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Review

Chocolate at heart: The anti-inflammatory impact of cocoa flavanols

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Chronic and acute inflammation underlies the molecular basis of atherosclerosis. Cocoa-based products are among the richest functional foods based upon flavanols and their influence on the inflammatory pathway, as demonstrated by several *in vitro* or *ex vivo* studies. Indeed, flavanols modify the production of pro-inflammatory cytokines, the synthesis of eicosanoids, the activation of platelets, and nitric oxide-mediated mechanisms. A relative paucity of data still characterizes the *in vivo* implications of these findings albeit there have been studies suggesting that the regular or occasional consumption of cocoa-rich compounds exerts beneficial effects on blood pressure, insulin resistance, vascular damage, and oxidative stress. Accordingly, rigorous controlled human studies with adequate follow-up and with the use of critical dietary questionnaires are needed to determine the effects of flavanols on the major endpoints of cardiovascular health.

Keywords: Atherosclerosis / Cocoa / Flavanols / Immunology / Inflammation

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1 Cocoa and health in a nutshell

Cocoa can be defined as a functional food due to its high content of monomeric (epicatechin and catechin) and oligomeric (procyanidins) flavanols [1–3]. These latter polymeric fractions are significantly more represented in cocoa when compared to other flavanol-rich foods such as red wine or green tea [4, 5]. During the past years, a growing number of studies, mostly conducted *in vitro* or *ex vivo* have demonstrated that flavanols share the capacity to modulate inflammation as well as other major metabolic and immunological pathways and these have been extensively reviewed most recently by Cooper *et al*. [6]. The flavanol beneficial effects on inflammation has implications to cardiovascular disease [7]. Importantly, inflammation and nitric oxide (NO) production play a major role in the development of the atherosclerotic plaque. For these reasons, molecules involved in the inflammatory cascade accompanying most cardiovascular diseases should be regarded as promising potential targets in the prevention and treatment of such conditions. Data from numerous studies suggest that cocoa-derived flavanols can effectively modify the inflammatory process [6] and thus potentially provide a benefit to individuals with cardiovascular risk factors. Most recently, data have demonstrated a significant effect of different formulations to determine the bioavailability of flavanols *in vivo* [8]. It has been observed that cocoa products influence specifically endothelium-derived NO synthesis and metabolism, cytokine production, and eicosanoid metabolism through a specific action on peripheral blood mononuclear cells (PBMC) [9, 10]. Cocoa products also share a beneficial effect on insulin resistance and hypertension [11], suggesting the possibility of further use in the management of the metabolic syndrome.

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Abbreviations: CAD, coronary artery disease; **Lt,** leukotriens; **NF-κB,** nuclear factor-kappaB; **NOS,** NO synthase; **NO,** nitric oxide; **PBMC,** peripheral blood mononuclear cells; **PHA,** phytohemagglutinin; **PG,** prostaglandins; **TGF,** tumor growth factor; **Tx,** thromboxanes

2 Inflammation and cardiovascular health

Chronic inflammation is now an established determinant in the onset and biology of coronary artery disease (CAD). In

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fact, immune cells found within the plaque (mostly macrophages, T cells, and mast cells) share an activated phenotype and secrete pro-inflammatory cytokines thus leading to the site recruitment of other immune cells and the production eicosanoids, NO, and changes in microvascular tone [12]. These mechanisms share the common grounds of altered signaling activation [13]. It remains to be determined what is the temporal and causal sequence of inflammation at the atherosclerosis site with other "unsuspected" players possibly triggering inflammation, as in the case of circulating cholesterol [14] or sex-related factors [15]. Further, long-term exposure to pro-inflammatory cytokines such as tumor necrosis factor alpha (TNFα) or IL6 is detrimental to the myocardium and associated with a poor prognosis in chronic heart failure [16, 17]. Based on these observations; however, novel therapeutic approaches with immunomodulatory molecules are encouraging in both prevention and treatment of atherosclerosis and CAD [18], particularly with the use of intravenous Igs [19]. We will herein, review aspects at the crossroad between cardiovascular diseases and inflammation that can be modulated by cocoa.

3 Cocoa flavanols and transcription factors

Nuclear factor-kappaB (NF-κB) is one of the major inducible transcription factors controlling the inflammatory response, cellular proliferation/growth, and cellular adhesion [20]. In particular, NF-κB regulates the transcription of several pro-inflammatory cytokines, prostaglandins (PG), and NO, so that the modulation of the NF-κB signaling is important in the modulation of the inflammatory response [21], as in the case of specific chemoprotective phytochemicals [22]. Recent data have shown that the major anti-inflammatory component of cocoa is constituted by (−)-epicatechin [23]. Similarly, comprehensive experimental evidence has demonstrated that cocoa-derived monomeric ((−)-epicatechin and (+)-catechin) and dimeric flavanols reduce NF-κB activation thus resulting in reduced IL2 production and oxidative burst [24, 25]. While further studies are awaited, we note that the hypothesized impact on NF-κB activation might constitute a common trait to all cocoa anti-inflammatory effects.

4 Cytokine production: The crossroad of inflammation

The production by immune cells of pro-inflammatory cytokines is a critical step in the establishment and maintenance of an inflammatory status and is therefore, a primary target for putative anti-inflammatory interventions. However, inflammation can also be counteracted by enhancing the impact of other molecules of opposite effect such as tumor growth factor (TGF)-β and IL-4. Cocoa flavanols exert a significant effect on both these mechanisms, although the major resulting effect appears to be anti-inflammatory, particularly by way of TNFα levels [26–28], although data appear to change with different flavanol molecules, as observed in other cytokines discussed below. Table 1 summarizes the available evidence on cytokine changes following flavanol supplementation. Importantly, the resulting effect in systemic inflammatory diseases remains to be determined since most evidence was obtained in experimental models.

The stimulation of resting T cells by mitogenic lectins such as phytohemagglutinin (PHA), is known to activate a cascade of signaling events that includes the upregulation of transcription factors (*i.e.*, NF-κB, AP-1, and NF-AT) leading to the secretion of IL-2 [29], the major determinant in the mounting of an immune response. Cocoa flavanol oligomers (pentamer, hexamer, and heptamer) dramatically inhibit the expression of IL-2 mRNA by cultured PHA-stimulated PBMC [9, 30] while polyphenols reduce IL2 protein levels [31, 32]. In this latter study, the preparation used contained approximately 50% polyphenols, 3% of which were in the form of (−)-epicatechin.

Other important pro-inflammatory cytokines include IL1β and TNFα that induce the production of endothelial adhesion molecules and biochemical mediators such as growth factors, eicosanoids, NO, and C reactive protein, ultimately increasing the thrombogenic risk [33, 34]. Our group has extensively investigated the impact of cocoa flavanols in this scenario and demonstrated that the effect varies with the molecular structure of flavanols. In fact, smaller flavanols fractions (monomer through tetramer) exert an anti-inflammatory response by suppressing IL-1β mRNA expression and protein secretion while larger *M*r fractions (pentamer through decamer) increase IL-1β production [9, 35]. On the other hand, trimeric through decameric fractions were the most active on the TNFα production [26]. More studies are needed to understand the resulting cumulative effects of different formulas of cocoa *in vivo* to finely tune the anti-inflammatory effect of a flavanol mixture. In a similar fashion, a *M*r dependant effect was demonstrated in the modulation of anti-inflammatory cytokines such as IL4 with larger molecules (pentamers through decamers) enhancing IL4 production and smaller ones being non-active on resting PBMC. When PBMC were challenged with PHA; however, the opposite was observed with monomers through tetramers stimulating IL4 production [10].

Flavanol effects on TGFβ warrant further discussion since this cytokine is critical not only to inflammation but also in tissue repair [36] and in maintaining the physiological function of the endothelium and smooth muscle cells [37]. *In vivo* evidence supports an inverse correlation between circulating levels of activated TGFβ and atherosclerosis progression [38] while the production of TGFβ1 can cause extracellular matrix accumulation that is unfavor-

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Table 1. Modulatory effects on cytokines by cocoa flavanols in different cell conditions

Cytokine [ref]	Flavanol(s)	Effect	Readout	Cell conditions
TNF-α [26 – 28, 102]	All	↓	Protein/mRNA	Resting/stimulated
IL-1β [9, 35]	1,2,3,4-Mer	↓	Protein/mRNA	PHA-stimulated
	Polyphenols	↑	Protein/mRNA	PHA-stimulated
IL-2 [9, 30 – 32]	Polyphenols	↓	mRNA	PHA-stimulated
	5,6,7-Mer	↓	mRNA	PHA-stimulated
	Catechin	↓	Protein	PMA-stimulated
IL-4 [9, 10]	5,6,7,8,9,10-Mer	↑	Protein	Resting
	Monomer	↑	Protein	PHA-stimulated
IL-5 [50]	1,2,3,4,5-Mer	↑	Protein	PHA-stimulated
	Oligomer	↓	Protein	PHA-stimulated
TGF-β [43]	All	↑/↓	Protein	Resting

able in the injured vessel wall, leading to cardiac fibrosis [39] and determining CAD severity [40, 41]. Based on these data, TGFβ was targeted using several pharmacological and alternative approaches such as resveratrol, a dietary plant polyphenol also found in red wine [42]. Our group widely investigated the effects of cocoa flavanols on TGFβ production and observed opposite responses based on baseline TGFβ levels in healthy subjects [43], a finding that remains of non-unique interpretation.

IL5 is a non-typical anti-inflammatory cytokine in the fact that it regulates eosinophil infiltration and maturation in the allergic inflammation [44] and induces the maturation of IgA-producing plasma cells [45]. As a result of its atypical activity, it has been suggested that IL5 significantly determines the risk of specific oral infectious episodes and ultimately the incidence of CAD; this is partially confirmed by epidemiological data [46, 47]. In particular, chronic periodontal infections may induce an inflammatory immune response that contributes to CAD, possibly through an abnormal humoral response involving IgA [48, 49]. Our group was thus interested in determining whether cocoa flavanols could influence IL5 production in human PBMC. Smaller molecules (monomers through pentamers) enhanced mitogen-induced IL5 secretion whereas larger flavanols (hexamers through decamers) inhibited its production in stimulated PBMC [50]. These findings support the hypothesis that specific cocoa flavanols may preferentially stimulate IgA which could in turn reduce the risk for dental caries and infections.

5 Eicosanoids in the cocoa spectrum

Eicosanoids derived from the arachidonic acid metabolism (*i.e.*, thromboxanes (Tx), PG, leukotriens (Lt), and epoxides) are involved at different levels in the inflammatory pathogenesis of atherosclerosis and CAD through vascular changes [19]. It is of interest that these molecules exert a wide range of effects within the inflammatory cascade and contribute to determining the pro- or anti-inflammatory results. As an example, while TxA2 promotes platelet aggregation and vasoconstriction, PG properties are essentially pro-inflammatory and include vasodilation and inhibition of platelet aggregation, as supported by the impact of cyclooxygenase inhibition [51]. In a complementary fashion, a PG/Tx imbalance has been linked to the cardiovascular thrombogenesis leading to myocardial infarction and stroke [52]. Although the effect of cocoa flavanols on the arachidonic acid metabolism remains to be completely defined, certain flavanols have been shown to effectively inhibit both isoforms of cyclooxygenase [53, 54] producing PG and Tx. Lipooxygenase is the key enzyme in the Lt synthesis and has been identified as a target in several diseases including asthma [55]. High-dose cocoa flavanols are associated with a lower levels of plasma Lt and increased levels of PG starting 2 h after consumption, possibly through inhibition of the lipooxygenase enzyme [56]. The observed flavanol-induced changes on eicosanoid profiles are summarized in Table 2.

6 Cocoa flavanols and platelets

When the endothelium is damaged it becomes exposed to circulating platelets that adhere to the matrix and secrete adenosine diphosphate (ADP) and Tx, thus promoting their aggregation and initiating the thrombosis cascade with erythrocytes and lymphocytes [6]. Platelet activation also contributes to the development of the atherosclerotic plaque and is currently being evaluated as a promising therapeutic target [57]. The effects of flavanol-rich cocoa on platelet activation and function in healthy human subjects have been extensively investigated. Pearson and Holt [58] recently demonstrated that the consumption of flavanol-rich cocoa products inhibit epinephrine- and ADP-induced expression of specific glycoproteins and selectins thus ultimately reducing platelet activation. Data demonstrate an inhibitory effect ensuing after 6 h from consumption [59] induced mainly by trimers and pentamers of flavanols [60] and paralleled by coronary artery diameter increase [61]. In

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Table 2. Eicosanoid- and NO-based anti-inflammatory effects of cocoa flavanols.

	Ref	Readout	Impact
Eicosanoid metabolism	[56, 103]	Lipoxygenase activity	↓
	[104]	Thromboxane A_2/prostacyclin ratio	=
	[105]	Leukotriene/prostacyclin ratio	↓
	[106]	Lipoxygenase activity	↓
NO	[79]	eNOS activity	↑
	[83]	iNOS-derived NO	↓
	[23, 75, 77, 79, 106]	NO-mediated vasodilation	↑
	[75]	Nitrosylated and nitrosated species	↑
	[80, 81]	Peroxynitrite-induced oxidation	↓
	[80, 81]	Nitration of tyrosine	↓
	[90]	Plasma NO levels	↑
	[27, 107]	NO release by macrophages	↓
	[108]	Age-dependent NO-mediated vasodilation	↑
	[76]	NO-mediated blood pressure	↓

addition, acute exposure to flavanol-rich cocoa also modulates the formation of platelet microparticles as a markers of platelet activation [62]. Long-term cocoa supplementation also reduced platelet aggregation [63] to a degree comparable to acetylsalicilic acid [64] and platelet clotting function was significantly increased also in the case of casual cocoa intake [65]. Of note, these effects of cocoa flavanols failed to manifest an additive effect in human subjects when combined with acetylsalicilic acid [66] while being more prominent in subjects with an active lifestyle compared to sedentary controls [67].

7 Flavanols and NO-based mechanisms

NO has multiple roles in the regulation of the cardiovascular system. It is of particular note that different NO synthase (NOS) isoforms exert their actions in a constitutive (endothelial NOS, eNOS) or inducible (iNOS) fashion which contributes to differences in their functional effects and subcellular impact [68, 69], while the effect of the neuronal form (nNOS) remains poorly understood in cardiovascular health. NO is a potent vasodilator and maintains vascular tone, as shown in animal models lacking specific NOS isoforms [70]. Further, endothelium-derived NO also counteracts leukocyte recruitment and platelet aggregation to the site of inflammation [71, 72] thus exerting a significant anti-inflammatory effect. In turn, attracted leukocytes stimulate local NO production [72]. On a different level, NO is critical in determining the oxidative stress in several conditions by reacting with the superoxide to produce more reactive oxygen species. Further, NO and reactive oxygen species may mediate the flavanol induced activation of the antioxidant response element (ARE)-driven transcription of phase II detoxifying and antioxidant defense enzymes in the endothelium [73]. Table 2 illustrates the NO-mediated changes induced by cocoa flavanols.

The effects of cocoa flavanols on NO-mediated pathways are multiple and are in some cases inferred from the observed endothelial changes, as exemplified by the impact of flavanols on the circulating levels of nitrosylated species as a marker of endothelial function [74]. A human study on the effects of a single dose of a cocoa-rich drink demonstrated a significant amelioration of the endothelial dysfunction in patients with at least one cardiovascular risk factor [75] while longer periods of supplementation led to significant lowering of blood pressure [76]. A longer cocoa supplementation (five days) also confirmed these observation and further, demonstrated that the effects were mediated by NO [77]. From an epidemiological standpoint, data from a study in San Blas, Panama, suggest that the high cocoa intake in this population might lead to significantly reduced incidence rates for cardiovascular and neoplastic diseases [78]. Larger flavanol molecules appear to be more active in this scenario compared to smaller ones [79]. When the NO-based oxidative stress changes were utilized as endpoints, flavanols elicited a significant benefit in an experimental model mainly by enhancing the anti-oxidant mechanisms [80–82]. In this latter evidence; however, tetramers appeared as the most active flavanol molecules. Finally, it is of note that macrophages challenged by bacterial stimuli produced lower amounts if NO in the presence of cocoa extract [83]. The mechanisms of flavanol action on NO-mediated pathways appear to be mediated by NADPH in the case of mono-*O*-methylated flavanols [84]. A growing amount of data from short term human studies are being reported and are consistent with an anti-oxidant effects of dark chocolate (Table 3). Of note, in specific cases, flavanol-free white chocolate was utilized as control and confirmed the superior impact [76].

8 Cocoa flavanols and neutrophils

Research efforts are currently dedicating a growing attention to the innate immunity compartment to discriminate novel approaches to enhance the response to infections. To further characterize cocoa direct influence on neutrophils,

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Table 3. Anti-oxidant effects of single doses or short supplementations of dark chocolate in healthy human subjects

Supplementation	Duration	*N*	Refs.	Change in oxidative stress
Dark chocolate	Single dose	20	[109]	=
Dark chocolate	Single dose	10	[60]	↓
Dark chocolate	Single dose	12	[110]	↓
Dark chocolate	Single dose	17	[111]	↓
Dark chocolate	2 wk	21	[112]	=
Dark chocolate	4 wk	23	[104]	↓
Dark chocolate	3 wk	45	[113]	=
Dark chocolate + cocoa powder	4 wk	23	[104]	↓
Dark chocolate + cocoa powder	6 wk	25	[114]	↓/↑
Milk chocolate with flavanols	14 days	28	[99]	↓
Dark chocolate	18 wk	22	[76]	↓
Cocoa powder	12 wk	13	[115]	↓

Different oxidative stress markers were used in these studies and the resulting effects are illustrated for clarity purposes.

our group most recently investigated the influence of cocoa flavanols on LPS-induced oxidative burst, adhesion molecule expression, and apoptosis. Our data demonstrate that cocoa monomer flavanols and procyanidins have a significant anti-inflammatory effect, inhibit the neutrophil oxidative bursts (one of the earliest events characterizing cell activation), and reduce the expression of adhesion molecules. These data provide evidence that cocoa flavanols can inhibit the LPS-induced production of ROS and oxidative burst, modulate adhesion molecules that characterize neutrophil activation, and restore the cell natural apoptosis rate. Mechanistically, this allows one to hypothesize that these effects are mediated by the MAPK pathway activation. The implications of atherosclerosis and immunity are also well illustrated in autoimmune disease [85 – 89].

9 *In vivo* studies of flavanols and cardiovascular endpoints

Most human evidence of the effects of cocoa flavanols is obtained from observational studies that cumulatively suggest that these decrease the risk of death from CAD and stroke is the result of a beneficial action on the cardiovascular physiology, in particular directed at arterial hypertesion, insulin resistance, endothelial dysfunction, and hyperlipidemia. Earlier studies failed to demonstrate significant changes on specific readouts such as blood pressure following short supplementation periods [77]. When longer supplementations with lower doses [11, 76] or specific readouts in acute consumption [23, 90] were studied, benefits were observed and a recent meta-analysis confirmed this conclusion while failing to reproduce a similar effect for tea [91]. These reports cumulatively suggest that a moderate consumption of cocoa-rich foods might produce long-term beneficial effects on blood pressure, possibly mediated by a reduction in arterial wall stiffness and central aortic impulse pressure [92]. Further, acute supplementation with dark chocolate was also found to ameliorate insulin tolerance [93], the common ground of the metabolic syndrome epidemic [94]. Similarly, flavanol (from grapefruits) consumption was found to be beneficial by improving glucose tolerance [95]. With respect to endothelial dysfunction, the study of flow-mediated vasodilation in patients with CAD demonstrated a significant amelioration [75] by increasing plasma nitrites and nitrates, integrated NO biomarkers. It intriguing to note that also insulin resistance has an NO-mediated mechanism [96] thus possibly providing a novel link between different aspects of the metabolic syndrome. This is the case, for example, of the inferred effects of acute cocoa consumption on cerebral perfusion through vasodilation [97].

Epidemiological data based on nutritional questionnaire also support the benefits of cocoa on different features of the syndrome, as well represented by the study of the Kuna Indian population of Panama [98]. In fact, his very interesting population is virtually free of hypertension and cardiovascular diseases and this appears to change with the changes in cocoa dietary content. Further, Fraga *et al*. [99, 100] investigated the effects of cocoa flavanols on dyslipidemia and reported significant decrements following short term consumption, a results similar to what observed with black tea, particularly on LDL cholesterol. Lastly, the study of over 30 000 postmenopausal woman indicated that the dietary intake of flavanols and other micronutrients were inversely correlated with the risk cardiovascular mortality [101].

10 Concluding remarks and a glimpse of the future

A growing body of experimental data are becoming available to support a beneficial role of cocoa flavanols on cardiovascular health in the general population. The impact of cocoa is often superior to other functional flavanol-rich

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foods such as green tea or red wine, although there are few comparative studies available. In the near future, rigorous controlled human studies are warranted to investigate other possible areas of interest, as in the case of postmenopausal women, immunodeficient subjects, or patients undergoing antiviral treatments for chronic infections. Such studies will be based on experimental *ex vivo* evidence and will need to account for individual and dietary confounding factors, particularly when metabolic endpoints are sought.

The authors have declared no conflict of interest.

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