Cocoa Flavonols and Procyanidins Promote Transforming Growth Factor-β_1 Homeostasis in Peripheral Blood Mononuclear Cells[1]

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Evidence suggests that certain flavan-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)-β_1 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-β_1. When cells from individuals with low baseline levels of TGF-β_1 (n = 7) were stimulated by individual FP fractions (25 μg/ml), TGF-β_1 release was enhanced in the range of 15%–66% over baseline (P < 0.05; monomer, dimer, and tetramer). The low-molecular-weight FP fractions (≤pentamer) were more effective at augmenting TGF-β_1 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-β_1 secretion from high TGF-β_1 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-β_1 release (17% and 23%, respectively). Given the vascular actions associated with TGF-β_1, 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. Exp Biol Med 228:93–99, 2003

Key words: cocoa; flavonols; procyanidins; TGF-β_1

Since the introduction of cocoa by the Mayan and Olmec civilizations of Central America, humans have been consuming cocoa as food, drink, and medicine (1). Derived from the seeds of *Theobroma cacao*, some cocoas and derivative products, e.g., chocolate, can offer a rich source of flavonoids. Particularly abundant in cocoa are the flavan-3-ol monomers, epicatechin and catechin, and their related oligomers, the procyanidins (FP) (2–4). It has been reported, in both humans and rats, that the flavan-3-ols can be rapidly absorbed from the gut, as can low amounts of the smaller oligomers (5–8). The presence of flavonoids in notable concentrations in certain foods (e.g., select fruits, vegetables, and legumes) has triggered numerous studies on their potential health benefits. Consumption of certain flavonoid-rich foods has been associated with anticarcinogenic and cardiovascular health effects (9–11). More recently, select FP have demonstrated activity *in vivo* and *in vitro* suggestive of cardiovascular benefits. The focus of this research has generally centered on the concept that FP-rich foods might modulate platelet function (12, 13) and vascular reactivity (14–16). The influence of select FP on mechanisms responsible, at least in part, for this vascular activity have also been investigated to some extent with respect to FP modulation of nitric oxide (NO) (14, 17) and select eicosanoid levels (18) as well as oxidant defense (6, 19–24). Complementing the above, select FP have demonstrated the potential to modulate the production of several immunomodulatory cytokines, including interleukin (IL)-1β (25), IL-2 (26), IL-4 (27), and IL-5 (28). Transforming growth factor (TGF)-β_1 is another immunomodulatory cytokine (29, 30) that is receiving attention as a possible mediator of cardiovascular health (31, 32).

TGF-β is a pleiotropic cytokine that plays an important role in regulating repair and regeneration after tissue injury (29, 30). It consists of a family of three isoforms (TGF-β1, 2, and 3) that are structurally and functionally related (29, 30). The most abundant isoform, TGF-β_1, is synthesized by a variety of cells in its latent form complexed with the latency-associated protein (29, 30). In a vascular context,

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studies have shown that optimal levels of TGF-β_1 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-β_1 can enhance atherogenesis by promoting excessive extracellular matrix accumulation, leading to cardiac fibrosis (38). Therefore, homeostatic levels of TGF-β_1 are important in maintaining cardiac function. In the present study, we examined the effects of purified cocoa FP fractions on TGF-β_1 secretion from resting peripheral blood mononuclear cells (PBMC).

Material and Methods

Cocoa Fraction Preparation. Water-soluble FP fractions were prepared from Cocoapro 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

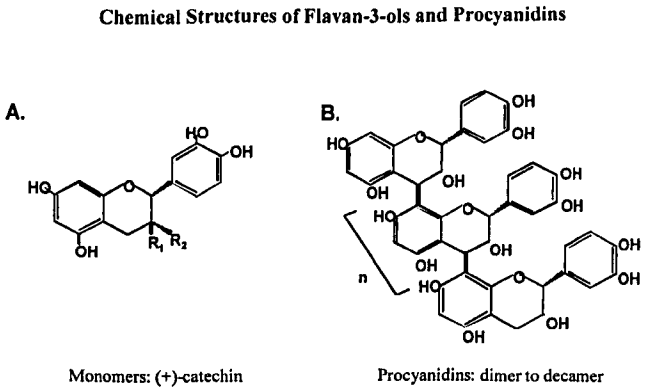

Figure 1. Chemical structures of flavan-3-ols and procyanidins.

Table I. Profile of the Individual Cocoa FP Fractions

Fration-name	Molecular weight (Da)	Procyanidin profile	%
Monomer	290	Monomer	95
Dimer	578	Dimer	98
Trimer	866	Trimer	93
Tetramer	1154	Tetramer	93
Pentamer	1442	Pentamer	93
Hexamer	1730	Hexamer	89
Heptamer	2018	Heptamer	79
		Hexamer	18
Octamer	2306	Octamer	76
		Heptamer	16
Nonamer	2594	Nonamer	60
		Octamer	28
Decamer	2882	Decamer	40
		Nonamer	17
		Octamer	22
		Heptamer	16

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 that 96%.

Culture of PBMC with Cocoa FP Fractions. Five hundred microliters of a 1.0×10^6 cell suspension was cultured with equal volumes of the various cocoa treatments at 37°C with 5% CO_2 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-β_1 (ELISA). Culture supernatant fractions were harvested after 72 hr and were stored at −20°C until analysis by ELISA. Unlike other cytokines, TGF-β_1 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-β_1 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 EIA 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 supernates containing the latent form of TGF-β_1 were activated in an acidic environment (0.5-ml sample + 0.1 ml 1 *N*

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-β_1 concentrations according to the manufacturers' recommendations. The lowest TGF-β_1 standard for the ELISA system was 31.3 pg/ml.

Statistics. The effects of different cocoa FP fractions on the secretion of TGF-β_1 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-β_1 (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

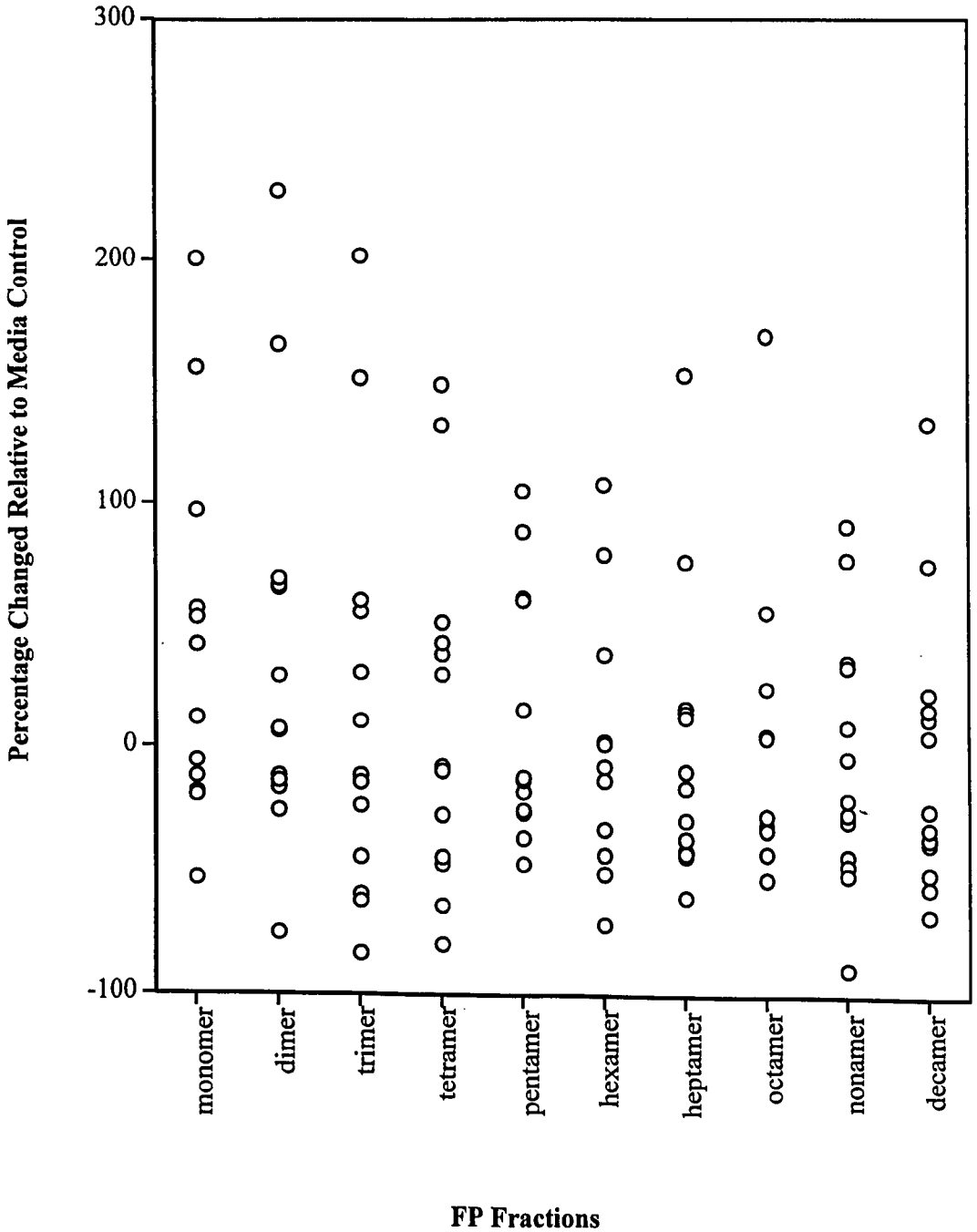

Figure 2. A scatter plot of the individuals ($n = 13$) tested and their responses to each cocoa FP fraction. Each open circle represents a value in the form of the percentage of change (relative to baseline control) from an individual.

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-β_1 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-β_1 above and below the median, clear trends could be observed in the way TGF-β_1 secretion was influenced by cocoa FP fractions. There were seven subjects in the LBP group whose baseline TGF-β_1 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-β_1 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-β_1 secretion relative to baseline (15%–20%; Table II). The monomeric and dimeric FP fractions markedly enhanced TGF-β_1 secretion in the LBP group, producing concentrations of 5981 ± 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-β_1 secretion in HBP (Fig. 4). The trimeric through decameric FP fractions significantly suppressed TGF-β_1 levels by 28%–42% relative

Table II. Effect of Cocoa FP Fractions on TGF-β_1 Secretion in Low (n = 7) and High (n = 6) Baseline Producers

FP fraction	Low baseline producers (3604 ± 568 pg/ml)	High baseline producers (7910 ± 695 pg/ml)
Monomer	+66% (5981 ± 666)	−17% (6559 ± 721)
Dimer	+68% (6062 ± 667)	−23% (6100 ± 1042)
Trimer	+42% (5104 ± 841)	−38% (4897 ± 1116)
Tetramer	+40% (5062 ± 731)	−38% (4912 ± 1126)
Pentamer	+30% (4698 ± 709)	−28% (5727 ± 858)
Hexamer	+17% (4214 ± 506)	−41% (4649 ± 710)
Heptamer	+20% (4311 ± 467)	−36% (5070 ± 557)
Octamer	+16% (4183 ± 421)	−39% (4813 ± 468)
Nonamer	+17% (4226 ± 732)	−34% (5212 ± 477)
Decamer	+15% (4147 ± 524)	−42% (4612 ± 680)

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.

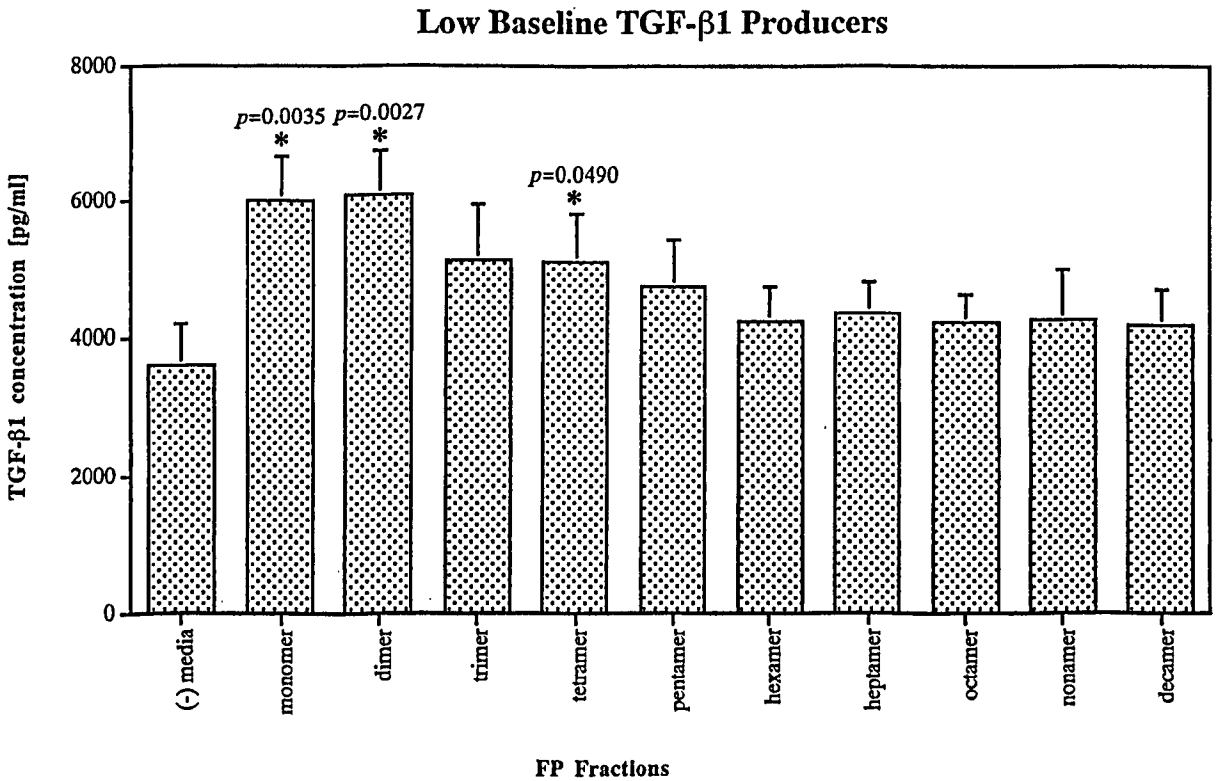

Figure 3. The effect of cocoa FP on secretion of TGF-β_1 in low baseline producers. PBMC were incubated in the presence of individual cocoa fractions (25 μg/ml) for 72 hr before supernates were extracted for ELISA analysis (mean ± SEM; n = 7). Values induced from cocoa treatment were compared with control values (i.e., media baseline without cocoa) using a student paired t test with a two-tailed P value. *Significance was taken as $P < 0.05$.

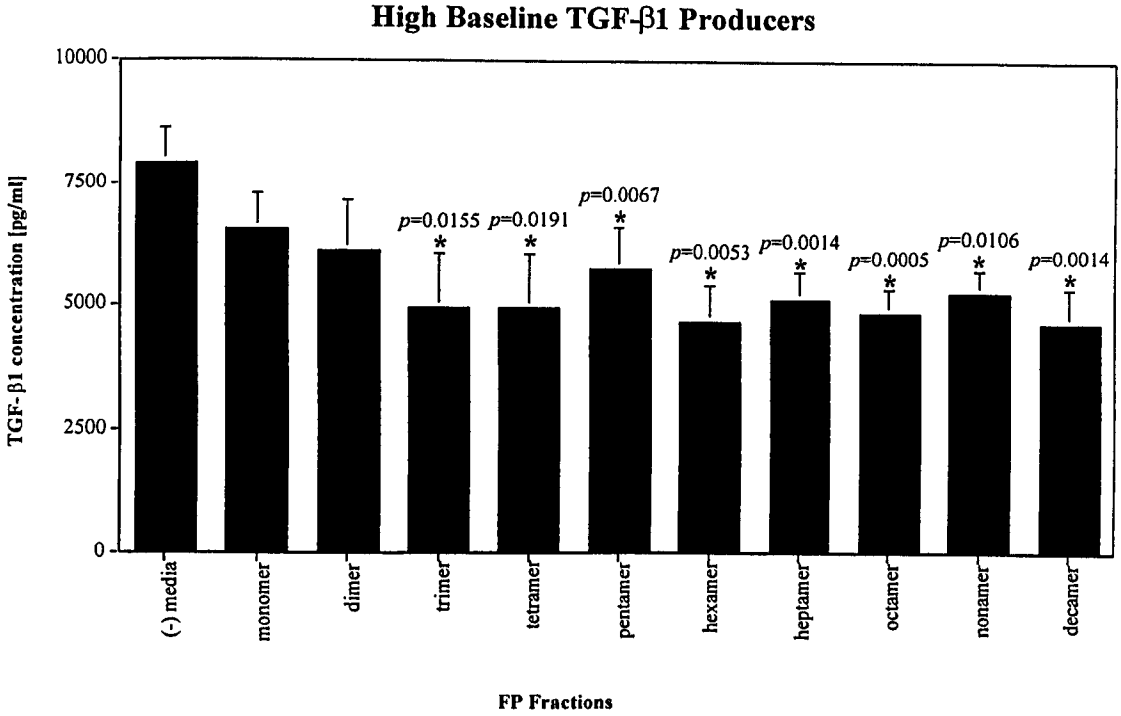

Figure 4. The effect of cocoa FP on secretion of TGF-β_1 in high baseline producers. PBMC were incubated in the presence of individual cocoa fractions (25 μg/ml) for 72 hr before supernates were extracted for ELISA analysis (mean ± SEM; $n = 7$). Values induced from cocoa treatment were compared with control values (i.e., media baseline without cocoa) using a student paired t test with a two-tailed P value. *Significance was taken as $P < 0.05$.

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-β_1 and have shown that cocoa FP fractions are able to promote homeostatic levels of TGF-β_1 by either augmenting or suppressing TGF-β_1 release depending on an individual's baseline level of TGF-β_1.

In the present study, an evaluation of baseline secretions of TGF-β_1 showed a large interindividual variability among the subjects examined. Grainger *et al.* (40) have shown that the circulating concentration of TGF-β_1 can vary considerably based on the genetic background of the individual. It is understandable that we saw such disparate baseline levels of TGF-β_1 given that polymorphisms in the TGF-β_1 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-β_1 protein secretion in PBMC from subjects whose baseline levels of TGF-β_1 were low (3604 ± 568 pg/ml). In contrast to LBP, PBMC from HBP individuals (7910 ± 695 pg/ml) showed suppressed TGF-β_1 production after incubation with FP fractions. It is possible that HBP were primed to produce TGF-β_1 prior to collecting blood from these subjects, and that cocoa fractions may have exacerbated the release of TGF-β_1 early on during the incubation period, leading to an inordinate amount of TGF-β_1 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-β_1 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-β_1 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-β_1. Taken together, our data suggests that FP fractions can directly stimulate the production of TGF-β_1 from LBP, while indirectly suppressing secretion from

HBP, possibly due to a regulatory feedback mechanism caused by excess production of TGF-β_1.

TGF-β_1 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-β_1 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-β_1 as an inhibitor of atherogenesis, *in vivo* studies have shown decreased levels of the active form of TGF-β_1 in subjects with advanced atherosclerosis (32). On the other hand, excess production of TGF-β_1 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-β_1 and coronary heart disease (CHD) demonstrated that an increase in the active form of TGF-β_1 was associated with the occurrence and severity of CHD (41). Furthermore, another investigation displayed a correlation between a high-producing TGF-β_1 genotype and an early onset of coronary vasculopathy after cardiac transplantation (42).

With TGF-β_1 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-β_1. Metcalfe *et al.* (43) suggested that tamoxifen reduced the formation of lipid lesions, in part by elevating circulating concentrations of TGF-β_1 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-β_1, suggesting a possible avenue to reduce the risk of cardiovascular disease. Contrary to screening agents for their ability to induce endogenous TGF-β_1, the discovery of antagonists for TGF-β_1 might be valuable in the treatment of fibrotic diseases. Decorin, a natural inhibitor of TGF-β_1, has been used to successfully suppress TGF-β_1-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-β_1 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-β_1 release was dependent not only on the molecular size of the FP fractions, but also by the capacity of the PBMC to secrete TGF-β_1. Some fractions were more active, with the general effect of cocoa fractions in each individual being similar in that they stimulated TGF-β_1 release from LBP, and inhibited TGF-β_1 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-β_1 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).

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