Transworld Research Network
37/661 (2), Fort P.O.
Trivandrum-695 023
Kerala, India

Recent Advances in Pharmaceutical Sciences, 2011: 121-132 ISBN: 978-81-7895-528-5
Editor: Diego Muñoz-Torrero

5. Effect of cocoa powder in the prevention of cardiovascular disease: Biological, consumption and inflammatory biomarkers. A metabolomic approach

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Abstract. Numerous health benefits have been attributed to cocoa and its derived products in the last decade including antioxidant, anti-platelet and positive effects on lipid metabolism and vascular function. Inflammation plays a key role in the initiation and progression of atherosclerosis. However, cocoa feeding trials focused on inflammation are still rare and the results yielded are controversial. Health effects derived from cocoa consumption have

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been partly attributed to its polyphenol content, in particular of flavanols. Bioavailability is a key issue for cocoa polyphenols in order to be able to exert their biological activities. In the case of flavanols, bioavailability is strongly influenced by several factors, such as their degree of polymerization and the food matrix in which the polyphenols are delivered. Furthermore, gut has become an active site for the metabolism of procyanidins (oligomeric and polymeric flavanols). Estimation of polyphenol consumption or exposure is also a very challenging task in Food and Nutrition Science in order to correlate the intake of phytochemicals with *in vivo* health effects. In the area of nutrition, modern analytical techniques based on mass spectrometry are leading to considerable advances in targeted metabolite analysis and particularly in Metabolomics or global metabolite analysis.

In this chapter we have summarized the most relevant results of our recent research on the bioavailability of cocoa polyphenols in humans and the effect of the matrix in which cocoa polyphenols are delivered considering both targeted analysis and a metabolomic approach. Furthermore, we have also summarized the effect of long-term consumption of cocoa powder in patients at high risk of cardiovascular disease (CVD) on the inflammatory biomarkers of atherosclerosis.

Introduction

Cocoa (*Theobroma cacao* L.) and its related products are rich sources of antioxidant flavonoids, containing higher amounts per serving than other polyphenol rich foods such as red wine or tea [1]. Flavanols are the most abundant flavonoids in cocoa and comprise monomeric flavanols [(+)-catechin and (-)-epicatechin], oligomeric (procyanidins B1, B2 and C1) and polymeric forms (procyanidins) [2].

Inflammation is central to cardiovascular disease. The process of atherosclerosis starts with inflammatory changes in the arterial endothelium, which in turn expresses a series of adhesion molecules that attract circulating monocytes, which migrate through the endothelial layer under the influence of various proinflammatory chemoattractants. Once within the arterial intima, the monocytes continue to undergo inflammatory changes, and through these inflammatory processes, the initial lesion of atherosclerosis, the fatty streak, is formed. Also, inflammation is central to the progression from fatty streak to complex plaque that can lead to fatal complications [3].

Evidence based on epidemiological studies supports the belief that regular consumption of cocoa-containing products may confer cardiovascular protection, reducing blood pressure and the risk of CVD mortality [4, 5]. In addition, a recent meta-analysis on the effectiveness of cocoa human feeding trials has suggested that cocoa and its derived products have a significant effect on endothelial function, increasing flow-mediated dilation and reducing systolic and diastolic blood pressures [6]. Other benefits from cocoa consumption are associated with their capacity to improve lipid levels and

insulin sensitivity, and to reduce platelet activation and function [7]. In addition, results from *in vitro* studies have shown that cocoa flavanols and procyanidins also present anti-inflammatory effects, suppressing the production of the proinflammatory molecules such as cytokines and chemokines [8, 9], which are produced in the initial phases of atherosclerosis. However, few human feeding trials have focused on the study of the anti-inflammatory effects of cocoa, and the results obtained have been contradictory, reporting either neutral effects or changes in a single inflammatory biomarker [10-12].

For these biological activities to be translated into real health effects *in vivo*, cocoa polyphenols need to be bioavailable. Bioavailability of flavanols is influenced by their degree of polymerization and the food matrix. While monomers are readily absorbed in the small intestine, oligomers and polymers need to be biotransformed by the colonic microbiota before absorption (Fig. 1). Equally important is the development of biomarkers of exposure in

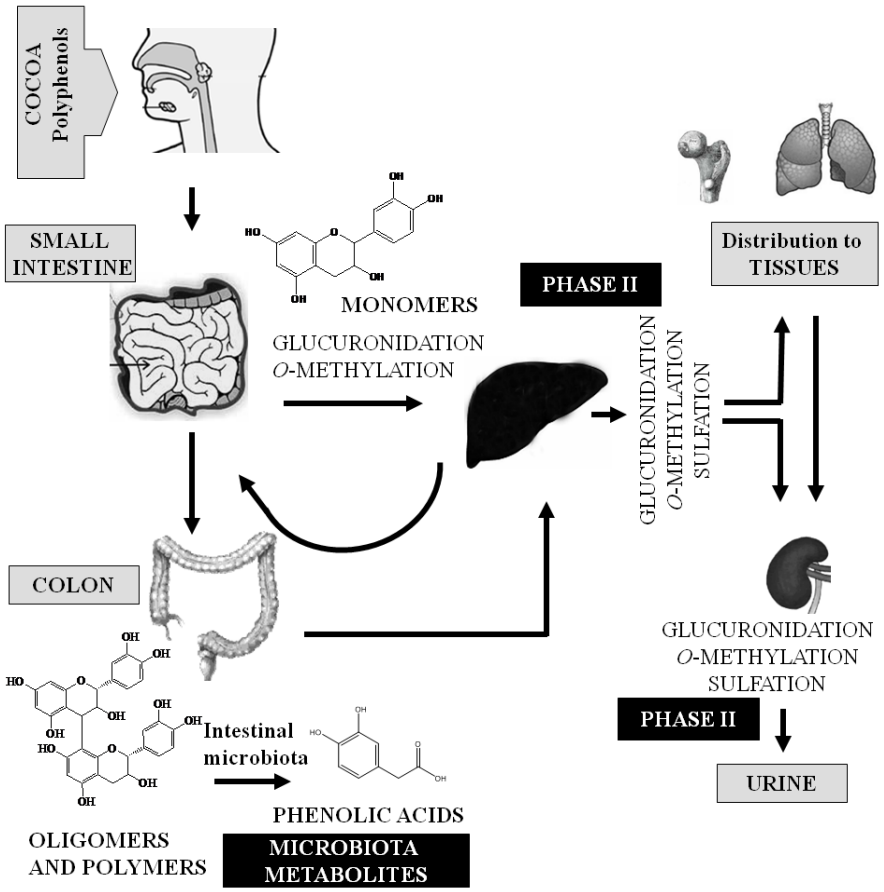

Figure 1. Scheme of bioavailability of cocoa flavanols showing phase II metabolites [conjugation by enzymes catechol-O-methyl transferases (COMT); uridine diphosphate glucuronyltransferases (UDPGT), and sulfotransferases (SULFT)], and microbial metabolites [degradation of oligomers and polymers by intestinal microbiota].

order to correlate the intake of flavanols with particular health effects *in vivo*. In this sense, global analysis of metabolites or metabolomics is becoming an important tool for the determination of new biomarkers of exposure to polyphenols.

The other important aspect that could modulate the bioavailability of polyphenols is the food matrix. Several studies have provided conflicting evidence about the effect of milk on the bioavailability of polyphenols, specifically flavanols, when looking at their phase II conjugate forms from cocoa [13-16]. Recently, it has been suggested that the possible influence of milk on cocoa flavonoid absorption, and consequently on the urinary excretion of phase II metabolites, is more relevant for drinks with lower flavanol content, which is typical of many commercial cocoas, than for drinks with higher content [17]. Therefore, as milk significantly lowered the excretion of urinary phase II metabolites of cocoa flavanols [17], there is a need to study the effect of milk on the colonic microbial metabolism of the non-absorbed cocoa flavanol fraction that reaches the colon [18].

Herein we will summarize the main results of our recent research on the bioavailability of cocoa polyphenols in two interventional studies: 1) A single dose study of cocoa powder whose main aims are: *a)* Effect of milk on the bioavailability of cocoa polyphenols [18], *b)* Metabolomic study to assess bioavailability and identify nutritional biomarkers of exposure after cocoa intake in humans [19]; 2) a long-term study of regular consumption of cocoa powder in patients at high risk of CVD whose main aims are: c*)* Effects of cocoa powder at clinical levels [20], and *d)* Targeted study of cocoa polyphenol metabolites to assess bioavailability and compliance [21].

1. Single cocoa powder intake: Short-term study

1.1. Effect of milk on the bioavailability of cocoa polyphenols

The short term study with single intake of cocoa powder was a randomized, crossover and controlled clinical trial. Twenty-one healthy volunteers (9 women and 12 men) were included in the study. Participants were instructed to abstain from polyphenol-rich foods for at least 48 h before the study and during the day of the study. The study was done on three different days with one week between the interventions. After overnight fasting, the volunteers consumed three different meals in random order: a) 40 g cocoa powder (Nutrexpa, Barcelona, Spain) and 250 mL whole milk (CC-M); b) 40 g cocoa powder and 250 mL water (CC-W); c) 250 mL whole milk as a control [18].

First, we evaluated the effect of milk on the absorption of (–)-epicatechin from cocoa powder. The only metabolite found in plasma at 2h after ingestion

of cocoa powder was epicatechin-glucuronide. The concentration of epicatechin glucuronide in plasma was ~20% higher after the intervention of CC-W in comparison to the intervention CC-M but these differences were almost significant (P=0.07) [14]. With respect to phase II metabolites of epicatechin in urine (three sulfates and one glucuronide conjugates), milk did not affect the total excretion of metabolites but affected the excretion profile of these conjugates [13].

We have also evaluated the metabolism of cocoa polyphenols at a colonic microbial level (microbiota metabolites) through the measure of the urinary excretion of microbial-derived phenolic acids and evaluated the effect of milk on urinary excretion of these compounds after the intake of a standard portion of cocoa powder with either water or milk in an acute intervention using a targeted quantitative procedure. We have carried out this targeted study through a developed and validated methodology that consist in solid-phase extraction (SPE) of biological samples (urine) followed by LC-MS/MS analysis [22].

We evaluated the excretion of 15 phenolic acids which had been described as microbial degradation products of flavanols [21, 23-26]. Of the 15 phenolic acids studied, the excretion of seven phenolic acids diminished after CC-M when compared with CC-W while two phenolic acids increased. Four phenolic acids of these seven, could come from the direct degradation of procyanidins by the intestinal microbiota following the scheme shown in Fig. 2. Procyanidins could be degraded forming 3,4-dihydroxyphenylacetic acid (Fig. 2) [18, 27] and this compound through α-oxidation could be degraded to protocatechuic acid [26]. Microbial dehydroxylation of protocatechuic acid could give rise to 4-hydroxybenzoic acid derivative that may undergo glycination in the liver and kidney being converted into 4-hydroxyhippuric acid [18, 24].

Two hydroxycinnamic acids, caffeic and ferulic acids, were also significantly reduced (50% and 80%, respectively) by the ingestion of CC-M when compared with CC-W. In cocoa, these compounds could come from several routes of metabolism, which could include dehydrogenation of 3,4-dihydroxyphenylpropionic acid as well as degradation from other phenolic derivatives present in cocoa powder, such as chlorogenic acid or *N*-phenylpropenoyl-L-amino acids [25, 28]. We observed a high concentration in the last urine fraction suggesting that a longer period of urine collection was required for their complete excretion.

Whereas a higher global urinary excretion of phenolic acids was observed with consumed CC-W, vanillic and phenylacetic acids showed an excretion pattern contrary to those of other metabolites. This result could mean that liposoluble vanillin, when ingested with CC-M, could be absorbed more easily

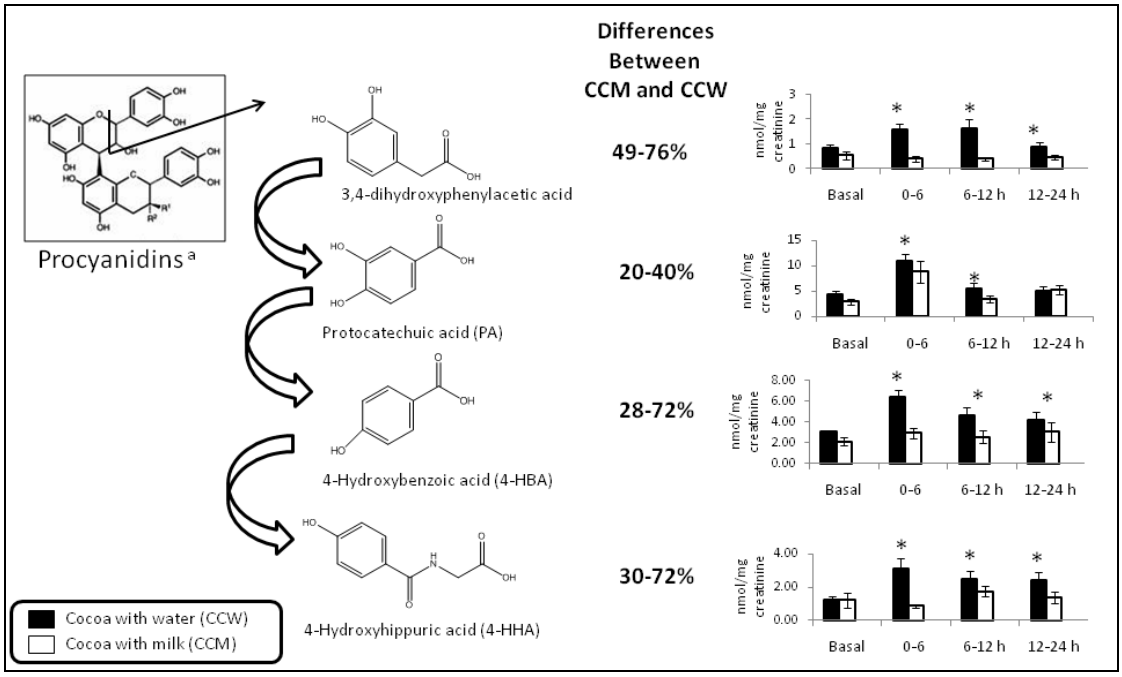

Figure 2. Urinary excretion [mean(SEM)] and percentage difference of four phenolic acids from cocoa procyanidins significantly affected by the consumption of cocoa powder with water or with milk [18]. Bars with an asterisk are significantly different ($P < 0.05$; Wilcoxon's test; $n = 21$) in the same time period between water and milk. [a]Described in [27].

and rapidly than after its intake with CC-W and could be oxidized to vanillic acid by aldehyde oxidase of the liver [29]. In the case of phenylacetic acid, its excretion was only significatively higher after CC-M than after CC-W in the last time period (12-24 h) although the same trend was observed in earlier urine fractions.

1.2. Metabolomic study to assess bioavailability and identify nutritional biomarkers of exposure after cocoa intake in humans

Metabolomics aims to assess metabolic changes in a global manner in order to provide the detailed biochemical responses of cellular systems and provide a comprehensive profile of all the metabolites present in a biological sample (metabolome). This approach has been applied to clinical, pharmaceutical and toxicological applications [30] and recently has also emerged as a field of increasing interest to Food and Nutrition Science [31, 32]. We have applied an HPLC-Q-TOF metabolomics approach followed by multivariate data analysis (PCA, OSC-PLS and OSC-PLSDA) to urine samples collected after a single intake of 40 g of cocoa powder with milk (250 mL) or with water (250 mL) or only milk (250 mL) as control, by

healthy subjects (n=10; 5 women and 5 men) in a randomized, crossover and controlled clinical trial [19]. Our aim in this metabolomic approach was to study the influence of cocoa intake on the urinary metabolic profile during the time course of a 24 h-urine excretion period (0-6, 6-12, 12-24 h) and to identify the most relevant biomarkers of intake.

The results revealed that modifications in the urine metabolome as a consequence of cocoa intake were explained by the excretion of a complex profile of cocoa-derived phytochemicals. A total of 27 metabolites related to cocoa intake were identified and were grouped as follows: a) purine alkaloid metabolites; b) polyphenol host metabolites; c) polyphenol colonic microbiota metabolites, d) cocoa flavour and taste compounds, and e) vitamins and amino acids.

Among purine alkaloid metabolites, the most important metabolites derived from theobromine metabolism, including 6-amino-5-[*N*-methylformylamino]-1-methyluracil (AMMU), 3- and 7-methyluric acid, 3- and 7-methylxanthine, among others, were identified and were found to be the main contributors to changes in the urine metabolome up to 24 h after cocoa intake. In the case of polyphenol host metabolites, epicatechin sulfate, *O*-methylepicatechin, vanillic acid, and its derivative vanilloglycine, identified for the first time after cocoa intake, were found to be present 6 h after cocoa powder consumption (Fig. 1) [25]. On the other hand, microbial-derived phenolic metabolites, such as phenylvaleric acid and phenylvalerolactone derivatives, were found to contribute to the urinary metabolome in the period between 6 and 12 h after cocoa ingestion (Fig. 1). Cocoa flavoured metabolites identified included 3,5-diethyl-2-methylpyrazine and hydroxyacetophenone, as well as the diketopiperazine derivatives. Finally, trigonellin and hydroxynicotinic acid were identified as metabolites derived from nicotinic acid (niacin) present in cocoa, whereas tyrosine was identified as a metabolite related to cocoa amino acids. Both types of metabolites were found to contribute to changes in urine metabolome in the fraction collected 6 h after cocoa intake.

These results demonstrate that metabolomics is an important tool for exploring the metabolism of cocoa phytochemicals. Metabolites from different origins and chemical structures could serve as biomarkers of cocoa powder intake and help us to understand if their presence could be related to afford health effects.

2. Regular cocoa powder intake: Long-term study

2.1. Effects of cocoa powder on inflammatory biomarkers of atherosclerosis

The long-term study with regular consumption of cocoa powder was a randomized, crossover and controlled clinical trial. The subjects included

were diabetic or had three or more of the following cardiovascular disease risk factors: tobacco smokers, hypertensives, hypercholesterolemics, and/or obese. Forty-two high-risk subjects were included in the study (19 men and 23 women, mean age of 69.7 years). The institutional review board of the hospital approved the study protocol and the trial was registered in the International Standard Randomized Controlled Trial Number, at controlled.trials.com (ISRCTN75176807). Subjects received two 20 g-sachets of soluble cocoa powder per day with 250 mL of skimmed milk each (C+M intervention) or only 500 mL skimmed milk per day (M intervention) for 4 weeks in a random order [20]. We have analyzed the expression of adhesion molecules [VLA-4 (very late activation antigen-4, CD49-d), LFA-1 (lymphocyte function-associated antigen-1, CD11a), Mac-1 (CD11b/CD18), SLex (Sialil-Lewis X, CD15s), CD36 and CD40] on the surface of peripheral blood mononuclear cells (PBMCs) for the first time after cocoa intake as inflammatory biomarkers of atherosclerosis. Our results showed significant ($p< 0.05$) lower values in the expression of adhesion molecules such as VLA-4 (-7.4%), CD40 (-6.6%) and CD36 (-21.2%) on monocyte surface after consumption of cocoa powder with skimmed milk.

Other circulating inflammatory biomarkers studied included serum levels of P-selectin, E-selectin, VCAM-1, ICAM-1, proinflammatory cytokine IL-6 and high-sensitivity C-reactive protein (hs-CRP). It was found that P-selectin and ICAM-I, which play an important role in leukocyte adhesion cascade, were significantly ($p< 0.05$) decreased by 10.8% and 9.7%, respectively, after the intake of cocoa powder with skimmed milk. Other biomarkers like MCP-1 and VCAM were also found to be at lower levels (although not significant) after cocoa consumption.

With respect to plasma lipids, the concentration of HDL cholesterol was significantly higher after the intervention of cocoa with skimmed milk in comparison to the skimmed milk intervention (4.2% increase, P=0.035). No significant changes were observed for total plasma cholesterol, triglycerides and LDL cholesterol. In addition, no significant changes were registered in systolic and diastolic blood pressure, or in heart rate between the two interventions [20].

All the above results strongly support a link between cocoa consumption and the modulation of inflammatory mediators in human subjects at high risk of CVD. These anti-inflammatory effects together with other previously reported effects, including antioxidant, anti-platelet and positive vascular effects may contribute to the overall benefits of cocoa consumption against atherosclerosis and could have significant implications for public health as far as cardiovascular disease is concerned.

2.2. Targeted study of cocoa polyphenol metabolites to assess bioavailability and compliance

In order to exert the beneficial effects, cocoa polyphenols need to be bioavailable. Monomeric flavanols are readily absorbed in the small intestine but polymers need to be degraded by the intestinal microbiota before absorption in the colon. We have carried out a targeted study of main cocoa polyphenol metabolites by HPLC-MS/MS in the plasma and urine samples collected from the clinical study in order to estimate their absorption *in vivo* and as a measure of protocol compliance [21]. Regular consumption of cocoa powder with skimmed milk resulted in a significant ($p < 0.05$) increase in the urinary excretion of phase II metabolites, including glucuronide and sulfate conjugates of (−)-epicatechin and *O*-methyl-epicatechin, derived from the absorption of epicatechin in the small intestine (Fig. 1). Colonic microbial-derived phenolic metabolites, including vanillic, 3,4-dihydroxyphenylacetic and 3-hydroxyphenylacetic acids, and particularly 5-(3,4-dihydroxyphenyl)-γ-valerolactone (Fig. 1), were also found in significant higher levels after consumption of cocoa powder with skimmed milk and represented the largest proportion of total phenolic metabolites excreted in urine. These results indicated that cocoa polyphenols are bioavailable and that the colon is an active site for the metabolism of cocoa polyphenols. *In vitro* studies performed by our group have suggested that some microbial-derived phenolic metabolites possess anti-inflammatory properties, suggesting that they could be partly responsible for the benefits observed in patients at high risk of CVD after cocoa consumption [33].

3. Conclusion

The results presented herein provide valuable information that supports the health benefits of cocoa consumption on CVD. New inflammatory biomarkers, such as the expression of adhesion molecules on leukocyte surface, have been used for the first time in a clinical cocoa trial focused on this issue. Although current state of the art does not allow an estimation of CVD risk associated with a given change in these or other inflammatory biomarkers, the positive effects observed after cocoa consumption may contribute together – along with other previously reported effects, in particular on vascular function – to the overall benefits of cocoa consumption against CVD.

Due to their high content in procyanidins, the colonic microbiota plays a key role in the metabolism of cocoa polyphenols, as demonstrated by the increase in certain lactones and phenolic acids after cocoa consumption in our targeted study.

Given that these metabolites could represent the largest proportion of all phenolic metabolites excreted in urine, more effort needs to be concentrated on the study of the biological properties of these microbial-derived phenolic metabolites, including their effects on the intestinal microbiota, which are still largely unknown.

Finally, our metabolomic approach, combined with visualization strategies, allowed the identification of a series of metabolites derived from cocoa polyphenols, alkaloids, flavour and aroma compounds, vitamins and amino acids that could serve as biomarkers of cocoa powder consumption, thus pushing nutritional information one step closer towards a better understanding of human health.

Acknowledgements

This research was supported by national grants CICYT (AGL: 2004-08378-C02-01/02, 2006-14228-C03-02/01 and 2009-13906-C02-01); CIBER 06/03 Fisiopatología de la Obesidad y la Nutrición is an initiative of the Instituto de Salud Carlos III, Spain; Centro Nacional de Investigaciones Cardiovasculares (CNIC-06) and Ingenio-CONSOLIDER program, Fun-c-food (CSD2007-063). M.U.-S. thanks the Sara Borrell postdoctoral program (CD09/00134), M.M. thanks the Ramon y Cajal program, R.Ll. thanks the Fondo de Investigación Sanitaria program (FIS, CD06/00161), and N.K. thanks the FPU fellowship program, all from the Ministry of Science and Innovation. M.R.-R. thanks the FI-DGR2010 fellowship program from the Generalitat de Catalunya. R.E. is recipient of a grant from FIS, Madrid, Spain.

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