Evidence that nitric oxide mediates the blood pressure lowering effect of a polyphenol-rich cocoa powder in spontaneously hypertensive rats

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Running title: Nitric oxide mediates a cocoa powder effect in hypertensive rats

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Abstract

The involvement of endothelial-relaxing factors on the antihypertensive effect of a polyphenol-rich cocoa powder named CocoanOx (CCX) was studied. Thirty 17-20-week-old male spontaneously hypertensive rats (SHR), weighing 314±3 g were used. They were divided into two groups of fifteen animals, that were respectively administered by gastric intubation distilled water or 300 mg/kg CCX dissolved in distilled water, between 9 and 10 am. Two hours after the oral administration, 5 of the animals in each group were intraperitoneally administered 1 ml saline. The remaining rats in both groups were divided into another two groups of 5 animals that were intraperitoneally administered 30 mg/kg Nw-nitro-L-arginine methyl ester (L-NAME) dissolved in 1 ml of saline or 5 mg/kg indomethacin also dissolved in 1 ml of saline. Systolic blood pressure (SBP) was recorded in the rats by the tail cuff method before the initial oral administration and also 4 hours after this administration. CCX caused a significant decrease in SBP. L-NAME caused a clear increase in SBP in the rats, and the effect of CCX was not observed in the SHR that were treated with L-NAME. Nevertheless, indomethacin treatment did not modify SBP in the SHR and this compound clearly failed to modify CCX antihypertensive effect in these animals

In conclusion, the antihypertensive effect of the polyphenol-rich cocoa powder, at least in the SHR strain, may be due to changes in endothelium-derived NO bioavailability. In particular, our results suggest that CCX affects endothelial NO synthesis in these animals.

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Key words: Cocoa, Nitric oxide, Polyphenols, Spontaneously hypertensive rats

Introduction

Hypertension is a major risk factor for stroke, myocardial infarction and kidney failure. In addition, worldwide hypertension is estimated to cause 7.1 million premature deaths and 4.5 % of disease burden (WHO, 1996). Treating hypertension has been associated with about a 40% reduction in the risk of stroke and about a 15% reduction in the risk of myocardial infarction (Collins et al., 1990). In spite of this, hypertension remains inadequately managed everyone (Mancia et al., 2002). The current and common method for controlling hypertension is the use of a long term drug therapy, but It is well known that drugs have many side effects which may complicate the patient's medical condition. In this context, the new strategies for treating hypertension based in natural products could greatly benefit the hypertensive patients.

Some human studies have demonstrated the antihypertensive properties of cocoa polyphenols (Taubert et al., 2003; Taubert et al., 2007a; Taubert et al., 2007b; Grassi et al., 2005a; Grassi et al., 2005b; Buijsse et al., 2006, Shroeter et al., 2006). However, it is worth noting that the preservation of polyphenols during the cocoa manufacturing is important to exhibit the health effects associated to cocoa consumption. It has been reported that high processing temperatures and longer roasting times, as well as alkali treatments, reduce the content of polyphenols in cocoa (Gu et al., 2006).

A polyphenol-rich cocoa powder, named CocoanOX™ (CCX), was produced by an innovative industrial patented process (Cienfuegos-Jovellanos et al., 2007). The antioxidant capacity of CCX (Schinella et al., 2010), and the

antihypertensive properties after short (Cienfuegos-Jovellanos et al., 2009) and long-term treatment (Quiñones et al., 2010) have been recently published.

In general terms, the antihypertensive properties of polyphenols have been associated with some conditions such as nitric oxide mediated-vasodilation (Emura et al., 2007; Yamamoto et al., 2008; Ichimura et al., 2006; Mukai et al., 2009), angiotensin converting enzyme inhibition (Li et al., 2005; Liu et al., 2003) and a reduced oxidative status caused by the antioxidant capacity of these compounds (Duarte et al., 2001; Negishi et al., 2004; Villar et al., 2002; Peng et al., 2005). Nevertheless, the underlying mechanisms involved in the antihypertensive effect of polyphenols have not been examined in detail. A better understanding of the mechanisms and the determining factors of the antihypertensive activity of polyphenols will allow a rational development of functional foods rich in polyphenols for blood pressure control.

Very recently, we have investigated some of the mechanism involved in the antihypertensive effect of CCX (Quiñones et al., 2010a; Quiñones et al., 2010b). The results obtained in these studies demonstrated that the antihypertensive effect of CCX could be endothelium dependent and mediated, at least in part, by endothelial release of NO and by a reduction of oxidative stress. In an attempt to go deep in the antihypertensive mechanisms of CCX, in this study we proposed to evaluate the *in vivo* participation of the endothelial relaxing factors nitric oxide and prostacyclin in SHR.

Material and Methods

Experimental procedure in rats

Products

CocoanOX was supplied by Natraceutical Group (Valencia, Spain). This product is a flavanol-rich cocoa powder produced from unfermented, blanch-treated, non-roasted cocoa beans using the procedure described by Cienfuegos-Jovellanos et al. (2009). Characterization of CCX including total polyphenol content and the corresponding flavan-3-ols profile, have been previously described (Cienfuegos-Jovellanos et al., 2009). The characterization of the CCX powder used in this study is presented in Table 1. Nw-nitro-L-arginine methyl ester (L-NAME) and indomethacin were purchased from Sigma Chemical, Co. (St. Louis, MO, USA).

General protocol

Thirty 17-20-week-old male spontaneously hypertensive rats (SHR), weighing 314±3 g were used. All these animals were obtained from Charles River Laboratories Spain. The rats were maintained at a temperature of 23º C with 12 hour light/dark cycles, and consumed tap water and a standard diet (A04 Panlab, Barcelona, Spain) *ad libitum* during the experiments. They were divided into two groups of fifteen animals, that were respectively administered by gastric intubation distilled water or 300 mg/kg CCX dissolved in distilled water, between 9 and 10 am. The total volume orally administered to the rats, either of water or of the CCX water solution, was always 1 ml. Two hours after the oral administration, 5 of the animals in each group were intraperitoneally administered 1 ml saline. The remaining rats in both groups were divided into

another two groups of 5 animals that were intraperitoneally administered 30 mg/kg Nw-nitro-L-arginine methyl ester (L-NAME) dissolved in 1 ml of saline or 5 mg/kg indomethacin also dissolved in 1 ml of saline. Systolic blood pressure (SBP) was recorded in the rats by the tail cuff method (Buñag et al., 1973) before the initial oral administration and also 4 hours after this administration. Before the measurements, the rats were kept at 38ºC for 10 minutes in order to detect the pulsations of the tail artery. To guarantee the reliability of the measurements we established a training period of two weeks before the actual trial time, and during this period the rats were accustomed to the procedure. Moreover, to establish the value of SBP five measurements were taken and the average of all of them was obtained, and to minimize stress-induced variations in blood pressure all measurements were taken by the same person in the same peaceful environment. Nevertheless, the researcher advised to carry out the measurements did not know the exact treatment of each animal.

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).

Statistical analysis

The results are expressed as mean values ± standard error of the mean (SEM) for 5 rats, and were analyzed by a one-way analysis of variance (ANOVA), using the GraphPad Prism software. Differences between the groups were assessed by the Bonferroni test. Differences between the means was considered to be significant when $P< 0.05$.

Results

The initial value of the SBP in the SHR was 223.3±2.4 mm Hg. The changes in this variable after the oral and the intraperitoneal administration of the different products are shown in Figures 1 and 2.

As expected, the animals that received only water and saline did not modify their SBP. 300 mg/kg CCX caused a significant decrease in SBP that could be appreciated 4 hours post-administration in the animals that received only saline after this cocoa powder (-49,53 ± 4,98 mm Hg). On the contrary, 30 mg of L-NAME caused a clear increase in the SBP in the water-treated rats (+16,16 ± 4,28 mm Hg y $p < 0,05$). The effect of L-NAME ~~could be~~ was clearly appreciated two hours after the intraperitoneal administration of this arginine derivative. As shown in Figure 1, the antihypertensive effect of CCX was completely abolished by intraperitoneal injection of 30 mg/kg L- Nevertheless, as shown in Figure 2, the intraperitoneal injection of 5 mg/kg indomethacin did not modify the SBP in the water-treated rats, and the antihypertensive effect of CCX was not altered in the rats that were treated with indomethacin after the cocoa administration.

Discussion

This study describes for first time the in vivo participation of endothelial relaxing factor, NO, in the blood pressure lowering effect of a polyphenol-rich cocoa powder, CCXWe have also used SHR rats in this study because are a well known experimental model for essential hypertension in humans

(FitzGerald et al., 2004). According to a previous research carried out in these animals (Cienfuegos-Jovellanos et al., 2009) a clear decrease in arterial blood pressure was observed 4 hours post-administration of 300 mg/kg CCX. Therefore, the present study corroborates the short-term antihypertensive effect of CCX in SHR. In previous studies CCX has demonstrated a high antioxidant capacity (Schinella et al., 2010) and in view of the displayed reasoning this is consistent with the antihypertensive and endothelial effect of this cocoa powder.

The first endothelium-derived relaxing substance described was prostacyclin, which is produced by the action of the cyclo-oxygenase enzyme (Vane et al., 1987). Nevertheless, NO was later identified as another important endothelial relaxing factor (Moncada et al., 1987). The role of NO in arterial tone was clearly established soon later (Moncada et al., 1988) and it was accepted that the source of endothelial NO was a guanidinium nitrogen of L-arginine and that the enzyme responsible for its formation was an oxygenase called endothelial NO synthase (eNOS) (Palmer et al., 1988). In fact, except for some apparent inconsistencies, it become generally accepted that NO was the main endothelium derived relaxing factor (Furchgott et al., 1990). NO is actually an important mediator of blood pressure homeostasis and the increase in arterial tone that characterizes the hypertensive state implies frequently an excess of free radicals that destroy this mediator. Enhanced endothelial superoxide anion production has been described in hypertension and these effects are related to impairment of endothelium-dependent relaxation (Kumar and Das, 1993; Sekiguchi et al., 2004). Nevertheless, it is also true that the hypertensive process in spontaneously hypertensive rats has been associated with the release of endothelial vasoconstrictor factors (mainly cyclo-oxygenase-

dependent endoperoxides and endothelin-1) (Félétou and Vanhoutte, 2006). Alterations in the function of arterial endothelium have been demonstrated also in prehypertensive SHR (Jameson et al., 1993).

L-NAME is an *in vivo* and *in vitro* inhibitor of eNOS (Moncada et al., 1991; Rees et al., 1990) and in the present study, a clear increase in SBP was observed after L-NAME treatment to the SHR. The inhibition of basal NO synthesis by this treatment in these animals could justify these results, but what is more important in order to fulfil the aim of this study is the impairment of CCX to reach antihypertensive effect that we have observed in the SHR administered the eNOS inhibitor two hours after cocoa treatment. The study carried out recently by Quiñones et al. (2010) already suggests that CCX may cause a decrease in arterial blood pressure by facilitating endothelial relaxing factors, but the results mentioned above provide clear evidence that CCX could facilitate in vivo NO release in the SHR. Nevertheless, the endothelium secretes other vasodilator agents different from NO such as prostacyclin, and we have also evaluate the effect of CCX in SHR intraperitoneally injected indomethacin, an inhibitor of endothelial prostanoid biosynthesis. However, indomethacin treatment did not modify SBP in the SHR and this compound clearly failed to modify CCX antihypertensive effect. Therefore, we could discard endothelial prostacyclin release as a mechanism implicated in the antihypertensive effect of this cocoa powder.

In this study, we have provided novel evidence to identify the mechanisms by which a polyphenol-rich cocoa lowers blood pressure in the hypertensive state. We have shown that the blood pressure lowering effect of CCX, at least in the SHR strain, may be due to changes in endothelium-derived

NO bioavailability, and in particular our results suggest that CCX antihypertensive effect is mediated through NO pathway affecting endothelial NO synthesis in these animals. In conclusion, CCX could be used as a antihypertensive food ingredient. However, further investigation should be carried out to characterize the signalling pathways involved in these effects, and to demonstrate their antihypertensive efficiency in humans.

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374 **Figure legends**

375

376 **Figure 1**. Changes in systolic blood pressure (SBP) caused in spontaneously
377 hypertensive rats after different treatments: water + saline (▭), 300 mg/kg CCX
378 + saline (■), water + 30 mg/kg L-NAME (▣) or 300 mg/kg CCX + 30 mg/kg L-
379 NAME (□). Data are expressed as mean ± SEM. The experimental groups
380 always have 5 animals. Different letters represent statistical differences
381 ($p<0.05$). P estimated by one-way ANOVA.

382

383 **Figure 2.** Changes in systolic blood pressure (SBP) caused in spontaneously
384 hypertensive rats after different treatments: water + saline (▭), 300 mg/kg CCX
385 + saline (■), water + 5 mg/kg indomethacin (▣) or 300 mg/kg CCX + 5 mg/kg
386 indomethacin (□). Data are expressed as mean ± SEM. The experimental
387 groups always have 5 animals. Different letters represent statistical differences
388 ($p<0.05$). P estimated by one-way ANOVA.

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393 **Figure 1**

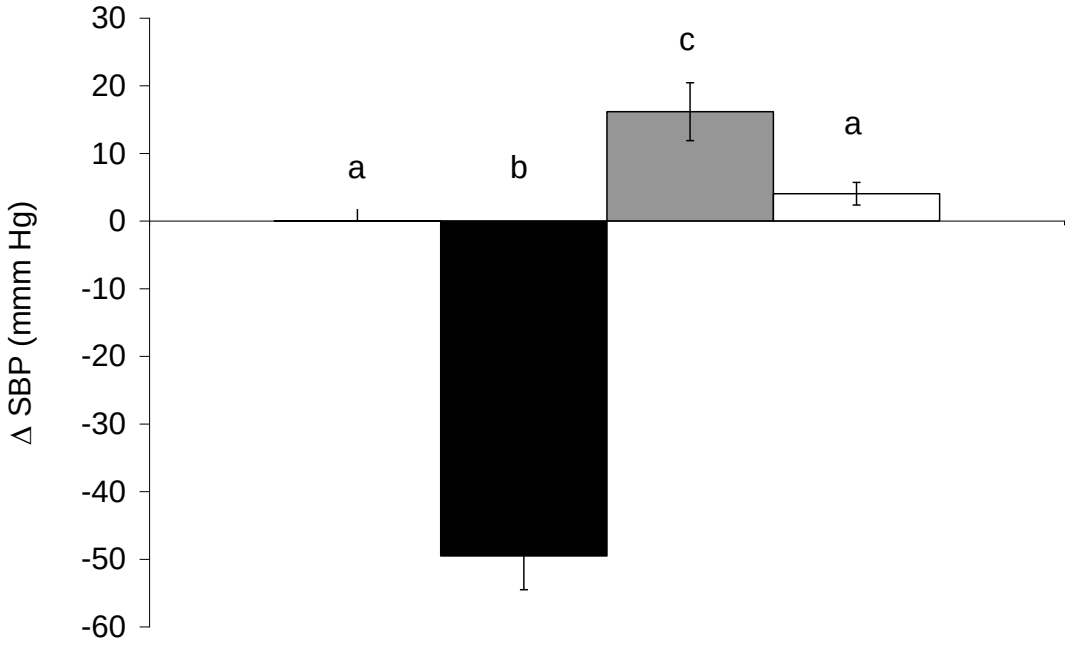

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Figure 2

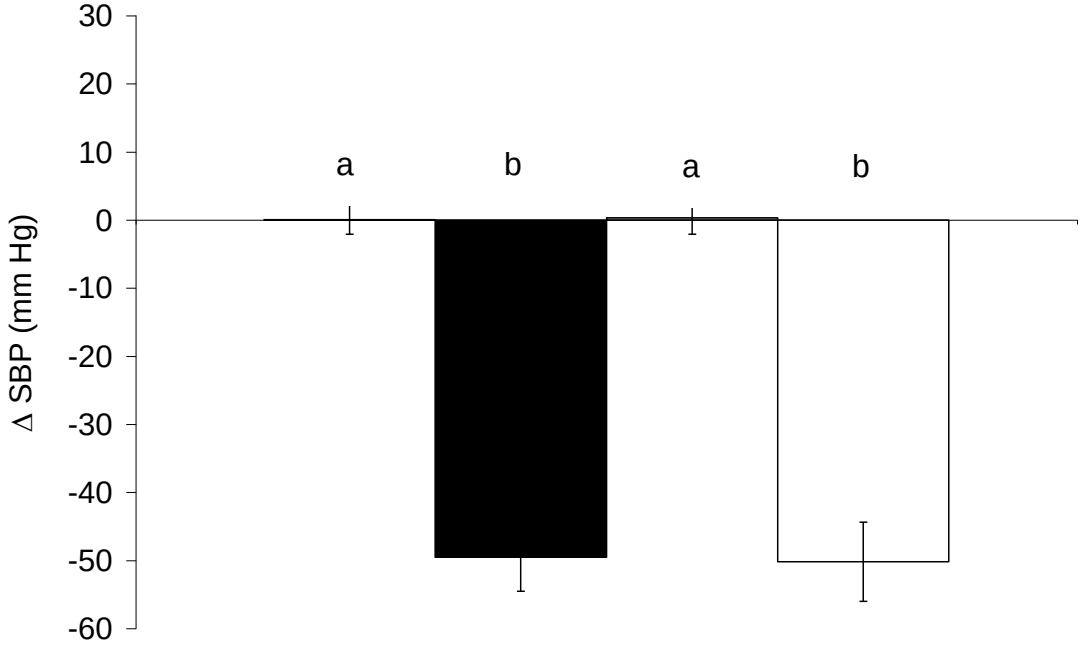

Table 1. Total polyphenols (mg/g) and flavan-3-ols (mg/g) of the CocoanOx powder used in this study.

Total Polyphenols[1]	**17.454 ± 0.0132**
Total flavan-3-ols [2]	**45.843 ± 0.270**
(+) Catechin	5.570 ± 0.096
(-) Epicatechin	20.834 ± 0.032
Procyanidin B2	18.128 ± 0.069
Procyanidin B1	1.351 ± 0.073

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

[1] Spectrophotometric method Folin-Ciocalteu. Results expressed as catechin equivalent. [2] DAD-HPLC