Diets rich in fruits and vegetables have been associated with benefits for human health. Those effects have been partially ascribed to their content in flavonoids, compounds that are present in many edible plants and its derived foods. In humans, a significant number of studies has been developed analyzing the effect of foods and beverages rich in flavonoids on the presence and progression of risk factors associated to cardiovascular disease. Epidemiological evidence demonstrates that diets rich in fruits and vegetables benefit heart and vascular health.[2–11] Molecularly, these beneficial effects of fruits and vegetables have been largely ascribed to their content in flavonoids. These compounds are synthesized in many edible plants and remain present when plants are processed to foods. Grapes and wine, cocoa and chocolate, black and green tea, and soy and soy-derived products, are among the most important sources of flavonoids in the human diet. A significant number of studies has been developed in humans, analyzing the effect of foods and beverages rich in flavonoids on the presence and progression of risk factors associated to cardiovascular disease. Cocoa derived products have been thoroughly studied and demonstrated to be efficient improving endothelial function and decreasing blood pressure in humans and animals. However, the final chemical species and the mechanism/s responsible for these effects have not been completely defined. In this paper we present data supporting the hypothesis that flavanols could define superoxide anion production and then, establish optimal nitric oxide levels and blood pressure.

Key Words: (−)-epicatechin, nitric oxide, oxidants, antioxidants

Lifestyle modifications, including dietary habits, have substantial effects on risk factors for cardiovascular disease such as hypertension.[3] Epidemiological evidence demonstrates that diets rich in fruits and vegetables benefit heart and vascular health.[2–11] Molecularly, these beneficial effects of fruits and vegetables have been largely ascribed to their content in flavonoids. These compounds are synthesized in many edible plants and remain present when plants are processed to foods. Grapes and wine, cocoa and chocolate, black and green tea, and soy and soy-derived products, are among the most important sources of flavonoids in the human diet. A significant number of studies has been developed in humans, analyzing the effect of foods and beverages rich in flavonoids on the presence and progression of risk factors associated to cardiovascular disease. Cocoa derived products have been thoroughly studied and demonstrated to be efficient improving endothelial function and decreasing blood pressure (BP).[12–14]

BP is influenced and regulated by a variety of conditions and chemical entities that interact among them in very complex ways. These entities include from atoms, e.g., sodium, to well-orchestrated systems, e.g., the renin-angiotensin system. In this complex scheme, nitric oxide (NO) appears to play a pivotal role in the regulation of vascular homeostasis, and then BP. We will focus the discussion about the effects of dietary flavonoids, mainly those provided by cocoa and cocoa products, on NO bioavailability as a result of regulating steady-state levels of superoxide anion (O₂−) and oxidants in cells and tissues.

Nitric Oxide and Oxidants in Biological Systems

NO is produced from L-arginine in a reaction catalyzed by the enzyme nitric oxide synthase (NOS). This enzyme is present in mammals in different isoforms: endothelial (eNOS), neuronal (nNOS), inducible (iNOS), which is expressed in response to different stimulus, and mitochondrial (mtNOS). NO reacts with several metal centers, molecular oxygen, thiol groups, and some oxygen radicals. NO fulfills its most important physiological action, i.e., vasodilation, by activating guanylyl cyclase through the reaction with its heme group. The guanylyl cyclase catalyzes the dephosphorylation of GTP to cGMP, which serves as a second messenger for signaling smooth muscle relaxation. Alternatively, NO reacts with superoxide anion, the one-electron reduction product of oxygen in a near diffusion controlled reaction to form peroxynitrite (NO + O₂− → ONOO⁻). This reaction has two physiological consequences: i) the generation of peroxynitrite which can oxidize cell components;[15] and ii) the elimination of NO. In this way, superoxide anion production is a modulator of NO steady-state levels. In mammalian cells, the principal source of superoxide anion is the mitochondrial electron transport, but other enzymatic sources include NADPH oxidase (NOX), xanthine oxidase, arachidonic acid metabolism by cyclooxygenase/lipoxygenase, and cytochrome P450.[16] In endothelial cells the NOX-dependent superoxide production is particularly relevant.

Flavanols

Cocoa, and foods and beverages made with, contain important amounts of flavan-3-ols or flavanols, a subfamily of flavonoids. Flavonoids include a large number of compounds that share a basic chemical structure and are classified in different subfamilies depending on specific substitution patterns.[17] Flavan-3-ols or flavanols are defined by the presence of a hydroxyl group at position 3 (ring C) and constitute the most complex subfamily of flavonoids, ranging from simple monomers to the oligomeric and polymeric forms, called procyandin.[17] Monomers could be present in the aglycone form or esterified with gallic acid to form the gallate derivatives. Studies on the composition of cocoa products have shown that non-esterified monomers of flavanols and procyanidins are the quantitatively most important type of flavonoids. Those chemical species have been identified as (−)-epicatechin (EC) (Fig. 1A), (+)-catechin (Cat), and mostly B-type procyanidins, that are oligomers of EC.[18,19] Importantly, regardless the manufacturing process applied and the raw material used to produce chocolate, the main flavanol present in chocolate is EC.[19,20]

In terms of the metabolism of flavanols by humans and animals, it has been demonstrated that the gastric environment does not affect flavanols and procyanidins stability, and they transit with minimal modification to the small intestine where can be absorbed.[21] EC reached detectable values in human plasma after dietary administration of chocolate or cocoa derived pro-
ducts. These results demonstrate that the EC present in the raw material, i.e. cocoa beans, even after the alterations suffered by manufacturing and metabolization processes, is present in tissues after its ingestion.

Flavanols and Human Blood Pressure

Pioneer observations by McCullough et al. (26) indicated that the Kuna Indians of Panama have a very low incidence of hypertension and cardiovascular disease, but when members of this tribe moved to Panama urban places, their BP increased. The migration leads to cultural changes including an important decrease in cocoa consumption, making cocoa the potential responsible for the observed changes in BP. Epidemiologically, a sub-study of the Zutphen population showed that cocoa (chocolate) consumption was associated with a decrease in BP and cardiovascular mortality. (21) Intervention trials with cocoa and cocoa products have included different groups of subjects: normotensive (young, old, overweight, hypercholesterolemic), pre-hypertensive, hypertensive stage 1, and hypertensive with impaired glucose tolerance. Most of the studies showed that cocoa consumption was associated with a decrease in BP. (27) Hooper et al. (13) reviewed the effectiveness of different flavonoid subclasses and flavonoid-rich food sources on cardiovascular disease, and one of the risk factors measured was BP. Through the analysis of 133 trials, their three significant conclusions were: i) chocolate decreased diastolic BP and systolic BP (SBP); ii) soy protein isolate (and no other soy derived products) decreased diastolic BP; and iii) acute ingestion of black tea increased SBP and diastolic BP. In the same direction, another meta-analysis about the effects of cocoa and tea on BP, showed that foods rich in cocoa may reduce, while tea intake appears to have no effect on BP. (12) A new and recent meta-analysis evaluating the effect of cocoa rich-foods on BP of hypertensive and normotensive individuals concludes that dark chocolate is superior to placebo in reducing systolic hypertension or diastolic prehypertension. (14) In contrast to studies using chocolate or cocoa preparations, only few studies have been developed on human subjects by using purified flavanols. One study was carried out by dietary supplementation with (+)-catechin in obese and near-obese Japanese children. (28) Results, stratified by the median of the initial BP values, showed that consumption of 75 mg (+)-catechin/d for 24 w was effective in reducing SBP in the above-median category. The studies with isolated flavanols are crucial to assess these.

Fig. 1. Dietary (−)-epicatechin (EC) and blood pressure. A: chemical structure of EC; B: systolic blood pressure (SBP) of rats subjected to L-NAME pre-treatment (40 mg/kg/d in drinking water) for 6 days; and C: SBP of rats administered with L-NAME (open square), or L-NAME + EC 0.4 g/100 g diet (closed square) during the subsequent 8 days. Sprague-Dawley rats (130–140 g) were maintained under controlled housing conditions. SBP was measured in preconditioned, conscious and restrained rats by tail-cuff plethysmography. Data are presented as means ± SEM. Groups were compared using ANOVA. *: p<0.05 respect to day zero of treatment (B), or to L-NAME treated rats (C).
compounds as responsible for the antihypertensive effects of flavanol containing foods.

**Flavanols, Nitric Oxide and Oxidative Stress in Human Studies**

The BP-lowering effect of cocoa-rich products have been correlated with: i) increases in plasma or urine NO-derived species; ii) improvement in NO-mediated flow-mediated dilation (FMD) as an indicative of vascular function; and iii) reduced oxidative stress. Plasma NO metabolites (measured as S-nitrosothiols) were increased in prehypertensive and hypertensive patients after 18 weeks consuming daily 6.3 g of chocolate (30 mg of polyphenols). The oral ingestion of a high-flavanol cocoa drink (917 mg of flavanols) resulted in a transient increase in NO-derived species concentration in plasma and urine of healthy subjects. This increase was associated with the improvement of FMD, and with plasma flavanol levels. More recently, the oral ingestion of 200 mg of quercetin or EC was also associated with increased plasma and urine NO metabolites. FMD was improved in hypertensive individuals (with and without impaired glucose tolerance) in association with decreased BP after 2 weeks consuming 100 g of chocolate. Engler et al. treated normotensive individuals for 2 weeks with chocolate and did not find any difference in BP and oxidative stress parameters, but a significant increase in FMD. However, Fraga et al. also working with a normotensive group and chocolate supplementation for 2 weeks, found positive results: decrease in BP associated to improvement in oxidative stress markers (plasma malondialdehyde, vitamin E/low density lipoprotein (LDL), vitamin E/cholesterol). More recently, an intervention study administering cocoa beverage to a group of subjects with essential hypertension for 2 weeks was developed. There were no changes in BP, but the insulin-mediated changes in brachial diameter were higher in cocoa treated patients than in placebo group. In another study, it was observed that two different doses of polyphenols (500 mg or 1000 mg during 2 weeks) were equally effective in reducing BP.

One important factor to consider when an integrative conclusion wants to be drawn from all these investigations is the composition of the chocolate used. Unfortunately, there is not a standardized chocolate or a consistent standardization (qualitative and/or quantitative) of the chocolate components through the different studies. Animal studies using purified compounds are a valid alternative to advance on the comprehension of the mechanisms underlying the BP lowering effect of cocoa derived products.

**Flavanols, Nitric Oxide and Oxidative Stress in Animal Studies**

Mechanistic aspects defining the beneficial effect of flavonoid-rich diets on endothelial function and BP have been studied in a variety of animal experimental models. However, only a few studies have been carried out by using cocoa derived products. A commercially available natural flavonoid-enriched cocoa powder was tested in spontaneously hypertensive rats (SHR). A single oral administration of different doses of the product (50–600 mg/kg) produced an antihypertensive effect in SHR without modifications in the BP of normotensive Wistar-Kyoto rats. The maximum effect on BP, caused by 300 mg/kg of powder, produced a BP decrease similar to the one obtained with 50 mg/kg captopril (an angiotensin converting enzyme inhibitor). More recently, the same flavonoid-enriched cocoa powder was studied as antihypertensive in SHR in a long-term treatment. Animals received the flavonoid-enriched cocoa powder in the drinking water at doses 100, 200 and 400 mg/kg/d during 20 weeks. All the doses attenuated the development of hypertension and improved the endothelial function in SHR. However, the higher antihypertensive effect was observed in the group treated with the lowest dose. A more mechanistic approach has been studied by using a model of type 2 diabetes, the Otsuka Long-Evans Tokushima Fatty (OLETF) rats, compared with nondiabetic Long-Evans Tokushima Otsuka (LETO) rats. All rats were daily treated with (+)-catechin (30 mg/kg/day) or saline for 12 weeks. OLETF rats showed an increase in BP, associated with higher NOX activity and expression of two NOX subunits (p22phox and p47phox) in aortic wall during the studied period. Treatment of the OLETF rats with Cat resulted in the maintenance of BP, arterial NOX activity and expression, and the oxidative stress conditions in values similar to that observed in saline-treated LETO rats.

Being EC (Fig. 1A) the most abundant flavanol in cocoa, the potential of this compound as responsible for the cocoa and chocolate BP-lowering effect is of singular relevance. Sprague-Dawley rats were pre-treated with the NO-synthase inhibitor N-nitro-L-arginine methyl ester (L-NAME) in the drinking water for 6 days. After that period, an increase of 25 mm Hg to 122 ± 6 mm Hg was observed (Fig. 1B). From that day, the rats were maintained during 8 days under the same L-NAME treatment, but receiving different diets: one group was fed with a control diet (L-NAME group) and the second group with an EC-enriched diet (0.4 g EC/100 g diet) (L-NAME + EC group). SBP was measured during the following 8 days in both dietary groups. At the end of that period, the group receiving control diet showed an additional increase in BP. Meanwhile, the group receiving the diet supplemented with EC maintained the initial BP values (Fig. 1C). These results suggest that the presence of EC in the diet can prevent the sustained BP increase induced by a deficiency in NO production associated to the L-NAME treatment.

**The Hypothesis of Nitric Oxide Bioavailability**

The concept of NO as a modulator of BP is centered in the fact that sufficient NO bioavailability is associated with normal vasodilatation and normal BP. On the other hand, decreases in NO steady-state concentration can lead to a failure in smooth muscle relaxation and the consequent hypertension. As indicated, under physiological conditions, most NO is produced by endothelial cells due to eNOS activity. In response to inflammatory stimuli NO can also be produced by iNOS in adventitia cells. The small size and lipophilic nature of NO allow the rapid diffusion of NO through cell membranes to reach its target cells, the smooth muscle cells.

The principal sources of superoxide anion in the vasculature are NOX activities, a family of oxidases that catalyze superoxide anion production using NADPH as the electron donor. Three, out of five NOX identified in animal tissues, have been reported in the rat vasculature: NOX1 in smooth muscle cells; NOX2 in endothelial and adventitial cells; and NOX4 in endothelial cells. Superoxide anion produced in the vascular wall will “consume” the NO diffusing from the endothelial cells being determinant of NO availability in the smooth muscle cells.

The processes involved in the maintenance of NO steady-state level have multiple steps susceptible to be regulated by flavonoids (Fig. 2): i) decrease in superoxide anion and related oxidants concentration in the cells by direct reaction (free radical scavenging action of EC); ii) decrease in superoxide anion concentration in the cell by diminishing NOX expression and/or activity; iii) increase in NO production by augmentation of eNOS expression and/or activity; iv) modulation of enzymes and receptors associated to ROS production, e.g., angiotensin II-mediated pathways, and protection of oxidative loss of tetrahydrobiopterin to avoid eNOS uncoupling (a condition in which eNOS produces large quantities of superoxide anion rather than NO). The final condition of optimal NO steady-state concentration can be reached by either, increasing its synthesis (without modification in its degradation), or by diminishing superoxide anion.
Increased NO levels in HUVEC. The authors observed a decrease in superoxide anion concentration as a result of a direct inhibition of NOX by EC metabolites. In addition, it was showed that while EC is an efficient superoxide anion scavenger, its O-methylated metabolites directly inhibit NOX. These metabolites have been reported as predominant species after EC ingestion by human and rats, and are structurally similar to apocynin, a well-known NOX inhibitor. Supporting this hypothesis, inhibition of the enzyme catechol-O-methyltransferase in HUVEC preceding the EC treatments, prevented the NO increase. This study indicates that methylation of EC could be essential to inhibit NOX, and as a consequence, superoxide anion production.

Finally, changes in eNOS and/or NOX expression in aorta have been reported in animals treated with purified compounds, such as Cat, the flavonol quercetin, the polyphenol resveratrol and dietary extracts rich in flavonoids. However, there is no data about EC modifying the expression of those enzymes in the aortic wall.

Conclusions

Flavanol containing foods, specially cocoa and cocoa-derived products have demonstrated to have BP-lowering effects in both, humans and animals. These effects could be related to the maintenance of optimal NO levels, and could be associated with lowering superoxide anion production in the vasculature.

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Abbreviations

BP blood pressure  
NO nitric oxide  
O− superoxide anion  
NOS nitric oxide synthase  
NOX NADPH oxidase  
EC (+)-epicatechin  
Cat (+)-catechin  
SBP systolic blood pressure  
FMD flow-mediated dilation  
LDL low density lipoprotein  
SHR spontaneously hypertensive rats  
OLETF rat Otsuka Long-Evans Tokushima Fatty rats  
LETO rat non-diabetic Long-Evans Tokushima Otsuka rats  
L-NAME N-nitro-L-arginine methyl ester  
HUVEC human umbilical vein endothelial cells

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