Cocoa consumption reduces NF-κB activation in peripheral blood mononuclear cells in humans

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Inflammation;
NF-κB;
Polyphenols;
Matrix effect

Abstract Background and aims: Epidemiological studies have demonstrated an association between high-polyphenol intake and reduced incidence of atherosclerosis. The healthy effects of cocoa-polyphenols may be due to their antioxidant and anti-inflammatory actions, although the exact mechanisms are unknown and depend on the matrix in which cocoa-polyphenols are delivered. Nuclear factor κB (NF-κB) is a key molecule in the pathophysiology of atherosclerosis involved in the regulation of adhesion molecules (AM) and cytokine expression and its activation is the first step in triggering the inflammatory process. The aim of this study was to evaluate the effect of acute cocoa consumption in different matrices related to the bioavailability of cocoa-polyphenols in NF-κB activation and the expression of AM.

Methods and results: Eighteen healthy volunteers randomly received 3 interventions: 40g of cocoa powder with milk (CM), with water (CW), and only milk (M). NF-κB activation in leukocytes and AM (sICAM, sVCAM, E-selectin) were measured before and 6h after each intervention. Consumption of CW significantly decreased NF-κB activation compared to baseline and to CM (P < 0.05, both), did not change after CM intervention, and significantly increased after M intervention (P = 0.014). sICAM-1 concentrations significantly decreased after 6h of CW and CM interventions (P ≤ 0.026; both) and E-selectin only decreased after CW intervention (P = 0.028). No significant changes were observed in sVCAM-1 concentrations.
Introduction

Several studies have shown that consumption of cocoa, a polyphenol-rich food [1], is related to a better health status because of its cardio protective, anticarcinogenic and neuro-preventive effects [2,3]. However, the exact mechanism by which cocoa intake produces these effects on health is not fully understood. Cardiovascular protection by flavonoid-rich diets has mainly been attributed to non-inflammatory mechanisms [4], such as vasodilatation [5], reduction in plasma cholesterol concentrations [6], insulin resistance [7] and blood pressure [8], as well as modulation of platelet function [9]. Some studies have also suggested anti-inflammatory mechanisms of polyphenol-rich diets through inhibition of proinflammatory molecules such as CRP, IL-6 and TNF-α [10]. A human clinical trial recently showed that regular cocoa consumption significantly decreased the expression of VLA-4 (very late antigen-4), CD40 and CD36 on monocyte surface and also decreased serum concentrations of endothelium-derived adhesion molecules such as P-selectin and ICAM-1 (intercellular adhesion molecule-1) [11]. In addition, in vitro studies have suggested that cocoa procyanidins and phenolic metabolites can also modify intracellular transduction pathways and thereby modulate the synthesis of inflammatory cytokines such as IL-1β and IL-2 [12] or even IL-6 [13].

The nuclear factor κB (NF-κB) has a central role in the development of inflammatory response because it is a crucial and regulating factor for adhesion molecules and cytokine expression [14]. In the last years NF-κB has been implicated in the pathophysiology of disorders such as atherosclerosis and cancer which have a significant impact on human health. This factor exists as an inactive form in the nucleus where it modulates the transcription of the inflammatory cytokines and adhesion molecules [15].

Thus, hypothetically, modulation of NF-κB activation could be a target to reduce inflammatory response and in this way decrease the injury to the cells which may be one of the first events in the development of certain disorders such as atherosclerosis, cancer or degenerative diseases [14]. In fact, some studies have demonstrated that acute intake of polyphenol-rich foods such as olive oil [16] and red wine [17] induce a reduction in NF-κB activation. However, no studies have evaluated the acute effect of cocoa on this pathway.

To date, several studies have evaluated the effect of cocoa intake on inflammatory markers (VCAM, ICAM) albeit with conflicting results [18–20]. This may be due to the different amount of polyphenols in cocoa products and to the effect of the different matrices in which the polyphenols are delivered [21]. In this sense, it is known that milk may reduce the bioavailability of cocoa-polyphenols [22,23] but the extent of the clinical effects is unknown.

Spain is the largest consumer of cocoa powder [reports by ACNielsen, Euromonitor International, and Caobisco Association of the Chocolate Biscuit and Confectionery Industries of the European Union (EU)] representing ~28% of the total cocoa consumption in this country [24], and cocoa powder is the main source of flavonoids in the young population, being mainly consumed with milk (Family-Food-Panel, Spain 2005–2006, Taylor Nelson Sofres).

Therefore, the aim of this study was to evaluate the effects of a single dose of cocoa consumed with water or milk on NF-κB activation in peripheral blood mononuclear cells (PBMC) and on the expression of downstream inflammatory molecules such as intercellular adhesion molecule-1 (ICAM-1), E-selectin and vascular cell adhesion molecule-1 (VCAM-1) in healthy subjects.

Methods

Subjects and study design

Eighteen healthy volunteers (9 men and 9 women, aged 19–49 years) were included in this clinical trial. None reported any of the following exclusion criteria: diabetes mellitus, tobacco smoking, hypertension, LDL-c levels > 160 mg/dL, HDL-c levels < 35 mg/dL, coronary heart disease (CHD), family history of premature CHD, cerebrovascular diseases, peripheral vascular diseases, human immunodeficiency virus infection, alcoholic liver disease, malnutrition, neoplastic or acute infection diseases. In addition, none were receiving any medication or vitamin supplements. Before inclusion in the trial, all the participants provided informed consent for the different procedures and none received any economic compensation. The Institutional Review Board of the Hospital Clinic approved the study protocol. This trial was registered in the Current Controlled Trials at London, International Standard Randomized Controlled Trial Number, at controlled-trials.com as ISRCTN75176807.

This study was an open, prospective, randomized, crossover, clinical trial. The participants followed a cocoa washout period of 7 days before each intervention and were instructed to abstain from alcoholic beverages and any polyphenol-rich foods for 48 h before and during the intervention days. Prior to the study a list of the allowed and forbidden foods was given to all the participants to ensure that the polyphenol free diet was strictly followed. Volunteers fasted overnight for at least 12 h before beginning the assessment period. All subjects performed the three interventions in a random order: i) 40g of cocoa powder with 250 mL of whole milk (CM), ii) 40 g of cocoa powder with 250 mL of water (CW), and iii) 250 mL of whole milk (M) as a control. The three interventions were performed during 3 consecutive weeks at 8:00 a.m. During the

Conclusions: The anti-inflammatory effect of cocoa intake may depend on the bioavailability of bioactive compounds and may be mediated at least in part by the modulation of NF-κB activation and downstream molecules reinforcing the link between cocoa intake and health.
days of the study the volunteers remained in the experimental clinical ward to ensure that they only consumed the diet prescribed in this study.

Cocoa powder composition

Table 1 details the cocoa powder phenolic content used in this trial [23]. The intervention beverages were always prepared following a standardized protocol to assess that caloric intake and macronutrient composition were the same. Sugar was added to M and CW preparations in ensure that the caloric content was the same as that of CM. The CM and CW macronutrient composition (in 250 mL) were 30.75 and 58.40 g for carbohydrates, 10.91 and 2.16 g for fat, 13.54 and 5.64 g for proteins and for energy 275.35 and 276.60 kcal, respectively, as described previously[23,25]. The M macronutrient composition (in 250 mL) was 42.75 g for carbohydrates, 7.75 g for fat, 18.11 g for proteins and 271.37 Kcal.

Clinical and laboratory measurements

Blood samples were obtained from all the volunteers before cocoa consumption (0 h or baseline) and 6 h after each intervention. The samples were coded with random numbers and processed immediately to perform lipid profile and immunological assays of PBMC. Blood lipid analysis, cholesterol and triglycerides were measured using enzymatic procedures and HDL cholesterol was quantified after precipitation with phosphotungstic acid and magnesium chloride. Samples were analyzed in duplicate. Serum was obtained after blood centrifugation and was immediately frozen at −80 °C until analysis.

In addition, phase II metabolites of epicatechin (ie, epicatechin–glucuronide and three epicatechin sulfates) [25] were measured in 2 h plasma and 0–6 h urine fraction samples and microbial metabolites of epicatechin (i.e., phenolic acids) [23] were measured in 0–6 h urine fraction samples by liquid chromatography tandem mass spectrometry evaluating the bioavailability of cocoa-polyphenols and as biochemical markers of compliance [23,25].

Table 1 Composition of the soluble cocoa powder (per 40 g) used in the study.

<table>
<thead>
<tr>
<th>Nutrient</th>
<th>Mean value (40 g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Carbohydrates</td>
<td>23 g</td>
</tr>
<tr>
<td>Fiber</td>
<td>6.4 g</td>
</tr>
<tr>
<td>Protein</td>
<td>5.6 g</td>
</tr>
<tr>
<td>Fat</td>
<td>2.2 g</td>
</tr>
<tr>
<td>Flavanols</td>
<td></td>
</tr>
<tr>
<td>(−)–Epicatechin</td>
<td>28.2 mg</td>
</tr>
<tr>
<td>(--)–Catechin</td>
<td>8.4 mg</td>
</tr>
<tr>
<td>Procyanidin B₂</td>
<td>25.5 mg</td>
</tr>
<tr>
<td>Flavonols</td>
<td></td>
</tr>
<tr>
<td>isoquercitrin</td>
<td>1.35 mg</td>
</tr>
<tr>
<td>quercetin</td>
<td>0.23 mg</td>
</tr>
<tr>
<td>quercetin-3-glucuronide</td>
<td>0.17 mg</td>
</tr>
<tr>
<td>quercetin-3-arabinoside</td>
<td>1.45 mg</td>
</tr>
</tbody>
</table>

* Measured by HPLC following the methodology of Andres-Lacueva et al., 2008 [24].

Total protein isolation of PBMC

PBMC were isolated from fresh blood samples by Ficoll-Hypaque (Pharmacia, Uppsala, Sw) density gradient [26]. Total protein from PBMC was isolated using the Tripure isolation reagent (Roche Molecular Biochemicals) following the manufacturer’s instructions. The quantification of total protein concentration from PBMC samples was carried out using the bicinchoninic acid protein assay (Pierce, Rockland, IL).

Determination of NF-κB activation by Western blot

An equal amount of proteins (20 µg) was loaded and separated into 10% SDS-PAGE gels and electro-transferred onto nitrocellulose membranes (Invitrogen, CA, USA). The blots were blocked with 5% non-fat dry milk 1 h and incubated overnight at 4 °C with a monoclonal antibody against p65 phosphorylated (P-p65) on serine 536 (Cell Signaling Technology Inc, Beverly, MA). To verify equal protein loading, the blots were reincubated with a monoclonal antibody against β-actin (Santa Cruz Biotechnology, Santa Cruz, CA). The antibody concentrations were used as indicated by the manufacturer’s instructions. Levels of P-p65 and β-actin expression were visualized by treating the blots with a quimioluminescent detection kit (Pierce, Rockland, IL) that enhanced the signal. The intensity of the signal was quantified with the Image-Gauge Software. Protein expression of NF-κB was assessed with the P-p65/β-actin ratio (P-p65 amount was normalized by actin content) in arbitrary units.

Determination of adhesion molecules by ELISA

Serum concentrations of soluble endothelial adhesion molecules sCAM-1, sVCAM-1 and sE-selectin were measured in duplicate by ELISA using commercial immunoassays for the quantitative detection of soluble human molecules (Bender MedSystems, Vienna, Au). Intra- and inter-assay variation coefficients of the methodology ranged from 3.1% to 5.4% for sCAM-1 and sVCAM-1 and from 5.2% to 7.6% for sE-selectin.

Statistical analysis

Statistical analyses were performed using the SPSS Statistical Analysis System (version 15.0; SPSS Inc, Chicago, IL). Continuous variables were expressed as mean ± SEM. One-factor analysis of variance for repeated measures with the LSD post-hoc test was used to compare changes in outcome variables in response to the intervention treatments. The level of significance was set at P < 0.05. To exclude the presence of a carryover effect for the three interventions, comparison of the outcome variables observed before the 3 intervention periods was performed. Within- and between group differences are expressed as means and 95% confidence intervals (CI).

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Results

Participant characteristics and bioavailability of cocoa-polyphenols

All the 18 participants (mean age 26 ± 7 years) completed the three interventions and none reported any adverse effect. The baseline lipid characteristics of the participants are detailed in Table 2 and no changes were observed between the three baseline periods. Nutrient intake was similar in all the study phases. There were no changes in body weight or lipid profile (data not shown).

Consumption of CW and CM resulted in urinary excretions of 0.64 and 0.55 nmol/mg creatinine, respectively, of cocoa phase II metabolites (Σ epicatechin–glucuronide and epicatechin sulfates) [25] representing an increased trend of 17% of excretion when cocoa was taken with water. The mean plasma levels of epicatechin–glucuronide 2 h after CW were higher, albeit not significantly, than those observed after CM (330 ± 156 nmol/L and 274 ± 138 nmol/L, respectively; P = 0.07). However, on separating the subjects by gender, the differences between the mean plasma levels of epicatechin–glucuronide 2 h after CW and CM achieved statistical significance in men (P = 0.04) [27]. Moreover, consumption of CW and CM resulted in the urinary excretion of 220.32 and 111.58 nmol/mg creatinine, respectively, of microbial metabolites (Σ 3,4-dihydroxyphenylacetic, protocatechuic, 4-hydroxybenzoic, 4-hydroxyhippuric, hippuric, caffeic and ferulic acids) derived from cocoa-polyphenols representing a significant increment of 97.5% (P < 0.001) after CW when compared with CM [23]. On analyzing phase II and microbial metabolites together, consumption of CW and CM resulted in urinary excretions of 220.96 and 112.13 nmol/mg creatinine, respectively, with a significant increment of 97.1% after CW intake (P < 0.001). Before the consumption of the test meals and after the washout periods, concentrations of phase II epicatechin metabolites in plasma and urine were under the detection limits of the technique [25,27]. Moreover, no significant differences were observed in urinary excretion of microbial metabolites before the interventions and after the washout periods [23].

NF-κB activation

Data on NF-κB activation before and after the three interventions are shown in Fig. 1. Changes in NF-κB activation measurements differed between the three interventions when analyzed by one-factor analysis of variance for repeated measures (P = 0.039). The effects of the interventions on NF-κB activation after 6 h were significantly greater in favor of cocoa (CM and CW) compared to the M intervention (P = 0.028 and P = 0.002, respectively) and were, moreover, also significantly greater in the CW compared to the CM intervention (P = 0.033). Compared to baseline levels, NF-κB activation was significantly decreased after the CW intervention (−28.8% [95%CI: −157.9% to −25.9%; P = 0.042]), being significantly increased after the M intervention (24.7% [95%CI: 11.4%−37.9%; P = 0.014]). No differences were observed after the CM intervention [2.6%, 95%CI: −29.4%−34.7%, P = 0.759].

Concentration of soluble adhesion molecules

Significant differences in ICAM-1 (P = 0.006) and E-selectin (P = 0.048) levels were also observed after analysis. The sICAM-1 concentrations significantly decreased 6 h after the CW and CM interventions when compared to baseline values [−12.18% (95% CI, −2.14% to −22.21%) and −8.19% (95% CI, −1.08% to −15.31%)] respectively; P < 0.026 both] (Fig. 2). No significant differences were observed after the M intervention, although it did show a trend to increase. On comparison between treatments no differences were observed between the baseline periods, despite significant differences after 6 h of treatment. Specifically, sICAM-1 concentrations were significantly lower after the CW compared to the CM intervention (P = 0.048). In the case of sE-selectin concentrations, a significant diminution was only observed 6 h after the CW intervention when

Table 2 Baseline lipid characteristics of the 18 participants in the study.

<table>
<thead>
<tr>
<th>Lipid baseline characteristics</th>
<th>Mean ± SEM</th>
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<tbody>
<tr>
<td>Cholesterol (mg/dL)</td>
<td>187.37 ± 24.53</td>
</tr>
<tr>
<td>Triglycerides (mg/dL)</td>
<td>60.44 ± 14.80</td>
</tr>
<tr>
<td>chDL (mg/dL)</td>
<td>52.41 ± 9.63</td>
</tr>
<tr>
<td>cLDL (mg/dL)</td>
<td>121.85 ± 24.05</td>
</tr>
<tr>
<td>LDL/HDL ratio</td>
<td>2.43 ± 0.66</td>
</tr>
<tr>
<td>Apo A - HDL (mg/dL)</td>
<td>128.81 ± 15.33</td>
</tr>
<tr>
<td>Apo B - LDL (mg/dL)</td>
<td>82.00 ± 12.50</td>
</tr>
</tbody>
</table>

Figure 1 NF-κB activation in the 18 volunteers at 0 h and 6 h after milk (M), cocoa with milk (CM) or cocoa with water (CW) intake. A) Representative example of Western blot of P-p65 protein expression (β-actin was used to verify equal protein loading) B) Densitometric quantification of P-p65 levels. Values are mean ± SEM. Bars with an * are significantly different (P < 0.05, LSD test) in the same intervention period (baseline vs. intervention). Bars with a † are significantly different (P < 0.05, LSD test) between 6 h periods.
Cocoa intake reduces NF-κB activation

In this clinical trial, acute consumption of 40 g of cocoa powder mixed with water significantly reduced phosphorylation of p65, the transcriptional active subunit of NF-κB, thereby significantly reducing the activation of NF-κB. On the other hand, the consumption of whole milk by volunteers resulted in a significant increment in NF-κB activation, whereas no changes in NF-κB activation were observed on consumption of 40g of cocoa powder mixed with milk. To our knowledge, there are no studies in human volunteers on the effect of cocoa powder on NF-κB activation.

NF-κB is a transcriptional modulator of genes involved in inflammation and has a crucial role in atherosclerosis and other inflammatory diseases. Nowadays, it is considered a major therapeutic target [28]. NF-κB activation is mediated by the stimulation of tumor necrosis factor alpha or interleukin-1β [29], although it may also be activated by some diet factors such as high-fat diets [30]. This activation leads to the transcription of mRNAs for ICAM-1, VCAM-1 and E-selectin resulting in high levels of ICAM-1, VCAM-1 and E-selectin proteins in the activated endothelial cells. These proteins are adhesion molecules involved in leukocyte-endothelial cell interaction [31]. Previous in vitro studies have shown that epicatechin, catechin and procyanidins isolated from cocoa already inhibit NF-κB activation [32].

The different effects of CM, CW and M on NF-κB activation are remarkable, and may be explained by the different fat and polyphenol content of each intervention. In fact, it has been reported that high-fat diets such as whole milk consumption, increase NF-κB activation [30]. However, this effect may be prevented by the simultaneous consumption of polyphenols [17]. The differences between consumption of CM and CW, both with 40 g of cocoa powder, could be attributable to the different bioavailability of polyphenolic compounds present in cocoa powder when taken with milk or water.

The effect of milk on the bioavailability of cocoa-polyphenols has previously been widely studied. Recent studies have shown that milk has minor or no effects on the plasma pharmacokinetics of phase II metabolites of epicatechin [22,25]. However, although greatly controversial, milk might diminish the urinary excretion of some phase II metabolites of epicatechin [22]. Cocoa-polyphenols that are not absorbed (mainly procyanidins) could reach the colon where they are degraded to phenolic acids by the intestinal microbiota and are absorbed in the organism [23]. The effect of milk on the excretion of microbial phenolic acids after acute ingestion of cocoa powder has been studied and showed that milk significantly diminishes the urinary excretion of some phenolic acids related to the metabolism of cocoa-polyphenols such as caffeic, ferulic, 3,4-dihydroxyphenylacetic, protocatechuic, 4-hydroxybenzoic, 4-hydroxyhippuric, hippuric acids [23]. Therefore, in general terms, metabolites derived from cocoa powder consumption seem to have greater bioavailability after CW than after CM intake. This fact could be directly related to the activation of NF-κB, since p65 phosphorylation levels after CM were significantly lower when compared with M and significantly higher when compared with CW. Moon et al. (2009) have recently shown that caffeic acid, a natural phenolic compound, reduces vascular inflammation inhibiting NF-κB activation in human umbilical vein endothelial cells [33]. Similarly, our results show that cocoa consumption, even in a single dose, might exert anti-inflammatory effects by modulating the NF-κB pathway and this could be attributable to the overall phenolic content of cocoa powder.

The activation of NF-κB may be the key step in increasing the levels of ICAM-1, VCAM-1 and E-selectin proteins in activated endothelial cells as previously reported [31]. Nevertheless, our results show that this activation does not have a direct impact on these three cytokines since only sICAM-1 concentrations decreased after the CM and CW interventions compared to baseline. Furthermore, sICAM-1 levels were significantly lower after the CW intervention when compared to the CM intervention which was also related to the bioavailability of cocoa-polyphenols. sE-Selectin concentrations only decreased after the CW intake.
intervention, whereas sVCAM concentrations were not modified after either intervention. These results are in accordance with the results of Kurlandsky et al., who showed a decrease in ICAM-1 concentrations without changes in VCAM-1 concentrations after dark chocolate consumption in healthy women [19]. In volunteers with cardiovascular risk factors, Monagas et al. [11] compared the effects of long-term consumption (1-month) of cocoa powder with skimmed milk (CSM) with those produced by skimmed milk (SM). Similar to our results they found a significant decrease in sICAM-1 concentrations after 1-month intake of CSM, and no differences were observed in s-Selectin and sVCAM-1 concentrations in high cardiovascular risk subjects. However, contrary to our results, long-term studies in non-healthy subjects and with consumption of chocolate showed no changes in ICAM-1, VCAM-1 and selectin levels [18, 34] or only a diminution in VCAM-1 levels [20]. The different results that we observed in vivo between NF-κB activation and the concentrations of the three cytokines may have two plausible explanations: the short time period between NF-κB activation and the measurement of adhesion molecules such as sVCAM-1, and also the direct effect of polyphenol metabolites of cocoa in the more bioavailable cytokines such as ICAM-1. However, further studies should be carried out in this field to determine the precise mechanism of how cocoa intake modulates NF-κB activation.

The main limitations of the current study are that only healthy subjects with low cardiovascular risk were included and we only evaluated the acute effects of cocoa intake on the mechanisms related to activation of inflammatory pathways. However, since the spectrum of the atherosclerotic lesions is a continuum, and high-risk patients show a higher inflammatory response in arterial wall [35], the protective effects of cocoa may be even greater in this type of subjects. Therefore, further studies are required to determine the time course regulation of NF-κB transcription and NF-κB activation by cocoa and other polyphenol-enriched foods. These new studies should include time course analysis of gene and protein expression profiling, which would provide information on the protective effect of cocoa during and after the postprandial phase.

In conclusion, cocoa consumption could confer beneficial anti-inflammatory effects mediated by inhibition of the NF-κB-dependent transcription pathway or by direct interaction with certain cytokines and the food matrix could play a crucial role in the modulation of this effect.

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