Mechanisms for antihypertensive effect of CocoanOX, a polyphenol-rich cocoa powder, in spontaneously hypertensive rats

Mar Quiñones[1], David Sánchez[1], Begoña Muguerza[3], Marta Miguel[2*], Amaya Aleixandre[1]

[1]Dpto. Farmacología, Fac. Medicina, U. Complutense, Avda. Complutense s/n, 28040 Madrid, Spain. [2]Instituto de Fermentaciones Industriales (CSIC). C/ Juan de la Cierva, 3 28006, Madrid, Spain. [3]Naturex Ingredients Spain, S.L.U., Autovía A-3, Salida 343, Camino de Torrent s/n, Quart de Poblet, 46930 Valencia, Spain.

Running title: Mechanisms of action of a polyphenol-rich cocoa powder

***Author for correspondence:**

Dr. Marta Miguel. Instituto de Fermentaciones Industriales (CSIC). C/ Juan de la Cierva, 3. 28006 Madrid, Spain. Tel: + 34 91 5622900. Fax + 34 91 5644853

e-mail: marta.miguel@ifi.csic.es

Abbreviations

spontaneously hypertensive rats (SHR); malonildialdehyde (MDA); angiotensin converting enzyme (ACE); N^{W}-nitro-L-arginine methyl ester (L-NAME).

Key words: Angiotensin converting enzyme, Cocoa, Nitric oxide, Oxidative stress, Polyphenols.

Abstract

We investigate the mechanisms involved in the long-term antihypertensive effect of a polyphenol-rich cocoa powder, named CocoanOx® (CCX), in spontaneously hypertensive rats (SHR). We have carried out two different batches of experiments. For the first batch of experiments, forty 3 week-old male SHR were randomly divided with *ad libitum* intake into four groups of 10 animals, that respectively received the following drinking fluids until the 20-week of life (treatment period): tap water (control), CCX 100 mg/kg/day, CCX 200 mg/kg/day and CCX 400 mg/kg/day. Five 20-weeks-old rats of each group were sacrificed by decapitation. From the 20th to 24 week of life all the remaining animals were given tap water (follow-up period), and all of them were sacrificed at the end of the follow-up period. Plasma malonildialdehyde (MDA), reduced-glutatione in the liver, plasma and aorta angiotensin converting enzyme (ACE) activity and plasma angiotensin II were determined in all the sacrificed SHR that were included in this batch of experiments. Plasma MDA decreased and reduced-glutatione increased in the liver from the 20 week-old CCX treated SHR. These effects were not observed in the rats that were sacrificed after the follow-up period. CCX treatment did not modify aorta ACE activity, but the activity of ACE and the levels of angiotensin II increased in the plasma of the SHR treated with the highest dose of CCX. ACE activity returned to basal values in the SHR that were sacrificed after the follow-up period. However, angiotensin II levels were slightly higher after withdrawal of CCX.

For the second batch of experiments we used aorta rings obtained from untreated SHR, and we evaluated the relaxation caused by CCX in different aorta preparations. CCX relaxed the intact aorta preparations but this cocoa did not relax the endothelium-denuded aorta rings from the untreated SHR. L-NAME, but not indomethacin, inhibited the relaxation caused by CCX in the SHR aorta rings. We postulate that the antihypertensive effect of CCX might be mediated by an improvement of endothelial release of nitric oxide and by a reduction of oxidative stress. The inhibition of ACE, could ~~also~~ be implicated in the antihypertensive effect of CCX.

1. Introduction

Several studies have shown that consumption of foods rich in flavonoid compounds is associated with lower incidence of cardiovascular disease (Hollenberg et al., 1997; Schroeter, Holt, Orozco, Schmitz, & Keen, 2003; Stoclet et al., 2004). Foods with high polyphenolic flavonoid content include many fruits and vegetables such as apples, onions, tea, red wine and cocoa. Cocoa and cocoa derivatives have been shown to have the highest content of flavanols, particularly flavan-3-ols and procyanidins (Arts, Hollman, & Kromhout, 1999; Lee, Kim, Lee, & Lee, 2003). An important epidemiological study related the high consumption of chocolate with lower blood pressure and lower risk of cardiovascular disease (Buijsse, Feskens, Kok, & Kromhout, 2006). Other studies carried out in humans have also shown an improvement in endothelial function after cocoa (Hung et al., 2004, Heiss et al., 2005) wine (Diebolt, Bucher, & Andriantsitohaina, 2001) and tea (Duffy et al., 2001) consumption. However, the biochemical mechanisms that explain the benefits of flavonoids on the cardiovascular system have not been completely elucidated.

Different mechanisms may justify the antihypertensive properties of polyphenols. The vasodilatation occasioned by these compounds has been related to the production of nitric oxide (Emura, Yokomizo, Toyoshi, & Moriwaki, 2007), the inhibition of angiotensin converting enzyme (ACE) (Li et al., 2005, Liu et al., 2003) and the reduction of oxidative stress (Duarte et al., 2001; Negishi et al., 2004; Peng et al., 2005). ~~Only~~ A better understanding of the mechanism and the determining factors of the antihypertensive activity of

polyphenols will allow a rational development of rich polyphenol functional foods for blood pressure control.

Cocoa beans are rich in polyphenols. In particular, they are rich in flavanols, but the concentration of these compounds in cocoa products not only depends on the initial flavanol content of the cocoa beans. The processing steps are also ~~very~~ important to condition the final content of flavanols in cocoa derivatives. CocoanOX (CCX) is a cocoa powder prepared by an industrial procedure to prevent polyphenol degradation (Tomás-Barberán et al., 2007). ~~CCX has therefore a high content in polyphenols (162 mg/g).~~ In these studies, the characterization of this product was carried out by our research group and we demonstrated that this product had a high content in polyphenols (162 mg/g). In this same work we also demonstrated the antihypertensive properties of CCX after a single oral administration in spontaneously hypertensive rats (SHR) (Cienfuegos-Jovellanos et al., 2009). Later, we also evaluated the long-term antihypertensive effect of CCX in SHR (Quiñones et al., 2010). Scientific evidence of bioactivity is required to market functional foods, and it is advisable to explain the mechanism involved in their beneficial effects. Since CCX is a polyphenol rich cocoa powder, the aim of this study was to evaluate different biomarkers implicated in the antihypertensive properties of these compounds.

2. Material and Methods

CocoanOX was supplied by Natraceutical Group (Valencia, Spain). This product, obtained via an enzymatic patented process, was previously characterized physico-chemically (Table 1). We have already demonstrated the short-term (Cienfuegos Jovellanos et al., 2009) and the long-term (Quiñones et al., 2010) antihypertensive effect of this product in SHR. In this study, we carried out two different batches of experiments. In the first one we used different tissues (plasma, aorta and liver) that had been obtained from CCX long-term treated SHR, and in the second one we used aorta ring preparations obtained from untreated SHR.

All the above-mentioned experiments were performed as authorized for scientific research (European Directive 86/609/CEE and Royal Decree 223/1988 of the Spanish Ministry of Agriculture, Fisheries and Food).

2.1. First batch of experiments

2.1.1. General protocol to treat the SHR and to obtain the tissues for the different determinations

After being weaned at 3 weeks, male SHR (Charles River Laboratories Spain S.A.) were housed in groups of five rats at a temperature of 23º C with 12 h light/dark cycles. They were in turn randomly divided with *ad libitum* intake into four groups of 10 animals, and during the experimental period (until the rats were 24-weeks-old), the SHR of these groups were fed on a solid standard diet (A04 Panlab, Barcelona, Spain). Until the rats were 20-week-old, the drinking fluids in these groups were tap water (control), CCX 100 mg/kg/day, CCX 200 mg/kg/day or CCX 400 mg/kg/day. Therefore, we

consider the period of time elapsed from 3 weeks of life until 20 weeks of life in the animals as the treatment period. Body weight, solid and liquid intake and systolic blood pressure values in these rats are described in detail in Quiñones et al. (Quiñones et al., 2010). After over-night fasting, five 20-week-old rats of each group were sacrificed by decapitation. Blood samples were obtained from the sacrificed rats to carry out the analysis of plasma malondialdehyde (MDA), plasma ACE activity and plasma angiotensin II. Aorta samples were also obtained to determine ACE activity in this tissue, and liver samples were also obtained to assess reduced-glutathione in this tissue. We describe below the procedures to evaluate all these parameters. The drinking fluid was always tap water in all groups of animals from the 20^{th} to 24^{th} week of life, and we consider this time as the follow-up period. At the end of the experimental period, the 24-week-old rats were sacrificed by decapitation after over-night fasting, and the same determinations and procedures described above were done

2.1.2 Plasma, aorta and liver preparations for biochemical determinations

Blood samples from the sacrificed animals were collected into tubes containing lithium heparin as anticoagulant. These samples were centrifuged at 2500 g for 20 minutes at 4ºC to obtain the plasma which was divided into aliquots and kept frozen at -80ºC until analysis of MDA and ACE activity. Aorta and liver tissue were homogenized at 4 ºC in a Potter with PBS (0.01 M PBS, 0.15 M NaCl, pH 7.4), the homogenates were centrifuged at 5000g for 15 min at 4 ºC and the supernatant was recovered. The supernatants of the

centrifuged samples were kept frozen at – 80 ºC until used for evaluate ACE activity and reduced-glutathione, respectively. The protein content of the homogenates was determined by the Bio-Rad protein assay (Bio-Rad Laboratories, Hercules, CA, USA), using bovine serum albumin as standard.

2.1.3 Malondialdehyde determination

Plasma malondialdehyde (MDA) levels were measured by a thiobarbituric acid assay based on that proposed by Rodríguez-Martínez et al. (Rodríguez-Martínez, & Ruiz-Torres, 1992) and modified as previously described Manso et al. (Manso et al., 2008). The plasma MDA values were expressed as nmol MDA.

2.1.4 Reduced-glutathione determination

Reduced-glutathione in the liver was measured by the monoclorobimane fluorimetric method (Kamencic, Lyon, Paterson, & Juurlink, 2000). For this, 90 μl of liver homogenizated supernatant were mixed with 10 μl of glutathione S-transferase solution (1U/ml), obtained from horse liver (Sigma-Aldrich, USA), and monoclorobimane (Fluka Biochemical, Switzerland) (100 mM). This reaction is catalysed by glutathione S-transferase. The levels of glutathione were quantified by a fluorimeter (Multiscan Asccent Labsystems, Spain) and were expressed as μmol/g tissue protein.

2.1.5 Determination of ACE activity in plasma and aorta

ACE activity in plasma and aorta were measured by a fluorimetric method as explained in (Miguel, Manso, Aleixandre, & López-Fandiño, 2007). ACE activity was expressed as mU ACE /ml in plasma samples, and as mU ACE/mg tissue protein in aorta samples.

2.1.6 Determination of angiotensin II in plasma

Angiotensin II (Ang II) was assayed by enzyme-linked immunosorbent assay (ELISA) using a commercial kit (Assay Pro, USA) according to the manufacturer's instructions. Angiotensin II levels were expressed as ng/ml plasma.

2.2 Second batch of experiments: experiments in aorta ring preparations

For these experiments we used 17-22 week old non-treated SHR. The animals were sacrificed by decapitation. The thorax was opened, and the aorta from the aortic arch to the diaphragm was rapidly excised and transferred to a beaker containing Krebs-Henseleit Solution with the following composition (mmol/L): NaCl, 118.2; KCl, 4.7; $CaCl_2$, 2.5; KH_2PO_4, 1.2; $MgSO_4$, 1.2; $NaHCO_3$, 25; and glucose, 10.0. Excess fat and connective tissue were removed from the aorta and the tissue was cut into rings (approximately 4 mm in length). The aortic rings were mounted between two steel hooks in isolated tissue chambers also containing Krebs-Henseleit Solution. The medium was maintained at 37ºC, and continuously bubbled with a 95% O_2-5% CO_2 mixture, which gave a pH of 7.4. An optimal resting tension of 2 g was applied to all aortic segments. This tension was adjusted every 15 min during a 60-90 min

equilibration period before adding drugs. Isometric tension was recorded by using an isometric force displacement transducer connected to an acquisition system (Protos 5, Panlab, Spain). After the equilibration period, the rings were first contracted by 80 mmol/L KCl to asses their functionality and when the contraction had reached the steady state (about 15 min. after the administration), the preparations were washed until the basal tension was recovered. Then the rings were exposed to 10^{-6} mol/L metoxamine, and dose-response curves to CCX (10^{-4} mg/ml – 1 mg/ml) were performed in the methoxamine-precontracted rings. Relaxant responses to CCX were expressed as a percentage of the precontraction induced by metoxamine.

The previously described procedure was applied to intact and endothelium-disrupted tissue. It was also applied to another two groups of intact preparations; one with the addition of N^W-nitro-L-arginine methyl ester (L-NAME) (10^{-4} M), an *in vivo* and *in vitro* inhibitor of NO-synthase, and the other one with the addition of indomethacin (10^{-5} M), a drug that inhibits cyclooxygenase and prostacyclin synthesis, to the bath solution 30 minutes before methoxamine administration. The denuded endothelium preparations were prepared by gently rubbing the tissue before it was cut into rings, and the efficacy of the procedure used to remove the endothelial cells was judged by the loss of acetylcholine-induced relaxation in the aorta preparations precontracted with methoxamine, as these cells, according to the studies published by Furchgott in 1980 and in 1999 (Furchgott, & Zawadzki, 1980; Furchgott, 1999), are necessary for this response.

2.8 Statistical analysis

The results are expressed as mean values ± S.E.M. for at least 5 rats or 5 determinations, and were analyzed by one or two-way analysis of variance (ANOVA), using the GraphPad Prism software. Differences between the groups were assessed by the Bonferroni test. Differences between the means were considered to be significant when $P< 0.05$.

3. Results

The concentration of MDA was significantly lower in the plasma of all the different groups of CCX treated rats than in the plasma of the non-treated rats. Nevertheless, the withdrawal of CCX caused an increase in plasma MDA concentration, and at the end of the follow up period, the concentration of this metabolite was similar in all the rats that had been treated with CCX and in the non-treated rats (Figure 1).

Long-term intake of CCX increased the levels of reduced gluthatione in the liver of the SHR. Reduced glutathione decreased after the follow-up period and returned to basal values in the liver from the animals that had received 100 mg/kg or 200 mg/kg of CCX. Nevertheless, the 24 week-old animals that had been treated with 400 mg/kg of CCX maintained high levels of reduced gluthatione in the liver after the follow-up period (Figure 2).

Long-term intake of CCX did not modify ACE activity in the aorta of the SHR (data not shown). However, ACE activity was higher in the plasma of the rats treated with 200 mg/kg or 400 mg/kg of CCX than in the plasma from the

non-treated SHR (Figure 3). Angiotensin II plasma levels also increased in the groups of rats treated with 200 mg/kg or 400 mg/kg of CCX. Plasma ACE activity decreased when we interrupted these treatments, and four weeks after the withdrawal of CCX the activity of this enzyme returned to basal values. However, angiotensin II levels were slightly higher after withdrawal of CCX (Figure 4).

CCX relaxed the intact aorta ring preparations from the untreated SHR, but CCX did not relax the endothelium-denuded aorta ring preparations from these animals. In addition, the relaxations caused by CCX in the 10^{-4} M L-NAME treated aorta ring preparations were lower than the relaxations caused by this cocoa powder in the non-treated aorta ring preparations, but the presence of 10^{-5} M indomethacin in the bath solution did not modify the relaxations induced by CCX in this tissue (Figure 5).

4. Discussion

Hypertension is a multifactorial pathology that is conditioned by different environmental and social factors, as well as by different endocrine, genetic and metabolic disorders. CCX is a cocoa powder that has demonstrated antihypertensive effects (Cienfuegos-Jovellanos et al., 2009; Quiñones et al., 2010). In this paper, we have ~~clearly~~ demonstrated that the long-term treatment with CCX could decrease plasma MDA and could also increase liver reduced gluthatione. These effects disappear when the treatment with CCX was removed. Therefore, the treatment with this cocoa powder, improves the

redox state and decreases oxidative stress. Since hypertension has been associated with free radical production, lipid peroxidation and oxidative stress (Pudu, Puddu, Cravero, Rosati, & Muscari, 2008; Harrison & Gongora, 2009). It is nowadays assumed that plasma MDA reflects all these alterations. On the contrary, reduced glutathione is a molecule which can scavenge reactive oxygen species and other free radicals. Its production is ~~very~~ important in the liver reaching other organs when levels are adequate. Therefore, the balance between plasma MDA and liver reduced gluthatione enable us to know the redox state and the antioxidant protection degree of the organism.

In addition, it is known that endothelial dysfunction could justify, at least in part, the increased blood pressure of hypertensive subjects. In 1980, Furchgott reported that endothelial cells have an obligatory role in the relaxation of arteries by acetylcholine and related muscarinic agonists (Furchgott, 1999). The relaxation results from the stimulation by the muscarinic agonist, of the release from the endothelial cells of a very labile diffusible factor that was later characterized as nitric oxide (NO) (Furchgott & Zawadzki, 1980). It has been demonstrated that polyphenol could improved endothelial function and could release NO (Grassi et al., 2008; Schmitt & Dirsch, 2009). We have previously demonstrated that the responses to acethylcholine improved in the aorta from the CCX treated SHR (Quiñones et al., 2010). The endothelial activity could be therefore implicated in the antihypertensive effect of CCX. In this paper, we have demonstrated that the vasorelaxant effect of this cocoa powder is, in fact, endothelium dependent, because this cocoa powder did not relax the endothelium denuded aorta preparations from SHR. Moreover, the

effect of CCX in the aorta preparations was impaired when NO synthase was inhibited. These results correlate CCX activity with the release of NO. On the contrary, prostacyclin seems not to participate in the vasodilator effect of CCX, because this cocoa powder clearly relaxed the aorta tissue when indomethacin, a COX inhibitor, was present in the organ bath.

It has been demonstrated that a variety of tissues, including blood vessels, heart and kidney contain all the essential components of the rennin-angiotensin system (Takai, Jin, Sakaguchi, & Miyazaki, 2004). In our study, we obtained plasma and aorta samples from CCX treated SHR to measure ACE activity. ACE activity in aorta samples from the CCX treated SHR was very similar to ACE activity in aorta from non treated rats. However, the ACE activity in the plasma from the SHR treated with 200 mg/kg/day or 400 mg/kg/day CCX, was significantly higher than the ACE activity in the plasma from non treated rats. Angiotensin II was also higher in the plasma obtained from the rats that had been treated with these doses of CCX than in the plasma from non treated SHR. CCX is a cocoa powder rich overall in monomers through trimers of flavonoids, and it has been described that the procyanidins are the flavonoids that mainly inhibits ACE (Actis-Goretta, Ottaviani, Keen, & Fraga, 2003; Ottaviani, Actis-Goretta, Villordo, & Fraga, 2006). Our results could be justified having in mind these ideas, because the long-term decrease in arterial blood produces compensatory mechanisms that try to increase this variable. Our results in fact indicate that CCX could also have ACE inhibitory properties. An elevation in plasma ACE concentration has been documented in humans and rats treated with ACE inhibitors (Fyhrquist, Forslund, Tikkanen, &

Grönhagen-Riska, 1980; Boomsma, Debruyn, Derkx, & Schalekamp, 1981; Wu & Ding, 2001). In previous works, our research group also demonstrated increased ACE activity in the plasma from SHR that had been long-term treated with captopril (Miguel et al., 2007). Costerousse et al. (1998) indicated that the increase in circulating ACE levels in rats treated with ACE inhibitors was associated to a generalized increase in ACE gene transcription and ACE synthesis in somatic cells. According to these researchers, this may be due to an adaptative response to the inhibition of the enzyme, but it could be independent of angiotensin II suppression. The consequences of ACE induction during inhibition of the enzyme are not known, but it does not seem to reduce the therapeutic effect of these drugs (Costerousse, Allegrini, Clozel, Ménard, & Alhenc-Gelas, 1998). It is also true those other mechanisms than ACE inhibition could justify the therapeutic effect of ACE inhibitors (Takai, Jin, Sakaguchi, & Miyazaki, 2004). In our study, ACE activity returned to basal values after the follow up period. However, angiotensin II levels were slightly higher after withdrawal of CCX. This could also be understood, because the feedback that could be produced when ACE is inhibited for a long time, disappears when the block of this enzyme is finished, and in turn a ~~massive~~ synthesis of this vasoconstrictor peptide occurs.

The results obtained in this study have demonstrated that the antihypertensive effect of CCX is endothelium dependent. CCX effect is mediated, at least in part, by endothelial release of NO and by a reduction of oxidative stress. Other mechanisms, like ACE inhibition could also justify the effect of this cocoa powder. In conclusion, we have described some

mechanisms implicated in the antihypertensive effect of CCX, a cocoa powder that could be used as a functional food ingredient for controlling blood pressure and other related disorders. However, we are aware that before CCX was marketed as antihypertensive ingredient, it would be necessary to carry out clinical studies to demonstrate their antihypertensive efficiency in humans.

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Figure legends

Figure 1. Histograms of plasma malondialdehyde (MDA) from 20 (■) and 24 week-old (□) spontaneously hypertensive rats. The animals had received the following different daily treatments from weaning until the 20th week of life: tap water, CocoanOX (CCX) 100 mg/kg, CCX 200 mg/kg and CCX 400 mg/kg. All rats drank tap water from the 20th week of life until the 24th week of life. The group that drank always tap water is considered the control group, and the other groups have been defined by the treatment that received until the 20th week of life. Data are mean values ± S.E.M. for 5 animals. * P < 0.05 vs the rats of the same age in the control group. # P < 0.05 vs 20 week-old rats.

He redactado otra vez la figura 1. Por mucho que el referee pretenda modificar la redacción esta debe incluir los tratamientos, y desde luego está mucho más claro lo que significa cada cosa aludiendo a la edad de los animales. No se deben quitar los tratamientos de las animales. He definido el grupo control y en las figuras debe ponerse en el eje de las X control NO tap water

HAY QUE HACER LA REDACCIÓN DE TODAS LAS FIGURAS EN BASE A LA 1 QUE HE REDACTADO.

HAY QUE QUITAR LA FIGURA 4 Y REHACER NÚMEROS

Figure 1. Histograms of plasma malondialdehyde (MDA). (■) = treatment, (□) wash-out. ~~The animals had received from weaning until the 20th week of life~~

~~different daily treatments: tap water (control), CocoanOX (CCX) 100 mg/kg, CCX 200 mg/kg and CCX 400 mg/kg. All rats drank tap water from the 20th week of life until the 24th week of life.~~ Data are mean values ± SEM. for 5 animals. *=P<0.05 vs tap water (control). #=P<0.05 vs 20 week-old treated animals. ~~There are not significative differences between 20 week-old and 24 week-old rats~~

Figure 2. REDACTAR COMO LA 1 Histograms of the reduced-glutathione in the liver from 20 ( ) and 24 week-old ( ) spontaneously hypertensive rats. The animals had received from weaning until the 20th week of life different daily treatments: tap water (control), CocoanOX (CCX) 100 mg/kg, CCX 200 mg/kg a nd CCX 400 mg/kg. All rats drank tap water from the 20th week of life until the 24th week of life. Data are mean values ± S.E.M. for 5 animals. *P<0.05 vs tap water (control); There are not significant differences between 20 and 24 week-old rats. ~~#P<0.05 vs 20 week-old treated rats.~~

Figure 3. REDACTAR como la 1 Histograms of the plasma angiotensin converting enzyme activity (ACE) from 20 ( ) and 24 week-old ( ) spontaneously hypertensive rats. The animals had received from weaning until the 20th week of life different daily treatments: tap water (control), CocoanOX (CCX) 100 mg/kg, CCX 200 mg/kg and CCX 400 mg/kg). All rats drank tap water from the 20th week of life until the 24th week of life. Data are mean values ± S.E.M. for 5 animals. *P<0.05 vs control tap water (control); #P<0.05 vs 20 week-old treated animals.

Figure 4. REDACTAR COMO LA 1 Histograms of angiotensin II plasma levels from 20 ( ) and 24 week-old ( ) spontaneously hypertensive rats. The animals had received from weaning until the 20th week of life different daily treatments: tap water (control), CocoanOX (CCX) 100 mg/kg, CCX 200 mg/kg and CCX 400 mg/kg). All rats drank tap water from the 20th week of life until the 24th week of life. Data are mean values ± S.E.M. for 5 animals. *P<0.05 vs tap water (control); #P<0.05 vs 20 week-old treated animals.

Figure 5. REDACTAR COMO LA 1. Cumulative dose-response curves to CocoanOX (CCX) (10^{-4} mg/ml – 1 mg/ml) in different aorta ring preparations from spontaneously hypertensive rats: intact (●), endothelium denuded (○), L-NAME (10^{-4} M)-treated (▲) and indomethacin (10^{-5} M)-treated (■). The results (mean ± SEM for at least 8 preparations and 3 animals) are expressed as the percentage of the previous methoxamine induced contraction. *P<0.05 vs intact preparations.

Table 1. Concentration of theobromine and flavan-3-ols (mg/g) of the industrial batch of CocoanOX™.

Compound	[mg/g]
Theobromine	15.95 ± 0.05
Total flavan-3-ols [1]	42.64 ± 0.25
(+) Catechin	5.18 ± 0.09
(-) Epicatechin	19.36 ± 0.03
Procyanidin B2	16.85 ± 0.06
Procyanidin B1	1.25 ± 0.07

The results are expressed on a wet basis as mean ± SD (n=2).

[1] DAD-HPLC

~~Taken from~~ Cienfuegos-Jovellanos et al., 2009.

559 **Figure 1**

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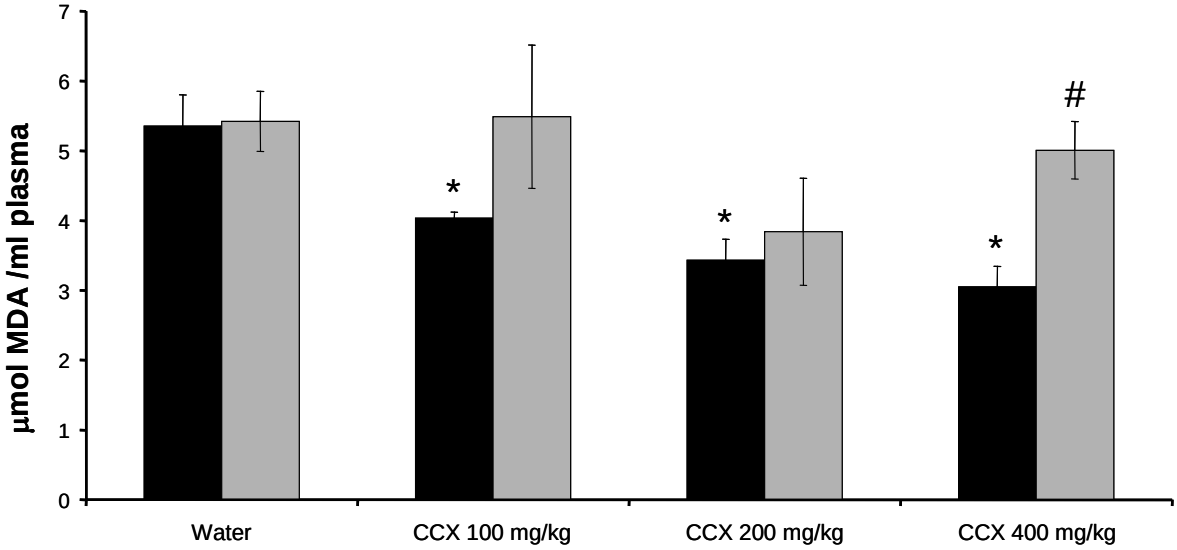

562 **Figure 2**

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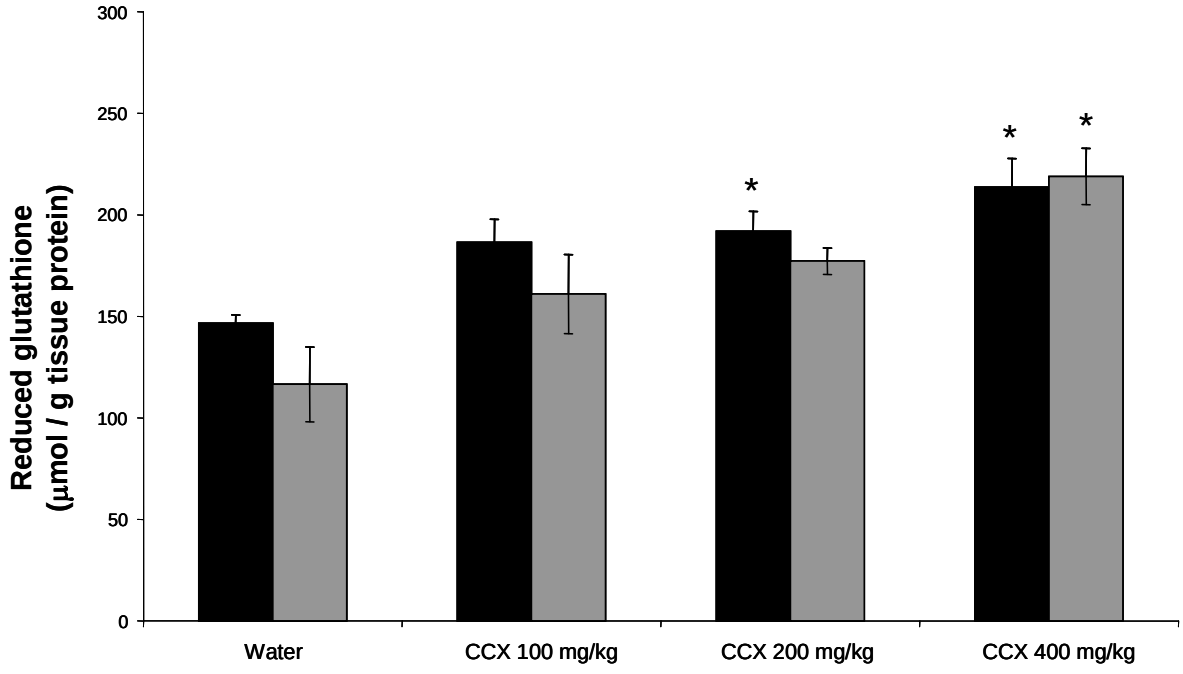

566 **Figure 3**

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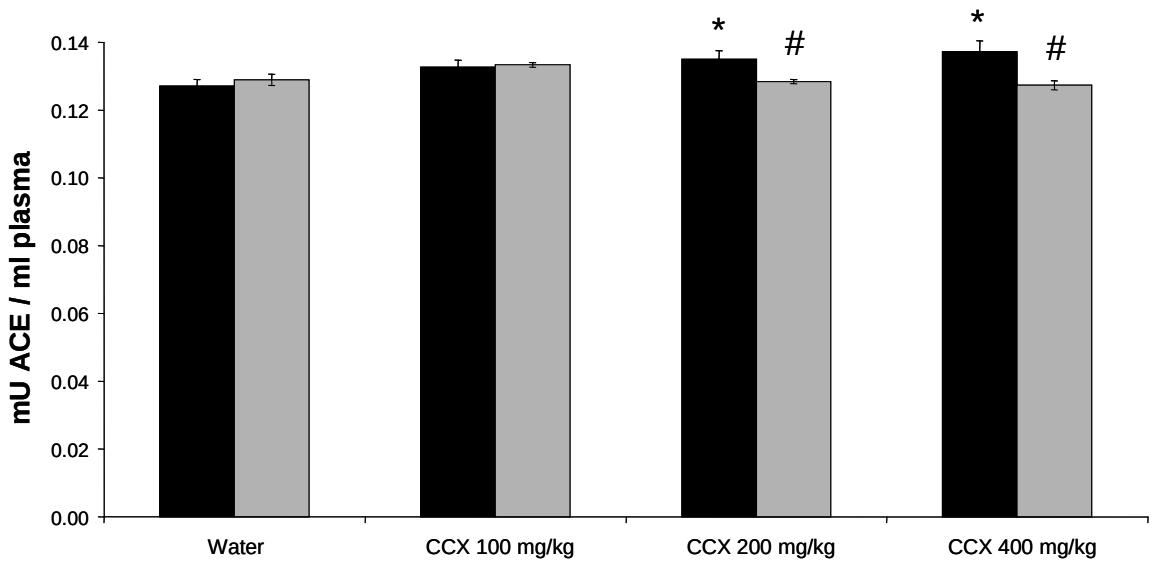

Figure 4

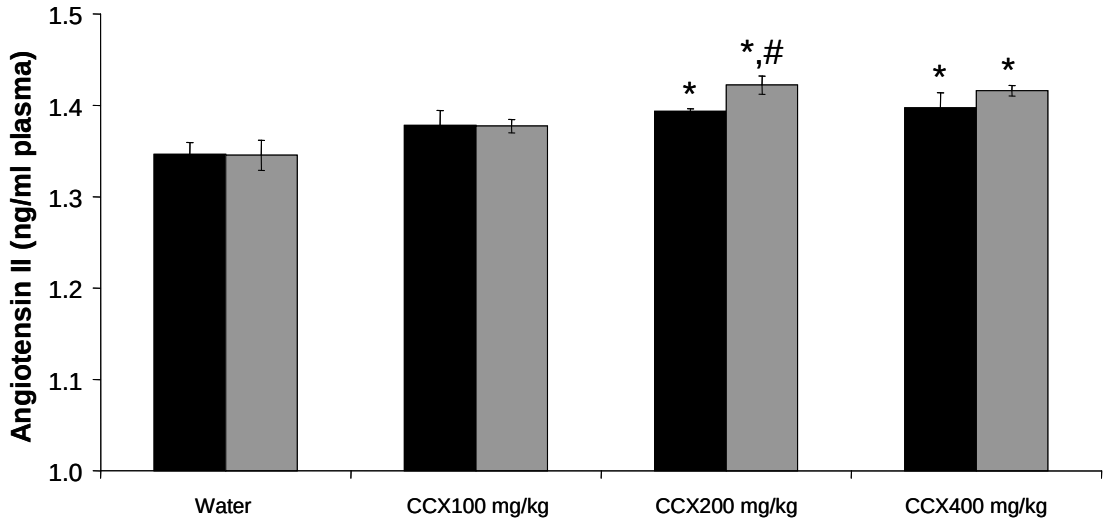
Angiotensin II (ng/ml plasma)
1.5
1.4
1.3
1.2
1.1
1.0
*
*,#
*
*
Water
CCX100 mg/kg
CCX200 mg/kg
CCX400 mg/kg

Figure 5