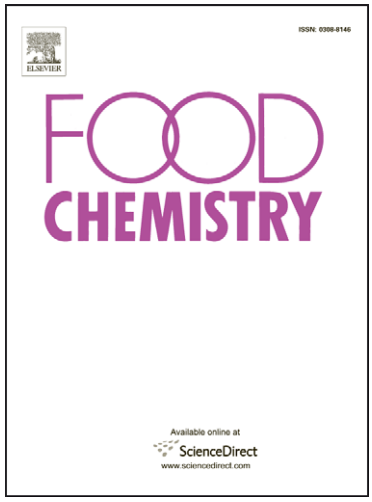

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Proanthocyanidins modulate triglyceride secretion by repressing the expression of long chain acyl-CoA synthetases in Caco2 intestinal cells

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ABSTRACT

In this work we determined the ability of grape seed proanthocyanidins (GSPE) to modulate triglyceride (TG) secretion by enterocytes in post-prandial and fasting states, using Caco2 cell line. GSPE did not modify TG secretion in the post-prandial state, even though it modulated the long chain acyl-CoA synthetase (ACSL) 5 and the carnitine-palmitoyl-transferase-1a gene expression. On the contrary, GSPE decreased TG secretion in the fasting state, significantly repressing ACSL3, ACSL5, I-FABP and PPARalpha gene expression. Intestinal cells can use two different pathways to carry out TG synthesis. Fatty acids (FA) delivered by ACSL3 and by ACSL5 would be directed towards the monoacylglycerol and glycerol-3-phosphate pathways, respectively. Therefore, proanthocyanidins repress the supply of FA towards the monoacylglycerol pathway in the post-prandial state, whereas they repress the supply of FAs towards both pathways in the fasting state, suggesting that the feeding state is a key factor regarding the effectiveness of proanthocyanidins to reduce triglyceridaemia.

Keywords: flavonoids; triglyceride; intestine; ACSL; PPARalpha.

1. Introduction

The disturbances of triglyceride (TG) metabolism are considered to be a substantial risk factor for obesity, atherosclerosis, insulin-dependent diabetes, breast and colon cancers (Alipour, Elte, van Zaanen, Rietveld & Castro Cabezas, 2008). Elevated levels of TG-rich lipoproteins (TRL), especially chylomicron (CM) remnants and very low density lipoprotein (VLDL) remnants, accumulate in the blood flow, and are strongly associated with an increased of the risk of myocardial infarction, ischaemic stroke and early death (Fujioka & Ishikawa, 2009).

The small intestine is the second most important source of plasma TRL, just after the liver (Levy, Mehran & Seidman, 1995). The intestine secretes several different lipoproteins, CM and VLDLs are the major ones (Mu & Hoy, 2004). VLDLs are the predominant lipoproteins during the fasting state and their assembly occurs constitutively (Hussain, 2000). VLDLs may serve to transport lipids derived from the bile and fatty acids of the plasma (Hussain, 2000). In the postprandial state, CM secretion is induced after fat ingestion and is impaired by the absence of bile acids (Hussain, 2000).

Most dietary TGs are absorbed by the enterocytes as fatty acids (FA) and monoacylglycerides (MAG). FA and MAG require reassembly to produce TG on the endoplasmatic reticulum by, predominantly, the progressive acylation of MAG *via* the monoacylglycerol pathway (Levy, Mehran & Seidman, 1995, Mansbach & Gorelick, 2007). Additionally, TG can also be synthesised by a separate route, by means of the acylation of glycerol-3-phosphate (G-3-P) (Mansbach & Gorelick, 2007). The MAG pathway would predominate in the postprandial period while the G-3-P pathway is the main one in the interprandial and fasted period (Petit, Niot, Poirier & Besnard, 2007/3). Oleate entering from the apical membrane is preferentially shunted to the MAG pathway to form TG, whereas oleate entering from the basolateral membrane *via* the

circulation, is shunted to the G-3-P acylation pathway. The required enzyme activating the FA prior to its incorporation into MAG or G-3-P is one of the five members of the acyl-CoA synthetase long chain family (ACSL) (Mansbach & Gorelick, 2007). Of these ACSLs, only ACSL3 and 5 are significantly expressed in the intestine (Mansbach & Gorelick, 2007). In this proposed scenario, oleate-CoA delivered by ACSL5 would be directed towards the MAG pathway and that delivered by ACSL3 would be directed towards the G-3-P pathway (Mansbach & Gorelick, 2007).

Proanthocyanidins (PA), the most abundant polyphenols in grapes, apples, red grape juice, red wine and chocolate (Crozier, Jaganath & Clifford, 2009, Mink et al., 2007) have been shown to reduce postprandrial hypertriglyceridaemia in animal models (Blade, Arola & Salvado, 2010) and improve plasma lipids in humans (Zern et al., 2005). The hypolipidaemic action of proanthocyanidins is attributable to a reduction of plasma levels TRL both in normolipidaemic (Del Bas et al., 2005) and dyslipidaemic rats (Quesada et al., 2009). Plasma TG levels are the result of the balance between the TRL secretion by the intestine and the liver and their uptake by the extrahepatic tissues through the lipoprotein lipase (LPL). Several studies demonstrated the role of the liver in the hypotriglyceridaemic response triggered by PA (Del Bas et al., 2008) and established the molecular mechanisms by which PA modulated lipid and lipoprotein metabolism in the liver (Del Bas et al., 2009). However, the molecular mechanism by which PA modulates lipid and lipoprotein metabolism in the intestine is largely unknown. Thus, the aim of this study was to gain further insights into the role that the intestine plays in the hypotriglyceride action of PA. Intestinal cells can use two different pathways to carry out the TG synthesis, the MAG pathway in the post-prandial state and the G-3-P pathway in the fasting state. Thus, we evaluated the effects of grape seed

proanthocyanidins extract (GSPE) on TG secretion and gene expression, by using the Caco2 cell line cultured in specific media simulating the two different feeding states.

2. Methods

2.1. Proanthocyanidin extract

GSPE was kindly provided by Les Dérives Résiniques et Terpéniques (Dax, France). This proanthocyanidin extract contained essentially monomeric (21.3%), dimeric (17.4%), trimeric (16.3%), tetrameric (13.3%) and oligomeric (5–13 units) (31.7%) proanthocyanidins.

2.2. Materials

Taurocholate acid sodium salt (TC), fatty-acid-free bovine serum albumin (BSA), and monoolein were obtained from Sigma. Oleic acid (OA) was purchased from Merck.

2.3. Cell Cultures

Caco2 cells (ATCC, Manassas, VA) were grown in Dulbecco's modified Eagle's medium (DMEM; BioWhittaker) supplemented with 100U/mL penicillin, 100 μg/mL streptomycin (BioWhittaker), 2 mM L-Glutamine (BioWhittaker) and 20% foetal bovine serum (BioWhittaker) in a 95% air, 5% CO_2 atmosphere at 37 ºC. The growth medium was replenished every 2 or 3 days. For gene expression analysis, cells at passages 58–61 were seeded in 12-well plates at 2.5×10^5 cells per well. For experiments on the secretion of TG from Caco2 cells, the cells at passages 60–63 were seeded in 12-well Millicell Hanging Cell Culture Inserts (Millipore, Billerica, MA) at 1×10^5 per insert. The experiments were performed at 18–21 days post-seeding.

111 *2.4. Preparation of feeding and fasting state*

112 In order to perform a post-prandial state (Luchoomun et al., 1999), the volume of the
113 culture medium was 0.4 mL of DMEM without Phenol Red (BioWhittaker)
114 supplemented with 100 U/mL penicillin, 100 µg/mL streptomycin (BioWhittaker), 2
115 mM L-Glutamine (BioWhittaker), 0.8 mM OA, 0.5 mM TC and 0.4 mM monoolein on
116 the apical side, and 1 mL of DMEM without Phenol Red (BioWhittaker) supplemented
117 with 100 U/mL penicillin, 100 µg/mL streptomycin (BioWhittaker), 2 mM L-glutamine
118 (BioWhittaker) and 0.4 mM BSA on the basolateral side.
119 In order to perform a fasting state (Luchoomun et al., 1999), the volume of the culture
120 medium was 0.4 mL of DMEM without Phenol Red (BioWhittaker) supplemented with
121 100U/mL penicillin, 100 µg/mL streptomycin (BioWhittaker), 2 mM L-glutamine
122 (BioWhittaker) supplemented with 0.4 mM BSA on the apical side, and 1 mL of
123 DMEM without Phenol Red (BioWhittaker) supplemented with 100U/mL penicillin,
124 100 µg/mL streptomycin (BioWhittaker), 2 mM L-Glutamine (BioWhittaker), 0.8 mM
125 OA and 0.4 mM BSA on the basolateral side.
126 Transepithelial electrical resistance (TEER) was measured just after removing the
127 growth medium by using the Millicell-ERS system (Millipore). The TEER value of
128 Caco2 cultured in each transwells chamber was around 308 ± 3.5 Ω cm^2 in the post-
129 prandial state and 319 ± 5.9 Ω cm^2 in the fasted state indicating the formation of tight
130 monolayers (Ho, Delgado & Storch, 2002).
131

132 *2.5. Triglyceride secretion*

133 Cells were cultured for 24 hours with the medium described above and treated with
134 different concentrations of GSPE diluted in ethanol. In all the experiments GSPE was
135 added into the apical side. The basolateral medium and cells were harvested after the

136 treatments. The medium was ultracentrifuged with Amicon Ultra-4 centrifugal filter
137 (Millipore) to concentrate it and the amount of TG secreted by the Caco2 cells was
138 measured by using an enzymatic colorimetric kit (QCA, Amposta, Spain). Values were
139 corrected per mg cell protein determined by colorimetric assay (Bradford, Sigma).
140
141 *2.6. Gene expression analyses*
142 Caco2 cells were cultured for an hour with the post-prandial or fasting medium; all of
143 them were treated with different concentrations of GSPE diluted in ethanol. Total RNA
144 was isolated by using an RNeasy Mini kit (Quiagen, Hilden, Germany) following the
145 manufacturer's instructions. cDNA was synthesised from 2 µg of total RNA using High
146 Capacity cDNA Reverse Transcription kit (Applied Biosystems, Foster City, CA). A
147 total 20 ng of cDNA was subjected to quantitative RT-PCR amplification using Taqman
148 Master Mix (Applied Biosystems). Specific Taqman probes (Applied Biosystems) were
149 used for different genes: microsomal triglyceride transfer protein (MTP:
150 Hs00165177_m1), Long-chain acyl-CoA synthetase 3 (ACSL3: Hs00244853_m1),
151 long-chain acyl-CoA synthetase 5 (ACSL5: Hs00212106_m1), diacylglycerol
152 acyltransferase 1 (DGAT1: Hs00201385_m1), apolipoprotein A4 (ApoA4:
153 Hs00166636_m1), carnitine palmitoyl transferase 1 (CPT-1a: Hs00157079_m1),
154 peroxisome proliferator-activated receptor alpha (PPARα: Hs00223686_m1), intestinal-
155 fatty acid binding protein (I-FABP: Hs00164552_m1). Cyclophilin (Ppia:
156 Hs99999904_m1) was used as an endogenous control. Real-time quantitative PCR
157 reactions were performed using the ABI Prism 7300 SDS Real-Time PCR system
158 (Applied Biosystems).
159
160 *2.7. Statistical analysis*

161 The results are reported as the mean ± S.E.M of three independent experiments for the
162 TG secretion and two independent experiments for the gene expression analyses. Each
163 independent experiment was performed in triplicate**.** The means were calculated
164 considering each well in the 2 or 3 independent experiments. Group means were
165 compared with an independent-samples Student's *t*-test ($p \leq 0.05$) using SPSS software
166 (SPSS Inc., Chicago, IL).
167

168 **3. Results**

169 *3.1. GSPE treatment repressed the TG secretion in a fasting state but not in a post-*
170 *prandial state in Caco2 intestinal cells*

171 Caco2 cells 21 days post-confluence were cultured for 24 hours with a post-prandial or
172 a fasted medium in 12-well Millicell Hanging Cell Culture Inserts (Millipore) and
173 treated with 25−100 mg/L GSPE always added into the apical side. As shown in Figure
174 1, only 100 mg/L of GSPE tended to decrease the TG secretion in the fasted state while
175 in a post-prandial condition there were no changes. Thus, GSPE repressed TG secretion
176 differently depending on the feeding state and it was more effective in the fasted state.
177

178 *3.2. GSPE treatment repressed ACSL3 and ACSL5 in a fasting state but only ACSL5 in*
179 *a post-prandial state*

180 In order to assess whether GSPE could modulate the expression of genes related to lipid
181 and lipoprotein metabolism depending on the feeding state, we measured the expression
182 of key proteins in Caco2 intestinal cells (Tables 1 and 2). We have chosen proteins
183 involved in the intracellular transport of the long-chain fatty acids (I-FABP), TG
184 synthesis (ACSL3, ACSL5, DGAT1), fatty acid oxidation (CPT-1a), and CM and
185 VLDL assembly (MTTP, ApoA4). Furthermore, the nuclear receptor PPARalpha was

186 also selected because proteins like CPT-1a, ACSLs and FABPs are its targets
187 (Alaynick, 2008, Storch & Corsico, 2008).
188 Caco2 cells cultured in post-prandial medium treated with 25 and 100 mg/L of GSPE
189 showed a significant repression of ACSL5 and a significant overexpression of CPT-1a,
190 when compared with the control group (Table 1.). This expression profile suggests an
191 increase of the channelling of fatty acid towards oxidation.
192 In contrast, Caco2 cells cultured in fasting medium treated with 100 mg/L of GSPE
193 (Table 2) showed a significant repression of ACSL5, ACSL3, I-FABP and PPARalpha,
194 when compared to the control group. Thus, Caco2 cells cultured in a medium that
195 simulates the fasted condition were more sensitive to GSPE in accordance with their
196 sensitivity to TG secretion (Figure 1). Altogether, these results suggest that, depending
197 on the feeding state, proanthocyanidins may act through different pathways to get the
198 beneficial effects that have been described.
199

200 **4. Discussion**

201 In the Western diet, lipids represent more than 40% of the daily caloric intake while the
202 nutritional advice is a 10% lower (Ros, 2000). This high fat supply associated with a
203 qualitative imbalance (excess of plasma TRL, saturated fatty acids and cholesterol, and
204 the absence of LPL) greatly contributes not only to the increase of obesity prevalence
205 among the population, but also to the appearance of a plethora of diseases, such as
206 atherosclerosis, non-insulin-dependent diabetes, breast and colon cancers (Besnard,
207 Niot, Poirier, Clement & Bernard, 2002). Therefore, dietary components that could
208 reduce TRL production by the intestine e extremely useful. PA, a group of flavonoids
209 that can be found in common foodstuffs (Crozier, Jaganath & Clifford, 2009, Mink et
210 al., 2007), actively reduces plasma TG and apolipoprotein B (ApoB) in

normolipidaemic rats (Del Bas et al., 2005), dyslipidaemic rats (Quesada et al., 2009), hamsters fed on a hypercholesterolaemic diet (Auger et al., 2004) and humans (Kar, Laight, Shaw & Cummings, 2006). In previous studies we showed the role of the liver in the hypotriglyceridaemic response triggered by PA (Del Bas et al., 2008) and established the molecular mechanisms by which PA modulated lipid and lipoprotein metabolism in the liver (Del Bas et al., 2009). This study, then, intended to determine the role that the intestine plays in the hypotriglyceridaemic action of GSPE. Some studies have indicated that intestines can supply 20% or more of the total plasma TG in the absence of dietary fat (Cenedella & Crouthamel, 1974) and 40% in fasted rats (Ockner, Hughes & Isselbacher, 1969). Thus, we studied the effectiveness of GSPE in both post-prandial and fasted states.

We have chosen Caco2 cells because these represent the sole enterocyte model capable of differentiating spontaneously under standard cell culture conditions and allow the study of lipoprotein processing (Levy, Mehran & Seidman, 1995). Caco2 cells secrete ApoB-containing particles that have similar flotation properties to those of plasma LDL (Hussain, 2000). However, supplementation of Caco2 cells with OA has generally been shown to result in the secretion of more VLDL-sized particles and fewer LDL-sized particles (Hussain, 2000). Since growing Caco2 cells in cell culture transwells, we have emulated the post-prandial state by supplementing the apical medium with OA, MAG and TC, and the fasted state by supplementing the basolateral medium with OA. The results of this study showed that Caco2 cells were more sensitive to a GSPE treatment in the fasted state. GSPE levels up to 100 mg/L did not modify TG secretion in post-prandial state but repressed TG secretion in fasted conditions. In the post-prandial state GSPE treatment repressed ACSL5 and overexpressed CPT-1a significantly.

Nevertheless, in the fasted state GSPE treatment repressed ACSL5, ACSL3, I-FABP and PPARalpha significantly.

ACSL are essential for *de novo* lipid synthesis, fatty acid catabolism and remodelling of membranes (Soupene & Kuypers, 2008). The ACSL isoforms differ in their substrate preferences, enzyme kinetics, intracellular locations and the direction of their acyl-CoA products towards independent downstream pathways (Ellis, Frahm, Li & Coleman, 2010, Mashek, Li & Coleman, 2006). Only ACSL3 and ACSL5 are significantly expressed in the intestine (Mansbach & Gorelick, 2007, Mashek, Li & Coleman, 2006). ACSL5 is the only isoform found in both mitochondrial membranes and endoplasmic reticulum (Mashek, McKenzie, Van Horn & Coleman, 2006), although it was detected also in the plasma membrane (Soupene & Kuypers, 2008). The overexpression of ACSL5 in rat hepatoma cell lines increases fatty acid (exogenous, not endogenous) incorporation into TG and without changes in β-oxidation or phospholipid synthesis (Bu, Mashek & Mashek, 2009, Mashek, Li & Coleman, 2006). Moreover, hepatic ACSL5 expression increases after applying an insulin treatment or a sterol regulatory element-binding protein (SREBP-1c) overexpression (Mashek, Li & Coleman, 2006). Nevertheless, ACSL3 was detected in lipase-activated lipid droplets but not in uninduced droplets (Soupene & Kuypers, 2008). Hepatic ACSL3 expression is upregulated in hyperlipidaemic hamsters (Wu, Liu, Chen, Fujimoto & Liu, 2009). Additionally, Yao H. et al (Yao & Ye, 2008) showed that ACSL3 played a crucial role in secretion of VLDL in human hepatoma Huh7 cells. Knockdown of ACSL3 in human hepatocytes decreases [1-^{14}C] oleic acid incorporation into phospholipids for VLDL synthesis (Bu, Mashek & Mashek, 2009). Despite there being no experimental evidence of the ACSL5 and ACSL3 roles in intestine, it has been postulated that FA delivered by ACSL5 would be directed towards the MAG pathway and by ACSL3 towards the G-3-P

pathway (Mansbach & Gorelick, 2007). Thus, in light of the results, it can be suggested that GSPE represses only the supply of FA towards the MAG pathway in the post-prandial state (ACSL5) but represses FA delivered towards both pathways in fasted state (ACSL5 and ACSL3). Therefore, GSPE could be more effective reducing TG secretion in the fasted than in the post-prandial state.

In the intestinal cells two fatty acid binding proteins (FABPs) are largely and equally expressed: liver-FABP (L-FABP), which is also expressed in the liver and intestinal-FABP (I-FABP), which is specially expressed in fully differentiated proximal absorptive enterocytes (Besnard, Niot, Poirier, Clement & Bernard, 2002). It has been suggested that there is a potential function of I-FABP, directing long-chain FA to specific subcellular sites of utilisation, such as β-oxidation and esterification into phospholipids and TG (Karsenty et al., 2009). Thus, the repression of I-FABP by GSPE in the fasted state could work together with ACSL5 and ACSL3 reducing TG synthesis and secretion.

PPARalpha, which is abundantly expressed in enterocytes, is an important nuclear receptor that mediates the effects of dietary lipids on gene expression (Bunger, Van Den Bosch, Van Der Meijde, Kersten, Hooiveld & Muller, 2007). Natural agonists of PPARalpha normally found in diet are oleic acid, eicosapentaenoic acid and docosahexaenoic acid (De Vogel-Van Den Bosch, Bunger, De Groot, Bosch-Vermeulen, Hooiveld & Muller, 2008). Cytoplasmatic FABP transfers and channels the FA and its CoA metabolites into nuclei, binds PPARalpha and activates the transcription of genes involved in fatty acid and glucose metabolism (Schroeder et al., 2008). PPARalpha controls the expression of several genes involved in fatty acid metabolism, such as those involved in the transport across the cell membrane, the intracellular binding (I-FABP), the formation of acyl-CoA (ACSL) and the

mitochondrial and peroxisomal β-oxidation (CPT-1a) (Alaynick, 2008). Therefore, the repression of ACSL and I-FABP by GSPE in the fasted state may be secondary for the reduction of PPARalpha expression.

In conclusion, Caco2 cells were more sensitive to GSPE treatment in the fasted state than in the post-prandial state. The repression of ACSL5, ACSL3, I-FABP and PPARalpha expressions in the fasted state may account for the lower levels of TG in the medium. Furthermore, the different modulation of gene expression by GSPE between the two feeding states suggests that the feeding state is an important condition in order that GSPE can carry out its hypotriglyceridaemic action in the intestine.

These results provide the first *in vitro* evidence of the fact that ACSL3 and ACSL5 are target genes of PA, supporting the idea that they are powerful agents for preventing and treating lipid-altered metabolic states.

Conflict of interest

The authors declare no conflict of interest.

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Table 1. mRNA of lipid-related genes in Caco2 cells grown in a medium simulating the post-prandial state and treated with 25 and 100 mg/L of grape seed proanthocyanidin extract (GSPE).

	Control	GSPE 25 mg/L	GSPE 100 mg/L
ACSL5	1.01 ± 0.07	0.68 ± 0.01 *****	0.72 ± 0.06 *****
ACSL3	1.00 ± 0.04	0.92 ± 0.01	0.94 ± 0.01
DGAT1	1.02 ± 0.09	1.11 ± 0.05	0.87 ± 0.07
ApoA4	1.01 ± 0.08	1.29 ± 0.16	1.05 ± 0.23
MTTP	1.05 ± 0.14	1.23 ± 0.16	1.06 ± 0.13
CPT-1a	1.03 ± 0.12	1.73 ± 0.17 *****	1.41 ± 0.04 *****
I-FABP	1.03 ± 0.10	0.86 ± 0.08	0.81 ± 0.09
PPARalpha	1.02 ± 0.06	1.06 ± 0.05	0.94 ± 0.08

The culture medium on the apical side was supplemented with 0.8 mM oleic acid, 0.5 mM taurocholate and 0.4mM monoolein. GSPE was added to the apical side. mRNA levels were measured after an hour of culture. Each value is the mean ± s.e.m of two independent experiments. * indicates significant differences ($p \leq 0.05$) between control cells and GSPE treated cells by Student's *t*-test.

Table 2. mRNA of lipid-related genes in Caco2 cells grown in a medium simulating the fasted state and treated with 100 mg/L of grape seed proanthocyanidin extract (GSPE).

	Control group	GSPE 100 mg/L
ACSL5	1.01 ± 0.06	0.75 ± 0.06 *
ACSL3	1.01 ± 0.03	0.82 ± 0.04 *
DGAT1	1.00 ± 0.03	0.94 ± 0.04
ApoA4	0.97 ± 0.05	0.78 ± 0.14
MTTP	1.05 ± 0.14	0.97 ± 0.03
I-FABP	1.01 ± 0.06	0.65 ± 0.04**
PPARalpha	1.00 ± 0.04	0.88 ± 0.02 *
CPT1a	1.01 ± 0.04	0.95 ± 0.04

The culture medium on the basolateral side was supplemented with 0.8 mM oleic acid and 0.4 mM BSA. GSPE was added to the apical side. mRNA levels were measured after an hour of culture. Each value is the mean ± s.e.m of two independent experiments. * indicates significant differences ($p \leq 0.05$) between control cells and GSPE treated cells by Student's *t*-test

446 **FIGURE LEGENDS**

447

448 **Figure 1: Triglyceride secretion by Caco2 cells grown in a medium simulating**
449 **post-prandial or fasted states and treated with a grape seed proanthocyanidin**
450 **extract (GSPE).**

451

452 In the post-prandial state, the culture medium on the apical side was supplemented with
453 0.8 mM oleic acid, 0.5 mM taurocholate and 0.4 mM monoolein. In the fasting state, the
454 culture medium on the basolateral side was supplemented with 0.8 mM oleic acid and
455 0.4 mM BSA. GSPE (25, 50 or 100 mg/L) was always added to the apical side.
456 Triglycerides were measured after 24 hours of culture. Each value is the mean ± s.e.m
457 of three independent experiments. # indicates significant differences ($p = 0.08$) between
458 control cells and GSPE-treated cells by Student's *t*-test.

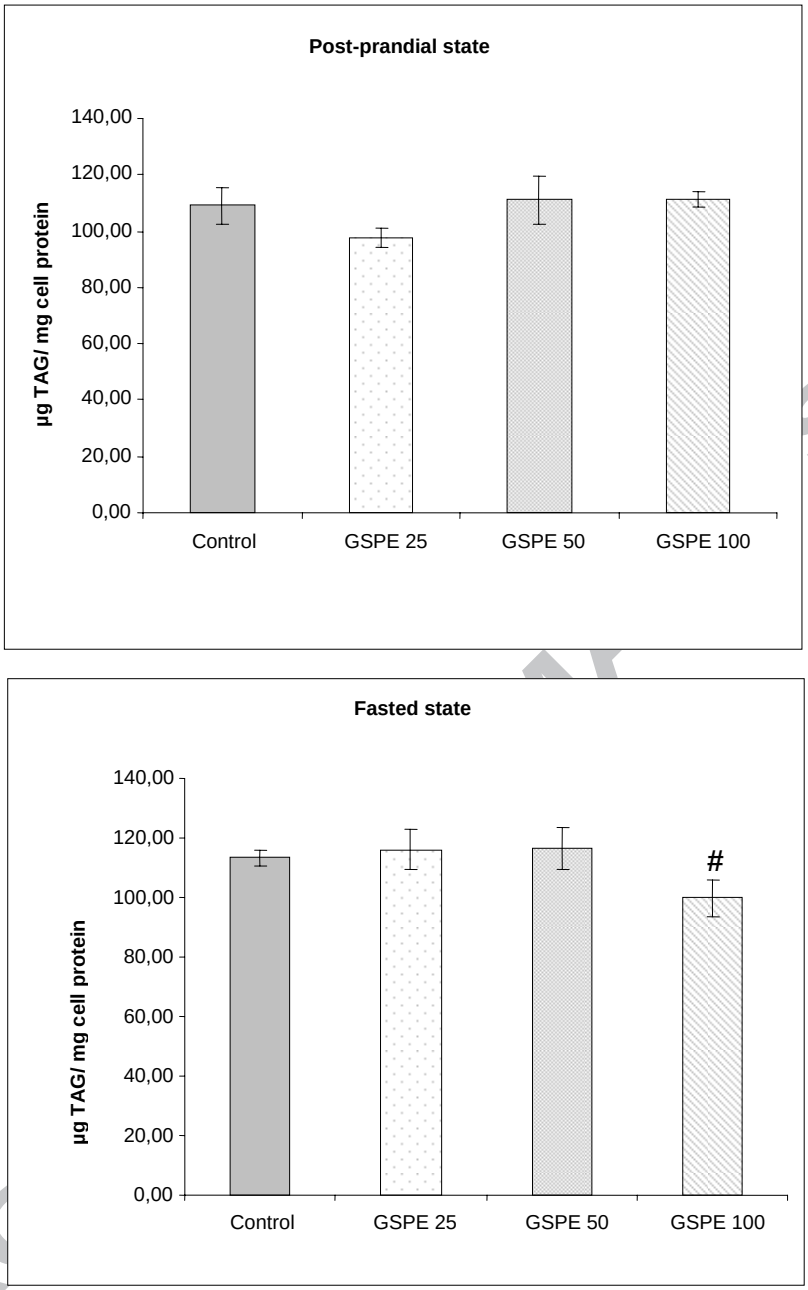
459

460 Figure 1

461

462

463



In Caco2 cells, proanthocyanidins only decrease triglyceride secretion in fasted sate > They repress ACSL3 and ACSL5 in fasting state but only ACSL5 in a postprandial state > ACSLs control the fate of fatty acids for triglyceride synthesis in intestinal cells > Feeding state could be a key factor for proanthocyanidin effectiveness on triglyceride