Cocoa Flavonols and Procyanidins Promote Transforming Growth Factor-β1 Homeostasis in Peripheral Blood Mononuclear Cells

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Evidence suggests that certain flavon-3-ols and procyanidins (FP) can have a positive influence on cardiovascular health. It has been previously reported that FP isolated from cocoa can potentially modulate the level and production of several signaling molecules associated with immune function and inflammation, including several cytokines and eicosanoids. In the present study, we examined whether FP fractions monomers through decamers modulate secretion of the cytokine transforming growth factor (TGF)-β1 from resting human peripheral blood mononuclear cells (PBMC). A total of 13 healthy subjects were studied and grouped according to their baseline production of TGF-β1. When cells from individuals with low baseline levels of TGF-β1 (n = 7) were stimulated by individual FP fractions (25 µg/ml), TGF-β1 release was enhanced in the range of 15%–66% over baseline (P < 0.05; monomer, dimer, and trimer). The low-molecular-weight FP fractions (pentamer) were more effective at augmenting TGF-β1 secretion than their larger counterparts (hexamer), with the monomer and dimer inducing the greatest increases (66% and 68%, respectively). In contrast to the above, TGF-β1 secretion from high TGF-β1 baseline subjects (n = 6) was inhibited by individual FP fractions (P < 0.05; trimer through decamer). The inhibition was most pronounced with trimeric through decameric fractions (28%–42%), and monomers and dimers moderately inhibited TGF-β1 release (17% and 23%, respectively). Given the vascular actions associated with TGF-β1, we suggest that in healthy individuals, homeostatic modulation of its production by FP offers an additional mechanism by which FP-rich foods can potentially benefit cardiovascular health.

Key words: cocoa; flavonoids; procyanidins; TGF-β1

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studies have shown that optimal levels of TGF-β1 are necessary to preserve endothelial function (33), inhibit smooth muscle cell proliferation (34), limit infarct size by attenuating cardiac myocyte apoptosis during early reperfusion (35), and prevent neutrophils and lymphocytes from adhering to endothelium (36, 37). However, when there is chronic vessel wall injury, excess production of TGF-β1 can enhance atherogenesis by promoting excessive extracellular matrix accumulation, leading to cardiac fibrosis (38). Therefore, homeostatic levels of TGF-β1 are important in maintaining cardiac function. In the present study, we examined the effects of purified cocoa FP fractions on TGF-β1 secretion from resting peripheral blood mononuclear cells (PBMC).

**Material and Methods**

**Cocoa Fraction Preparation.** Water-soluble FP fractions were prepared from Cocoa pro cocoa and were quantified by liquid chromatography (LC)-mass spectrometry (MS) as detailed by Adamson et al. (39), and were provided by Mars, Inc. (Hackettstown, NJ). The monomer fraction contains the flavan-3-ols, (+)-epicatechin, and (+) -catechin, whereas the oligomers of these monomeric units are known as procyanidins (Fig. 1). Purified fractions of monomer through decamers were investigated. The purified FP fractions contained less than 0.5% (w/w) of total alkaloids (theobromine and caffeine). The monomer and procyanidin composition, estimated by high-performance liquid chromatography (HPLC), and molecular weights of these preparations are shown in Table I. All samples were suspended in RPMI-1640 (Gibco-BRL, Gaithersburg, MD) with 10% heat-inactivated fetal bovine serum (Atlanta Biologicals, Norcross, GA). They were then diluted with the same medium to final concentrations of 25 μg/ml. This dose was shown to have the maximum stimulatory/inhibitory effects on the secretion of IL-1β, IL-2, IL-4, and IL-5 from PBMCs, as reported elsewhere (25–28). Therefore, to standardize our present investigation with our previous studies, we used the same dose for the analysis of TGF-β production. However, we are aware that this does place limits on our current study design.

**PBMC Isolation.** Peripheral blood from healthy volunteers was collected into sodium citrate-containing tubes and was mixed 1:1 with Hanks’ Balanced Salt Solution (HBSS; Gibco-BRL) without calcium chloride, magnesium chloride, or magnesium sulfate. The diluted blood was then layered over a Histopaque-1077 gradient (Sigma, St. Louis, MO) and was centrifuged at 500 g for 30 min at room temperature. PBMC were harvested from the interface layer, washed twice with HBSS, and then counted. The cells were resuspended in RPMI-1640 containing 10% fetal bovine serum and supplemented with 0.1% of a 50 mg/ml gentamicin solution (Gibco-BRL). PBMC concentration was adjusted to 2 × 10^6 viable cells/ml after estimation of viability by trypan blue exclusion assay. Viability was consistently greater than 96%.

**Culture of PBMC with Cocoa FP Fractions.** Five hundred microliters of a 1.0 × 10⁶ cell suspension was cultured with equal volumes of the various cocoa treatments at 37°C with 5% CO₂ in 48-well plates. Resting PBMC were incubated with individual cocoa FP fractions at 25 μg/ml. All treatments were performed in duplicate. Following a 72-hr incubation, the supernatant fractions were harvested for enzyme-linked immunoassay (ELISA) analysis.

**TGF-β1 (ELISA).** Culture supernatant fractions were harvested after 72 hr and were stored at −20°C until analysis by ELISA. Unlike other cytokines, TGF-β1 is secreted in a latent form complexed to a latency-associated protein for stabilization, allowing it to circulate extracellularly for long periods of time. Hence, extended incubation time will not significantly enhance TGF-β1 degradation. Rather, we believe that analysis at the 72-hr time point will be more representative of the true effects of the cocoa procyanidins. Ninety-six well Costar ELISA plates (catalog number 2592; Cambridge, MA) were coated with mouse anti-TGF-β1 supplied in the DuoSet Human TGF-β1 ELISA Development kit (R&D Systems, Minneapolis, MN). Cell culture supernatants containing the latent form of TGF-β1 were activated in an acidic environment (0.5-ml sample + 0.1 ml 1 N

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**Table I. Profile of the Individual Cocoa FP Fractions**

<table>
<thead>
<tr>
<th>Fration-name</th>
<th>Molecular weight (Da)</th>
<th>Procyanidin profile</th>
<th>%</th>
</tr>
</thead>
<tbody>
<tr>
<td>Monomer</td>
<td>290</td>
<td>Monomer</td>
<td>95</td>
</tr>
<tr>
<td>Dimer</td>
<td>578</td>
<td>Dimer</td>
<td>98</td>
</tr>
<tr>
<td>Trimer</td>
<td>866</td>
<td>Trimer</td>
<td>93</td>
</tr>
<tr>
<td>Tetramer</td>
<td>1154</td>
<td>Tetramer</td>
<td>93</td>
</tr>
<tr>
<td>Pentamer</td>
<td>1442</td>
<td>Pentamer</td>
<td>93</td>
</tr>
<tr>
<td>Hexamer</td>
<td>1730</td>
<td>Hexamer</td>
<td>89</td>
</tr>
<tr>
<td>Heptamer</td>
<td>2018</td>
<td>Heptamer</td>
<td>79</td>
</tr>
<tr>
<td>Octamer</td>
<td>2306</td>
<td>Octamer</td>
<td>76</td>
</tr>
<tr>
<td>Nonamer</td>
<td>2594</td>
<td>Nonamer</td>
<td>60</td>
</tr>
<tr>
<td>Octamer</td>
<td>28</td>
<td></td>
<td>28</td>
</tr>
<tr>
<td>Decamer</td>
<td>2882</td>
<td>Decamer</td>
<td>40</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>17</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>22</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>16</td>
</tr>
</tbody>
</table>

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**Chemical Structures of Flavon-3-ols and Procyanidins**

**A.**

[Chemical Structure Image]

**B.**

[Chemical Structure Image]

**Figure 1.** Chemical structures of flavon-3-ols and procyandins.
HCl) and were neutralized with 0.1 ml of 1.2 N NaOH/0.5 M HEPES. Subsequently, the activated supernates were measured for TGF-β₁ concentrations according to the manufacturers’ recommendations. The lowest TGF-β₁ standard for the ELISA system was 31.3 pg/ml.

**Statistics.** The effects of different cocoa FP fractions on the secretion of TGF-β₁ were examined in unstimulated resting PBMC. Results were compared by Student paired t test with a two-tailed P value (i.e., control cells without cocoa flavonoids versus cells treated with individual FP fractions). Significance was taken as P < 0.05.

**High versus Low Baseline Producers.** Individual baseline production of TGF-β₁ (measured at 72 hr) were divided into two groups based on terms of frequency above or below the median. Because we have an odd number of subjects (n = 13), the median value is assigned to the individual producing 7th [(n + 1)/2 th] largest baseline. One group lies on or below the median concentration of 5944 pg/ml in which we termed low baseline producers (LBP; 1609–5944 pg/ml), whereas the remaining subjects belong to a group of high baseline producers (HBP; 6,519–11,166 pg / ml) that lies above the median value. In addition the

![Figure 2](image)

**Figure 2.** A scatter plot of the individuals (n = 13) tested and their responses to each cocoa FP fraction. Each open circle represents a value in the form of the percentage of change (relative to baseline control) from an individual.
mean baseline values of LBP (3604 ± 568 pg/ml) and HBP (7910 ± 695 pg/ml) were statistically significant (P = 0.002) when the values from LBP were compared with that of HBP by Student paired $t$ test with a two-tailed $P$ value.

Results

Unstimulated resting PBMC were prepared and incubated with individual cocoa FP fractions at 25 µg/ml. TGF-β1 production was assessed in the supernatant fractions after 72 hr of incubation. ELISA analysis showed that interindividual variability was high among the 13 subjects tested. Figure 2 depicts the fluctuating response of these individuals to cocoa FP fractions in the form of percentage of change relative to the media baseline for each subject. However, when individuals were categorized based on their baseline production of TGF-β1 above and below the median, clear trends could be observed in the way TGF-β1 secretion was influenced by cocoa FP fractions. There were seven subjects in the LBP group whose baseline TGF-β1 concentrations ranged from 1609 to 5944 pg/ml (3604 ± 568 pg/ml). The HBP group displayed a mean baseline level (7910 ± 695 pg/ml; $n = 6$) of over twice the mean value observed with the LBP. Individual cocoa FP fractions were stimulatory for TGF-β1 release in the low LBP group (Fig. 3). In general, low-molecular-weight FP fractions (≤pentamer) were more effective than the larger oligomers in augmentation, inducing increases ranging from 30% to 68% over baseline (Table II), whereas the larger oligomers (≥hexamer) only moderately increased TGF-β1 secretion relative to baseline (15%–20%; Table II). The monomeric and dimeric FP fractions markedly enhanced TGF-β1 secretion in the LBP group, producing concentrations of 5913 ± 666 ($P = 0.0035$) and 6062 ± 667 ($P = 0.0027$) pg/ml, respectively. In contrast to the LBP group, individual cocoa FP fractions were inhibitory for TGF-β1 secretion in HBP (Fig. 4). The trimeric through decameric FP fractions significantly suppressed TGF-β1 levels by 28%–42% relative

![Low Baseline TGF-β1 Producers](image)

**Figure 3.** The effect of cocoa FP on secretion of TGF-β1 in low baseline producers. PBMC were incubated in the presence of individual cocoa fractions (25 µg/ml) for 72 hr before supernates were extracted for ELISA analysis (mean ± SEM; $n = 7$). Values induced from cocoa treatment were compared with control values (i.e., media baseline without cocoa) using a student paired $t$ test with a two-tailed $P$ value. *Significance was taken as $P < 0.05$.  

<table>
<thead>
<tr>
<th>FP fraction</th>
<th>Low baseline producers (3604 ± 568 pg/ml)</th>
<th>High baseline producers (7910 ± 695 pg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Monomer</td>
<td>+66% (5913 ± 666)</td>
<td>−17% (6559 ± 721)</td>
</tr>
<tr>
<td>Dimer</td>
<td>+68% (6062 ± 667)</td>
<td>−23% (6100 ± 1042)</td>
</tr>
<tr>
<td>Trimer</td>
<td>+42% (5104 ± 841)</td>
<td>−38% (4897 ± 1116)</td>
</tr>
<tr>
<td>Tetramer</td>
<td>+40% (5062 ± 731)</td>
<td>−38% (4912 ± 1126)</td>
</tr>
<tr>
<td>Pentamer</td>
<td>+30% (4698 ± 709)</td>
<td>−28% (5727 ± 858)</td>
</tr>
<tr>
<td>Hexamer</td>
<td>+17% (4214 ± 506)</td>
<td>−41% (4649 ± 710)</td>
</tr>
<tr>
<td>Heptamer</td>
<td>+20% (4311 ± 467)</td>
<td>−36% (5070 ± 557)</td>
</tr>
<tr>
<td>Octamer</td>
<td>+16% (4183 ± 421)</td>
<td>−39% (4813 ± 468)</td>
</tr>
<tr>
<td>Nonamer</td>
<td>+17% (4226 ± 732)</td>
<td>−34% (5212 ± 477)</td>
</tr>
<tr>
<td>Decamer</td>
<td>+15% (4147 ± 524)</td>
<td>−42% (4612 ± 680)</td>
</tr>
</tbody>
</table>

*Note. In each group, the mean values from cocoa-treated samples are compared with corresponding mean baseline value and are expressed as the percentage of change from media baseline control.*
to baseline (Table II), whereas the monomer and dimer showed moderate reductions (17% and 23%, respectively).

Discussion

FP have demonstrated the potential to modulate a wide variety of factors associated with vascular health. This includes antioxidant actions (9, 19–24), modulation of eicosanoids and NO and peroxynitrate levels (17, 18), and modulation of cytokine production (25–28). Here, we have extended these findings to TGF-β1 and have shown that cocoa FP fractions are able to promote homeostatic levels of TGF-β1 by either augmenting or suppressing TGF-β1 release depending on an individual’s baseline level of TGF-β1.

In the present study, an evaluation of baseline secretions of TGF-β1 showed a large interindividual variability among the subjects examined. Grainger et al. (40) have shown that the circulating concentration of TGF-β1 can vary considerably based on the genetic background of the individual. It is understandable that we saw such disparate baseline levels of TGF-β1 given that polymorphisms in the TGF-β1 gene can influence its production. Unfortunately, in the current study, we were unable to perform genotypic analysis on the subjects tested. Moreover, the subjects tested were healthy and free of any illness leading up to the blood draw. However, a prefasting period was not required for the volunteers, and, therefore, food consumption could have contributed to the observed variability.

Nevertheless, it is clear that in our study, cocoa FP fractions were stimulatory for TGF-β1 protein secretion in PBMC from subjects whose baseline levels of TGF-β1 were low (3604 ± 568 pg/ml). In contrast to LBP, PBMC from HBP individuals (7910 ± 695 pg/ml) showed suppressed TGF-β1 production after incubation with FP fractions. It is possible that HBP were primed to produce TGF-β1 prior to collecting blood from these subjects, and that cocoa fractions may have exacerbated the release of TGF-β1 early on during the incubation period, leading to an inordinate amount of TGF-β1 that would have negatively inhibited the further release of this protein and resulted in reduced levels displayed at the 72-hr time point. Therefore, our measurements from HBPs treated with cocoa represent cells that, at the 72-hr time point, are refractory to the stimulatory properties of cocoa. Contrary to HBPs, the cells from LBPs were not primed to release TGF-β1 prior to culture, and are therefore capable of induction after cocoa stimulation. The ability of cocoa to enhance secretion over baseline of LBPs ranged from 4182 ± 421 pg/ml (octomer) to 6062 ± 667 pg/ml (dimer). This range of stimulation is still below the baseline levels of HBPs (6,519–11,166 pg/ml), suggesting that the measurements taken from cocoa-treated LBPs is likely to represent a point on the incline of the production curve. If we were to perform an kinetic analysis of TGF-β1 secretion after the 72-hr time point from LBPs, we suspect that the protein levels would continue to climb past the baseline levels detected in the HBPs until a threshold is reached where a negative feedback loop prevents further secretion of TGF-β1. Taken together, our data suggests that FP fractions can directly stimulate the production of TGF-β1 from LBP, while indirectly suppressing secretion from HBP.

Figure 4. The effect of cocoa FP on secretion of TGF-β1 in high baseline producers. PBMC were incubated in the presence of individual cocoa fractions (25 µg/ml) for 72 hr before supernates were extracted for ELISA analysis (mean ± SEM; n = 7). Values induced from cocoa treatment were compared with control values (i.e., media baseline without cocoa) using a student paired t test with a two-tailed P value. *Significance was taken as P < 0.05.

COCOA AND TGF-β1
HBP, possibly due to a regulatory feedback mechanism caused by excess production of TGF-β1.

TGF-β1 is a multifunctional protein thought to be involved in a variety of physiological processes (29, 30). In particular, it has received attention as a potential mediator of cardiovascular protection since Grainger and Metcalfe proposed their protective cytokine hypothesis (32, 40). This hypothesis is based on the evidence that TGF-β1 actively maintains the normal physiological phenotype of endothelial cells and smooth muscle cells in the arterial vessel wall, thereby inhibiting activation of endothelial cells, as well as suppressing migration, dedifferentiation, and proliferation of smooth muscle cells induced by atherogenic agents. In support of TGF-β1 as an inhibitor of atherogenesis, in vivo studies have shown decreased levels of the active form of TGF-β1 in subjects with advanced atherosclerosis (32). On the other hand, excess production of TGF-β1 can cause extracellular matrix accumulation that is unfavorable in the injured vessel wall, consequently leading to cardiac fibrosis (38). A study exploring the association between TGF-β1 and coronary heart disease (CHD) demonstrated that an increase in the active form of TGF-β1 was associated with the occurrence and severity of CHD (41). Furthermore, another investigation displayed a correlation between a high-producing TGF-β1 genotype and an early onset of coronary vasculopathy after cardiac transplantation (42).

With TGF-β1 being a potent modulator of the cardiovascular system, it is understandable that considerable research has been devoted to the manipulation of its production and activity for therapeutic purposes. A variety of agents have been suggested to augment the production of TGF-β1, Metcalfe et al. (43) suggested that tamoxifen reduced the formation of lipid lesions, in part by elevating circulating concentrations of TGF-β1 in mice subjected to a high-fat diet; although consistent with this, Djurovic et al. (44) reported that postmenopausal women undergoing hormone replacement therapy showed increased plasma concentrations of TGF-β1, suggesting a possible avenue to reduce the risk of cardiovascular disease. Contrary to screening agents for their ability to induce endogenous TGF-β1, the discovery of antagonists for TGF-β1 might be valuable in the treatment of fibrotic diseases. Decorin, a natural inhibitor of TGF-β1, has been used to successfully suppress TGF-β1-mediated tissue fibrosis in the rat kidney (45). In addition, resveratrol, a dietary plant polyphenol, was reported to have a protective effect against dysfunctions in vascular smooth muscle cells, in part due to its ability to inhibit TGF-β1 mRNA (46).

In our previous studies on the effects of cocoa FP on cytokine production, a biphasic type effect was observed, with the larger and smaller procyanidin fractions showing differential effects on cytokine production. In resting PBMC, the larger FP oligomers (≥hexamer) markedly stimulated IL-1β and IL-4 release, whereas the smaller fractions inhibited their secretion (25, 27). However, in the present investigation, we observed that the effect of FP on TGF-β1 release was dependent not only on the molecular size of the FP fractions, but also by the capacity of the PBMC to secrete TGF-β1. Some fractions were more active, with the general effect of cocoa fractions in each individual being similar in that they stimulated TGF-β1 release from LBP, and inhibited TGF-β1 secretion from HBP. Given the above, we suggest that cocoa FP, in concert with their effects on platelet reactivity (12, 13), eicosanoid production (18, 47), and vascular reactivity (14–16), may have protective effects on the cardiovascular system by promoting the maintenance of homeostatic TGF-β1 levels.

Although cocoa FP have demonstrated interesting properties in vitro, the critical question remains whether the same effects can be observed in vivo. Indeed, the bioavailability of procyanidins have been documented through radiolabeling techniques (48). However, those studies did not address the issue of whether the procyanidins were intact or depolymerized before absorption. In a recent report, Holt et al. (5) showed that cocoa procyanidin dimer B2 [epicatechin-(4β-8)-epicatechin] can be detected in the plasma of humans within 30 min of consuming a cocoa beverage. Clearly, further in vivo studies are needed to document the efficacy of cocoa flavonols as a cardiovascular modulator. Nevertheless, this study provides additional data in favor of in vivo analysis of the health benefits of dietary FP from a variety of foods, including FP-rich cocoa and chocolate (9–11).