The prothrombotic state associated with obesity-induced hypertension is reduced by cocoa and its main flavanols

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Background. Little is known about the effects of cocoa and its main flavanols on the prothrombotic state associated with the development of hypertension in diet-induced obesity models. Purpose. To evaluate the effects of cocoa powder, cocoa extract and their main flavanols on plasma biomarkers related to impaired coagulation and fibrinolysis and its association with hypertension and obesity-related metabolic disorders in rats fed a hypercaloric diet. Methods. Male Wistar rats were randomly assigned to 7 treatment groups (n = 7): normal diet (ND); hypercaloric diet control group (HCD); HCD + cocoa powder (CO); HCD + cocoa extract (CO-EX); HCD + (−)-epicatechin (EPI); HCD + (+)-catechin (CAT); and HCD + procyanidin B2 (PB2). Blood pressure was measured using the tail-cuff method (week 7). At the end of the experimental period (week 8), rats were sacrificed and blood samples were collected immediately for coagulation and biochemical analyses. Results. Oral administration of CO, CO-EX and their main flavanols significantly decreased plasma biomarkers related to impaired coagulation and fibrinolysis (vWF, FVIII, fibrinogen and PAI-1) in rats fed a hypercaloric diet. These effects were associated with decreased systolic and diastolic blood pressure, aortic oxidative stress (MDA levels) and improvement of dyslipidemia, insulin resistance and circulating markers of inflammation (TNF-α, IL-6 and CRP) compared to the HCD group. Conclusion. Our results showed that cocoa and its main flavanols may improve endothelial dysfunction and exert their antihypertensive effects by decreasing the prothrombotic state in rats fed a hypercaloric diet. Moreover, improvement of obesity-related metabolic disorders may also contribute to their BP-lowering effect.

Introduction

Obesity is a major worldwide health problem, closely associated with the development of hypertension and cardiovascular disease (CVD). Although the underlying mechanisms have not been fully elucidated, endothelial dysfunction has been proposed as a key factor linking obesity with hypertension.1–3

Given that impaired nitric oxide (NO) bioavailability is considered as a major determinant of vascular resistance and hypertension, the vasodilator effect of endothelium-derived NO is often used to evaluate endothelial function.4 However, there has been growing interest in the measurement of several biomarkers of endothelial activation in order to have a broader understanding of the multiple features of endothelial dysfunction.5,6

Recent research has demonstrated that central obesity contributes to endothelial dysfunction not only by decreasing NO-dependent vasodilation, but also by disrupting the balance between coagulation and fibrinolytic systems, thus predisposing to a prothrombotic state.2,3,7

The impairment of hemostatic balance in obesity involves platelet hyperaggregability and hypercoagulability and is associated with increased levels of von Willebrand factor (vWF), factor VIII (FVIII) and fibrinogen. vWF is a multimeric glycoprotein, synthesized exclusively in endothelial cells and megakaryocytes and released from Weibel-Palade bodies in response to endothelial damage. It plays a vital role in mediating platelet adhesion to the subendothelium by linking to specific platelet membrane receptors (glycoprotein IIb-IX complex) and to constituents of subendothelial connective tissue. Since vWF is required for stability of FVIII, both factors circulate as a complex and coordinate the clot formation at the site of endothelial cell injury.6,8,9 Likewise, fibrinogen is an acute-phase protein, synthesized in the liver, that promotes platelet aggregation and formation of tight clots by binding to platelet glycoprotein IIb/IIIa receptors.9–11

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On the other hand, hypofibrinolysis is characterized by decreased fibrin degradation within the thrombus and removal from the circulation. Inhibition of the fibrinolytic system is mediated by α2-antiplasmin at the level of plasmin and by plasminogen activator inhibitors (PAI-1 and PAI-2) at the level of the plasminogen activators. PAI-1 has been identified as the primary inhibitor of the fibrinolytic process and thus is considered a key player in the prothrombotic state. It binds to fibrin and forms a covalent complex with tissue-type (tPA) or urokinase-type plasminogen activators (uPA), inactivating them and inhibiting plasmin generation. Although PAI-1 is synthesized by different cell types, visceral adipose tissue has been shown to be a major contributor to its elevated plasma levels observed in obesity.12

Accumulating evidence shows that prothrombotic tendency in obesity, characterized by an increase in the biomarkers of hypercoagulability and hypofibrinolysis, contributes to the development and progression of hypertension, which is considered one of the most powerful risk factors for CVD.9,13

Antihypertensive drug therapy is usually successful but may be associated with negative side effects. Hence, there has been increasing interest in studying bioactive compounds from natural sources that may show therapeutic potential against the prothrombotic state and hypertension.

Previous studies show that cocoa and cocoa extract have a potential beneficial effect on obesity and metabolic disorders, through its main flavanols such as catechin, epicatechin and procyanidin B2, which significantly decreased body weight gain and adipose tissue and lowered blood glucose, insulin, cholesterol, triacylglycerols and c-LDL. Additionally they decreased the expression of proinflammatory genes such as TNF alpha and IL-6 and increased PPARγ, CD36 and ACC in rats with obesity induced by high-calorie diet.14

Cocoa and its main flavanols have been intensively studied for their potential antihypertensive effects. Several studies have proposed that an increase in NO production by enhancing eNOS expression and/or activity is a potential mechanism by which cocoa flavanols exert their antihypertensive effects.15,16 However, the antioxidant and anti-inflammatory effects of cocoa flavanols have also been considered as potential mechanisms contributing to their blood pressure-lowering effect. In this context, the aim of this study was to evaluate the effects of cocoa powder, a flavanol-rich cocoa extract and its main flavanols (epicatechin, catechin and procyanidin B2) on plasma biomarkers related to impaired coagulation and fibrinolysis (vWF, FVIII, fibrinogen and PAI-1) and its association with hypertension and obesity-related metabolic disorders in rats fed a hypercaloric diet.

Materials and methods

Experimental study design

All procedures and care of animals were undertaken in compliance with the guiding principles of the Ethics Code for Animal Studies of the Escuela Nacional de Ciencias Biológicas (ENCB) and the Guide for the Care and Use of Laboratory Animals of the Mexican Council for Animal Care (NOM-062-ZOO-1999). Male Wistar rats (180 ± 5 g of body weight) were purchased from the Animal House of Autonomous Metropolitan University, Xochimilco Campus and were housed individually in stainless steel cages with mesh bottoms and maintained under controlled ambient conditions (temperature, 22 ± 2 °C; and relative humidity, 40–60%). The light–dark cycle was 12/12 h: lights were turned off at 20:00 h and turned on at 08:00 h.

Rats were allowed to acclimate for five days before being randomly assigned to 7 treatment groups (n = 7): normal diet control group (ND); high-fat hypercaloric diet control group (HCD); HCD + cocoa powder (CO; 1 g per kg bw); HCD + cocoa extract (CO-EX; 100 mg per kg bw); HCD + (−)-epicatechin (EPI; 10 mg per kg bw); HCD + (+)-catechin (CAT; 10 mg per kg bw); and HCD + procyanidin B2 (PB2; 10 mg per kg bw). Since the biological activity of natural bioactive compounds is highly dependent on their absorption and bioavailability after ingestion of foods rich in these compounds, pharmacokinetic studies of cocoa flavan-3-ols were considered for dose selection of cocoa powder and pure test compounds. A wide range of doses used varies from 1–325 mg per kg of body weight, as well as different routes of administration.17–23 For this study the oral dose of cocoa powder was set at 1 g per kg body weight.24 Considering that this study sought to evaluate whether beneficial effects of cocoa powder could be mediated by a particular flavanol or the synergy between them, cocoa extract and epicatechin doses (100 and 10 mg per kg body weight, respectively) were selected based on their estimated content per gram of cocoa powder.

Control groups received purified water as a vehicle. Dose selection for each compound was based on our previous studies demonstrating their positive influence on obesity-related metabolic risk factors.14

All rats had unlimited access to purified water and the experimental diets throughout the whole experimental period. After 7 weeks of treatment, blood pressure was measured in preconditioned, conscious and restrained rats, using the non-invasive tail-cuff method. At the end of the study, after an overnight fast (12 h), rats were exsanguinated from the abdominal aorta under pentobarbital sodium anesthesia (32 mg kg−1 IP). Blood samples were collected immediately in plasma and serum-separating tubes for coagulation and biochemical analyses.

Chemicals and diets

Normal diet (ND) (Rodent Diet 2018) and high-fat hypercaloric diet (HCD) (TD. 88137) were purchased from Teklad Global Harlan Laboratories, Inc. (Madison, WI). Unsweetened cocoa powder (CocoaVia®) and cocoa extract (CocoaVia®) were purchased from Mars, Inc. (Hackettstown, NJ). The nutritional composition of experimental diets and total polyphenol, flavonoids and main flavanols content of cocoa powder and extract have been reported previously.14 (−)-Epicatechin (E1753; ≥90%) and (+)-catechin hydrate (C1251; ≥98%) were purchased...
from Sigma-Aldrich Co. Ltd (Toluca, Estado de Mexico). Procyanidin B2 (51656; 95.25 purity) was purchased from Chempacific Corp. (Baltimore, Maryland).

Blood pressure measurement

Blood pressure measurements were recorded with a tail-cuff computerized blood pressure system (MRBP-R, IITC Life Science Inc., CA, USA). Three readings were taken consecutively on conscious rats and the average was then calculated and taken as a final reading for systolic and diastolic blood pressure and heart rate determinations.

Sample preparation

Blood samples were collected from the abdominal aorta into two tubes: light blue-top BD Vacutainer® tubes containing 3.2% sodium citrate (Cat. no.: 363080) to perform coagulation testing and sterile gold BD Vacutainer SST™ test tubes (Cat. no.: 367983) to determine inflammation markers serum levels.

Thoracic aorta was removed, placed in a Petri dish filled with ice-cold PBS and carefully cleaned off adherent fat and connective tissue in an ice-bath for immediate MDA measurement.

Biochemical analyses

Blood samples from Vacutainer SST™ tubes were allowed to clot for 15 minutes, and centrifuged at 1500g for 15 minutes at 4 °C. Serum samples were separated, frozen and stored at −80 °C until assays were performed.

Haemostatic parameters

Platelet-poor plasmas were obtained by centrifugation of blood samples (sodium citrate tubes) at 2000g for 10 min at 4 °C. Fibrinogen (PT-Fibrinogen HS Plus HemosILTM Werfen, Instrumentation Laboratory) and blood coagulation FVIII (Factor VIII HemosILTM Werfen, Instrumentation Laboratory) were determined using an ACL ELITE PRO instrument (Instrumentation Laboratory Company, Milano, Italy). Immunological assay of the von Willebrand factor (vWF:Ag) was performed using an immune-turbidimetric test (von Willebrand Factor Antigen HemosILTM Werfen, Instrumentation Laboratory). All results were expressed as a percentage of coagulometric activity.

Adipocytokines and inflammation markers determination

Serum levels of leptin (catalogue no. EZRL-83 K; EMD Millipore, St Charles, Missouri), adiponectin (ApN) (catalogue no. EZRADP-62 K; EMD Millipore), TNF-α (catalogue no. EZRTNFα; EMD Millipore, St Charles, Missouri), IL-6 (catalogue no. EZRIL6; EMD Millipore), C-reactive protein (catalogue no. CYT294; EMD Millipore, St Charles, Missouri) and plasminogen activator inhibitor-1 (Cat. no. CSB-E07948r, Cusabio Biotech Co. Ltd, Mexico City) were determined using commercial enzyme-linked immunosorbent assay (ELISA) kits according to the manufacturer’s instructions.

Lipid peroxidation in the aortic wall

Lipid peroxidation in the aortic wall was determined indirectly by measuring the content of malondialdehyde (MDA), one of the final products of lipid peroxidation. Aortic tissue (50 mg) was homogenized in 0.5 mL cold phosphate buffer (pH 7.0), followed by the addition of 1 mL of a reagent containing tri-chloroacetic acid (TCA), thiobarbituric acid (TBA) and HCl (15% (w/v), 0.375% (w/v), and 0.25 N, respectively). The mixture was heated at 100 °C for 30 min and then centrifuged at 1000g for 10 min to remove the flocculent precipitate. Absorbance was measured at 532 nm (Bio-Mini Shimadzu Spectrophotometer) and the MDA concentration was calculated using an extinction coefficient of 1.56 × 10⁻⁵ M⁻¹ cm⁻¹ and expressed as nmol of malondialdehyde per mg protein. Tissue protein was estimated using the Coomassie blue method, with bovine serum albumin (BSA) as the standard.

Statistical analyses

Data were tested for normality (Shapiro–Wilk test) and homogeneity (Levene tests) of variances prior to statistical analysis. Data were expressed as mean values ± SEM. One-way ANOVA was performed followed by the Holm–Sidak test, for multiple comparisons in all quantitative variables. Pearson correlation coefficients were calculated to determine the degree to which two continuous variables were related. The pooled data of all groups were used to calculate the correlation between the selected variables. A value of p < 0.05 was considered significant. The statistical analyses were performed using SigmaPlot version 12.0 from Systat Software, Inc., San Jose California, USA.

Results

Effect of cocoa powder, cocoa extract and their main flavonols on adipokines, inflammatory markers and aorta MDA levels

Serum levels of ApN were significantly decreased by 26.3% (p < 0.001) in the HCD group compared to the ND group (Fig. 1A), whereas all treatments significantly increased the ApN levels (~26%; p < 0.001) compared to the HCD group, being flavonoid extract which presents the best effect, with equal levels to the ND group, followed by catechin and the remaining groups. As shown in Fig. 1(B, C, D and E) the HCD group significantly increased serum leptin (12.1-fold; p < 0.001), IL-6 (2.4-fold; p < 0.001), TNF-α (31%; p < 0.001) and CRP levels (20%; p < 0.001) compared to the ND group. Conversely, all treated groups significantly decreased IL-6 (2.6-fold; p < 0.001), TNF-α (11.8%; p < 0.001) and CRP levels (13.6%; p < 0.001) compared to the HCD group, but still higher than the ND group. Only CO-EX, CO and EPI treatments showed a significant decrease in leptin levels (48.3, 43.5 and 17.7%, respectively; p < 0.001) compared to the HCD group, significant reduction but remaining at high levels compared to the ND group. Fig. 1F shows that lipid peroxidation in the aorta (MDA levels) was significantly higher (1.8-fold; p < 0.001) in the HCD group than in the ND group, whereas all treated groups significantly reduced arterial MDA.
levels ($p < 0.001$) compared to the HCD group, reaching normal levels (ND group). CO, EPI and CAT treatments showed the highest decrease in MDA levels ($\sim 40.3\%; p < 0.001$) compared to the HCD group, followed by CO-EX and PB2 groups ($\sim 33\%; p < 0.001$).

**Effect of cocoa powder, cocoa extract and their main flavanols on prothrombotic markers**

Fig. 2(A and B) show that the HCD group significantly increased the plasma levels of the von Willebrand factor (vWF) and factor VIII (FVIII) by 29.2 and 37.6%, respectively, compared to the ND group. In contrast, CO, CO-EX and CAT treatments were highly effective in decreasing vWF and FVIII levels ($\sim 18$ and 14%, respectively; $p < 0.001$) compared to the HCD group. In addition, the HCD group significantly increased the plasma levels of fibrinogen (9.2%; $p < 0.001$) and plasminogen activator inhibitor-1 (PAI-1) levels (4-fold; $p < 0.001$) compared to the ND group (Fig. 2C and D), showing that CO-EX, EPI and PB2 groups reached normal levels.

Interestingly, all four prothrombotic markers (vWF, FVIII, fibrinogen and PAI-1) were positively correlated with the obesity-related adiposity index, insulin resistance (HOMA-IR), aortic oxidative stress (MDA levels), inflammatory markers (TNF-α, IL-6 and CRP) and blood pressure (SBP and DBP) (Table 1).

<table>
<thead>
<tr>
<th></th>
<th>PAI-1</th>
<th>FVIII</th>
<th>vWF</th>
<th>Fibrinogen</th>
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<tr>
<td>ApN (μg mL$^{-1}$)</td>
<td>−0.82</td>
<td>&lt;0.001</td>
<td>−0.81</td>
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<tr>
<td>CRP (μg mL$^{-1}$)</td>
<td>0.90</td>
<td>&lt;0.001</td>
<td>0.79</td>
<td>&lt;0.001</td>
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<tr>
<td>TNF-α (μg mL$^{-1}$)</td>
<td>0.87</td>
<td>&lt;0.001</td>
<td>0.89</td>
<td>&lt;0.001</td>
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<tr>
<td>IL-6 (pg mL$^{-1}$)</td>
<td>0.79</td>
<td>&lt;0.001</td>
<td>0.64</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>PAI-1 (ng mL$^{-1}$)</td>
<td>−</td>
<td></td>
<td>0.91</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>FVIII (%)</td>
<td>0.91</td>
<td>&lt;0.001</td>
<td>−</td>
<td></td>
</tr>
<tr>
<td>vWF (%)</td>
<td>0.68</td>
<td>&lt;0.001</td>
<td>0.74</td>
<td>&lt;0.001</td>
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<tr>
<td>Fibrinogen (mg dL$^{-1}$)</td>
<td>0.65</td>
<td>&lt;0.001</td>
<td>0.54</td>
<td>&lt;0.001</td>
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<td>SBP (mmHg)</td>
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<td>&lt;0.001</td>
<td>0.85</td>
<td>&lt;0.001</td>
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<td>DBP (mmHg)</td>
<td>0.80</td>
<td>&lt;0.001</td>
<td>0.88</td>
<td>&lt;0.001</td>
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<tr>
<td>Aorta MDA (nM)</td>
<td>0.87</td>
<td>&lt;0.001</td>
<td>0.72</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>

Average fibrinogen levels in CO-EX, EPI, CAT and PB groups did not differ from those of the ND group. Moreover, all treated groups significantly decreased PAI-1 levels by $\sim 1.8$-fold ($p < 0.001$) compared to the HCD group, showing no difference between the treatments.
Effect of cocoa powder, cocoa extract and their three main flavanols on systolic (SBP) and diastolic blood pressure (DBP) and heart rate (HR)

Fig. 3(A and B) show that the HCD group significantly increased SBP (23.4 ± 2.3 mmHg; p < 0.001), DBP (23.9 ± 1.6 mmHg; p < 0.001) and HR (43.7 ± 2.2 bpm; p < 0.001) compared to the ND group. Conversely, all treated groups significantly decreased blood pressure compared to the HCD group; however, CO was the most effective treatment in reducing both SBP (∼18 ± 1.7 mmHg; p < 0.001) and DBP (∼17 ± 1.8 mmHg; p < 0.001). CO-EX, CAT and PB2 groups significantly reduced SBP by ∼15.2 ± 1.7 mmHg and DBP by ∼14 ± 1.5 mmHg, followed by the EPI group (SBP: −6 ± 1.5 and DBP: −8 ± 1 mmHg). Heart rate levels of all treated groups were similar to those of the ND group. Comparing the effect of the CO-EX with the different pure flavanols assayed on blood pressure, no additive effects or synergies were observed.

Discussion

This study shows that oral administration of cocoa powder, a flavanol-rich cocoa extract and its main flavanols (epicatechin, catechin and procyanidin B2) decreases plasma biomarkers related to impaired coagulation and fibrinolysis (vWF, FVIII, fibrinogen and PAI-1) in rats fed a hypercaloric diet.

These effects showed to be associated with an attenuation of metabolic disorders, circulating markers of inflammation (CRP, TNF-α and IL-6), aortic lipoperoxidation and hypertension.

In the current study, consumption of a hypercaloric diet led to significantly higher serum leptin levels than the ND group and, in accordance with other studies, were strongly correlated with adiposity. CO and CO-EX were the most effective treatments in reducing hyperleptinemia, body weight gain and adiposity index compared to the HCD group, suggesting that these treatments can ameliorate leptin resistance.

We previously reported that all treatments exerted favorable effects on metabolic risk factors for the development of cardiovascular diseases (CVD), such as obesity, dyslipidemia, hyperglycemia, hyperinsulinemia and insulin resistance (IR) induced by hypercaloric diet intake.

Obesity-associated adipocyte hypertrophy leads to increased expression of chemokines, such as monocyte chemotactic protein-1 (MCP-1), that enhances the recruitment and infiltration of macrophages into white adipose tissue (WAT), thus increasing the production and secretion of proinflammatory adipocytokines that have been suggested to be a causative link between obesity-related adipose tissue dysfunction and metabolic abnormalities, such as insulin resistance and dyslipidemia.

TNF-α and IL-6-induced impairment of insulin signaling in WAT has been shown to reduce its lipid-buffering capacity, leading to ectopic lipid accumulation and systemic IR. Conversely, ApN is an anti-inflammatory and insulin-sensitizing adipocytokine that has been shown to be negatively correlated with obesity-related metabolic disorders.

Since adipose tissue inflammation is causally linked to the pathogenesis of obesity-associated metabolic disorders, previous in vitro and in vivo studies have shown that cocoa flavanols decrease WAT inflammation and improve IR, through decreased proinflammatory cytokines production and modulation of TNF-α-induced activation of key inflammatory signaling cascades (nuclear factor kappa B (NF-kB), mitogen-activated kinases (MAPKs), extracellular-signal-regulated kinases (ERK1/2) and c-jun N-terminal kinases (JNK)).

In addition, we have previously shown that upregulation of PPARγ gene expression and attenuated inflammation in WAT by cocoa and its main flavanols may improve systemic IR. In line with these findings, our results demonstrated that CO, CO-EX and their main flavanols significantly attenuated low-grade inflammation by increasing ApN and decreasing TNF-α and IL-6 serum levels.

The elevation of circulating proinflammatory cytokines in obesity, particularly IL-6, has been associated with an increased hepatic production of the C-reactive protein (CRP). This acute-phase protein has also been used as a marker of systemic inflammation and has been implicated in the development of obesity-related metabolic disorders, such as IR and oxidative stress.

The results of our study showed that all treated groups significantly decreased serum CRP levels compared to the HCD group, and that this effect was associated with decreased levels of proinflammatory cytokines and IR. Moreover, it has been reported that reduction of CRP is associated with a lower risk of cardiovascular events, such as hypertension.

Even though the precise mechanisms linking obesity to hypertension are not fully explored, a large part of this association has been attributed to endothelial dysfunction.

Many mechanisms are involved in these effects, such as the anti-platelet effect due partly to the inhibition of thromboxane...
A₂ (TXA₂) formation through the suppression of arachidonic acid liberation and TXA₂ synthase activity,⁴⁴ angiotensin converting enzyme (ACE) inhibition activity, and inhibition of angiotensin II-induced vascular smooth muscle cell proliferation via the mitogen-activated protein kinase pathway.⁴⁵ Another mechanism is the suppressing Ang II-induced cell growth and division into VSMC, mediated by the AT1 receptor. Reducing blood pressure by cocoa flavonoids seems to be due among others to interfere with the metabolism of NO at different levels.⁴⁶

Different flavonoids including catechin and epicatechin have been shown to inhibit from 17–95% of ACE activity, and the ability of this is related to the main structural features and included the catechol group in the B-ring and the ketone group at the C₄ carbon on the C-ring as well as concentration.⁴⁷

There is evidence supporting that obesity-related inflammation, IR and oxidative stress play a major role in the pathogenesis of endothelial dysfunction, as all of them reduce endothelium-derived NO production and progressively deteriorate its function by decreasing the expression and/or activity of endothelial nitric oxide synthase (eNOS), disrupting the balance between NO-dependent vasodilator actions and endothelin-1 (ET-1)-dependent vasoconstrictor actions and increasing the expression of MCP-1 and adhesion proteins (VCAM-1, ICAM-1, P-selectin and E-selectin) on endothelial cells.⁴⁸–⁵³

Endothelial dysfunction is commonly demonstrated by an impaired vasodilatation in response to physiological and pharmacological stimuli of endothelial NO production. However, measurement of specific biomarkers that increase during endothelial activation can provide insight into the proinflammatory and prothrombotic processes that occur during endothelial damage.⁵⁴

von Willebrand factor (vWF), factor VIII (FVIII), fibrinogen and plasminogen activator inhibitor-1 (PAI-1) are among the most common hemostatic factors associated with endothelial dysfunction and cardiovascular risk. Hence, in the present study, elevated levels of these 4 factors in rats fed the hypercaloric diet may indicate an obesity-related prothrombotic state, characterized by platelet hyperactivity, hypercoagulability and hypofibrinolysis.⁵⁵,⁵⁶

vWF and FVIII are two closely related coagulation proteins involved in primary hemostasis that promote adhesion and aggregation of platelets and subsequent clot formation. Since vWF is synthesized exclusively in endothelial cells and rapidly released from endothelial Weibel–Palade bodies in response to endothelial damage, it has been considered as a useful marker of endothelium dysfunction.⁶,⁵⁶,⁵⁷

In addition, fibrinogen has shown to play a pivotal role not only in the coagulation cascade but also in the inflammatory response on the vascular endothelium. High plasma fibrinogen levels have been associated with increased blood viscosity and platelet aggregation that lead to endothelial and immune cells activation and upregulation of adhesion molecules.¹⁰,⁵⁸

In agreement with these findings, our results suggest that the reduced plasma levels of vWF, FVIII and fibrinogen in all treated groups may reduce the risk of thrombus formation in rats fed the hypercaloric diet. Moreover, since most coagulation factors are synthesized in the liver, epidemiological and experimental evidence has demonstrated an association between obesity-related hepatic steatosis and a pro-coagulant phenotype.⁵⁹–⁶¹

So far, little evidence exists regarding the effect of cocoa polyphenols on markers such as vWF, FVIII, fibrinogen and PAI-1, and their relationship with hypertension and inflammation markers. This study explains in part, how cocoa flavonols reduce the prothrombotic state. Therefore, based on our previous research demonstrating that cocoa and its main flavonols are effective treatments in decreasing hepatic steatosis and inflammation in rats fed a hypercaloric diet,⁶² the results of the current study suggest that hepatoprotective effects of cocoa flavonoids may contribute to reduced plasma fibrinogen, FVIII and CRP levels.

Impaired fibrinolysis also contributes to endothelial dysfunction by disrupting the balance between the tissue plasminogen activator (tPA) and PAI-1 levels. Therefore, elevated levels of PAI-1 result in deficient plasminogen activation, decreased removal of fibrin clots, and thus increased predisposition to thrombosis.¹⁹,⁶³ Endothelial and vascular smooth muscle cells are the main sources of PAI-1; however, it has been shown that WAT is an important contributor to the elevated plasma PAI-1 levels observed in diet-induced obesity.⁶⁴,⁶⁵

In the current study, all treated groups significantly decreased PAI-1 levels and this effect showed to be strongly related to the decreased levels of inflammatory markers (CRP, TNF-α and IL-6) rather than the level of adiposity. Moreover, given the association between obesity-related non-alcoholic fatty liver disease (NAFLD) and the impaired fibrinolytic response,⁵⁹,⁶⁶ we suggest that modulation of hepatic steatosis and inflammation by cocoa flavonoids may also contribute to decreased PAI-1 levels in rats fed the hypercaloric diet.⁶²

In agreement with previous studies, our results indicate that hypercaloric diet promotes a hypercoagulable state that may contribute to the development of hypertension, since vWF, FVIII, fibrinogen and PAI-1 showed a strong association with systolic (SBP) and diastolic (DBP) blood pressure. Conversely, oral administration of CO, CO-EX and their three main flavonols reduced SBP and DBP in rats fed the hypercaloric diet, and these results were associated with decreased levels of vWF, FVIII, fibrinogen and PAI-1.

Hypertension is a complex multifactorial disease; thus several experimental animal models have been developed to mimic the etiological factors that contribute to human hypertension. Spontaneously hypertensive rats, DOCA-salt and NO-deficient (using L-NAME) hypertensive rats are the most commonly used experimental models for studying the antihypertensive effects of several flavonoids, including cocoa flavanols.⁶⁷–⁶⁹

Previous evidence has demonstrated that cocoa and cocoa-derived products decrease BP through several mechanisms,
including (i) induction of endothelial nitric oxide synthase (eNOS) expression and activity, (ii) decrease in serum inflammatory markers, (iii) interference with the NF-kB signaling pathway, (iv) downregulation of cell adhesion molecules expression and (v) inhibition of NADPH oxidase-dependent superoxide production.\(^{70,71}\)

Whilst obesity is a well-established risk factor that contributes to the development of hypertension, there is little information on the mechanisms that may be mediating the antihypertensive effects of cocoa flavanols in animal models of obesity. Hence, in the present study, we showed that cocoa and its main flavanols reduce obesity-related hypertension probably by decreasing the prothrombotic state in rats fed a hypercaloric diet.

Epicatechin has been shown to be the major flavanol in human and animal plasma after cocoa consumption, hence several studies have focused their attention in demonstrating its BP-lowering effect on different rat models of hypertension.\(^{72–74}\) However, given the heterogeneity among studies (regarding dose administration and experimental animal models), the results cannot yet be considered conclusive.

The evidence of the absorption and bioavailability of cocoa flavanols is continuously growing, showing that EPI is better absorbed than CAT, and the last one more than PB2. In combination, (+)-CAT and (−)-EPI might be absorbed in the gastrointestinal tract competitively of rats, however, this is modified by the type of food matrix, concentration, administered dose, and the presence of other components like fat, that can improve it. Moreover, the association with the observed effects may be due to compounds or their metabolites. CAT is found exclusively in the plasma as methyl, sulfate and glucuronic acid conjugates and generally has a shorter half-life. EPI was metabolized mainly to sulfate conjugates and not glucuronidated by the liver, small intestine or large intestine.\(^{75}\)

Whereas the concentration of CAT is smaller than the EPI in plasma, pharmacokinetic studies are required at different doses, target organs, compounds alone and in combination, which may explain on what signaling cascades are acting as well as metabolic pathways affecting on, for a better understanding of the effects found.

Although several aspects of \textit{in vivo} intestinal absorption, metabolism and bioavailability of procyanidin oligomers (particularly procyanidin B2) still remain to be clarified, to date, evidence from laboratory animal and human intervention studies suggests that procyanidin B2 biological properties (e.g. antioxidant, antiplatelet aggregation) may be mainly mediated by low-molecular-weight phenolic acids resulting from colonic catabolism.\(^{76–79}\)

Despite being less efficiently absorbed than epicatechin, it is also possible that plasma procyanidin B2 concentration (directly absorbed by passive paracellular transport in the small intestine) may work in an additive manner with microbial metabolites to support its beneficial effects.\(^{76–79}\) Moreover, \textit{in vitro} and \textit{ex vivo} research has shown that procyanidin B2 may be degraded into epicatechin after cleavage of the interflavan bond in acidic gastric juice and in the colon. Thus, we may hypothesize that epicatechin (unconjugated or subsequently metabolized into conjugated methyl, sulfated and glucuronidated epicatechin) may also exert synergistic effects with procyanidin B2 or its colonic phenolic acids.\(^{76–80}\)

Furthermore, since investigations using specific and chemically pure naturally occurring cocoa flavanols are rare, their beneficial effects on endothelial function and CVD cannot be excluded. In the present study, we showed that, besides EPI treatment, chronic administration of CAT and PB2 reduces hypertension in rats fed a hypercaloric diet. Therefore, these flavanols could also be considered as potential agents to treat hypertension. Moreover, we suggest that, together with EPI, CAT and PB2 might synergistically contribute to the antihypertensive effect of the CO group. More studies are needed to confirm their vascular protective effects and the mechanisms underlying their BP-lowering effect.

**Conclusions**

Our results show that cocoa and its main flavanols may improve endothelial dysfunction and exert their antihypertensive effects by decreasing the prothrombotic state in rats fed a hypercaloric diet. Moreover, given the association between prothrombotic factors (vWF, FVIII, fibrinogen and PAI-1) and markers of inflammation (TNF-IL-6 and CRP), lipid peroxidation (aortic MDA) and IR (HOMA-IR), our results suggest that improvement of obesity-related metabolic disorders may also contribute to the antihypertensive effect of cocoa and its main flavanols (Fig. 4).

Accumulating evidence shows multiple potential mechanisms underlying the antihypertensive effect of cocoa flavano-
nols. However, since the prothrombotic tendency in obesity is a major risk factor for the development of hypertension, further research is required to elucidate the mechanisms by which cocoa flavanols improve alterations of the hemostatic balance in animal models of obesity-related hypertension.

The Bioethics Committee of the National School of Biological Sciences of the National Polytechnic Institute approved the experiments described here.

Conflict of interest

The authors of this manuscript have no conflicts of interest to declare.

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