumder et al., 1999b; Ghoshal et al., 2000; Ghoshal and Jacob, 2001; Ghoshal et al., 2002a; Ghoshal et al., 2002b; Majumder et al., 2002; Datta et al., 2005; Majumder et al., 2006). In support of this hypothesis, it was previously shown that GSPE induces the expression of the orphan nuclear receptor Small Heterodimer Partner (SHP/NR0B2) (Del Bas et al., 2005; Del Bas et al., 2008) a promiscuous transcriptional repressor that has been shown to recruit histone deacetylases, histone methyltranferases and DNA methyltransferases to the promoters of several of their target genes, both *in vivo* and *in vitro* (Hider et al., 2001; Boulias and Talianidis, 2004; Bavner et al., 2005; Gobinet et al., 2005; Fang et al., 2007a; Fang et al., 2007b). We have here shown that the upregulation of SHP occurs concomitantly with the downregulation of MT genes in HepG2 cells upon the addition of GSPE. It is however not yet known whether metallothionein genes are targets of SHP. To address this issue we took advantage of *in vivo* experiments done by our research group with SHP KO and FXR KO mice (Del Bas et al., 2008; Del Bas et al., 2009) and showed that oral administration of GSPE elicits downregulation of hepatic MT expression in WT mice and also in SHP-null mice, but not in FXR-null mice. These results indicate that the observed MT repression elicited by GSPE in mice livers does not require the intervention of SHP but is FXR-dependent. We have also

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shown that chenodeoxycholic acid (CDCA), a bile acid that induces the expression of SHP by activating FXR in HepG2 cells (Del Bas et al., 2009), also repressed the expression of MT genes concomitantly with the induction of SHP, in a similar way as GSPE. These results also suggest that GSPE might act through FXR to repress MT expression in HepG2 cells. These set of experiments let us to discard the hypothesis of the intervention of SHP in recruiting epigenetic machinery to silence MT genes, and revealed MT genes as novel targets of the nuclear receptor FXR. Thus, the nuclear receptor FXR, its activators (bile acids) and its co-activators (procyanidins) (Del Bas et al., 2009) may be considered as regulators of MT expression and, hence, of zinc homeostasis in hepatic cells.

2) An alternative explanation for the repression of MT expression elicited by GSPE in HepG2 is a **diminished bioavailability of zinc** ions to the cells, caused by GSPE and, consequently, the transcriptional activity of MTF-1 in the promoters of MT genes, since transcriptional activity of MTF-1 is sensitive to the availability of intracellular zinc ions. Our results have clearly shown that accumulation of total intracellular zinc in HepG2 cells was hindered upon addition of 150 mg/L GSPE to the standard culture medium (5 μM zinc) as well as when cells were stimulated to accumulate zinc by the addition of 100 μM zinc or 50 μM copper to the medium or by treatment with IL-6 in basal zinc conditions. In addition, toxic effects of 300 μM zinc in the medium were prevented by non-toxic amounts of GSPE. Reversion of excess zinc toxicity occurred despite the fact that zinc-induced expression of MT, a major defense against zinc toxicity (Coyle et al., 2002b; Haq et al., 2003), was hindered by GSPE. A diminished zinc availability may also explain the weaker induction of MT transcription by extra zinc, ROS generated by tBOOH, and by copper, and the complete repression of IL-6 action after the incubation of HepG2 cells with procyanidins. Thus, zinc is necessary for complete induction of MT transcription by ROS. In addition, to activate MT transcription via Nrf2 /ARE, ROS promote the oxidation and degradation of metallothionein, with concomitant release of metallothionein-bound zinc, which then binds MTF-1 and activate

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MT transcription. Therefore, zinc-saturated metallothionein is necessary to completely activate MT gene transcription by ROS (Andrews, 2000; Zhang et al., 2003; Bi et al., 2004; Laity and Andrews, 2007). Also full induction of MT transcription by IL-6 is dependent on zinc availability and MTF-1 activity. IL-6 induces the transcription of its target genes via the signal transducers gp130 leading to the activation of the JAK/STAT (Janus kinase/signal transducer and activator of transcription) and the MAPK (mitogen-activated protein kinase) cascades: ERK1/2, p38 and JNK. Both pathways lead to the activation of transcription factors involved in MT transcription: STAT members, activated directly by JAK kinases, and C/EBP members, activated through the MAPK pathways, and hence MT genes are highly inducible by IL-6 (Schroeder and Cousins, 1990; Baumann et al., 1991; Baumann et al., 1992; Lee et al., 1999; Hernandez et al., 2000; Ghoshal and Jacob, 2001; Ghoshal et al., 2001; Heinrich et al., 2003). It is however known that hepatic MT induction in rats by IL-6 is also dependent on an adequate dietary zinc supply (Huber and Cousins, 1993). The way by which zinc uptake influences induction of MT transcription by IL-6 have been elucidated (Liuzzi et al., 2005). IL-6 upregulates zinc uptake in hepatic cells by increasing expression of zinc transporters ZIP1, ZIP6, ZIP7 and ZIP14, and downregulating ZnT1 and ZnT6, concomitant with upregulation of MTF-1 in the liver of mice treated with LPS or turpentine. This response depends on IL-6 production and is avoided in IL-6 null mice (Liuzzi et al., 2005). Control of hepatic zinc uptake and accumulation by IL-6 has been therefore claimed to be responsible for the plasma hypozincemia associated to the acute phase of the inflammatory response (Liuzzi et al., 2005). We may argue that the same mechanism could also be responsible for postprandial hypozincemia. We have shown that GSPE completely blocks the induction of MT genes by IL-6 in HepG2 cells (Figure V.1.4E). The inhibition by GSPE of zinc uptake by HepG2 cells in basal conditions (Figure V.1.19) strongly suggest that diminished zinc availability is also responsible, at least in part, of the null response of MT expression to IL-6 treatment, since MT upregulation by IL-6 is dependent upon zinc availability. Repression of IL-6 signaling by procyanidins may well be relevant in the postprandial situation. It has previously been described that changes in hepatic gene expression profile triggered by GSPE in the

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postprandial phase are consistent with procyanidins blocking the action of IL-6, but not of other proinflammatory cytokines. IL-6 inducible genes which were downregulated in the liver of GSPE-treated animals are the secretory leukocyte protease inhibitor (Slpi), Foxa2, MT-1 and MT-2, and several genes that encode acute phase proteins: retinol-binding protein, haptoglobin, fibrinogen B and alpha-1- antitrypsin. C-reactive protein (CRP), another IL-6 responsive, acute-phase protein (Gabay and Kushner, 1999; van Oostrom et al., 2004) has also been shown to be decreased in response to chronic GSPE intake in rats fed a high fat diet (Terra et al., 2009).

The demonstrated diminished zinc bioavailability also supports the concept that catechins/procyanidins of GSPE form complexes with zinc in the culture medium thereby preventing the entrance of zinc into the cells through plasma membrane zinc transporter. In this regard, it is known that metal complexation may cause the concatenation of monomeric flavonoids with the metal cations, yielding the flavonoids less prone to partition into membranes (Hider et al., 2001). It may be inferred that the metal cations that link together the flavonoid subunits will also be unable to enter the cell through transmembrane metal ion transporters. Actually, catechins and procyanidins have been shown to inhibit the entrance of iron in human intestinal Caco-2 cells (Scalbert et al., 1999) and long term consumption of high doses of GSPE may lower serum iron levels in rats (Wren et al., 2002). Also consistent with this mechanism, it has also been reported that the ratio of zinc to EGCG, as well as its way of administration, determines the rate of EGCG uptake by PC-3 cells: when EGCG is complexed with zinc by precipitation of saturated solutions of the flavonoid and the metal, the entrance of EGCG (80 μM) in the cells is less than half that when the same amount of only EGCG is provided to the cells; on the contrary, when EGCG is supplied in solution with and equimolar amount of zinc, intracellular accumulation of EGCG is enhanced more than twofold (Sun et al., 2008). The authors inferred that the variable structure and stoichiometry of EGCG-zinc complexes determines different permeability of the flavonoids to the cell membrane.

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In order to elucidate weather catechins/procyanidins of GSPE could form complexes with zinc, we have used several physical-chemical assays. Given that GSPE is a mixture of different catechins and procyanidins with different grade of polymerization, we also tested pure substances contained in the extract such as: flavan-3-ol monomers, (+)-catechin (C), (-)-epicatechin (EC), (-)-epicatechin gallate (ECG), EGCG; dimeric procyanidins B1, B2, B3 and B4 and the procyanidin trimer C1. We also tested different fractions obtained from GSPE. In general, all substances tested (with the exception of dimer B3) suing UV-ViS and fluorescence spectra showed the ability to interact with zinc ions in solution. Also, all catechins and procyanidins tested display an affinity for zinc cations in solution high enough to make them dissociate from the zinc-specific chelator Zinquin, even at very low concentrations and a molar ratio of only 0.1 μM flavonoid to 10 μM Zinquin and 1 μ M zinc. Furthermore, GSPE was also able to remove zinc ions bound to TPEN. This strongly suggests that these flavonoids would have enough strength to displace zinc loosely bound to proteins in cell culture media and, once internalized, within the cell cytoplasm, as does Zinquin and TPEN (Coyle et al., 1994; Reaves et al., 2000). In the cytosol of most cell types, free zinc concentrations are in the picomolar to nanomolar range, and those of Zinquin-detectable, labile zinc are in the range of 1-10 nM, whereas total intracellular zinc, which includes the fixed or structural pool of zinc within metalloproteins, is several hundreds (usually 100-300) μM (Coyle et al., 1994; Hirano et al., 2008; Murakami and Hirano, 2008; Maret, 2009). In human plasma, Zinquin-chelatable zinc is roughly 8 μM, constitutes about >90% of albumin-bound zinc and is considered to be in route for uptake by the different tissues (Chung et al., 2006). Thus, chelation of labile zinc by catechins and procyanidins of GSPE is likely to be relevant *in vivo* since, following oral administration of 1 mg GSPE per kg of body weight to rats, parent unmetabolized catechins, and dimeric and trimeric procyanidins reach plasma concentrations of 2, 2 and 8 μM, respectively, 2 hours after GSPE administration. Glucuronidated catechin and epicatechin reach plasma concentrations of 23.9 and 20.5 μM respectively in these animals (Serra et al., 2009). These metabolites are expected to retain the ability to bind zinc cations since they still have a strong bidentate metal

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site (two adjacent hydroxyl groups) in the B ring and the hydroxyl group in the A ring (see Figure I.1.7). Tetrameric and pentameric procyanidins are also absorbed in rats and may reach plasma concentrations of 7 μg/mL each one (Shoji et al., 2006). Likewise, EGCG may reach concentrations of up to 1.5 μM in human plasma after oral intake of a single 800 mg dose of EGCG (Chow et al., 2001). In this context, it is noteworthy that 5 h after GSPE administration, MT mRNA levels are drastically reduced to 30% of control values in the liver of rats fed a single oral dose of 250 mg GSPE/Kg body weight (Quesada et al., 2007), a dose that effectively lowers postprandial triglyceridemia (Del Bas et al., 2005).

The changes elicited by GSPE in the expression of MT, ZnT and ZIP genes in different conditions are also consistent with a diminished availability of extracellular zinc for HepG2 cells, as it closely resemble the changes elicited by zinc deprivation described in different cell models. Thus, GSPE exerted and effect contrary to those of zinc supplementation on the expression of MT1X, MT2A and zinc efflux transporter ZnT1, which where simultaneously upregulated by addition of zinc and repressed by GSPE in basal and zinc overload conditions. The expression of zinc importers ZIP1 and ZIP4 was also simultaneously repressed by GSPE in conditions of basal and increased zinc concentrations in the medium. Similarly, in cultured mouse fibroblasts, mRNA levels of MT and ZnT1 are elevated upon addition of zinc to the medium, a response mediated by MTF-1, and are downregulated when cells are cultured in zinc depleted medium (Langmade et al., 2000). Also Caco-2 cells respond to supplementation of zinc elevating the expression of MT and ZnT1, whereas treatment with the zinc chelator TPEN results in enhanced expression of ZIP4 and downregulation of ZnT1 and MT1 (Shen et al., 2008). In HeLa cells, supplementation of zinc enhances expression of ZnT1, whereas administration of TPEN upregulates ZnT7 and ZnT5 (Devergnas et al., 2004), as shown here in HepG2 cells treated with GSPE in basal zinc conditions. These expression changes have been interpreted as an homeostatic response of the cells to compensate for reduced zinc availability and directed to maintain adequate zinc

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levels in the cytoplasm and within the Golgi network (Cousins et al., 2003; Devergnas et al., 2004; Shen et al., 2008).

Microarray gene expression data obtained from HepG2 cells treated with GSPE not only yielded information about zinc homeostasis, but also about copper status. The results have shown that the copper transporter Ctr2 was highly upregulated by the addition of GSPE to standard cultured medium in HepG2 cells. Given that Ctr2 is mostly located in intracellular compartments such as endosomes and lysosomes (van den Berghe PV, 2007) and it is partially located to the plasma membrane (Bertinato J, 2008), this results strongly suggest that total intracellular copper might be diminished. Measurements of total intracellular copper content in the same condition confirmed this hypothesis. As seen previously with zinc, procyanidins would also complex copper in the medium making concatenamers less permeable to the cell and thus, leaving less copper bioavailable. Therefore, augmentation of Ctr2 mRNA levels could be a compensatory response to the lack of copper in the cell, since Ctr2 facilitate copper release from intracellular copper stores and mobilize it to the cytoplasm for incorporation into cuproproteins (Bertinato J, 2008). As expected, addition of copper to the cultured medium resulted in an increase of both total intracellular copper content and MT expression in HepG2 cells. GSPE added to this copper supplemented medium was also able to hinder intracellular copper accumulation and elevation of MT mRNA levels. The addition of copper ions to the medium also elicited an augmentation of total intracellular zinc content. This could be due to the displacement of zinc from zinc loaded metallothionein (Zn-MT) by copper ions. This mechanism explains the rise in MT gene expression elicited by copper. Thus, zinc ions released from Zn-MT become available to bind MTF-1 and promote transcription of MT genes (Zhang et al., 2003). GSPE was also able to chelate copper ions in copper supplemented medium, since it could hinder both total intracellular zinc content and MT expression.

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Strikingly, incubation of HepG2 cells with GSPE, in spite of diminishing total intracellular zinc concentrations relative to control cells in conditions of basal (5 μM) and excess (100 μM) zinc concentrations, as well as in cells treated with IL-6, always produced an augmentation of Zinquin-detectable labile pool of intracellular zinc. To our knowledge, only two reports have described the effect of dietary polyphenolic compounds on labile zinc. The glycone isoflavone genistin, applied at 100 μM, enhances the pro-apoptotic effects of zinc in HepG2 cells and up-regulate the expression of MT and ZnT1 concomitantly increasing the labile zinc pool detectable by FluoZin-3 (Chung et al., 2006). The effect of genistin on total zinc content and on zinc toxicity was not reported. The stilbene resveratrol, at physiological concentrations (10 μM), efficiently chelates zinc in solution and, when applied to normal prostate epithelial cells cultured in 16 or 32 μM zinc, arrests cell growth and enhances Zinquin-detectable zinc, while not affecting total zinc nor MT expression (Zhang et al., 2009). The authors propose that the increment of labile zinc elicited by resveratrol is due to the cellular uptake of resveratrol-zinc complexes, followed by the intracellular dissociation of the complexes (Zhang et al., 2009).

As mentioned above, GSPE is a mixture of dozens of different catechins and procyanidins, so we tested the effect of three pure substances (a monomeric procyanidin, EGCG; a dimeric procyanidin, B1 and a trimeric procyanidin, C1) in different aspects of zinc homeostasis. Results with EGCG showed that this compound exhibited the same effects on cellular zinc homeostasis as GSPE did. Strikingly, trimer C1 behaved opposite to GSPE and EGCG in MT and zinc transporter expression, total zinc accumulation and on cell viability in basal and zinc overload conditions. However, trimer C1 enhanced cytoplasmatic labile zinc as did GSPE and EGCG. Trimer C1 behaves as genistein in augmenting MT expression and increasing the labile pool of zinc (Chung et al., 2006). Preliminary results with dimer B1 showed that this compound acts in the same way as trimer C1 in modulating MT and increasing total zinc accumulation. Further experiments are needed to know its effects on cytoplasmatic labile zinc and other aspects of zinc homeostasis.

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Considering these data, it appears that flavonoids may enhance intracellular labile zinc levels independently of their effect on MT and ZnT1 expression and total intracellular zinc content.

A plausible explanation for the increment of cytoplasmic labile zinc exerted by GSPE and EGCG is that membrane-permeable flavonoid-zinc complexes are always formed in addition to membrane-impermeable flavonoid-zinc concatenamers. Those zinc atoms that enter the cells complexed with the flavonoids (and hence through a ZIP-independent way) will add to the pool of labile zinc and latter, once the flavonoid has been metabolized, to the pool of free zinc (Figure V.1). This increment in free/labile zinc, within the nanomolar range of concentrations, should not suffice to produce significant increments of total zinc content, which amounts concentration of few hundreds micromolar. An alternative, though not excluding possibility is that, once internalized, the flavonoids mobilize zinc from intracellular zinc stores such as MT, endoplasmic reticulum, vesicles, or zincosomes, as have been shown for Zinquin itself, that is able to retrieve some of the MT-bound zinc cations (Coyle et al., 1994). This could be the case for trimer C1, that upregulates MT gene expression and increase both total and labile zinc in HepG2 cells (Figure V.3.1). Mobilization of copper ions from the nuclear compartment has been shown in lymphocytes treated with EGCG (Shamim et al., 2008). The proportion of the different types of flavonoid-zinc complexes formed will depend on the absolute and relative concentrations of flavonoid and zinc, on competing metals and zinc-binding proteins, temperature, pH, etc.

In any case, the increment in labile zinc elicited by GSPE and EGCG should not be immediately available or sufficient to stimulate the transcriptional activity of MTF-1 on the promoters of MT and ZnT1, given the observed downregulation of MT and ZnT1 mRNA levels in GSPE treated cells. It could however suffice to enhance the transcription of GCLC, also under the control of MTF-1. MTF-1 discerns between different MRE in response to different zinc load (Laity and Andrews, 2007). Alternatively, upregulation of GCLC by GSPE and EGCG may be independent of MTF-1. EGCG is known to induce the expression of GCLC, and manganese superoxide dismutase (MnSOD) by activating the transcriptional

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activity of the redox-sensitive nuclear factor erythroid 2 p45-related factor (Nrf2) which recognizes the antioxidant response element (ARE) in the promoter of these antioxidant genes (Na and Surh, 2008).

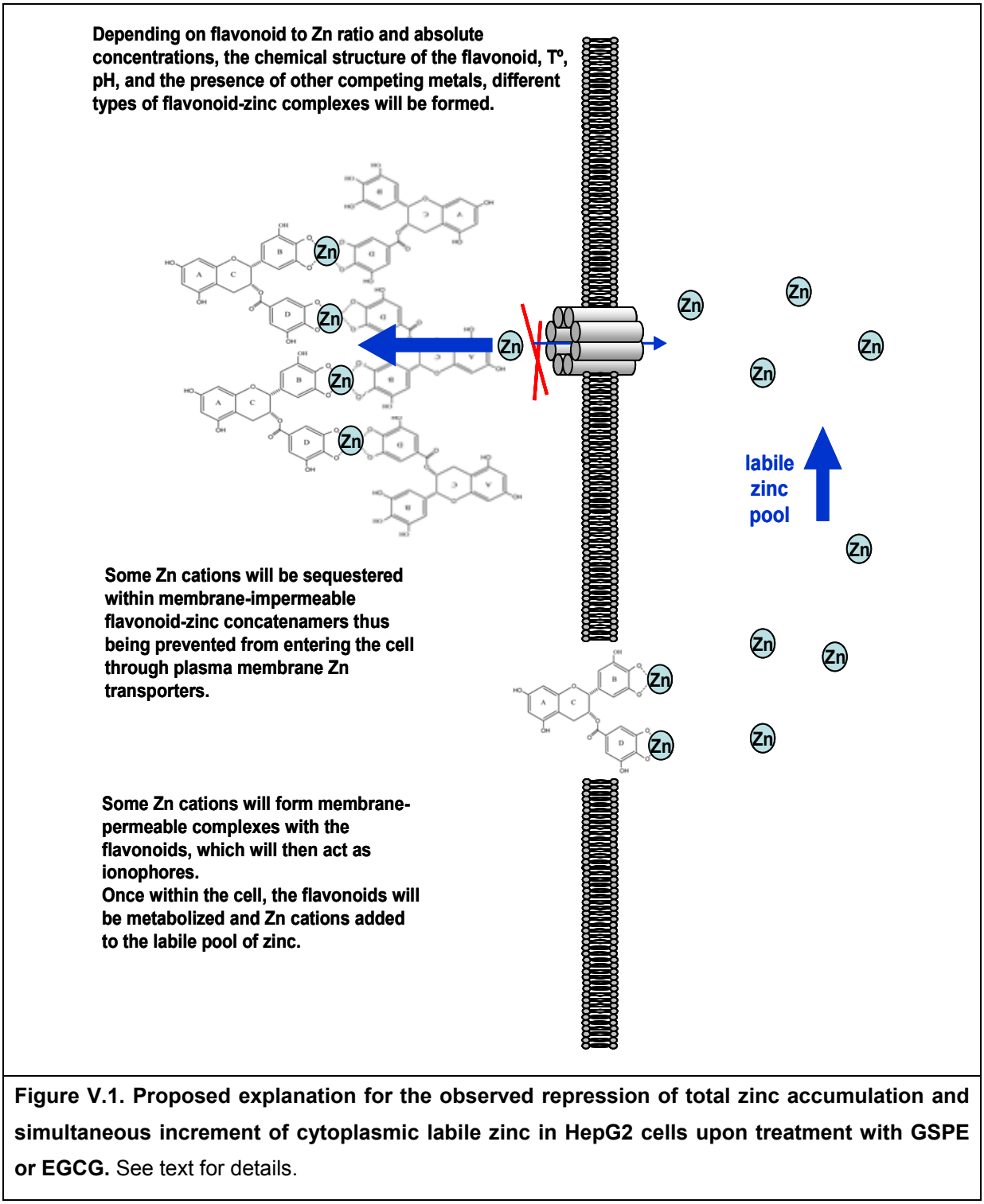

Figure V.1. Proposed explanation for the observed repression of total zinc accumulation and simultaneous increment of cytoplasmic labile zinc in HepG2 cells upon treatment with GSPE or EGCG. See text for details.

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Whatever the mechanism behind, the increment of labile, Zinquin-detectable cytoplasmic zinc described here for GSPE, EGCG and trimer C1 may be relevant to explain the bioactivity of these flavonoids. Zinquin-detectable zinc is considered a measure of the pool of this metal that can be exchanged between proteins and is endowed with a regulatory and signaling function, i.e., may modulate the activity of components of signal transduction pathways and key enzymes of multiple metabolic pathways (Coyle et al., 1994; Beyersmann and Haase, 2001; Hirano et al., 2008; Murakami and Hirano, 2008; Maret, 2009). Thus, increments within the nanomolar range in the levels of free cytoplasmic zinc inhibit the activity of cyclic nucleotide phosphodiesterases (PDE) and protein tyrosine phosphatases (PTP), and activate mitogen-activated protein kinase (MAPK), protein kinase C (PKC), and calcium-calmodulin activated protein kinase-2 (CaMPK-2), leading to changes in the phosphorylation state of numerous downstream cell signaling and transcription factors. For instance, inhibition of PTP 1B by zinc (IC50 17 nM) results in enhanced net phosphorylation of the insulin receptor and activation of downstream signalling cascades pathways such as MAP, PI-3 and Akt kinases, and is thought to contribute to the insulin-mimetic effects of zinc and zinc-complexes (Haase and Maret, 2005; Basuki et al., 2007) (Figure V.2). Likewise, inhibition of PDE by zinc (IC50 20 nM) enhances cGMP and cAMP signaling (Beyersmann and Haase, 2001). Free zinc may also directly activate transcription factors, as is the case for MTF-1, or inhibit its transcriptional activity, as for nuclear factor-kappa beta (NF-KB) (Beyersmann and Haase, 2001). It is remarkable that many actions described for diverse flavonoids on signaling pathways overlap with those described for fluctuations of free and labile zinc. As an example, GSPE displays insulinomimetic effects, enhancing phosphorylation of IR and downstream kinases (Montagut et al., 2009). Likewise, many flavonoids, including EGCG and procyanidins inhibit the transcriptional activity of NF-KB (Mackenzie et al., 2004; Terra et al., 2007; Na and Surh, 2008). Similarly, many flavonoids have been shown to inhibit PDE activity, consequently elevating cytoplasmic cAMP levels (Peluso, 2006).

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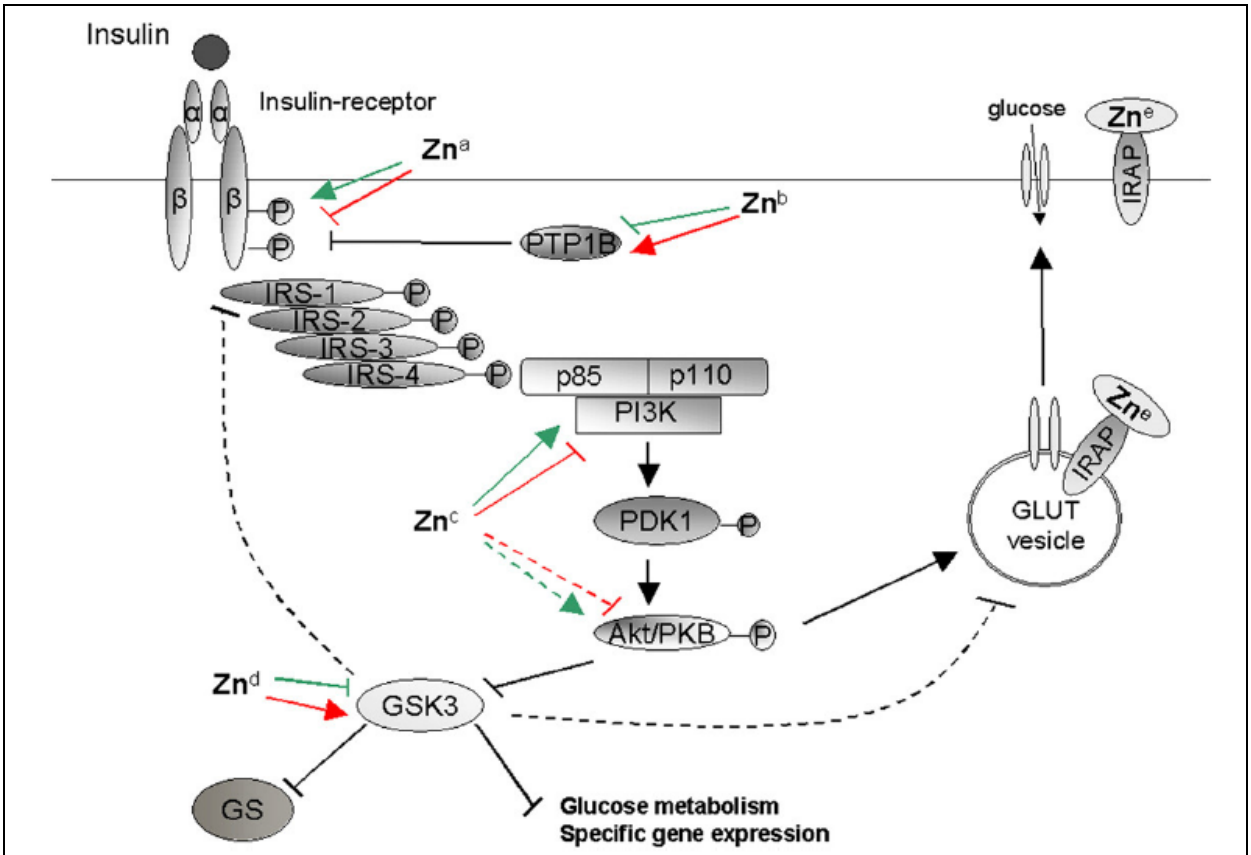

Figure V.2. Influence of zinc on the insulin signaling pathway. (→) Activation; (⊣) inhibition; (- - →) activation/inhibition of Akt by zinc/zinc deficiency might not be a direct effect of zinc on Akt but might be mediated by stimulation of PI3K; (- - ⊣) inhibition under certain conditions described in the review; green: insulinomimetic effects of zinc; red: effects of zinc deficiency leading to insulin resistance. Some of the insulinomimetic effects of zinc can be explained by the influence of zinc on insulin signaling. In brief, after binding of insulin to the α subunits of the tetrameric insulin receptor, the kinase activity of the β subunit is stimulated, which results in transphosphorylation of the β subunit This induces phosphorylation of members of the IRS family and subsequent interaction with signaling molecules like the p85 subunit of the PI3K. PI3K in turn triggers phosphorylation of PDK1, a serine kinase that activates Akt/PKB. Akt leads to stimulation of GLUT 4 translocation in adipocytes and to inhibition of GSK-3, thereby allowing activation of glycogen synthase in adipocytes, translocation of GLUT to the cell surface and induction of glucose metabolism. In addition, inhibition of GSK-3 results in enhanced protein synthesis and gene expression. Zinc leads to tyrosine phosphorylation of the β subunit of the insulin-receptor and to inhibition of PTP1B which dephosphorylates the insulin receptor, thus increasing phosphorylation of the receptor Akt is activated by zinc in a PI3K-dependent way and zinc inhibits GSK-3, just like insulin. Moreover, zinc plays a role in glucose transport since it is part of IRAP, a molecule probably required for maintenance of normal GLUT levels. Figure taken from (Jansen et al., 2009).

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3) Another mechanism of transcriptional repression of MT has been recently described in primary human hepatocellular carcinomas (HCCs), including HepG2. Activation of phosphatidylinositol 3-kinase (PI3K) and protein kinase B (PKB/AKT) inactivates glycogen synthase kinase (GSK). This kinase activates MT expression by phosphorylating the CCAAT/Enhancer Binding Protein α (C/EBPα) that interacts with MTF-1 and/or basal transcription factors in the promoter of MT genes (Datta et al., 2007) (Figure V.3). On the other hand, increased levels of cytoplasmic free/labile zinc, brought about by zinc ionophores such as pyrithione, enhance phosphorylation and activation of PI3K and AKT in many cell types, including hepatic cells (Haase and Maret, 2005; Jansen et al., 2009). By combining these two mechanisms, it appears that increments in labile zinc may occur simultaneously with repression of MT expression, a situation that should help the cell to keep intracellular zinc available for essential functions when extracellular zinc becomes temporally unavailable, and is consistent with an homeostatic response of cells to low zinc availability. Experiments done in HepG2 cells grown 12 h in standard culture medium (5 μM zinc) supplemented with 150mg/L GSPE, revealed that expression of C/EBPα was decreased to 15%, using oligonucleotide microarray hybridization (Figure V.4). Diminished C/EBPα expression would also reduce MTF1 activity. In this context, it is also remarkable that GSPE has been shown to elicit phosphorylation of PI3K and AKT, a mechanism that might underlie the known insulinomimetic effect of procyanidins (Montagut et al., 2009) and is consistent with the hypothesis that flavonoids may work as zinc ionophores.

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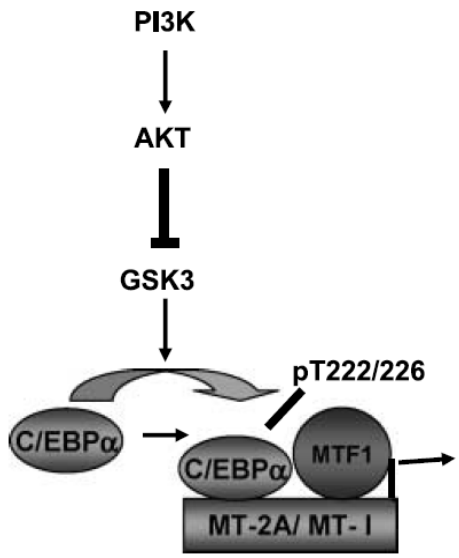

Figure V.3. A model depicting regulation of MT genes in HCC cells by PI3K and its downstream effectors. Metallothionein expression is suppressed in primary human hepatocellular carcinomas and is mediated through inactivation of CAAT/Enhancer binding protein α by PI3K signaling cascade. Figure taken from (Datta et al., 2007).

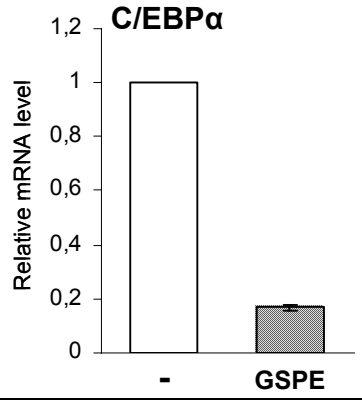

Figure V.4. C/EBPα is downregulated by GSPE in HepG2 cells. Either vehicle (control) or GSPE (150 mg/L) were added to HepG2 cells. Samples were collected for microarray analysis of mRNA levels 12 hours after treatment.

In summary, we have presented evidence supporting that interaction of catechins and procyanidins with zinc cations modulate zinc absorption and metabolism, resulting in increased levels of cytoplasmic labile zinc. Repression of MT expression, elicited by the co-activation of FXR by procyanidins, and possibly requiring phosphorylation of Akt and CAAT/EBPα, allows HepG2 cells to keep the cytoplasmic levels of labile zinc elevated. We forward the hypothesis that elevation of labile zinc by these flavonoids may be a relevant mechanism by which they affect multiple metabolic and cell signaling pathways that respond to intracellular fluctuations of labile zinc. Further research is necessary to asses the mechanisms by which these flavonoids enhance cytoplasmic labile zinc and the consequences of this enhancement on modulation of zinc signaling and metabolic pathways.

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VI. CONCLUDING REMARKS

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The main conclusions obtained in this PhD Thesis are:

1. **GSPE inhibits the expression of MT genes in hepatic cells, both *in vivo* and *in vitro*, in human and rat hepatocytes,** implying that GSPE act directly on hepatic MT, excluding the intervention of other tissues. Therefore, MT genes are bona fide targets of procyanidin action and, hence, procyanidins modulate zinc homeostasys in hepatic cells.

2. **GSPE hinders the activation of MT genes elicited by all tested inducers that mimic the postprandial situation *in vitro*, namely: zinc and copper cations, the proinflammatory cytokine IL-6, the ROS generator tBOOH and the glucocorticoid receptor agonist dexamethasone.** Therefore, GSPE elicits repression of hepatic MT expression through a general mechanism that interferes with the different signal transduction pathways used by the inducers of MT expression.

3. **The repressing effect of GSPE on hepatic MT expression *in vivo* is mediated by the bile acids receptor FXR and is independent of SHP. Bile acids act as repressors of MT expression *in vitro* in HepG2 cells.** These facts reveal MT genes as novel targets of FXR and, consequently, confirm FXR as a novel regulator of zinc homeostasis in hepatic cells. Consequently, FXR activators and coactivators –namaly bile acids, and procyanidins, respectively- act as modulators of MT expression and zinc homeostasys.

4. **Catechins and procyanidins complex with zinc cations in solution.** At physiological concentrations, they display an affinity for zinc ions high enough to make them dissociate from the zinc specific chelator Zinquin. Thus, the formation of flavonoid-zinc complexes may play a relevant role in modulating metal and flavonoid bioavailability.

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4. Procyanidins affect all aspects of zinc homeostasys in hepatic cells, *in vitro*.

4.1. In addition to repress MT expression, GSPE inhibits the expression of zinc-efflux transporters, induces the expression of zinc-influx transporters and of zinc importers of the Golgi network, and inhibits intracellular zinc accumulation in HepG2 cells cultured in basal zinc conditions. All these changes are similar to that elicited by known zinc sequesters and are considered as a compensatory homeostatic response of cells to diminish extracellular zinc availability.

4.2. GSPE counteracts the effects of excess zinc and IL-6 on expression of zinc transporters and intracellular zinc accumulation, and hinders the toxic effects of excess zinc on viability in HepG2 cells. Thus, procyanidins prevent the entrance of zinc into the cells through plasma membrane ZIP transporters, supporting the concept that GSPE complexes with zinc in the culture medium rendering most zinc cations unavailable to the cell.

5. GSPE affects copper homeostasis in HepG2 cells. GSPE represses the expression of copper-efflux transporters, induce the expression of copper-influx transporters and diminishes intracellular copper content. These results strongly suggest that procyanidins display a sequestering effect on extracellular copper and trigger a compensatory transcriptional response on genes controlling cellular copper homeostasis.

6. EGCG reproduces the major effects of GSPE on zinc homeostasis in HepG2 cells. EGCG modulates MT and zinc transporters expression, diminishes intracellular zinc accumulation and prevent zinc toxicity in the same way as GSPE.

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7. Dimeric procyanidin B1 and trimeric procyanidin C1 behave opposite to GSPE and EGCG regarding modulation of MT expression and intracellular zinc accumulation in basal conditions in HepG2 cells; C1 enhances the toxic effects of excess zinc. These results suggest that B1 and C1 do not form membrane-impermeable complexes with zinc, and therefore do not impede MT induction nor zinc toxicity elicited by extracellular zinc cations.

8. GSPE, EGCG and trimer C1 elevate cytoplasmatic labile zinc in HepG2 cells. It appears that these flavonoids enhance cytoplasmatic labile zinc levels independently of their effects on MT and zinc transporters expression and on accumulation of total intracellular zinc.

9. We postulate that extracellular cheletion of zinc cations by EGCG and EGCG may explain the enhancement of labile zinc cocurrently with the inhibition of total zinc content:

9.1. On the one hand, the vast majority of **GSPE and EGCG** molecules, in the **micromolar** concentrations range, **form membrane-impermeable concatenamers with zinc cations** in the extracellular milieu, thus sequestering most of the available extracellular zinc and impeding its entrance into the cell through plasma membrane zinc transporters.

9.2. On the other hand, a tiny fraction of **GSPE and EGCG**, in the **nanomolar** concentrations range, **form membrane-permeable complexes with zinc cations**, thus acting as metal **ionophores**, and thereby increasing the cytoplasmic labile pool of zinc.

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10. **GSPE and EGCG may modulate the multiple signaling and metabolic pathways that are targets of zinc signaling,** i.e., that are susceptible to be regulated by fluctuations in the cytoplasmic pool of labile zinc. Thus, the modulation of zinc signaling may underlay many of the effects exerted by GSPE and EGCG *in vivo* and *in vitro*.

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VII. ANEXES

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PROCEEDINGS

Grape seed procyanidins inhibit the expression of metallothione in genes in human HepG2 cells

Isabel María Quesada · Josep María Del Bas · Cinta Bladé · Anna Ardèvol · Mayte Blay · María Josepa Salvadó · Gerard Pujadas · Juan Fernández-Larrea · Lluis Arola

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Abstract Procyanidins are the most abundant polyphenols in red wine and are also found in cereals, fruits, chocolate and tea. They exert many beneficial health effects, especially on the cardiovascular system (Bagchi et al. in Mutat Res 523–524:87–97, 2003; Williams et al. in Free Radic Biol Med 36:838–849, 2004; Dell'Agli et al. in Cardiovasc Res 63(4):593–602, 2004; Del Bas et al. FASEB J 19:479–480, 2005). Here, we show that oral administration of a grape seed procyanidins extract (GSPE) to healthy rats results, 5 h after treatment, in a 70% inhibition of metallothionein (MT) gene expression in the liver, as determined by oligonucleotide microarray hybridization. Similarly, in cultured human hepatocytes HepG2, GSPE downregulate the expression of MT genes at the mRNA level, as evaluated by quantitative RTPCR. Thus, mRNA levels of six functional MT genes, MT1A, 1E, 1F, 1G, 1X and MT2A, are diminished between 50 and 80% when HepG2 cells are treated during 12 h with GSPE. Only the expression of two human MT genes, MT1G and MT1E, is transiently increased during the first 2 h of treatment. GSPE-induced inhibition of MT genes expression is dose dependent, at concentrations that are not toxic for the cells. Our findings demonstrate that metallothionein genes are direct targets of procyanidins action, both in vivo and in vitro, in hepatic cells. Thus, this study will help to elucidate the mechanisms by which procyanidin exert their beneficial actions.

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Keywords Metallothionein · Procyanidins · Gene expression · Rat · Liver · HepG2

Introduction

Procyanidins (PCs) are the most abundant polyphenols in red wine and they are also found in cereals, fruits, chocolate and tea. Many epidemiological studies have demonstrated that moderate consumption of red wine is associated with reduced mortality and risk of cardiovascular disease. Also, numerous in vivo and in vitro studies have shown that PCs of red wine and other foods contribute to cardioprotection through different ways: they have a strong antioxidant capacity, act as anti-inflammatory agents, decrease arterial pressure and modify plasma lipid profile by lowering the atherogenic risk index. Some PCs have been shown to interact with specific cell surface membrane receptors and with proteins of intracellular signaling pathways, finally leading to the modulation of gene expression in many cell types and tissues [1, 2].

Metallothioneins (MTs) are a family of low molecular weight, cysteine-rich metal-binding proteins widely distributed and highly conserved among bacteria, fungi, plants and animals. They are important for the detoxification of heavy metals such as Cd and Hg, but in physiological conditions they are mainly involved in the control or Zn and Cu homeostasis. For instance, they are able to interchange Zn with zinc-finger-containing transcription factors. Basal expression of mammalian MT genes may be up regulated by heavy metals, oxidative stress (reactive oxygen species), pro-inflammatory cytokines (TNF-a, IL-1, IL-6), glucocorticoids and glucagon. Conversely, MT expression can be repressed by agents that promote DNA methylation or histone deacetylation. All these agents

modulate MT gene expression through specific elements located within the promoter region of every MT gene [3]. In this study, we show that grape seed procyanidin extracts (GSPE) inhibit basal and metal-induced expression of MT genes in hepatic cells, both in vitro and in vivo. Thus, MT genes are direct targets of PCs bioactivity and MT gene expression may be used to analyze the mechanisms by which dietary PCs exert beneficial effects on health.

Materials and methods

Cell culture

Human hepatoblastoma HepG2 cells (American type culture collection, ATCC) were cultured in 12 well plates in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum, 1% penicillin, 1% streptomycin, 1% L-glutamine, 1% non-essential amino acids, in a humidified atmosphere with 5% CO_2 at 37°C. In all experiments, 105 cells were seeded per well, the medium was changed 6 h later and cells were allowed to recover 15 h more before the administration of either vehicle (control cultures), grape seed procyanidins extract (GSPE) at different final concentrations (PCtreated cells, see figure legends), or $CuCl_2$ at 50 mM final concentration (Cu-treated cells).

Quantification of mRNA levels

Total RNA was purified from HepG2 cells using the NucleoSpin RNA II kit (Machery-Nagel, Düren, Germany) following the instructions of the manufacturer. Single-stranded cDNA was generated from total RNA using TaqMan reverse transcription reagents (Applied Biosystems). Quantitative PCR amplification and detection were performed using specific TaqMan assay-on-demand probes (Applied Biosystems) and the Applied Biosystems Real Time 7000 PCR System termocycler and software. Three biological samples were taken for each experimental condition, and three technical replicas were performed for each biological sample. ActinB was taken as the control gene used in MT basal expression experiments, and cyclophilin was the control gene for Cu-induction experiments.

The grape seed procyanidins extract (VitaFlavan) was kindly provided by Les Dérives Résiniques et Terpéniques, Cedex, France.

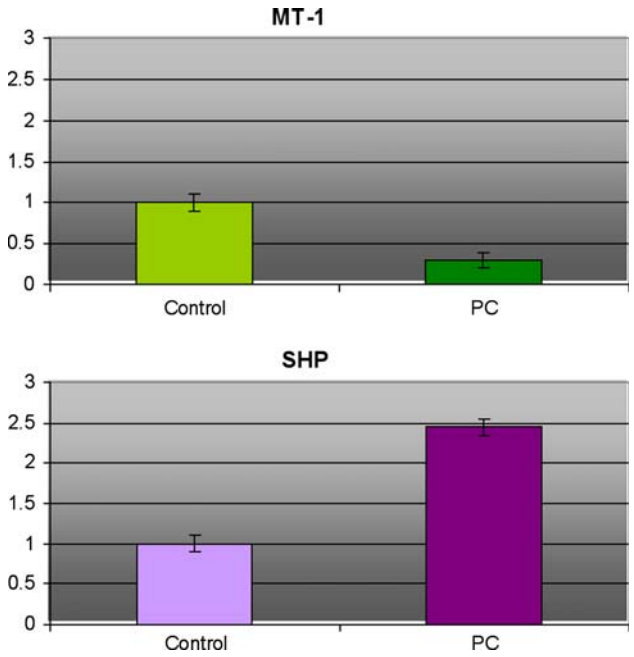

Fig. 1 Effect of PCs administration on MT and SHP gene expression in the liver of healthy rats

Results

Procyanidins down regulate hepatic MT gene expression in healthy rats. In order to identify genes whose expression in the liver is modulated by PCs, healthy rats were given a single oral dose of GSPE and, 5 h after treatment, changes in the pattern of gene expression in hepatic cells were determined by differential oligonucleotide microarray hybridization (Fig. 1) [4]. These analyses revealed that MT gene expression is down regulated to 30% in PCs treated versus untreated control animals. At the same time, mRNA levels of the orphan nuclear receptor small heterodimer

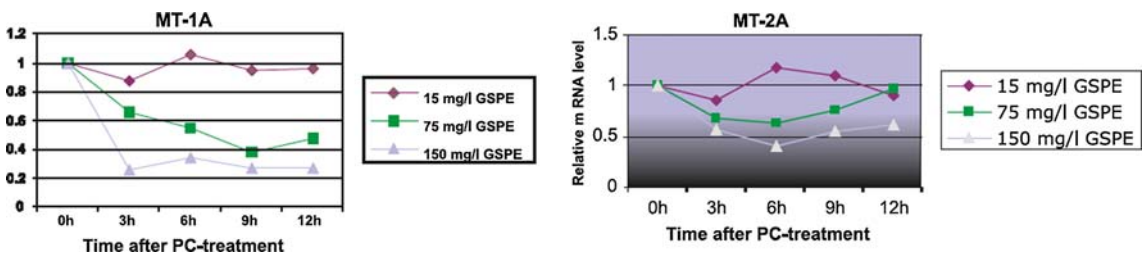

Fig. 2 Dose effect of PCs on MTs mRNA level in HepG2 cells

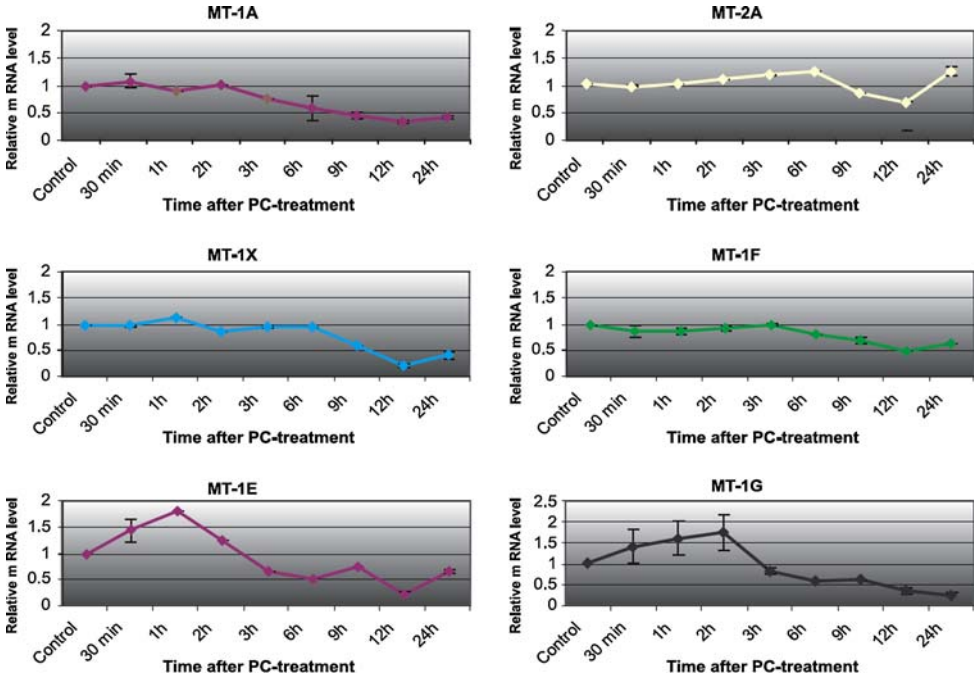

Fig. 3 Time course effects of PCs on MTs mRNA level in HepG2 cells

partner (SHP, Nr0b2), increases twice in PCs treated rats in comparison to control animals [4]. Thus, the expression of both MT and SHP genes are targets of PCs action in hepatic cells in vivo. Procyanidins repress basal MT gene expression in cultured human hepatocytes.

In order to test weather PCs may down regulate hepatic MT gene expression in other mammalian species and without the intermediation of peripheral tissues, human hepatocytes of the HepG2 cell line were administered different doses of a GSPE during several periods of time (Fig. 2) and mRNA levels of the MT1-A and MT2A genes were analyzed by RT-PCR. GSPE inhibited the expression of MT1-A and MT2-A genes in a dose-dependent manner, at PCs concentrations that are not toxic for the cells. Next, we used the highest non-toxic and effective concentration of GSPE to monitor PCs effect on the expression of other hepatic MT genes, along different times of treatment (Fig. 3). We found that, although with different kinetics, the mRNA level of all hepatic MT genes tested (MT1-A, E, E, F, G, X and MT2-A) were already decreased 6 h after treatment, and reached a minimum level after 12 h. Only the expression of two MT genes, MT1E and MT1G was transiently increased during the first 2 h of treatment. The decrease in MT mRNA level upon 12 h of PCs treatment was highest for the MT1-X gene (28% of basal level) and lowest for the MT2-A gene (36% lower than control level). Procyanidins repress metal-induced MT gene expression in cultured human hepatocytes.

In order to test weather the inhibition of MT gene expression triggered by PCs in basal, non-induced conditions could also be effective in induced conditions, we first treated HepG2 cells with GSPE and then applied copper salts to stimulate transcription of MT genes (Fig. 4) When human HepG2 cells were treated with 50 μM $CuCl_2$ alone, an increase of all MTs mRNAs was observed. The highest effect was on the MT1X gene, whose mRNA increased 3.5-fold 12 h after treatment with $CuCl_2$. This induction capacity of copper salts was prevented by the treatment with GSPE. Thus, MT1X mRNA levels increased only 1.5-fold when GSPE were added prior to the induction with copper salts.

Discussion

We have shown here that:

1. Procyanidins inhibit the expression of metallothionein genes in hepatic cells, both in vivo and in vitro. Simultaneously, procyanidins enhance de expression of the transcriptional repressor SHP.
2. The effect of procyanidins on metallothionein gene expression in HepG2 cells is dose dependent, at concentrations that are not toxic for the cells.
3. In addition, procyanidins can prevent the increase of copper induced expression of metallothionein genes.

Our results imply that MT genes are targets of PC actions and that MT gene expression may be used to monitor bioactivity of dietary PC. In addition, the study of the different pathways by which PCs inhibit MT expression,

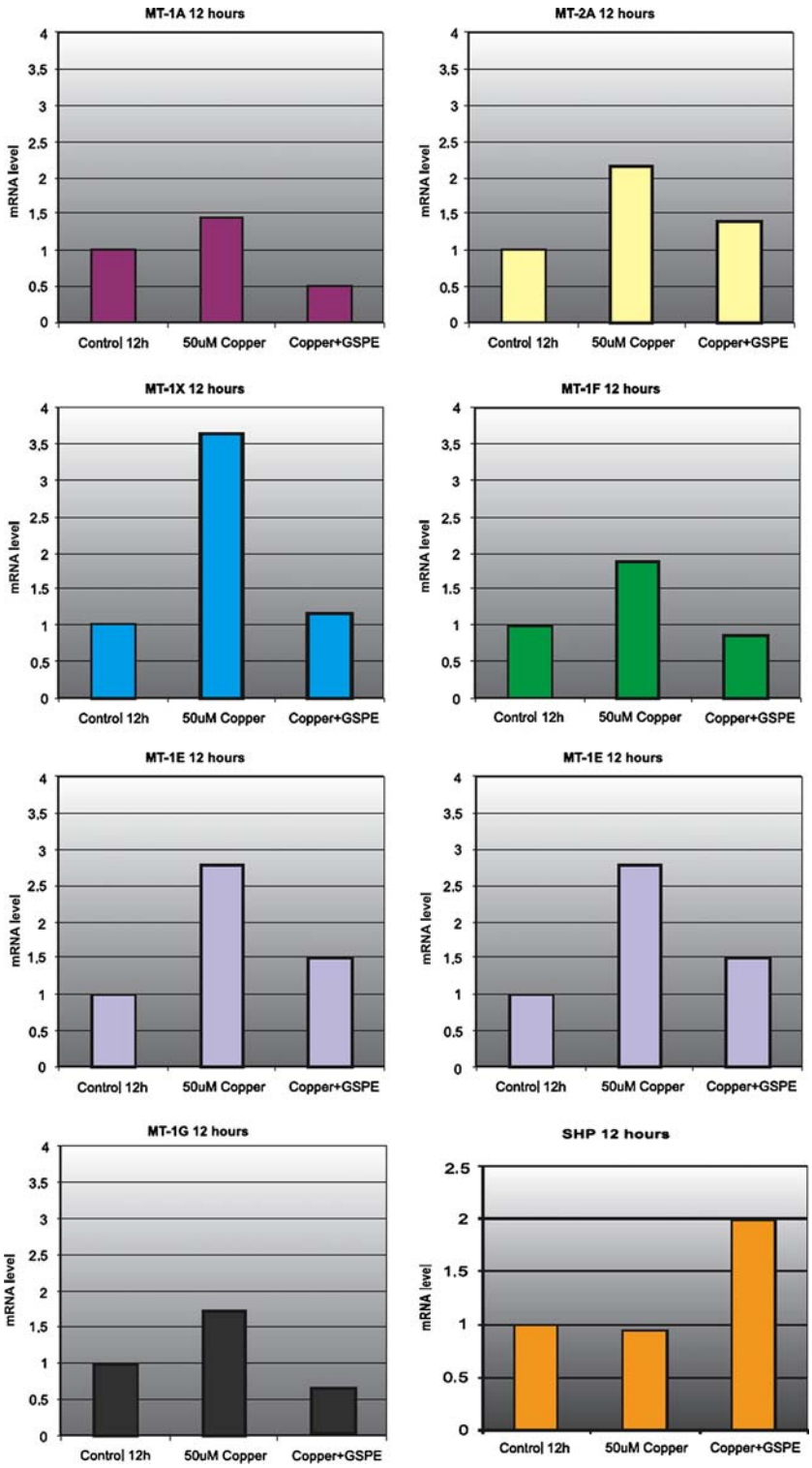

Fig. 4 Effect of PCs on copper-induced expression of MT genes and on SHP mRNA in HepG2 cells

may be a useful tool to identify the mechanisms by which dietary PC modify cellular metabolism in a way that is beneficial for health. We propose that the decreased expression of MT genes triggered by administration of PC, reflects the antioxidant, anti-inflammatory and insulin-like activities already described for procyanidins. The concomitant induction of SHP, a transcriptional repressor that acts by recruiting histone deacetylases [5], suggest that SHP could mediate the PC-induced repression of MT gene expression.

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Dietary Catechins and Procyanidins Modulate Zinc Homeostasis in Human HepG2 Cells

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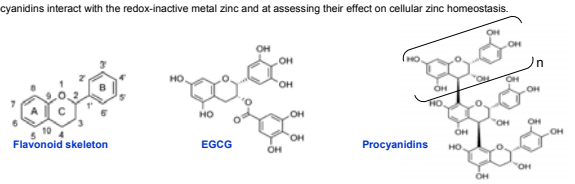
Introduction

Catechins and their polymers procyanidins are health-promoting flavonoids found in edible vegetables and fruits [1,2]. They act as antioxidants by scavenging reactive oxygen species and by chelating the redox active metals iron and copper [3,4]. They also behave as signaling molecules, modulating multiple cell signalling pathways, including that of insulin, and gene expression, including that of antioxidant enzymes [5-8]. This study was aimed at determining whether catechins and procyanidins interact with the redox-inactive metal zinc and at assessing their effect on cellular zinc homeostasis.



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Results

Catechins and procyanidins interact with zinc in solution

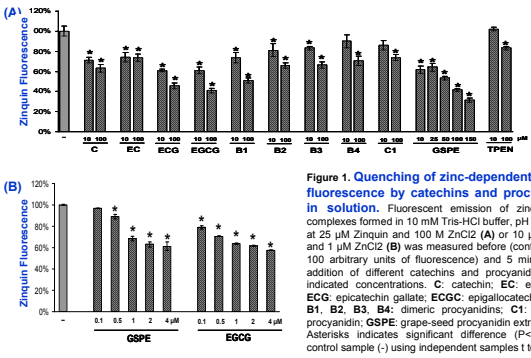

Figure 1. Quenching of zinc-dependent Zinquin fluorescence by catechins and procyanidins in solution. Fluorescent emission of zinc- Zinquin complexes formed in 10 mM Tris-HCl buffer, pH 7.4, 25 ºC, at 25 µM Zinquin and 100 M ZnCl2 **(A)** or 10 µM Zinquin and 1 µM ZnCl2 **(B)** was measured before (control values, 100 arbitrary units of fluorescence) and 5 minutes after addition of different catechins and procyanidins at the indicated concentrations. **C**: catechin; **EC**: epicatechin; **ECG**: epicatechin gallate; **ECGC**: epigallocatechin gallate; **B1**, **B2**, **B3**, **B4**: dimeric procyanidins; **C1**: a trimeric procyanidin; **GSPE**: grape-seed procyanidin extract. Asterisks indicates significant difference (P<0.05) with control sample (-) using independent samples t test.

GSPE modulates the expression of genes involved in zinc homeostasis in a similar way as zinc chelators and diminishes total intracellular zinc in HepG2 cells cultured in basal zinc conditions.

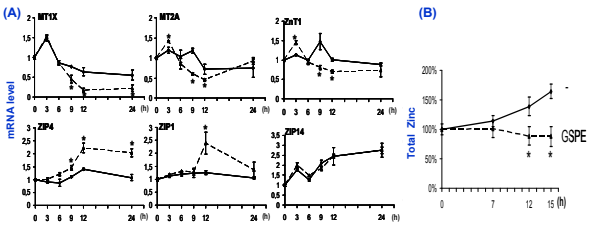

Figure 2. A Kinetics of mRNA levels of MTs and plasma membrane zinc transporters. Black lines: control cells, added fresh medium (5 µM Zn) at time 0h. Dashed lines: cells added fresh medium containing GSPE (150 mg/L). mRNA levels were quantified by RT-QPCR using specific TaqMan probes (Applied Biosystems) and Cyclophilin A (PpiA) as control gene. **Figure 2.B Progression of total intracellular zinc content**. The total amount of zinc within cells was determined by FAAS, and values normalized per total protein content of the cells. The zinc to protein ratio in cells just at the beginning of the treatment with 150 mg/L GSPE was 3,355 ± 0,125 nmol zinc per mg protein and is assigned the arbitrary value of 100. Asterisks denote significant difference (P<0.05) in treated cells versus control cells at the same time point using independent samples t test.

GSPE counteracts the effect of excess zinc on expression of MT and zinc transporters, on intracellular zinc accumulation and on cell viability

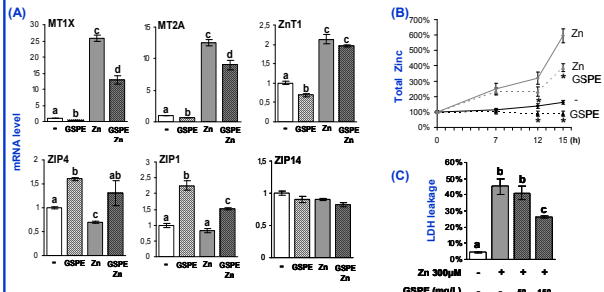

Figure 4. Effects of GSPE on zinc homeostasis in HepG2 cells treated with excess zinc. **(A) mRNA levels** of **MT** and palsma membrane **zinc transporter** genes upon 12 hours incubation with 150 mg/L GSPE (GSPE), 100 µM $ZnCl_2$ (Zn), or both (GSPE Zn), relative to the levels in untreated cells (-). **(B)** Evolution of **total intracellular zinc**, normalized per protein content, in the same cell cultures. **(C)** Prevention of toxicity of 300 µ M zinc in the culture medium by the addition of GSPE, assessed by the **LDH test** after 24 hours of treatment. Asterisks as in Fig.3. Different letters denote different values after one way ANOVA test (P<0.05).

GSPE elevates intracellular labile zinc in HepG2 cells

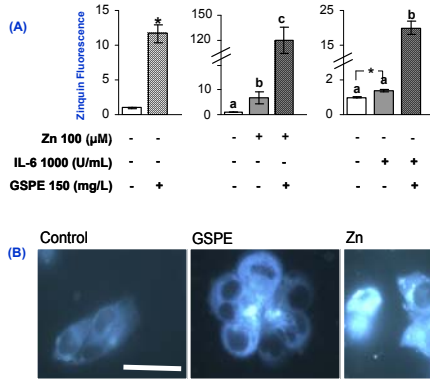

Figure 6. Effect of GSPE on cytoplasmic labile zinc in HepG2 cells. **(A)** Fluorescence of **Zinquin**-loaded cells was normalized to protein content in each sample, and untreated cells were assigned the arbitrary value of 1. Treatments with GSPE, zinc and IL-6 were as in Fig. 2, 3 and 4, respectively. **(B)** Images of Zinquin-loaded HepG2 cells. To visualize intracellular Zinquin fluorescence, cells were seeded in wells containing glass coverslips, treated as above, and loaded with Zinquin ester; images at 1000x magnification were acquired with a Leica DM 4000B microscope using UV light illumination (λex = 340-380 nm) and a blue emission filer (λem ≥ 425nm). Barr: 50 µm.

EGCG reproduces the major effects of GSPE on zinc homeostasis

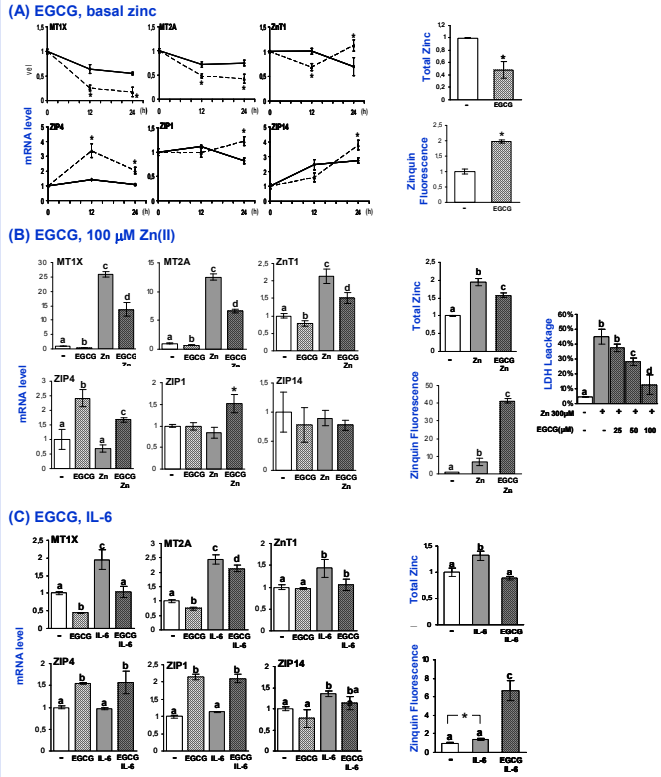

Figure 6. Effects of EGCG on zinc homeostasis in HepG2 cells. Effect of EGCG (100 µM) on gene expression, total intracellular zinc and Zinquin-detectable cytoplasmic labile zinc (both normalized per protein content) in HepG2 cells: **(A)** cultured in standard conditions; **(B)** treated 12 h with additional (100 µM) zinc; **(C)** treated 15 h with IL-6 (1000 U/mL). Asterisks and letters as in previous figures.

Discussion

Interaction of catechins and procyanidins with zinc cations modulate zinc absorption and metabolism, resulting in increased levels of cytoplasmic labile zinc. We forward the hypothesis that elevation of labile zinc may mediate the effect that these flavonoids exert on multiple metabolic and cell signalling pathways. The schematic models shown below depict putative mechanisms by which flavonoids may enhance cytosolic labile zinc while keeping MTs expression downregulated.

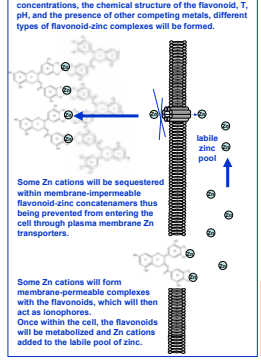

Fig. 1. Influence of zinc on the insulin signaling pathway.
Activation (—>) or inhibition(—⊣) by high (green) or low (red) levels of labile zinc.
From: ***Zinc and diabetes — clinical links and molecular mechanisms.***
J Jansen, W Karges, L Rink. Journal of Nutritional Biochemistry (2009) 20 399–417.

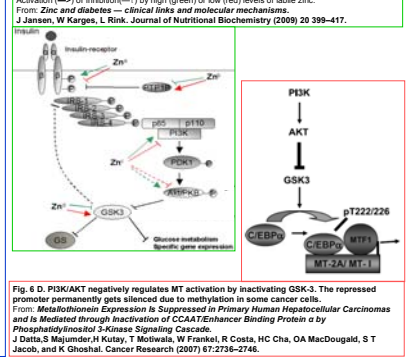

Fig. 6 D. PI3K/AKT negatively regulates MT activation by inactivating GSK-3. The repressed promoter permanently gets silenced due to methylation in some cancer cells.
From: ***Metallothionein Expression Is Suppressed in Primary Human Hepatocellular Carcinomas and Is Mediated through Inactivation of CCAAT/Enhancer Binding Protein α by Phosphatidylinositol 3-Kinase Signaling Cascade.***
J Datta,S Majumder,H Kutay, T Motiwala, W Frankel, R Costa, HC Cha, OA MacDougald, S T Jacob, and K Ghoshal. Cancer Research (2007) 67:2736–2746.

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Dietary catechins and procyanidins modulate zinc homeostasis in human HepG2 cells☆

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Abstract

Catechins and their polymers procyanidins are health-promoting flavonoids found in edible vegetables and fruits. They act as antioxidants by scavenging reactive oxygen species and by chelating the redox-active metals iron and copper. They also behave as signaling molecules, modulating multiple cell signalling pathways and gene expression, including that of antioxidant enzymes. This study aimed at determining whether catechins and procyanidins interact with the redox-inactive metal zinc and at assessing their effect on cellular zinc homeostasis. We found that a grape-seed procyanidin extract (GSPE) and the green tea flavonoid (−)-epigallocatechin-3-gallate (EGCG) bind zinc cations in solution with higher affinity than the zinc-specific chelator Zinquin, and dose-dependently prevent zinc-induced toxicity in the human hepatocarcinoma cell line HepG2, evaluated by the lactate dehydrogenase test. GSPE and EGCG hinder intracellular accumulation of total zinc, measured by atomic flame absorption spectrometry, concomitantly increasing the level of cytoplasmic labile zinc detectable by Zinquin fluorescence. Concurrently, GSPE and EGCG inhibit the expression, evaluated at the mRNA level by quantitative reverse transcriptase-polymerase chain reaction, of zinc-binding metallothioneins and of plasma membrane zinc exporter ZnT1 (SLC30A1), while enhancing the expression of cellular zinc importers ZIP1 (SLC39A1) and ZIP4 (SLC39A4). GSPE and EGCG also produce all these effects when HepG2 cells are stimulated to import zinc by treatment with supplemental zinc or the proinflammatory cytokine interleukin-6. We suggest that extracellular complexation of zinc cations and the elevation of cytoplasmic labile zinc may be relevant mechanisms underlying the modulation of diverse cell signaling and metabolic pathways by catechins and procyanidins.

Keywords: Epigallocatechin gallate; Procyanidins; Flavonoids; Labile zinc; Metallothionein; Zinc transporters

1. Introduction

The flavan-3-ols (+)-catechin and (−)-epicatechin, their gallated derivatives and their polymeric forms, procyanidins, constitute the most abundant polyphenols of flavonoid type present in edible fruits, red wine, chocolate and tea. They are considered bioactive micronutrients whose consumption entails benefits for human health as they may reduce the risk of cardiovascular diseases and metabolic disorders such as hypertriglyceridemia, diabetes and inflammation, and cancer [1–7]. Many beneficial effects of flavonoids have been ascribed to their antioxidant activity, which they exert directly by scavenging reactive oxygen species (ROS) and by chelating the redox-active transition metals iron and copper, that may act as ROS generators [8,9]. Flavonoids also act indirectly as antioxidants by inhibiting redox-sensitive transcription factors and pro-oxidant enzymes as well as through induction of phase II and antioxidant enzymes [10–12]. Beyond their antioxidant actions, flavonoids modulate multiple cell signaling pathways and, ultimately, gene transcription and metabolic fluxes [13–16].

Some flavonoids have been shown capable of complexation with the redox-inactive metal zinc [9,17–19], an essential micronutrient whose deficiency causes multiple dysfunctions, including alterations of glucidic and lipid metabolisms [20,21]. The majority of cellular zinc is tightly bound to proteins, functioning as a catalytic or structural component of an estimated 300 mammalian enzymes and proteins involved in virtually all cellular processes; some intracellular zinc exists in its free ionic form or loosely bound to proteins, and acts as a second messenger that modulates multiple signaling and metabolic pathways [22–26]. In mammalian cells, zinc from the extracellular milieu and from intracellular compartments enters the cytoplasm through 14 specialized transmembrane proteins of the ZIP/SLC39 family, whereas cytoplasmic extrusion of zinc is performed by 10 transporters of the ZnT/SLC30 family, being ZnT1, located at the plasma membrane, the primary regulator

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of cellular zinc efflux [27–29]. Within the cytoplasm, zinc is bound by metal-free apo-metallothionein (apo-MT) and by protonated glutathione to generate Zn-MT and G-SZn, respectively; the apo-MT/MT ratio controls free and labile zinc concentrations [30,31]. MT also serves as ROS scavenger and as heavy metal chelator, and its transcription responds, in addition to zinc, to stress stimuli such as ROS, heavy metals and proinflammatory cytokines [32,33]. The zinc-sensor transcription factor MTF1 (metal response element-binding transcription factor 1) coordinately up-regulates the transcription of MTs, ZnT1 and glutamate-cysteine ligase catalytic subunit (GCLC), the rate-limiting enzyme in glutathione biosynthesis, in response to elevated extracellular and intracellular labile zinc concentrations [34]. Dysfunctions of MT and zinc transporters act as promoting factors in several chronic pathologies including diabetes and cancer [35–37].

Research of flavonoids effects on zinc homeostasis has been scarce and focused on MT expression, regarded as an antioxidant enzyme. For instance, in human intestinal Caco-2 cells, genistein enhances expression of MT, whereas quercetin has the opposite effect, albeit enhances copper induction of MT [38–40]. Also, long-term consumption of flavonoids may affect mineral status, and for instance, iron, copper and zinc levels are diminished in the liver of rats fed with rutin and baicalin [41]. Conversely, zinc has been shown to influence the bioactivity of some flavonoids. Thus, zinc yields (−)-epigallocatechin-3-gallate (EGCG) effective in protecting cultured rat hepatocytes against hepatotoxin-induced cell injury [17] and enhances its antiproliferative effects on prostate cancer cells [18]. Zinc also stimulates the apoptotic effect of genistein in osteoclastic cells [42]. Therefore, bioactivity of flavonoids and zinc metabolism might be interconnected.

The aim of this work was to gain insight into the modulation of zinc homeostasis by catechins and procyanidins in the human hepatocarcinoma HepG2 cells, used as a model of hepatic cell. We found that a grape-seed procyanidins extract (GSPE) and the green tea polyphenol EGCG chelate zinc in solution and dose-dependently prevent zinc toxicity in HepG2 cells. GSPE and EGCG hindered the induction of MT genes and ZnT1 expression and the accumulation of total intracellular zinc elicited by treatment of cells with zinc and interleukin 6 (IL-6) but, on the contrary, enhanced the expression of plasma membrane ZIP transporters and the elevation of cytoplasmic labile zinc elicited by zinc and IL-6. Given the relevance of zinc in cell signaling and control of metabolic pathways, we forward the hypothesis that modulation of zinc homeostasis might underlay some of the health-promoting actions of catechins and procyanidins.

2. Materials and methods

2.1. Chemicals

GSPE was from Les Dérives Résiniques et Térpeniques (Dax, France). This extract consist of monomeric catechins (16.55%), dimeric (18.77%), trimeric (16%), tetrameric (9.3%) and oligomeric (5–13 U) (35.7%) procyanidins and phenolic acids (4.22%). Pure procyanidin C1 was provided by Prof. Jean-Michel Mérillon and Dr. Xavier Vitrac (Polyphénols Biotech, Bordeaux, France). All other procyanidins, catechins, TPEN [*N*,*N*,*N*′,*N*′-tetrakis(2-phridylmethyl) ethylenediamine], $ZnCl_2$, dexamethasone, Zinquin ethyl ester and dimethyl sulfoxide (DMSO) were from Sigma, and IL-6 was from Roche.

2.2. Interaction of catechins and procyanidins with zinc in solution

UV-Vis absorption spectra of flavonoids (10 μM) in phosphate-buffered saline (PBS) at pH 7.4, was recorded before and after addition of 5 μM $ZnCl_2$, as previously reported [38], using a Hitachi U-1900 Spectrophotometer, with a 4-nm slit width. Zinc-dependent fluorescent emission of Zinquin (485-490 nm) dissolved in 50 mM Tris-HCl buffer and 0.1 M NaCl, pH 7.4 [43], was recorded in a Perkin Elmer LS 50 spectrofluorimeter, with excitation set at 365–370 nm, at 25°C. Quenching of zinc-dependent Zinquin fluorescence by flavonoids was monitored 5 min after addition of different amounts of the flavonoids to the solution containing 10 μM Zinquin and 1 μM zinc, or 25 μM Zinquin and 100 μM zinc.

2.3. Cell cultures, treatments and cytotoxicity assays

HepG2 cells (ATCC code HB-8065) were grown in Dulbecco's modified Eagle medium (DMEM; BioWittaker) supplemented with 10% fetal bovine serum (BioWittaker), 2 mM glutamine and 1% nonessential amino acids. This medium contains 4.9±0.2 μM zinc as determined by flame atomic absorption spectrometry (FAAS). Cells were incubated at 37°C in a humidified, 5% CO_2-enriched atmosphere and routinely splitted at a 1:5 ratio upon reaching 80% confluence. For treatments, cultures at 80% confluence were trypsinized and resuspended at a density of $0.5 \cdot 10^6$ cells/ml, and 1-ml aliquots were seeded per well in 12-well plates (Orange Scientific). Twenty-four hours later, medium was replaced with 1 ml of fresh medium containing the different test substances or vehicle (final 0.2% ethanol). Cytotoxicity of GSPE, EGCG and $ZnCl_2$ was assessed by measuring lactate dehydrogenase (LDH) leakage in cells treated 24 h with different concentrations of the test substances as previously described [12].

2.4. Measurements of intracellular total and labile zinc

To quantify total intracellular zinc, cells were thoroughly washed with PBS and lysated with 0.01 M NaOH and 0.01% sodium dodecyl sulfate. Aliquots of the cell lysates were used to quantify zinc by FAAS as previously described [44], using a Hitachi Z-8200 Polarized Zeeman AA Spectrophotometer, and protein content, by the Bradford method. To measure changes in intracellular labile zinc, cells were washed with PBS, incubated 30 min at 37°C in 25 μM Zinquin ethyl ester in PBS, washed again and finally suspended in PBS; protein content and Zinquin fluorescence were determined in cell aliquots. Background fluorescence of Zinquin-unloaded cells was substracted from readings to derive Zinquin-dependent fluorescence as described [22].

2.5. Gene expression analysis

Total RNA was isolated from HepG2 cells using NucleoSpin RNA 2 kit (Macherie-Naegel, Germany). To quantify relative mRNA levels of specific genes in different RNA samples, cDNAs were generated from total RNAs using TaqMan Reverse Transcription Reagents, and quantitative reverse transcriptase-polymerase chain reactions (RT-PCRs) were performed using the TaqMan PCR Core Reagent Kit, specific TaqMan Assay-on-Demand Probes and the Real-Time 7000 PCR System, all from Applied Biosystems. Cyclophilin peptidylprolyl isomerase A (cyclophilin A) (PPIA) was used as reference gene. For microarray analysis, RNAs were obtained from four independent control or GSPE-treated cells and pooled. Integrity of pooled RNA was assessed with the Agilent 2100 Bioanalyzer and the RNA 6000 LabChipR. For microarray hybridization, Cy3- or Cy5-labeled cRNA was obtained from each RNA pool using the Agilent Low RNA Input Fluorescent Linear Amplification Kit. Fluorescent probes of each labeled cRNA were pooled and hybridized against Agilent Whole Human Genome Microarrays following the Agilent 60-mer Oligo Microarray processing protocol. Fluorescence signals of hybridized microarrays were acquired with the Agilent G2505B scanner and quantified with the Agilent G2567AA Feature Extraction Software. Duplicate hybridizations with a dye-swap labeling were performed for the pair of RNA samples being compared.

2.6. Statistical analysis

For statistical analysis in cytotoxicity assays, zinc and protein quantification, quantitative RT-PCRs and fluorescence measurements, *t* test and one-way analysis of variance (ANOVA) analyses were performed using SPSS software. Except for microarray analysis, all data are the result of at least three independent experiments. Differences were considered significant for $P \leq .05$.

3. Results

3.1. Catechins and procyanidins interact with zinc in solution

In order to test whether catechins and procyanidins may interact with zinc cations in solution, we monitored the modification of the UV-Vis absorption spectrum of GSPE and EGCG in the presence of $ZnCl_2$. Based on the previously described 2:1 stoichiometry in flavonoid-Fe(II) complexes [38], the concentration of GSPE and EGCG was set at 10 μM, and that of Zn(II) was 5 μM (Fig. 1A). GSPE and EGCG rapidly changed their spectral properties upon addition of $ZnCl_2$ to the solution, implying that they complex with zinc cations when these are free in solution. Subsequently, we tested the ability of GSPE; (+)-catechin (C); (−)-epicatechin; (−)-epicatechin gallate; EGCG; dimeric procyanidins B1, B2, B3 and B4 and the procyanidin trimmer C1 to quench the zinc-dependent fluorescence of Zinquin, a zinc-specific fluorescent chelator widely employed to measure concentrations of labile (free plus loosely bound) zinc within cells and in biological fluids [22,43,45]. GSPE and the

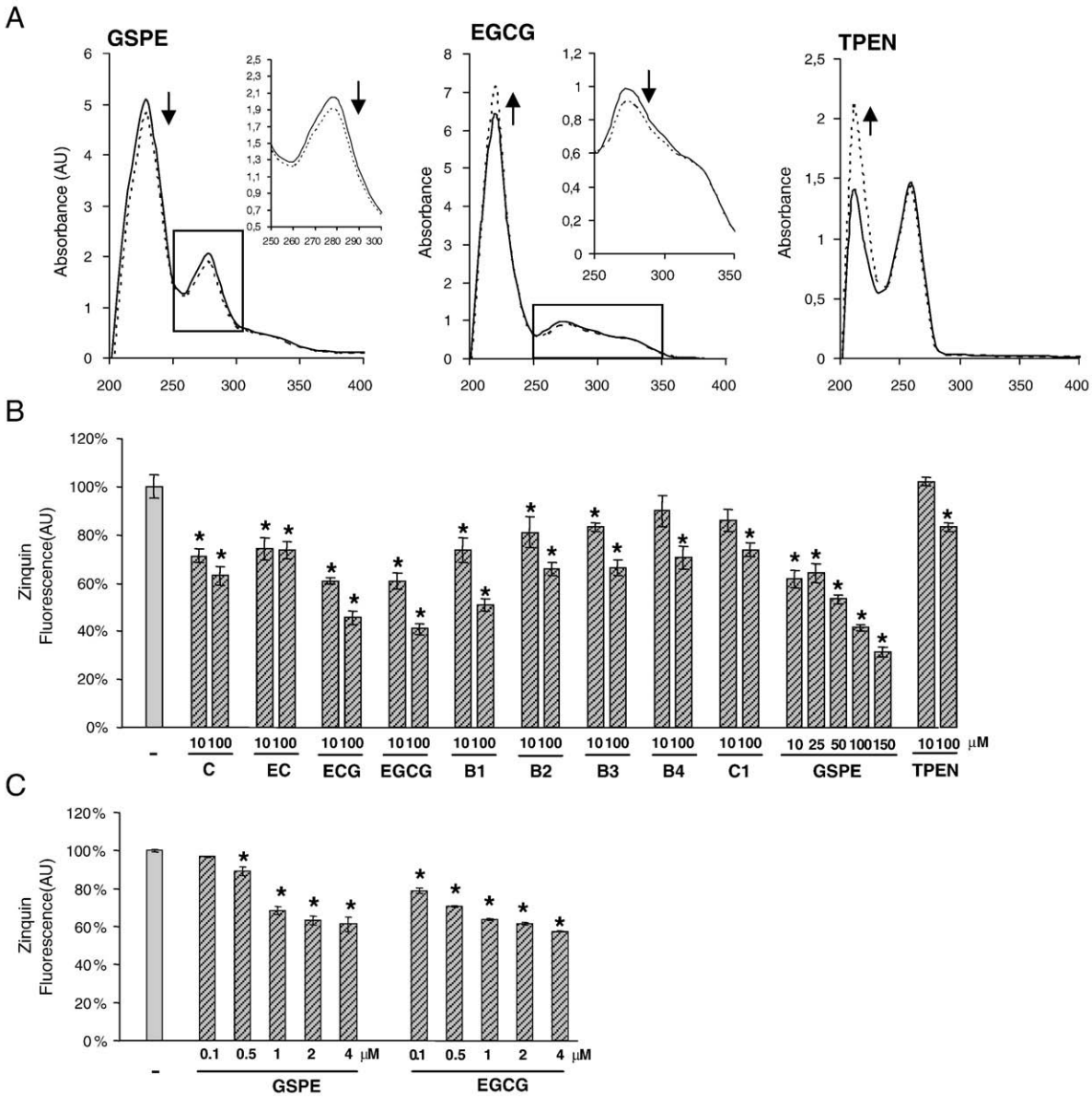

Fig. 1. Interaction of catechins and procyanidins with zinc cations in solution. (A) UV-Vis absorption spectra of GSPE and EGCG coexisted in solution with Zn(II). Continuous lines represent the UV-Vis spectra of 10 μM GSPE, EGCG or the zinc chelator TPEN dissolved in PBS, recorded 5 min after the substances have been added to the buffer. Dashed lines represent the corresponding spectra when the substances were dissolved in PBS containing 5 μM $ZnCl_2$. AU, arbitrary units. (B) Quenching of zinc-dependent fluorescence of Zinquin by catechins and procyanidins in solution. Fluorescent emission of zinc-Zinquin complexes formed in 10 mM Tris-HCl buffer, pH 7.4, 25°C, at 25 μM Zinquin ethyl ester and 100 μM $ZnCl_2$ (B1) or 10 μM Zinquin ethyl ester and 1 μM $ZnCl_2$ (B2) was measured before (control values, 100 arbitrary units of fluorescence) and 5 min after addition of different catechins and procyanidins at the indicated micromolar concentrations. Asterisks indicate significant difference ($P<.05$) versus control sample (−) using independent-samples *t* test.

individual catechins and procyanidins quenched zinc-dependent Zinquin fluorescence rapidly and dose-dependently, with higher efficacy than the zinc chelator TPEN, at flavonoid to Zinquin ratios of 10:25 in the presence of 100 μM zinc (Fig. 1B) and even at 0.1:10 flavonoid to Zinquin ratio in the presence of 1 μM zinc, for both GSPE and EGCG (Fig. 1C). These results imply that these flavonoids bind zinc cations in solution with enough affinity to cause its displacement from Zinquin-zinc complexes.

3.2. GSPE regulates the expression of MT and zinc transporter genes in HepG2 cells in a time- and dose-dependent manner

Zinc chelators are known to affect the expression of genes involved in the cellular uptake, storage and extrusion of zinc cations [43,46,47]. In order to asses whether the ability of catechins and procyanidins to complex with zinc may affect cellular zinc homeostasis, we monitored changes in the expression profile of genes involved in zinc transport and storage in HepG2 cells grown 12 h in standard culture medium (5 μM zinc) supplemented with 150 mg/L GSPE, using oligonucleotide microarray hybridization. Expression of MT genes and zinc transporters of the ZnT and ZIP families were profoundly affected by GSPE (Table 1). mRNA levels of all MT genes expressed in HepG2 were down-regulated by GSPE. Among the zinc transporters of the ZnT family (efflux of zinc from the cytoplasm), only ZnT1 and ZnT10 (extrusion of zinc out of the cell) were clearly down-regulated, whereas the expression of ZnT5 and ZnT7 (uptake of zinc into the Golgi network) were up-regulated. Regarding the ZIP family of zinc transporters (import of zinc into the cytoplasm), mRNA

Table 1
Effect of GSPE on mRNA levels of genes involved in zinc homeostasis in HepG2 cells

Genebank ID	Gene symbol/s; name	Mean fold change GSPE vs. control	
		Microarray	RT-PCR
Metallothioneins			
NM_005946	MT1A	0.54	0.34
NM_005947	MT1B	0.61	0.26
NM_175617	MT1E	0.45	0.48
NM_005949	MT1F	0.64	
NM_005950	MT1G	0.69	0.34
NM_005951	MT1H	0.74	
NM_175622	MT1J	0.65	
NM_176870	MT1K	0.46	
NR_001447	MT1L	0.77	
NM_005952	MT1X	0.54	0.26
NM_005953	MT2A	0.70	0.64
SLC30A family of zinc transporters (cytoplasmic zinc export)			
NM_021194	SLC30A1/ZnT1	0.46	0.68
NM_001004434	SLC30A2/ZnT2	-	
NM_003459	SLC30A3/ZnT3	1.23	
NM_013309	SLC30A4/ZnT4	-	
NM_022902	SLC30A5/ZnT5	1.80	
NM_017964	SLC30A6/ZnT6	2.09	
NM_133496	SLC30A7/ZnT7	4.19	
NM_173851	SLC30A8/ZnT8	-	
NM_006345	SLC30A9/ZnT9	0.93	
NM_018713	SLC30A10/ZnT10	0.51	
SLC39A family of zinc transporters (cytoplasmic zinc import)			
NM_014437	SLC39A1/ZIP1	1.77	2.25
NM_014579	SLC39A2/ZIP2	-	
NM_144564	SLC39A3/ZIP3	0.51	
NM_130849	SLC39A4/ZIP4	1.45	1.60
NM_173596	SLC39A5/ZIP5	0.61	
NM_012319	SLC39A6/ZIP6	2.04	
NM_006979	SLC39A7/ZIP7	0.65	
NM_022154	SLC39A8/ZIP8	-	
NM_018375	SLC39A9/ZIP9	1.57	
NM_020342	SLC39A10/ZIP10	4.97	
NM_139177	SLC39A11/ZIP11	0.51	
NM_152725	SLC39A12/ZIP12	-	
NM_152264	SLC39A13/ZIP13	1.80	
NM_015359	SLC39A14/ZIP14	1.10	0.90
Glutathione biosynthesis			
NM_001498	GCLC; glutamate-cysteine ligase, catalytic subunit	3.73	3.35
NM_002061	Glutamate-cysteine ligase, modifier subunit	2.73	
Plasma zinc carriers			
NM_000477	ALB; albumin	2.62	
NM_000014	A2M; alpha-2-macroglobulin	1.34	
Transcription factors			
NM_005955	MTF1; metal-regulatory transcription factor 1	0.98	
Antioxidant enzymes			
NM_000454	SOD1; Cu/Zn-SOD; superoxide dismutase 1, soluble	0.89	
NM_000636	SOD2; Mn-SOD; superoxide dismutase 2, mitochondrial	7.07	
NM_001752	CAT; catalase	0.67	

HepG2 cells cultivated in standard culture medium (5 μM zinc) were incubated 12 h with 150 mg/L GSPE. Control cells where given only vehicle (final 0.1% ethanol). Total RNA from each group of cells (4 samples per group) were pooled and processed to obtain microarray hybridization data. Mean fold change refers to the mRNA levels of each gene in GSPE-treated cells relative to that in untreated cells, and are the mean of two independent hybridizations with dye-swap labeling of RNA samples.
–, signal is similar to background fluorescence. MFC in bold characters were obtained by quantitative RT-PCR performed with non-pooled RNA samples.

levels of ZIP1, ZIP4, ZIP6, ZIP10 and ZIP13 (located in the plasma membrane) became elevated upon GSPE treatment, whereas ZIP3, ZIP5, ZIP7 (which extrude zinc form the Golgi apparatus) and ZIP11 (unknown location) were down-regulated. GCLC and glutamate-cysteine ligase, modifier subunit, which encode the catalytic and regulatory subunit, respectively, of the rate-limiting enzyme in glutathione biosynthesis, were strongly up-regulated by GSPE treatment. Also, albumin and alpha-2-macrogobulin, the main carriers of zinc in plasma, became up-regulated by GSPE. Therefore, the expression of the main genes involved in zinc homeostasis is modulated by catechins and/or procyanidins present in GSPE.

Next, in order to confirm microarray data and asses time and dose dependency of the response of zinc-related genes to GSPE, we cultured HepG2 cells in the presence of 15, 75 or 150 mg/L GSPE, and monitored changes in gene expression of six MT genes and four plasma membrane zinc transporters at various times, using quantitative RT-PCR. The results show (Fig. 2A) that all six MT genes tested were progressively down-regulated by 150 mg/L GSPE with respect to control cells, reaching relative minimal values between 9 and 12 h after addition of GSPE. Only mRNA levels of MT2A returned to control expression after 24 h of treatment. Time-dependent expression of ZnT1 closely paralleled that of MT2A, suggesting a common regulatory mechanism for the down-regulation by GSPE, whereas the levels of ZIP1 and ZIP4 mRNAs, which where up-regulated by GSPE, progressed with an inverse tendency to that of MT2A and ZnT1. The expression of ZIP14 resulted unaffected by GSPE, and mRNA levels of GCLC increased steadily in GSPE-treated cells. Dose dependency was shown for changes in the expression of MT1A, MT1X, MT2A, ZnT1, ZIP1, ZIP4 and GCLC in HepG2 cells treated 12 h with 15, 75 or 150 mg/L GSPE (Fig. 2B).

3.3. GSPE diminishes the accumulation of zinc in HepG2 cells cultured in basal zinc conditions

The modification elicited by GSPE in the expression of MTs, ZnT1, ZIP1 and Zip4, as well as ZnT5 and ZnT7, in HepG2 cells is remarkably similar to that described in other cell lines grown in conditions of reduced zinc availability, i.e., treated with the zinc chelator TPEN [43,46–48] or in zinc-depleted medium [49] that results in a reduction of total intracellular zinc. To address this point, we measured the total zinc content of control and GSPE-treated cells at different times (Fig. 2C). In control cells, total intracellular zinc increased steadily from 3.355 (S.D. 0.125) to 5.511 (S.D. 0.373) nmol of zinc per milligram of protein 15 h after the addition of fresh medium to the cells. In contrast, in cells treated with 150 mg/L GSPE, total intracellular zinc remained roughly constant from the time of GSPE administration until the end of cultivation. Therefore, GSPE hinders the normal entrance of zinc cations into HepG2 cells.

3.4. GSPE counteracts the effect of excess zinc on expression of MT and zinc transporters, on intracellular zinc accumulation and on cell viability

Next, we tested the capacity of GSPE to inhibit MT and ZnT1 expression and intracellular zinc accumulation in conditions of zinc overload. Zinc in excessive amounts is toxic to all types of cells and induction of MT and ZnT1 expression, mediated by the zinc-sensing transcription factor MTF1, followed by chelation of zinc by the newly synthesized apo-thionein and extrusion of zinc out of the cell, is recognized as a defense mechanism against zinc toxicity [33,34]. As shown in Fig. 3A, addition of 100 μM zinc to the culture media, a concentration that is not toxic to HepG2 cells (Fig. 3C), resulted in a 25-fold induction of MT1X and a 13-fold induction of MT2A, 12 h after addition of zinc. Concomitantly, expression of ZnT1 was enhanced twofold, whereas ZIP4 was down-regulated to 60% of control value. Expression of ZIP1 and ZIP14 was unaffected by addition of zinc. Total intracellular zinc was 3.2-fold higher in zinc-treated cells than in

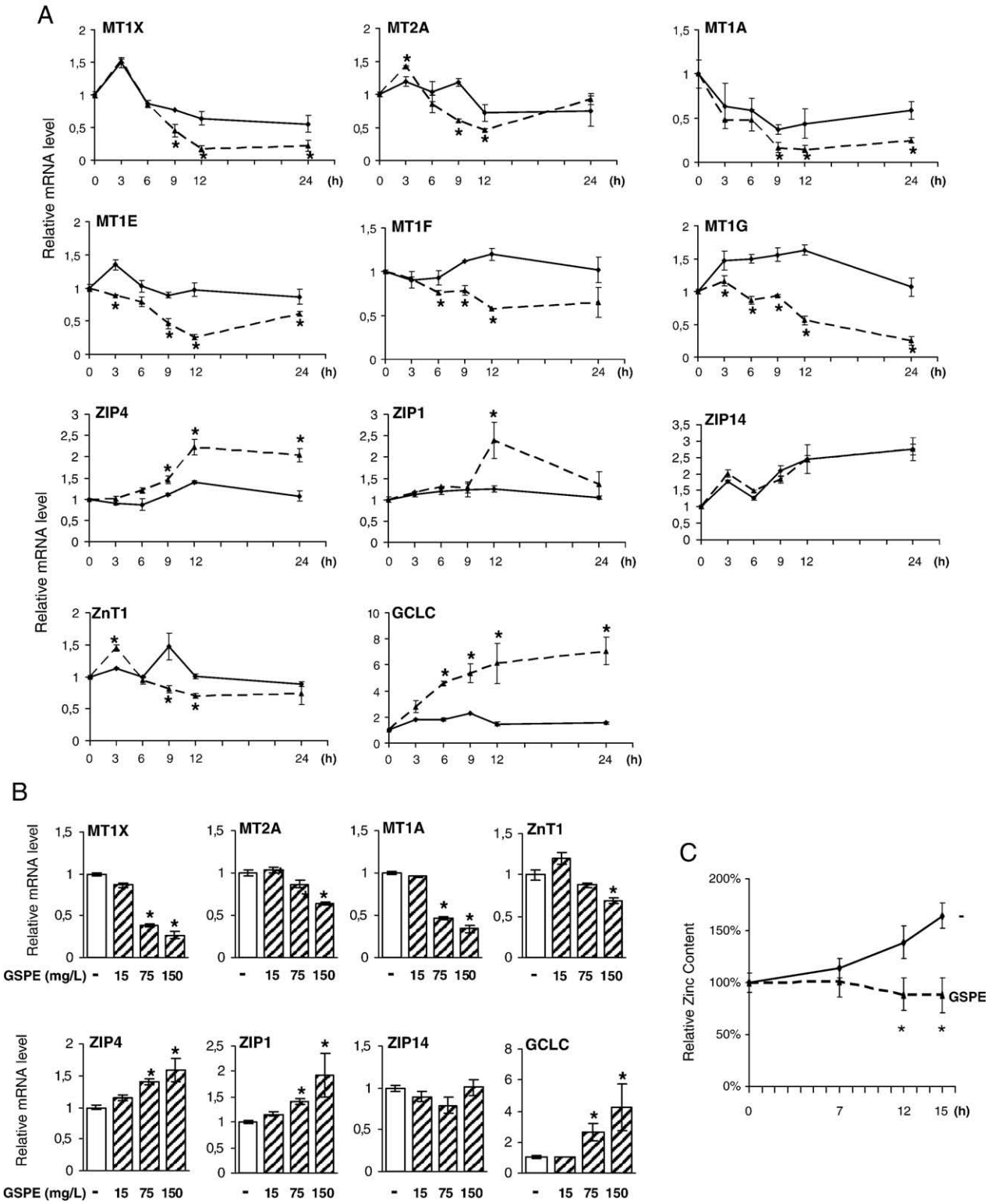

Fig. 2. GSPE modulates the expression of zinc-homeostasis genes and total zinc content in HepG2 cells cultured in standard medium. (A) Kinetics of mRNA levels of MT, GCLC and plasma membrane zinc transporters. Black lines represent the evolution of the mRNA level of each gene in control cells, relative to the mRNA level of that gene in untreated cells just at the beginning of the treatment with 150 mg/L GSPE (dashed lines), determined by quantitative RT-PCR. (B) Dose-dependency of the effects of GSPE on expression of zinc-homeostasis genes. HepG2 cells were treated with either vehicle (−), 15, 75 or 150 mg/L GSPE for 12 h, and relative mRNA levels of the indicated genes determined by RT-PCR. (C) Progression of total intracellular zinc content. The total amount of zinc within cells was determined by FAAS, and values normalized per total protein content of the cells. The zinc to protein ratio in the cells just before the beginning of the treatment with 150 mg/L GSPE was 3.355 nanomoles of zinc per mg of protein (0.125 S.D.), and is assigned the arbitrary value of 100. Asterisks indicates significant difference ($P<.05$) in treated cells versus control cells at the same time point using independent-samples t test.

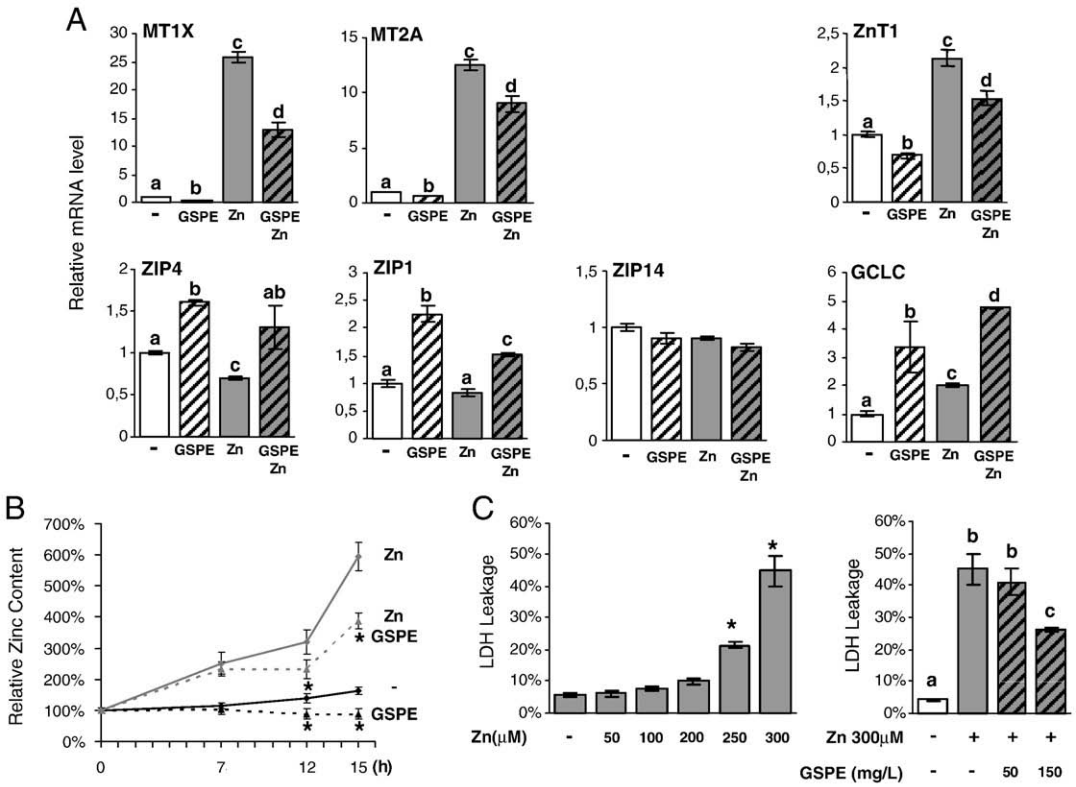

Fig. 3. Effects of GSPE on zinc homeostasis in HepG2 cells treated with excess zinc. (A) mRNA levels of MT and zinc transporter genes upon 12-h incubation with 150 mg/L GSPE (GSPE), 100 μM $ZnCl_2$ (Zn) or both (GSPE Zn), relative to the levels in untreated cells (−). (B) Evolution of total intracellular zinc, normalized per protein content, in the same cell cultures. (C) Dose-dependent toxicity of zinc on HepG2 cells and prevention of toxicity of 300 μM zinc in the culture medium by the addition of GSPE, assessed by the LDH test after 24 h of treatment. Numbers indicate the percentage of total LDH activity of a cell culture which is present in the culture medium. Asterisks indicate significant difference ($P<.05$) from control cells. Different letters denote different values after one-way ANOVA test ($P<.05$).

control cells. Addition of GSPE (150 mg/L) to the media significantly hindered the zinc-induced up-regulation of MT1X and MT2A, whereas it was unable to impede induction of ZnT1. In the presence of excess zinc, GSPE still up-regulated ZIP1 and ZIP4 as in control cells, but to a lesser extent. Therefore, GSPE counteracts the effects of additional zinc on MTs, ZnT1 and ZIP4 expression, whereas zinc counteracts the effects of GSPE on MT, ZnT1, ZIP1 and ZIP4. Concomitantly, zinc-stimulated accumulation of intracellular zinc was severely inhibited by GSPE (Fig. 3B). These results suggested that GSPE should be able to counteract toxics effects of zinc. To test this, we performed LDH tests to evaluate the effect of increasing amounts of zinc on cell viability (Fig. 3C). Zinc concentrations above 200 μM resulted in significant LDH leakage, reaching 80% upon 24 h incubation of cells with 300 μM zinc. Addition of 150 mg/L GSPE to the cells prevented the noxious effects of 300 μM zinc by 50% after the LDH test. Thus, even if inhibiting MT expression, nontoxic amounts of GSPE are able to counteract the toxic effects of excessive zinc, concomitantly reducing intracellular accumulation of zinc, strongly suggesting that GSPE renders zinc in the culture medium unavailable and, hence, nontoxic, to cells.

3.5. GSPE hinders the induction of MTs and ZIP14 and the intracellular zinc accumulation elicited by IL-6

Subsequently, we tested whether GSPE may affect de expression of MT and zinc transporters and intracellular zinc accumulation when cells are stimulated to take up zinc by stimuli different from zinc itself. In murine hepatocytes, IL-6 induces the expression of MT and Zip14 signaling through the STAT (signal transducer and activator of transcription) pathway; subsequently, uptake of zinc mediated by Zip14 further increases the activation of MT expression via MTF1; this response has been associated to the hypozincemia that accompanies the acute-phase response in infectious and inflammatory processes [27,50]. In HepG2 cells, IL-6 induced a marked increase in the expression of MT1X, MT2A, ZIP14 and ZIP1 (Fig. 4A), concomitantly increasing intracellular zinc levels to 150% of untreated cells (Fig. 4B). ZnT1 and ZIP4 mRNA levels were not affected by IL-6 at this time. When GSPE was added to HepG2 cells together with IL-6, induction of MT genes was completely blocked (Fig. 4A), and so was intracellular zinc accumulation (Fig. 4B). IL-6 induction of ZIP14 was unaffected by GSPE, consistent with direct regulation of ZIP14 by IL-6 in a way independent of zinc availability. As in standard zinc conditions, ZnT1 was down-regulated, and Zip4 was up-regulated by GSPE independently of IL-6. Effect of GSPE on ZIP1 was not evident at this time. Therefore, inhibition of zinc uptake and MT expression by GSPE is relevant in standard and zinc-overload conditions but also in the cellular response to extracellular signals, such as those that mediate the acute-phase response in inflammatory processes.

3.6. GSPE elevates intracellular labile zinc in HepG2 cells

We next monitored the effect of GSPE treatment on cytoplasmic levels of labile zinc, measured as zinc-dependent Zinquin fluorescence. As shown in Fig. 5A, GSPE increased the cytoplasmic levels of Zinquin-detectable zinc in HepG2 cells in all conditions tested. After 12 h of GSPE treatment, Zinquin fluorescence was enhanced by 12-fold compared with cells cultured in standard conditions. Addition of 100 μM zinc resulted in a 7-fold increase in Zinquin fluorescence, and coincubation with 100 μM zinc and 150 mg/L GSPE produced a further increase of up to 120-fold compared to untreated cells. IL-6

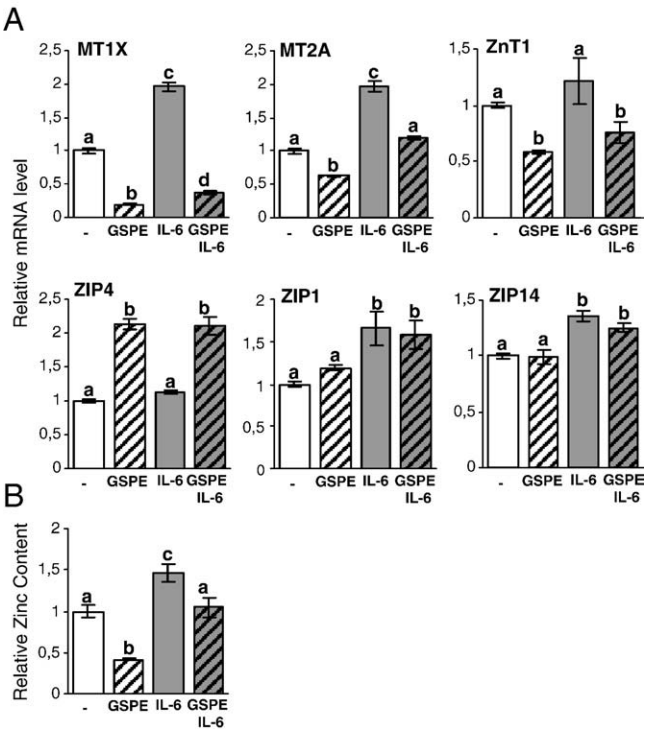

Fig. 4. Effects of GSPE on zinc homeostasis in HepG2 cells treated with IL-6. (A) mRNA levels of MT and zinc transporter genes after 15-h incubation with 150 mg/L GSPE, 1000 U/ml IL-6, or both treatments simultaneously, relative to control cells. Dexamethasone 1 μM was added to all cultures. (B) Total intracellular zinc content, normalized per protein content, in the same cell cultures. Different letters denote different values after one-way ANOVA test (P<.05).

treatment of HepG2 cells cultured in standard zinc conditions enhanced the labile pool of zinc by 1.4-fold, and addition of GSPE further increased it up to 18-fold. Microscopic observations of Zinquin-loaded HepG2 cells were done to visualize this effect of GSPE on cytoplasmic labile zinc in HepG2 cells grown in basal zinc concentrations as well as in conditions of zinc excess (Fig. 5B). Thus, in spite of hindering intracellular zinc accumulation, GSPE produced an increase in the cytoplasmic pool of labile zinc in HepG2 cells, in standard growth conditions as well as when cells are stimulated to accumulate zinc.

3.7. EGCG reproduces the major effects of GSPE on zinc homeostasis

GSPE consist of a mixture of dozens of different catechins and procyanidins which, expectedly, will display different affinities for zinc. For the convenience of characterizing an individual flavonoid, we tested the effect of EGCG on the different parameters of zinc homeostasis previously tested with GSPE in HepG2 cells. The results show that EGCG behaved essentially as GSPE in all conditions studied. Thus, in basal zinc conditions (Fig. 6A), EGCG repressed MT1X, MT2A and ZnT1 expression while enhancing that of ZIP1 and ZIP4; concomitantly, EGCG hindered the normal augmentation of total intracellular zinc but enhanced the pool of cytoplasmic labile zinc. In conditions of zinc excess (Fig. 6B), EGCG reverted the zinc-induced up-regulation of MT and ZnT1 genes and the zinc-induced down-regulation of ZIP1 and ZIP4, concomitantly slowing down the associated accumulation of intracellular zinc. Likewise, EGCG dose-dependently reverted zinc-induced toxicity. EGCG also impeded the induction of MT1X, MT2A and ZIP14 by IL-6 and hampered the associated accumulation of total intracellular zinc (Fig. 6C). Finally, like GSPE, EGCG produced a large increase in the intracellular, Zinquin-detectable, labile pool of zinc in all conditions tested (Fig. 6A-C).

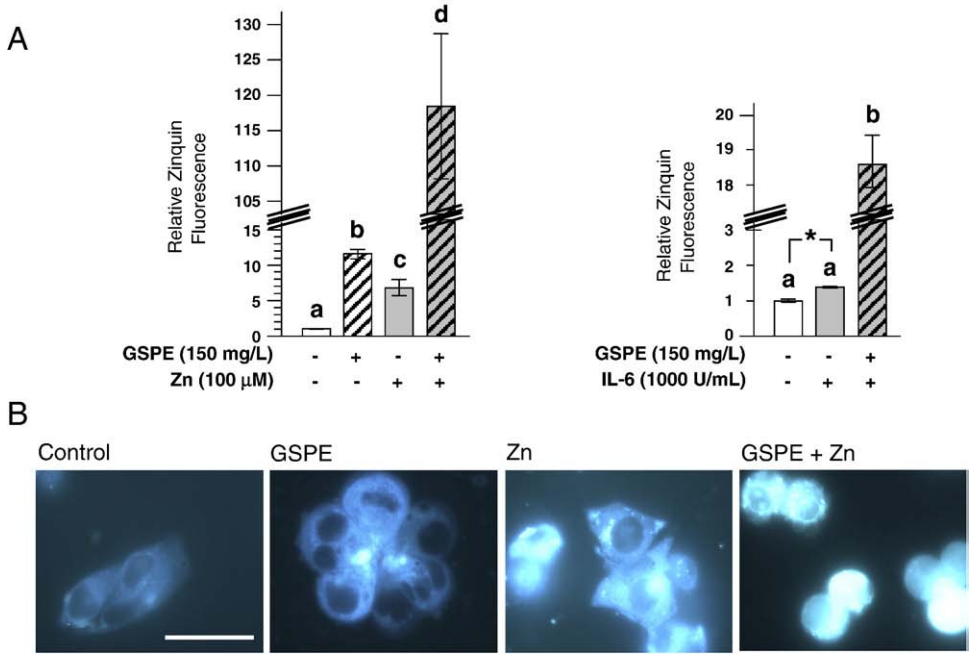

Fig. 5. Effect of GSPE on cytoplasmic labile zinc in HepG2 cells. (A) Fluorescence (arbitrary units) of Zinquin-loaded cells was normalized to protein content in each sample, and untreated cells were assigned the arbitrary value of 1. Treatments with GSPE, zinc and IL-6 were as in Figs. 3 and 4, respectively. (B) Images of Zinquin-loaded HepG2 cells. To visualize intracellular Zinquin fluorescence, cells were seeded in wells containing glass coverslips, treated as above, and loaded with Zinquin ethyl ester as described [22]; images at 1000× magnification were acquired with a Leica DM 4000B microscope using UV light illumination (λ_{ex}=340–380 nm) and a blue emission filer (λ_{em}≥425nm). Bar indicates 50 μm.

4. Discussion

We have shown here that GSPE, a mixture of catechins and procyanidins, as well as individual catechins and procyanidins, display an affinity for zinc cations in solution high enough to make them dissociate from the zinc-specific chelator Zinquin, even at very low concentrations and at molar ratios of 0.1 μM flavonoid to 10 μM Zinquin and 1 μM zinc. This strongly suggests that these flavonoids will

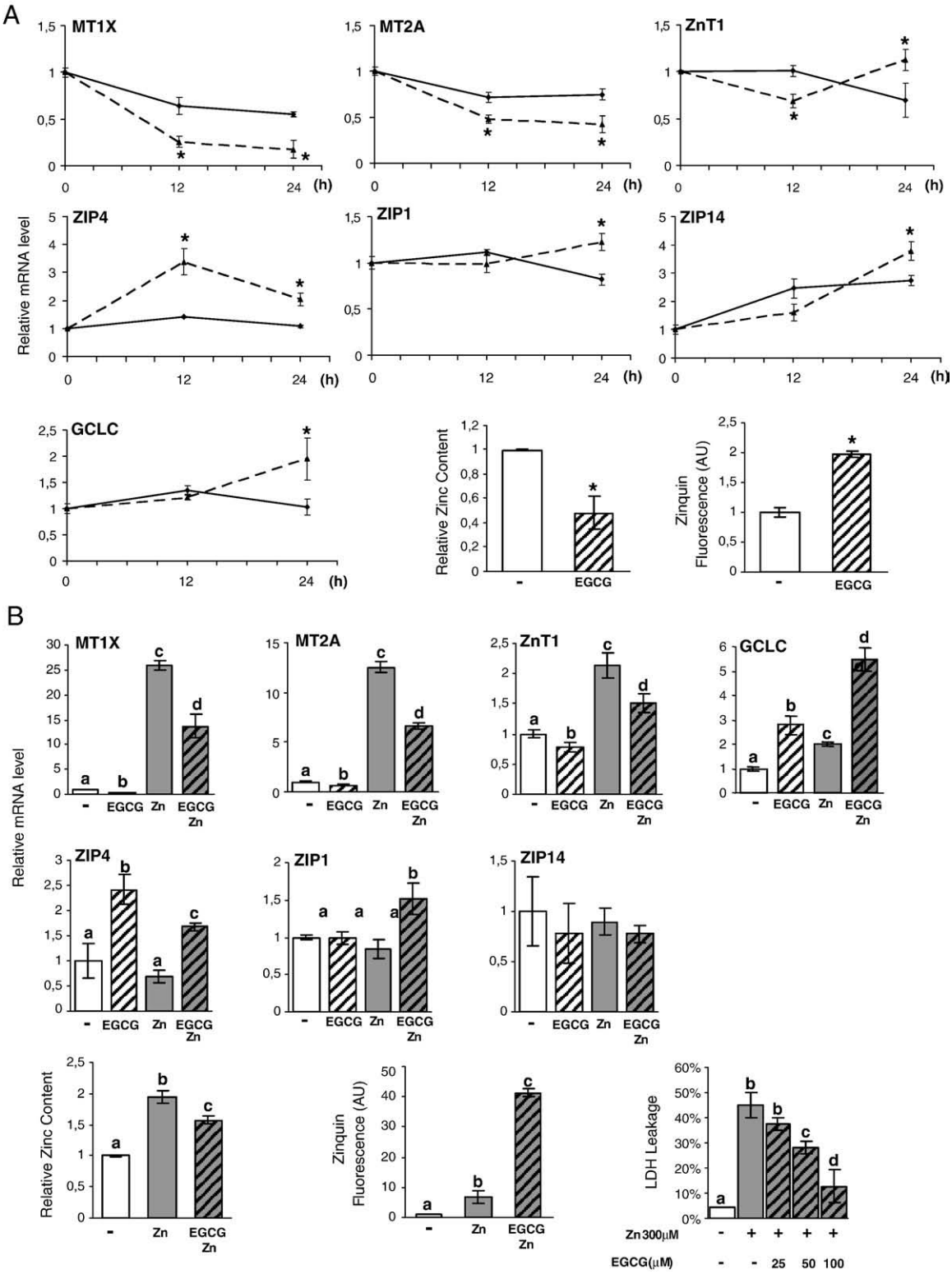

Fig. 6. Effects of EGCG on zinc homeostasis in HepG2 cells. Effect of EGCG on gene expression, total intracellular zinc and cytoplasmic labile zinc in HepG2 cells cultured in standard conditions (A), in cell cultures treated 12 h with additional (100 μM) zinc (B) and in cells treated 15 h with IL-6 (C). Asterisks indicates significant difference from control value with $P \leq .05$. Different letters denote different values after one way ANOVA test ($P < .05$).

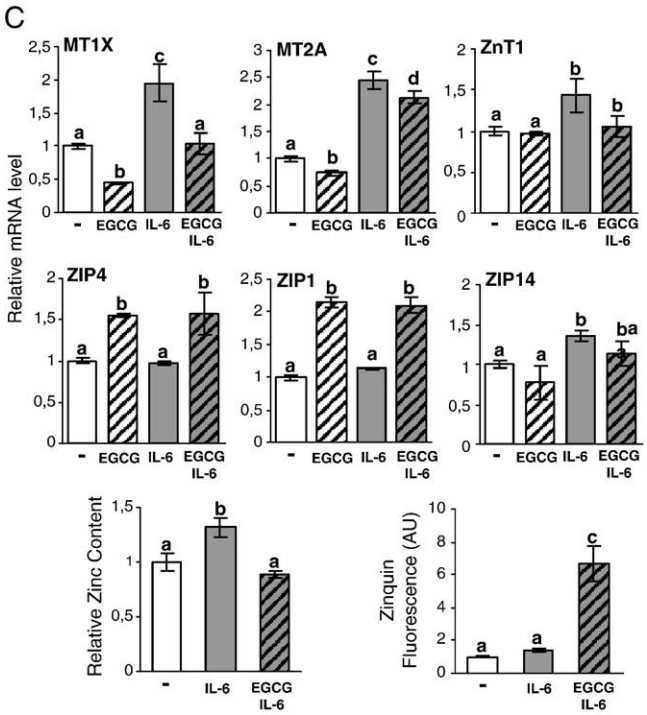

Fig. 6. (*continued*)

be able to displace zinc loosely bound to proteins in cell culture media and, once internalized, within the cell cytoplasm, as does Zinquin itself [22,45]. In the cytosol of most cell types, free zinc concentrations are in the picomolar to nanomolar range, and those of Zinquin-detectable, labile zinc are in the range of 1–10 μM, whereas the concentration total intracellular zinc, which includes the fixed or structural pool of zinc within metalloproteins, is several hundred (usually 100–300) μM [22,24–26]. In human plasma, Zinquin-chelatable zinc is roughly 8 μM, constitutes about >90% of albumin-bound zinc and is considered in route for uptake by the different tissues [45]. Thus, chelation of labile zinc by catechins and procyanidins of GSPE is likely to be relevant in vivo since, following oral administration of 1 g GSPE per kilogram of body weight to rats, parent unmetabolized catechins and dimeric and trimeric procyanidins reach plasma concentrations of 2, 2 and 8 μM, respectively, 2 h after GSPE administration. Glucuronidated catechin and epicatechin reach plasma concentrations of 23.9 and 20.5 μM, respectively, in these animals [51]. These metabolites are expected to retain the ability to bind zinc cations as they still have a strong bidentate metal binding site (two adjacent hydroxyl groups) in the B ring and a hydroxyl group in the A ring (see Ref. [9]). Tetrameric and pentameric procyanidins are also absorbed in rats and may reach plasma concentrations of 7 μg/ml each one [52]. Likewise, EGCG may reach concentrations of up to 1.5 μM in human plasma after oral intake of a single 800 mg dose of EGCG [53]. In this context, it is noteworthy that 5 h after GSPE administration, MT mRNA levels are drastically reduced to 30% of control values in the liver of rats fed a single oral dose of 250 mg/GSPE per kilogram of body weight [54], a dose that effectively lowers postprandial triglyceridemia [4].

We have shown that accumulation of total intracellular zinc in HepG2 cells was hindered upon addition of 150 mg/L GSPE or 100 μM EGCG to the standard culture medium (5 μM zinc) as well as when cells were stimulated to accumulate zinc by the addition of 100 μM zinc to the medium or by treatment with IL-6 in basal zinc conditions. In addition, toxic effects of 300 μM zinc in the medium were prevented by non-toxic amounts of GSPE and EGCG. Reversion of excess zinc toxicity occurred despite the fact that zinc-induced expression of MT, a major defense against zinc toxicity [32,33], was hindered by GSPE and EGCG. Taken together, these results supports the concept that EGCG and catechins/procyanidins of GSPE form complexes with zinc in the culture medium, thereby preventing the entrance of zinc into the cells through plasma membrane zinc transporters. In this regard, it is known that metal complexation may cause the concatenation of monomeric flavonoids with the metal cations, yielding the flavonoids less prone to partition into membranes [9]. It may be inferred that the metal cations that link together the flavonoid subunits will also be unable to enter the cell through transmembrane metal ion transporters. Actually, catechins and procyanidins have been shown to inhibit the entrance of iron in human intestinal Caco-2 cells [8], and long-term consumption of high doses of GSPE may lower serum iron levels in rats [55]. Also consistent with this mechanism, it has also been reported that the ratio of zinc to EGCG, as well as its way of administration, determines the rate of EGCG uptake by PC-3 cells: when EGCG is complexed with zinc by precipitation of saturated solutions of the flavonoid and the metal, the entrance of EGCG (80 μM) in the cells is less than half that when the same amount of only EGCG is provided to the cells; on the contrary, when EGCG is supplied in solution with and equimolar amount of zinc, intracellular accumulation of EGCG is enhanced more than twofold [18]. The authors inferred that the variable structure and stoichiometry of EGCG-zinc complexes determines different permeability of the flavonoids to the cell membrane.

The changes elicited by GSPE and EGCG in the expression of MT, ZnT and ZIP genes in different conditions are also consistent with a diminished availability of extracellular zinc for HepG2 cells, as they closely resemble the changes elicited by zinc deprivation described in different cell models. Thus, GSPE and EGCG exerted an effect contrary to those of zinc supplementation on the expression of MT1X, MT2A and zinc efflux transporter ZnT1, which where simultaneously up-regulated by addition of zinc and repressed by GSPE in basal and zinc overload conditions. The expression of zinc importers ZIP1 and ZIP4 was also simultaneously repressed by GSPE and EGCG in conditions of basal and increased zinc concentrations in the medium. Similarly, in cultured mouse fibroblasts, mRNA levels of MT and ZnT1 are elevated upon addition of zinc to the medium, a response mediated by MTF1 and are down-regulated when cells are cultured in zinc depleted medium [49]. Also, Caco-2 cells respond to supplementation of zinc elevating the expression of MT and ZnT1, whereas treatment with the zinc chelator TPEN results in enhanced expression of ZIP4 and down-regulation of ZnT1 and MT1 [47]. In HeLa cells, supplementation of zinc enhances expression of ZnT1, whereas administration of TPEN up-regulates ZnT7 and ZnT5 [43], as shown here in HepG2 cells treated with GSPE in basal zinc conditions. These expression changes have been interpreted as a homeostatic response of the cells to compensate for reduced zinc availability and directed to maintain adequate zinc levels in the cytoplasm and within the Golgi network [43,47,48]. Strikingly, incubation of HepG2 cells with GSPE and EGCG, in spite of diminishing total intracellular zinc concentrations relative to control cells in conditions of basal (5 μM) and excess (100 μM) zinc concentrations, as well as in cells treated with IL-6, always produced an augmentation of Zinquin-detectable labile pool of intracellular zinc. To our knowledge, only two reports have described the effect of dietary polyphenolic compounds on labile zinc. The glycone isoflavone genistin, applied at 100 μM, enhances the proapoptotic effects of zinc in HepG2 cells and up-regulate the expression of MT and ZnT1 concomitantly increasing the labile zinc pool detectable by FluoZin-3 [56]. The effect of genistin on total zinc content and on zinc toxicity was not reported. The stilbene resveratrol, at physiological concentrations (10 μM), efficiently chelates zinc in solution and, when applied to normal human prostate epithelial cells cultured in 16 or 32 μM Zn(II), arrests cell growth and enhances Zinquin-detectable zinc, while not affecting total zinc nor MT expression [57]. The authors

propose that the increment of labile zinc elicited by resveratrol is due to the cellular uptake of resveratrol-zinc complexes, followed by the intracellular dissociation of the complexes [57]. Considering these published data and those presented here, it appears that flavonoids may enhance intracellular labile zinc levels independently of their effect on MT and ZnT1 expression, which would rather correlate with total intracellular zinc content. A plausible explanation for the increment of cytoplasmic labile zinc is that membrane-permeable flavonoid-zinc complexes are always formed in addition to membrane-impermeable flavonoid-zinc concatenamers. Those zinc atoms that enter the cells complexed with the flavonoids, that would be, in this way, acting as ionophores, will add to the pool of labile zinc and latter, once the flavonoid has been metabolized, to the pool of free zinc. This increment in free/labile zinc should not suffice to produce significant increments of total zinc content, as seen in HepG2 cells treated with EGCG and GSPE. An alternative, though not excluding, possibility is that, once internalized, the flavonoids mobilize zinc from intracellular zinc stores such as MT, endoplasmic reticulum, vesicles or zincosomes, as has been shown for Zinquin itself, that is able to retrieve some of the MT-bound zinc cations [22]. Mobilization of copper ions from the nuclear compartment has been shown in lymphocytes treated with EGCG [58]. In any case, the increment in labile zinc elicited by GSPE and EGCG should not be immediately available or sufficient to stimulate the transcriptional activity of MTF1 on the promoters of MT and ZnT1, given the observed down-regulation of MT and ZnT1 mRNA levels in GSPE-treated cells. It could, however, suffice to enhance the transcription of GCLC, also under the control of MTF1. MTF1 discerns between different metal response element (MRE) in response to different zinc load [34]. Alternatively, up-regulation of GCLC by GSPE and EGCG may be independent of MTF1. EGCG is known to induce the expression of GCLC and manganese superoxide dismutase by activating the transcriptional activity of the redox-sensitive nuclear factor erythroid 2 p45-related factor which recognizes the antioxidant response element in the promoter of these antioxidant genes [11]. A known mechanism for repression of MT expression in hepatocarcinoma cell lines, including HepG2, lays on the activation of phosphatidylinositol 3-kinase (PI3K) and the serine/threonine kinase Akt, also called protein kinase B (PKB), that in turn-inactivates glycogen synthase kinase 3; this kinase activates MT expression by phosphorylating the CCAAT/enhancer binding protein α that interacts with MTF1 and/or basal transcription factors in the promoter of MT genes [59]. On the other hand, increased levels of cytoplasmic free/labile zinc, brought about by zinc ionophores such as pyrithione, enhance phosphorylation and activation of PI3K and Akt/PKB in many cell types, including hepatic cells [60,61]. By combining these two mechanisms, it appears that increments in labile zinc may occur simultaneously with repression of MT expression, a situation that should help the cell to keep intracellular zinc available for essential functions when extracellular zinc becomes temporally unavailable, and is consistent with a homeostatic response of cells to low zinc availability. In this context, it is remarkable that GSPE has been recently shown to elicit phosphorylation of PI3K and Akt/PKB, a mechanism that might underlie the known insulinomimetic effect of procyanidins [5].

Whatever the underlying mechanism, the increment of labile, Zinquin-detectable cytoplasmic zinc described here for GSPE and EGCG may be relevant to explain the bioactivity of these flavonoids. Zinquin-detectable zinc is considered a measure of the pool of this metal that can be exchanged between proteins and is endowed with a regulatory and signaling function, i.e., it may modulate the activity of components of signal transduction pathways and key enzymes of multiple metabolic pathways [22–26]. Thus, increments within the nanomolar range in the levels of free cytoplasmic zinc inhibit the activity of cyclic nucleotide phosphodiesterases (PDE) and protein tyrosine phosphatases (PTP), and activate mitogen-activated protein kinase (MAPK), protein kinase C and calcium-calmodulin activated protein kinase-2, leading to changes in the phosphorylation state of numerous downstream cell signaling and transcription factors. For instance, inhibition of PTP 1B by zinc (IC_{50} 17 nM) results in enhanced net phosphorylation of the insulin receptor and activation of downstream signalling cascades pathways such as MAPK, PI3K and Akt/PKB and is thought to contribute to the insulin-mimetic effects of zinc and zinc complexes [60–62]. Likewise, inhibition of PDE by zinc (IC_{50} 20 nM) enhances cGMP and cAMP signalling [23]. Free zinc may also directly activate transcription factors, as is the case for MTF1, or inhibit its transcriptional activity, as for nuclear factor-kappa B (NF-κB) [23]. It is remarkable that many actions described for diverse flavonoids on signaling pathways overlap with those described for fluctuations of free and labile zinc. As mentioned above, GSPE has been recently shown to elicit phosphorylation of PI3K and Akt/PKB independently of insulin, a mechanism that might underlie the known insulinomimetic effect of procyanidins [5]. Likewise, many flavonoids, including EGCG and procyanidins inhibit the transcriptional activity of NF-κB [6,7,10]; similarly, many flavonoids have been shown to inhibit PDE activity, consequently elevating cytoplasmic cAMP levels [15].

In summary, we have presented evidence supporting that interaction of catechins and procyanidins with zinc cations modulate zinc absorption and metabolism, resulting in increased levels of cytoplasmic labile zinc. We forward the hypothesis that modulation of labile zinc by these flavonoids may be a relevant mechanism by which flavonoids affect multiple metabolic and cell signalling pathways that respond to intracellular fluctuations of labile zinc. Further research is necessary to asses the mechanisms by which these flavonoids enhance cytoplasmic labile zinc and the consequences of this enhancement on modulation of zinc signaling and metabolic pathways, as well as to asses the relevance that zinc chelation by individual catechin/procyanidins and their metabolites may have in vivo.

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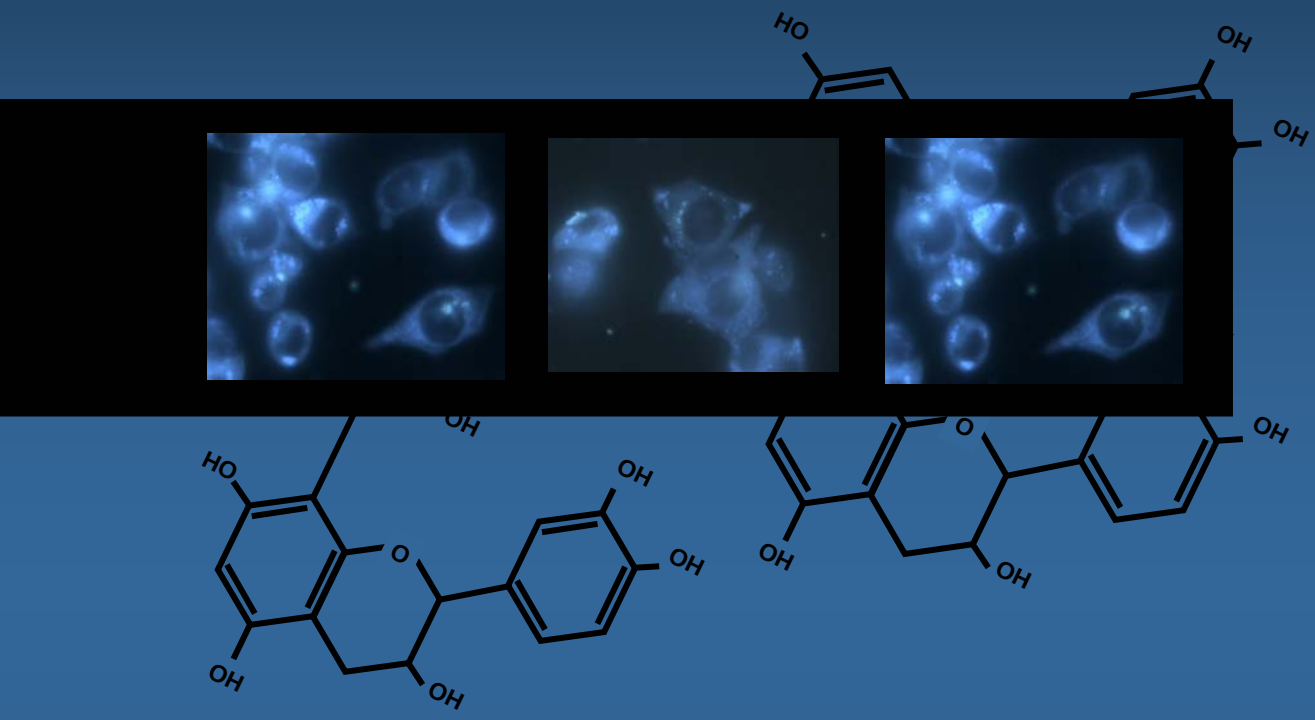

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