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Clovamide-Type Phenylpropenoic Acid Amides, *N*-Coumaroyldopamine and *N*-Caffeoyldopamine, Inhibit Platelet-Leukocyte Interactions via Suppressing P-Selectin Expression

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ABSTRACT

N-Coumaroyldopamine and *N*-caffeoyldopamine are clovamide-type phenylpropenoic acid amides found in *Theobroma cacao*. In this article, *N*-coumaroyldopamine and *N*-caffeoyldopamine were investigated to determine their effects on P-selectin expression and platelet-leukocyte interactions in vitro and in vivo models. At the concentration of 0.05 μM, they were able to inhibit P-selectin expression on the platelets by 33 ($P < 0.011$) and 30% ($P < 0.012$), respectively. The inhibition was partially blocked by β_2-adrenoceptor antagonists, suggesting that β_2 receptors are probably engaged in the inhibition. *N*-Caffeoyldopamine and *N*-coumaroyldopamine could also suppress platelet-leukocyte interactions in blood samples by 36 ($P < 0.013$) and 32% ($P < 0.011$), respectively, at the same concentration (0.05 μM). In an animal study, mice administrated orally with *N*-caffeoyldopamine (50 and 100 μg/35 g of body weight) also showed great reduction in the P-selectin expression and platelet-leukocyte interactions by 31 to 45% ($P < 0.011$) and 34 to 43% ($P < 0.014$), respectively. These data suggest that the clovamide-type phenylpropenoic acid amides are able to suppress platelet-leukocyte interactions via inhibiting P-selectin expression.

Activated platelets and platelet-leukocyte interactions are pathophysiologically involved in the progresses of several cardiovascular diseases such as atherosclerosis, angina, acute myocardial infarction, and ischemic cerebral stroke (Bouchard and Tracy, 2001; Krieglstein and Granger, 2001; Schror, 2003; Andrews et al., 2004; Huo and Ley, 2004). P-Selectin is a 140-kDa type-1 transmembrane glycoprotein belonging to the selectin family of cell adhesion receptors. The protein is involved in platelet-leukocyte interactions and platelet-endothelium interactions via binding to P-selectin ligand (PSGL-1) on leukocytes and endothelium (Blann et al., 2003; Merten and Thiagarajan, 2004). P-Selectin is stored in the α-granules of platelets. By the exposure of platelets to ADP (adenosine diphosphate), collagen, thrombin, thromboxane A2, or arachidonic acid, P-selectin is translocated to the cell surface, facilitating adhesion to leukocytes and/or endothelium and eliciting the cells to produce cytokines, tissue factors, and procoagulants (Andre, 2004; Vandendries et al., 2004; Chirinos et al., 2005). Because of the significant implication of P-selectin in the pathogenesis of coronary artery diseases and stroke, compounds able to suppress P-selectin expression and platelet-leukocyte interactions have been explored relentlessly.

Clovamide-type phenylpropenoic acid amides are phytochemicals belonging to a group of phenylpropenoic acid amides found in plants such as *Lycium* spp., *Capsicum* spp., *Cannabis* spp., and *Theobroma cacao* (Yamamoto et al., 1991; Schmidt et al., 1996; Back et al., 2001; Alemanno et al., 2003; Cutillo et al., 2003; Wu et al., 2003). Clovamide-type phenylpropenoic acid amides were originally discovered as phytoalexins accumulating in response to wounding and pathogen attacks (Yamamoto et al., 1991; Cutillo et al., 2003; Wu et al., 2003). In our laboratory, clovamide-type phenylpropenoic acid amides found in *Theobroma cacao* have been studied to determine their effects on platelet functions, because the consumption of cocoa-derived products has been suggested to have beneficial effects on cardiovascular diseases, but little is known about the effects of the clovamide-type phenylpropenoic acid amides on the diseases (Rein et al., 2000; Visioli et

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ABBREVIATIONS: PSGL-1, P-selectin ligand; ICI 118551, (±)-1-[2,3-(dihydro-7-methyl-1*H*-inden-4-yl)oxy]-3-[(1-methylethyl)amino]-2 butanol; FITC, fluorescein isothiocyanate; PE, phycoerythrin.

al., 2000; Park, 2005). In this study, two clovamide-type phenylpropenoic acid amides (*N*-caffeoyldopamine and *N*-coumaroyldopamine; Fig. 1) were mainly investigated to determine their effects on P-selectin expression and platelet-leukocyte interactions using in vitro and in vivo models. The outcomes of this study may provide information regarding potential effects of plants with clovamide-type phenylpropenoic acids on cardiovascular diseases.

Materials and Methods

Materials

Alprenolol, propranolol, atenolol, metoprolol, butoxamine, ICI 118551, and other chemicals were purchased from Sigma Chemical Co. (St. Louis, MO). *N*-Coumaroyldopamine and *N*-caffeoyldopamine were synthesized and purified as described previously (Park and Schoene, 2002; Park, 2005). Collagen was obtained from Chrono-Log Corp. (Hampton, PA). U937 cells were purchased from American Type Culture Collection (Manassas, VA). Swiss-Webster mice 7 to 9 weeks old were purchased from Charles River Laboratories (Wilmington, MA).

Methods

Detection of P-Selectin Expression. Blood was collected from tails of mice and placed in siliconized microcentrifuge tubes containing 15% EDTA. The modified Tyrode's buffer [134 mM NaCl, 0.34 mM Na_2HPO_4, 2.9 mM KCl, 12 mM $NaHCO_3$, 20 mM HEPES, 5 mM glucose, and 0.35% (w/v) bovine serum albumin, pH 7.0] was added to bring the sample volume to 100 μl. From these diluted samples, aliquots were placed in 12 $\times$ 75-mm polypropylene tubes along with the appropriate antibody, collagen (2.5 μg/ml), and the modified Tyrode's buffer in a final volume of 200 μl. In vitro experiments, *N*-coumaroyldopamine and *N*-caffeoyldopamine were dissolved in ethanol and added to diluted blood samples, where the final ethanol volume never exceeded 0.5% (v/v) in both control and test tubes. Samples were analyzed for P-selectin (CD62p) expression on platelets within 1 h of the collection by flow cytometry (Chen et al., 2003; Nieswandt et al., 2005). Data were acquired for 10,000 platelets, and the extent of exposure of CD62p was determined as the measure of platelet activation (FACSCalibur flow cytometer and Cell Quest Pro software; BD Biosciences, San Jose, CA). Fluorescein isothiocyanate (FITC)-conjugated rat anti-mouse CD62p (P-selectin) monoclonal antibody and the isotype control were obtained from BD Biosciences (Tárnok et al., 1999; Ahn et al., 2005).

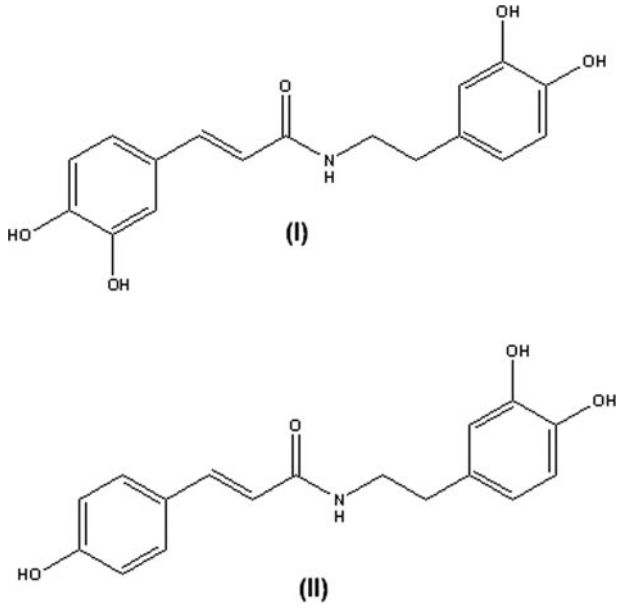

Fig. 1. Chemical structures of *N*-caffeoyldopamine and *N*-coumaroyldopamine. Chemical structures for *N*-coumaroyldopamine (I) and *N*-caffeoyldopamine (II).

Platelet-Leukocyte Interactions in Whole Blood. Blood samples were collected from mice tail in microcentrifuge tubes containing 3.8% sodium citrate (10 μl) and immediately adjusted to 100 μl with the modified Tyrode's buffer. The samples were treated with *N*-caffeoyldopamine or *N*-coumaroyldopamine before staining with antibodies to identify platelets and leukocytes. Antibodies used to identify blood cells were as follows: *R*-phycoerythrin (PE) rat anti-mouse CD41 and isotype control *R*-PE-conjugated rat $IgG_{1\kappa}$ (BD Biosciences) for platelets and FITC rat anti-mouse CD45 and isotype control FITC-conjugated rat IgG_{2b} (Serotec, Raleigh, NC) for leukocytes. Platelet-leukocyte interactions were determined by flow cytometry as described previously (Tárnok et al., 1999).

Animal Experiments. Swiss-Webster mice 3 to 4 weeks old were purchased from Charles River. Mice were placed in standard cages and housed in the environmentally controlled Beltsville Human Nutrition Research Center Animal Facility. The animal room was maintained at 20°C and 55% relative humidity. On arrival, mice were fed AIN-76A purified diet that provides the recommended allowance of all nutrients required for maintaining optimal health but lacking *N*-caffeoyldopamine tested in the study, where the diet was analyzed by high-performance liquid chromatography to confirm that *N*-caffeoyldopamine was not in the diet. After 8 weeks, mice were assigned and remained to three groups ($n = 5$) for 10 weeks. Mice in the first group (control) were administrated orally using a dosing needle with distilled water (100 μl), and mice in the second group (T1) were administrated orally with distilled water (100 μl) containing *N*-caffeoyldopamine (50 μg) and mice in the last group (T2) with *N*-caffeoyldopamine (100 μg). Blood was collected from mice once a week for 10 weeks after the group assignment. Blood was collected via tail-bleeding technique 30 min after the oral administration, and blood samples from each group were used for P-selectin and platelet-leukocyte aggregation assays.

Statistical Analysis. Treatments effects on the parameters measured were compared by analyzing the means for differences using either analysis of variance or analysis of variance by ranks as appropriate. Differences were considered to be significant when $p < 0.05$. Data points represent the mean $\pm$ S.D. of three or more samples.

Results

Effects of *N*-Coumaroyldopamine and *N*-Caffeoyldopamine on Collagen-Induced and Basal P-Selectin Expression on Platelets. The effects of *N*-coumaroyldopamine and *N*-caffeoyldopamine on P-selectin expression were determined by measuring P-selectin (CD62p) expression on platelets. Blood samples were prepared as described under *Materials and Methods*. Then, blood samples were analyzed for CD62p exposure on platelet membranes by flow cytometry. As shown in Fig. 2, both *N*-caffeoyldopamine and *N*-coumaroyldopamine at the concentration of 0.05 μM were able to suppress P-selectin expression on mice platelets by 33 ($P < 0.011$) and 30% ($P < 0.012$), respectively. Although *N*-caffeoyldopamine was a little more potent than *N*-coumaroyldopamine in suppressing P-selectin expression on the platelets, both compounds were able to suppress P-selectin expression significantly at relatively low concentrations (0.05, 0.25, and 0.50 μM). We also studied the effects of *N*-coumaroyldopamine and *N*-caffeoyldopamine on basal P-selectin expression (without collagen treatments) in blood samples because the basal expression is often more relevant physiologically than the collagen-induced expression to consequent platelet inter-

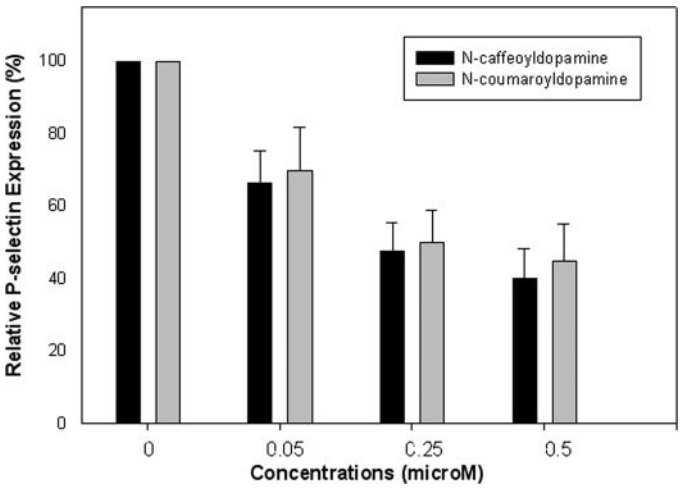

Fig. 2. Effects of *N*-caffeoyldopamine and *N*-coumaroyldopamine on collagen-induced P-selectin expression on platelets. Platelets were incubated with *N*-caffeoyldopamine or *N*-coumaroyldopamine for 10 min and treated with collagen (2.5 μg/ml). P-Selectin expression was determined as described under *Materials and Methods*. Data points represent the mean ± S.D. of three or more samples. All treatment levels resulted in a significant reduction in the relative expression of P-selectin on platelets compared with control samples ($P < 0.015$).

actions with leukocytes and/or endothelial cells in the blood. *N*-Coumaroyldopamine and *N*-caffeoyldopamine were also able to inhibit the basal P-selectin expression as much as 30% ($P < 0.017$) at the concentration of 0.05 μM (Fig. 3). These data indicate that *N*-coumaroyldopamine and *N*-caffeoyldopamine can inhibit significantly the basal and collagen-induced P-selectin expression on platelets at relatively low concentrations (0.05, 0.25, and 0.50 μM).

Effects of β-Blockers on P-Selectin Expression Inhibition by *N*-Caffeoyldopamine. Upon the exposure of platelets to collagen and other activators, P-selectin is translocated to the surface of platelets. The P-selectin expression can be inhibited by increasing intracellular cyclic AMP (cAMP) (Ryningen et al., 1999; Libersan et al., 2003). Previously, *N*-caffeoyldopamine and *N*-coumaroyldopamine were reported to produce cAMP via β_2 adrenoceptor in our laboratory (Park, 2005). Therefore, the involvement of cAMP was investigated related to the inhibition of P-selectin expression. In this experiment, *N*-caffeoyldopamine was used because of its better efficacy in inhibiting P-selectin expression on platelets, and three different types of β-blockers (nonselective β-adrenoceptor antagonists, β_1-specific antagonists, and β_2-specific antagonists) were used to block effects of *N*-caffeoyldopamine on P-selectin expression. For blocking β adrenoceptors on the platelets, nonselective β-adrenoceptor antagonists (alprenolol and propranolol), β_1-specific antagonists (atenolol and metoprolol) and β_2-specific antagonists (butoxamine and ICI 118551) were pretreated before the addition of *N*-caffeoyldopamine to blood samples. As shown in Fig. 4, A and C, the pretreatment of nonselective β-adrenoceptor antagonists (alprenolol and propranolol) and β_2-specific antagonists (butoxamine and ICI 118551) significantly blocked the inhibitory effects of *N*-caffeoyldopamine on the P-selectin expression induced by collagen. However, the pretreatment of β_1-specific antagonists (atenolol and metoprolol) did not block the inhibitory effects of *N*-caffeoyldopamine (Fig. 4B). The similar experiments were performed using platelets without the treatment of collagens, and similar data were obtained that the β-adrenoceptor antagonists and the β_2-specific antagonists partially blocked the inhibitory effects of *N*-caffeoyldopamine on the P-selectin expression but not the β_1-specific antagonists. These data indicate that the inhibitory effects of the clovamide-type phenylpropenoic acid amides on P-selectin expression are likely to be via producing cAMP via β_2 adrenoceptors. However, it should be noticed that the inhibition of P-selectin expression by *N*-caffeoyldopamine may be from more than producing cAMP via β_2 adrenoceptors, because β_2-specific antagonists (butoxamine and ICI 118551) could not prevent the inhibitory effects completely in spite of high concentrations (2.5 and 5 μM) of β_2-specific antagonists used in the experiments (Table 1).

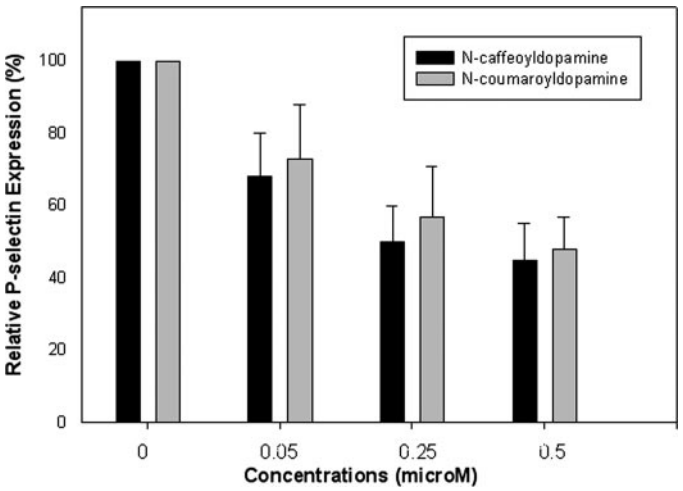

Fig. 3. Effects of *N*-caffeoyldopamine and *N*-coumaroyldopamine on basal P-selectin expression on platelets. Platelets were incubated with *N*-caffeoyldopamine or *N*-coumaroyldopamine for 10 min. P-Selectin expression was determined as described under *Materials and Methods*. Data points represent the mean ± S.D. of three or more samples. All treatment levels resulted in a significant reduction in the relative expression of P-selectin on platelets compared with control samples ($P < 0.017$).

Effects of Cilostazol on P-Selectin Expression. The data suggest that the cAMP production is involved in the inhibition of P-selectin. If so, the inhibition may be also effected by compounds to modulate levels of cAMP such as phosphodiesterase III inhibitors (Kariyazono et al., 2001; Ito et al., 2004). Therefore, we evaluated the effects of cilostazol, a phosphodiesterase III inhibitor, on the P-selectin expression. As shown in Fig. 5, the data indicate that cilostazol is able to inhibit P-selectin expression in a concentration-dependent manner. This result also suggests the involvement of cAMP in the inhibition of P-selectin in platelets.

Effects of *N*-Caffeoyldopamine and *N*-Coumaroyldopamine on Platelet-Leukocyte Interactions in Whole Blood. Recent studies suggest that platelet-leukocyte interactions may be a better marker of platelet activation than P-selectin expression because the interactions reflect a series of pathophysiological processes of platelets implicated in cardiovascular diseases (Bouchard and Tracy, 2001; Krieglstein and Granger, 2001). Therefore, the effects of *N*-caffeoyldopamine and *N*-coumaroyldopamine on platelet-leukocyte interactions were investigated. Blood samples were prepared, and platelet-leukocyte interactions were determined using flow cytometry as described under *Materials and Methods*. As shown in Fig. 6A, both *N*-caffeoyldopamine and *N*-cou-

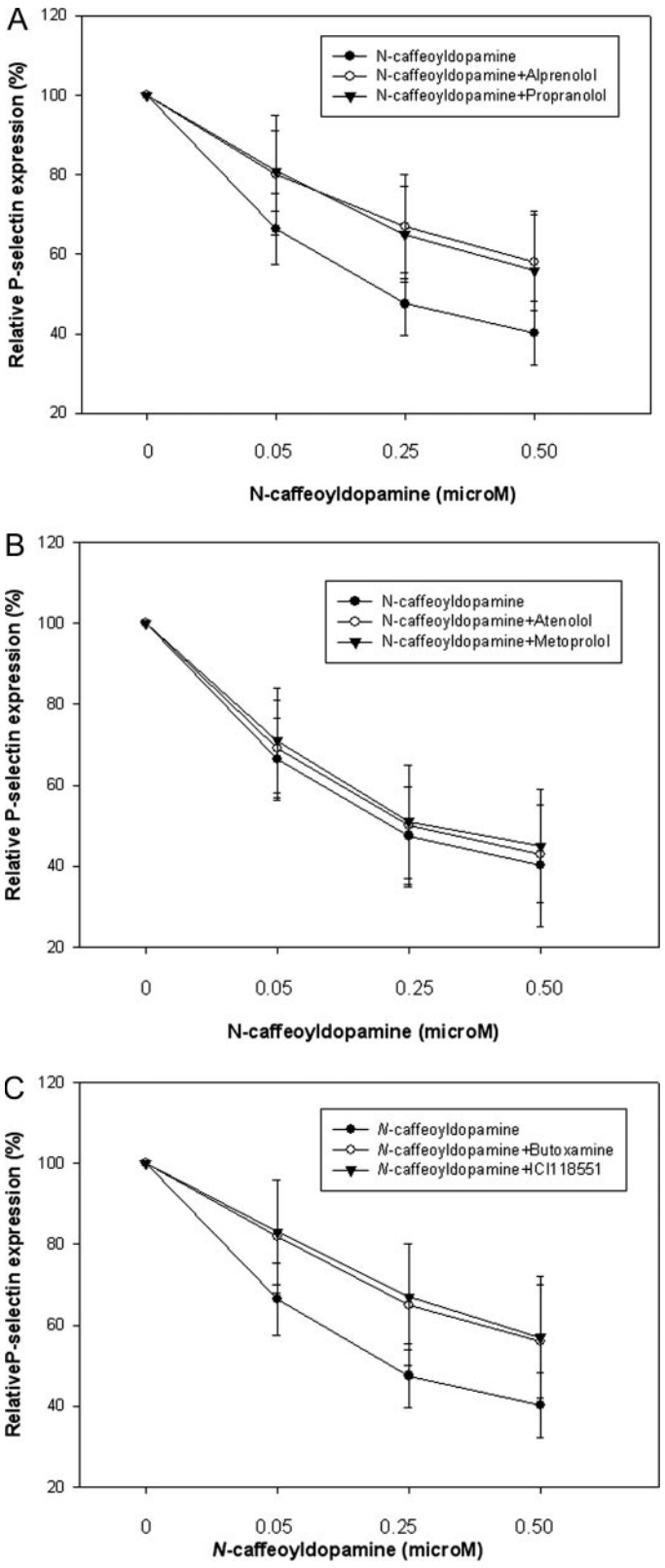

Fig. 4. The effects of β-blockers on P-selectin expression inhibited by *N*-caffeoyldopamine. Platelets were pretreated for 5 min with β-adrenoceptor antagonists before the incubation with *N*-caffeoyldopamine and treated with collagen (2.5 μg/ml). Control was not pretreated with the antagonists. A, platelets were treated with 1 μM nonselective β-adrenoceptor antagonists (alprenolol and propranolol). B, platelets were treated with 1 μM β_1-specific antagonists (atenolol and metoprolol). C, platelets were treated with 1 μM β_2-selective β-adrenoceptor antagonists (butoxamine and ICI 118551). Data points represent the mean ± S.D. of three or more samples. All treatment levels resulted in a significant reduction in the relative expression of P-selectin on platelets compared with control samples ($P < 0.05$).

maroyldopamine at the concentrations of 0.05 μM were able to suppress platelet-leukocyte interactions in blood samples by 36 ($P < 0.013$) and 32% ($P < 0.011$), respectively. The suppression of platelet-leukocyte interactions was not as significant as when using blood samples treated with β_2 antagonists (butoxamine and ICI 118551) (Fig. 6B). These data suggest that *N*-caffeoyldopamine and *N*-coumaroyldopamine may suppress platelet-leukocyte interactions via inhibiting P-selectin expression on platelets.

In Vivo Effects of *N*-Caffeoyldopamine on P-Selectin Expression and Platelet-Leukocyte Interactions in Mice. Because of their potent in vitro efficacy, animal experiments were conducted to confirm the inhibitory effects of the clovamide-type phenylpropenoic acid amides on P-selectin expression and platelet-leukocyte interactions in vivo. In our animal study, *N*-caffeoyldopamine was used because of its greater potency than *N*-coumaroyldopamine. For the experiments, mice were assigned to three groups: control, T1, and T2 groups. Mice in the control group were orally provided with distilled water (100 μl), and mice in the T1 and T2 groups were orally administrated with distilled water (100 μl) containing *N*-caffeoyldopamine (50 μg) and (100 μg), respectively. Because the average body weight of mice during the experiment was around 35 g, mice were likely to be provided orally with *N*-caffeoyldopamine (50 or 100 μg/35 g of body weight). Blood samples were collected and analyzed as described under *Materials and Methods*. As expected, mice administrated orally with *N*-caffeoyldopamine (both 50 and 100 μg) showed great reduction in both the P-selectin expression and platelet-leukocyte interactions by 31 to 45% ($P < 0.011$) and 34 to 43% ($P < 0.014$), respectively, compared with the control mice (Fig. 7, A and B). To verify the presence of *N*-caffeoyldopamine in the plasma of the mice administrated orally with *N*-caffeoyldopamine (both 50 and 100 μg), the quantity of *N*-caffeoyldopamine was determined using the high-performance liquid chromatography method, and the plasma concentrations of *N*-caffeoyldopamine were found between 0.020 to 0.060 μM. In vitro, approximately 30% P-selectin inhibition was observed at the concentrations of 0.020 to 0.060 μM, which is well compatible to the in vivo data described herein. These data suggest that *N*-caffeoyldopamine is able to inhibit P-selectin expression, thereby suppressing platelet-leukocyte interactions because platelet-leukocyte interactions were not suppressed without the inhibition of P-selectin expression as demonstrated in β-blocker experiments. All together, the data suggest that *N*-caffeoyldopamine may provide inhibitory effects on platelet activation and platelet-leukocyte interactions in vitro and in vivo.

Discussion

Coronary artery disease is a major cause of human mortality. The disease is caused by multiple cellular events such as fat deposits, plaque formation, and consequent inflammatory processes. P-Selectin is a member of the selectin family of cell adhesion molecules expressed on stimulated endothelial cells and activated platelets. The protein mediates leu-

TABLE 1
Effects of butoxamine on the inhibition of P-selectin expression by *N*-caffeoyldopamine at different concentrations of collagen
A, 50 nM *N*-caffeoyldoapmine; B, 50 nM *N*-caffeoyldoapmine and 2.5 μM butoxamine; and C, 50 nM *N*-caffeoyldoapmine and 5 μM butoxamine. All treatment levels resulted in a significant reduction in the relative expression of P-selectin on platelets compared with control samples ($P < 0.05$).

	CD62p	CD62p + A	CD62p + B	CD62p + C
			%	
Collagen (1 μg/ml)	18.2	12.9	16.8	17.1
Collagen (2.5 μg/ml)	25.2	16.4	19.9	20.4

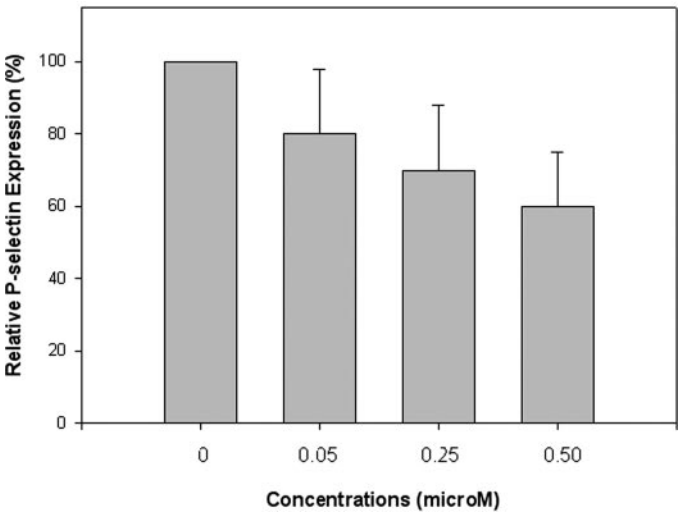

Fig. 5. Effects of cilostazol on P-selectin expression. Platelets were incubated with cilostazol for 10 min. P-Selectin expression was determined as described under *Materials and Methods*. Data points represent the mean ± S.D. of three or more samples. All treatment levels resulted in a reduction in the relative expression of P-selectin on platelets compared with control samples ($P < 0.01$).

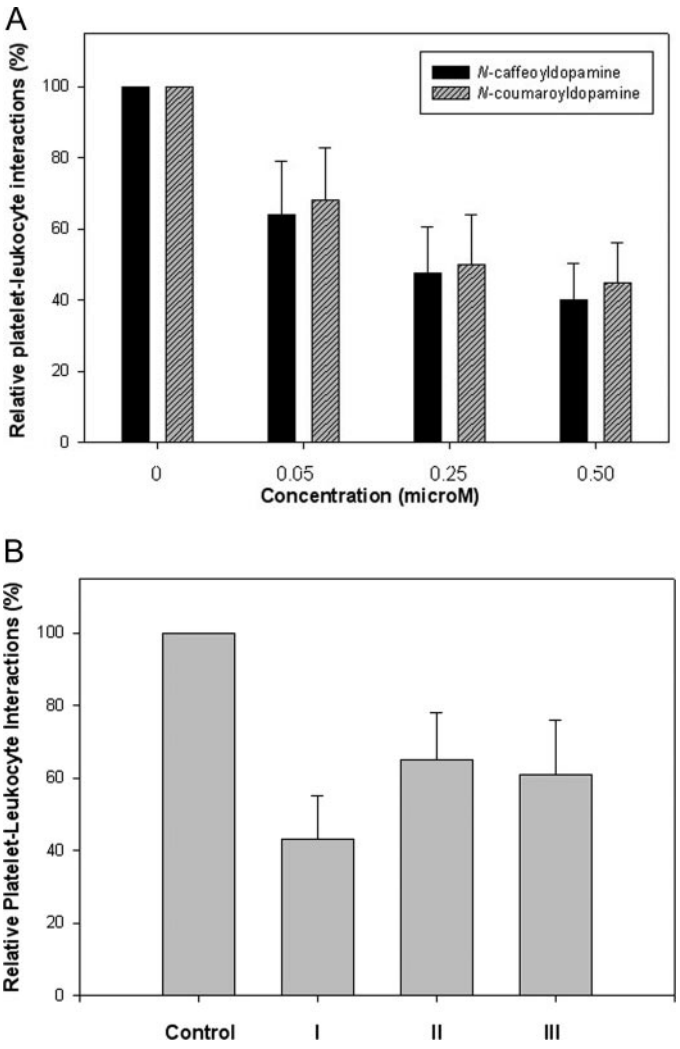

Fig. 6. The effects of *N*-coumaroyldopamine and *N*-caffeoyldopamine on platelet-leukocyte interactions in whole blood. Blood samples were prepared from tail blood collections, and the samples were incubated with antibodies to identify platelets and leukocytes: *R*-PE rat anti-mouse CD41 (the antibody for platelets) and FITC rat anti-mouse CD45 (the antibody for leukocytes) (BD Biosciences). A, blood samples were pretreated for 10 min with *N*-coumaroyldopamine or *N*-caffeoyldopamine. Platelet-leukocyte interactions were determined by flow cytometry as described under *Materials and Methods*. B, blood samples were pretreated for 5 min with 1 μM β-adrenoceptor antagonists (butoxamine and ICI 118551) and then treated for 10 min with *N*-caffeoyldopamine (0.50 μM); (I) *N*-caffeoyldopamine, (II) *N*-caffeoyldopamine + butoxamine, and (III) *N*-caffeoyldopamine + ICI 118551. Data points represent the mean ± S.D. of three or more samples. All treatment levels produced a significant reduction in the relative percentage of platelet-leukocyte interactions compared with control samples ($P < 0.01$).

kocyte rolling on stimulated endothelial cells and heterotypic aggregation of activated platelets onto leukocytes. The significance of P-selectin-mediated cell-adhesive interactions in the pathogeneses of coronary heart disease, including the acute coronary syndrome, has been well documented, and compounds able to modulate P-selectin-mediated cell-adhesive interactions have been searched and/or developed for many years (Geng et al., 2004; Kappelmayer et al., 2004; Vandendries et al., 2004).

Traditionally, plants and plant-derived products have been used for preventing and/or treating many diseases, including cardiovascular diseases. Indeed, some plants were found to contain phytochemicals with beneficial effects on the diseases. *Theobroma cacao* is one of the plants investigated because of its potential effects on coronary heart disease. However, the studies are still inconclusive, and more studies are required (Kondo et al., 1996; Sanbongi et al., 1997; Kris-Etherton et al., 2000; Weisburger, 2001). Interestingly, several clovamide-type phenylpropenoic acid amides such as *N*-caffeoyldopamine, clovamide (*N*-coumaroyltyrosine), deoxyclovamide, and *N*-caffeoyltyrosine were recently identified in cocoa seeds, but little is known about the effects of the clovamide-type phenylpropenoic acid amides on the diseases. Therefore, effects of two clovamide-type phenylpropenoic acid amides (*N*-caffeoyldopamine and *N*-coumaroyldopamine) on P-selectin expression and platelet-leukocyte interactions were investigated in this study. The data indicate that *N*-caffeoyldopamine and *N*-coumaroyldopamine are potent compounds to suppress platelet-leukocyte interactions via inhibiting P-selectin expression.

P-Selectin expression is regulated by numerous cellular events. In platelets, P-selectin expression is up-regulated by thrombin, histamine, and ADP activation; meanwhile, P-selectin expression can be down-regulated by TXI2 producing cAMP in the cells. cAMP is currently considered as a potent molecule leading to the down-regulation of platelet P-selectin expression. The down-regulation is likely to be mediated mainly through activation of cAMP-dependent protein kinase (Fisch et al., 1997; Libersan et al., 2003). Therefore, cellular events leading to producing cAMP are probably able to mod-

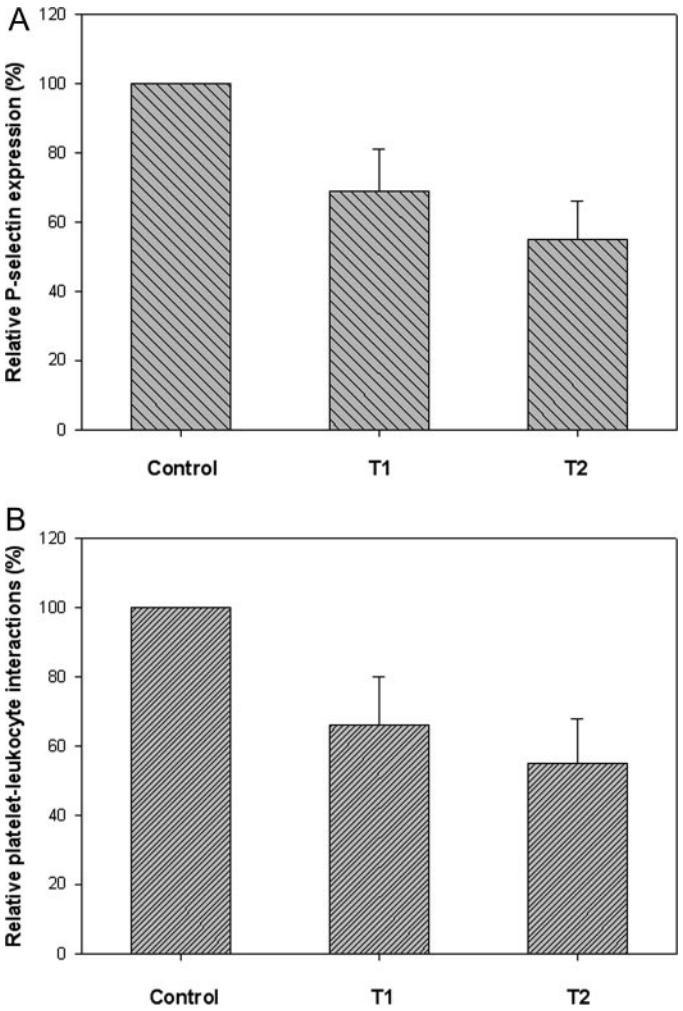

Fig. 7. In vivo effects of *N*-caffeoyldopamine on P-selectin expression and platelet-leukocyte interactions in mice. Swiss-Webster mice (11–12 weeks old) were assigned to three groups ($n = 5$). Mice in the first group (control) were administrated orally using a dosing needle with distilled water (100 μl), and mice in the second (T1) and third (T2) groups were administrated orally with distilled water (100 μl) containing *N*-caffeoyldopamine (50 and 100 μg), respectively. Blood was collected via tail-bleeding technique, and blood samples from each group were used for P-selectin (A, $n = 5$) and platelet-leukocyte aggregation assays (B, $n = 3$). Reduction in the relative expression of P-selectin and platelet-leukocyte interactions was significant for both T1 and T2 groups compared with the control group ($P < 0.01$).

ulate P-selectin expression on platelets. In this study, *N*-caffeoyldopamine and *N*-coumaroyldopamine were demonstrated to suppress P-selectin expression on platelets, and the suppression was partially reversed by the treatment of β_2-adrenoceptor antagonists (butoxamine and ICI 118551). These data suggest clearly that the cAMP production is involved in the inhibition of P-selectin. The involvement of cAMP was also demonstrated by cilostazol, a phosphodiesterase III inhibitor, modulating levels of cAMP. However, the effect of cilostazol was less potent than *N*-caffeoyldopamine and *N*-coumaroyldopamine. These data, in fact, support that the inhibitory effects of *N*-caffeoyldopamine and *N*-coumaroyldopamine are more than producing cAMP in platelets, because β-antagonists can not block the P-selectin inhibition completely and because the inhibition of P-selectin expression by cilostazol was less potent than *N*-caffeoyldopamine and *N*-coumaroyldopamine. In the future, the mechanism representing the rest should be investigated.

Although P-selectin is a good marker of platelet activation, platelet-leukocyte interactions are considered to be a better marker of platelet activation because P-selectin on the surface of platelets disappears quickly in the degranulated platelets and because the interactions reflect biological processes of platelets implicated in cardiovascular diseases (Bouchard and Tracy, 2001; Krieglstein and Granger, 2001). In this study, the effects of the clovamide-type phenylpropenoic acid amides (*N*-caffeoyldopamine and *N*-coumaroyldopamine) on P-selectin were demonstrated to be very significant. Therefore, it is a rational approach to investigate the effects of the clovamide-type phenylpropenoic acid amides (*N*-caffeoyldopamine and *N*-coumaroyldopamine) on PSGL-1 (the ligand for P-selectin) on leukocytes. A preliminary experiment was performed using human myelocytic U937 cells because the specific antibody for mouse PSGL-1 is not good in mouse whole blood. As expected, the two clovamide-type phenylpropenoic acid amides were unable to change the level of PSGL-1 expression on the U937 cells (unpublished data), suggesting that the suppression of platelet-leukocyte interaction is likely to be from the inhibition of P-selectin expression rather than that of PSGL-1 expression. PSGL-1 is the disulfide-bonded homodimeric mucin-like glycoprotein on leukocytes interacting with not only P-selectin but also L- and E-selectins. By binding to P-selectin expressed on activated endothelium and platelets, PSGL-1 mediates leukocyte-endothelial and leukocyte-platelet adhesion; by binding to L-selectin expressed on apposing leukocytes, PSGL-1 mediates leukocyte-leukocyte adhesion; and by binding to E-selectin expressed on vascular endothelial cells, PSGL-1 mediates leukocyte-endothelial adhesion (Snapp et al., 1998). In this study, *N*-caffeoyldopamine and *N*-coumaroyldopamine suppressed platelet-leukocyte interactions via inhibiting P-selectin, not PSGL-1. However, the effects of the amides on L- and E-selectins are still unknown and yet to be investigated.

An animal study was performed to verify the potent in vitro effects in vivo. As described under *Materials and Methods*, the duration of the animal study was 10 weeks. During the period, no sign of abnormality of behavioral, body weight, or food consumption pattern had been observed at the concentrations provided in this study, indicating no acute effects of *N*-caffeoyldopamine on mice. As demonstrated in the animal study, the potent inhibitory effects of a clovamide-type phenylpropenoic acid amide, *N*-caffeoyldopamine, on P-selectin expression and platelet-leukocyte interactions were confirmed in mice administrated orally with *N*-caffeoyldopamine (both 50 and 100 μg). Currently, several sets of animal studies are being conducted to assess long-term effects (6 months, 1 year, and whole life span) of clovamide-type phenylpropenoic acid amides on both P-selectin expression and platelet-leukocyte interactions. Preliminary data suggest that the amides are still able to suppress platelet-leukocyte interactions via inhibiting P-selectin expression in the long term, and there is no significant abnormality of behavior, body weight, or food consumption pattern. In summary, all the data indicate that the intake of *N*-caffeoyldopamine and *N*-coumaroyldopamine may provide potentially beneficial effects on P-selectin expression and platelet-leukocyte interactions (Ito et al., 2004; Vandendries et al., 2004).

References

Ahn KC, Jun AJ, Pawar P, Jadhav S, Napier S, McCarty OJ, and Konstantopoulos K (2005) Preferential binding of platelets to monocytes over neutrophils under flow. *Biochem Biophys Res Commun* **329:**345–355.

Alemanno L, Ramos T, Gargadenec A, Andary C, and Ferriere N (2003) Localization and identification of phenolic compounds in *Theobroma cacao* L. somatic embryogenesis. *Ann Bot (Lond)* **92:**613–623.

Andre P (2004) P-selectin in haemostasis. *Br J Haematol* **126:**298–306.

Andrews RK, Berndt MC, and Berndt MC (2004) Platelet physiology and thrombosis. *Thromb Res* **114:**447–453.

Back K, Jang SM, Lee BC, Schmidt A, Strack D, and Kim KM (2001) Cloning and characterization of a hydroxycinnamoyl-CoA:tyramine *N*-(hydroxycinnamoyl) transferase induced in response to UV-C and wounding from Capsicum annuum. *Plant Cell Physiol* **42:**475–481.

Blann AD, Nadar SK, and Lip GY (2003) The adhesion molecule P-selectin and cardiovascular disease. *Eur Heart J* **24:**2166–2179.

Bouchard BA and Tracy PB (2001) Platelets, leukocytes and coagulation. *Curr Opin Hematol* **8:**263–269.

Chen Y, Davis-Gorman G, Watson RR, and McDonagh PF (2003) Platelet CD62p expression and microparticle formation in murine acquired immune deficiency syndrome and chronic ethanol consumption. *Alcohol* **38:**25–30.

Chirinos JA, Heresi GA, Velasquez H, Jy W, Jimenez JJ, Ahn E, Horstman LL, Soriano AO, Zambrano JP, and Ahn YS (2005) Elevation of endothelial microparticles, platelets and leukocyte activation in patients with venous thromboembolism. *J Am Coll Cardiol* **45:**1467–1471.

Cutillo F, D'Abrosca B, DellaGreca M, Di Marino C, Golino A, Previtera L, and Zarrelli A (2003) Cinnamic acid amides from Chenopodium album: effects on seeds germination and plant growth. *Phytochemistry* **64:**1381–1387.

Fisch A, Tobusch K, Veit K, Meyer J, and Darius H (1997) Prostacyclin receptor desensitization is a reversible phenomenon in human platelets. *Circulation* **96:** 756–760.

Geng JG, Chen M, and Chou KC (2004) P-selectin cell adhesion molecule in inflammation, thrombosis, cancer growth and metastasis. *Curr Med Chem* **11:**2153–2160.

Huo Y and Ley KF (2004) Role of platelets in the development of atherosclerosis. *Trends Cardiovasc Med* **14:**18–22.

Ito H, Miyakoda G, and Mori T (2004) Cilostazol inhibits platelet-leukocyte interaction by suppression of platelet activation. *Platelets* **15:**293–301.

Kappelmayer J, Nagy B Jr, Miszti-Blasius K, Hevessy Z, and Setiadi H (2004) The emerging value of P-selectin as a disease marker. *Clin Chem Lab Med* **42:**475–486.

Kariyazono H, Nakamura K, Shinkawa T, Yamaguchi T, Sakata R, and Yamada K (2001) Inhibition of platelet aggregation and the release of P-selectin from platelets by cilostazol. *Thromb Res* **101:**445–453.

Kondo K, Hirano R, Matsumoto A, Igarashi O, and Itakura H (1996) Inhibition of LDL oxidation by cocoa. *Lancet* **348:**1514.

Krieglstein CF and Granger DN (2001) Adhesion molecules and their role in vascular disease. *Am J Hypertens* **14:**44S–54S.

Kris-Etherton PM, Pelkman CL, Zhao G, and Wang Y (2000) No evidence for a link between consumption of chocolate and coronary heart disease. *Am J Clin Nutr* **72:**1059–1061.

Libersan D, Rousseau G, and Merhi Y (2003) Differential regulation of P-selectin expression by protein kinase A and protein kinase G in thrombin-stimulated human platelets. *Thromb Haemost* **89:**310–317.

Merten M and Thiagarajan P (2004) P-selectin in arterial thrombosis. *Z Kardiol* **93:**855–863.

Nieswandt B, Schulte V, and Bergmeier W (2005) Flow-cytometric analysis of mouse platelet function. *Methods Mol Biol* **272:**255–268.

Park JB (2005) *N*-Coumaroyldopamine and *N*-caffeoyldopamine increase cAMP via beta 2-adrenoceptors in myelocytic U937 cells. *FASEB J* **19:**497–502.

Park JB and Schoene N (2002) Synthesis and characterization of *N*-coumaroyltyramine as a potent phytochemical which arrests human transformed cells via inhibiting protein tyrosine kinases. *Biochem Biophys Res Commun* **292:**1104–1110.

Rein D, Paglieroni TG, Wun T, Pearson DA, Schmitz HH, Gosselin R, and Keen CL (2000) Cocoa inhibits platelet activation and function. *Am J Clin Nutr* **72:**30–35.

Ryningen A, Jensen BO, and Holmsen H (1999) Role of autocrine stimulation on the effects of cyclic AMP on protein and lipid phosphorylation in collagen-activated and thrombin-activated platelets. *Eur J Biochem* **260:**87–96.

Sanbongi C, Suzuki N, and Sakane T (1997) Polyphenols in chocolate, which have antioxidant activity, modulate immune functions in humans in vitro. *Cell Immunol* **177:**129–136.

Schmidt A, Grimm R, Schmidt J, Scheel D, Strack D, and Rosahl S (1996) Cloning and expression of a potato cDNA encoding hydroxycinnamoyl-CoA:tyramine *N*-(hydroxycinnamoyl)transferase. *J Biol Chem* **274:**4273–4280.

Schror K (2003) Antithrombotic drugs in vascular medicine: a historical perspective. *Semin Vasc Med* **3:**97–105.

Snapp KR, Ding H, Atkins K, Warnke R, Luscinskas FW, and Kansas GS (1998) A novel p-selectin glycoprotein ligand-1 monoclonal antibody recognizes an epitope within the tyrosine sulfate motif of human PSGL-1 and blocks recognition of both P- and L-selectin. *Blood* **91:**154–164.

Tárnok A, Mahnke A, Müller M, and Zotz RJ (1999) Rapid in vitro biocompatibility assay of endovascular stents by flow cytometry using platelet activation and platelet leukocyte aggregation. *Cytometry* **38:**30–39.

Vandendries ER, Furie BC, and Furie B (2004) Role of P-selectin and PSGL-1 in coagulation and thrombosis. *Thromb Haemost* **92:**459–466.

Visioli F, Borsani L, and Galli C (2000) Diet and prevention of coronary heart disease: the potential role of phytochemicals. *Cardiovasc Res* **47:**419–425.

Weisburger JH (2001) Chemopreventive effects of cocoa polyphenols on chronic diseases. *Exp Biol Med* **226:**891–897.

Wu PL, Su GC, and Wu TS (2003) Constituents from the stems of Aristolochia manshuriensis. *J Nat Prod* **66:**996–998.

Yamamoto I, Matsunaga T, Kobayashi H, Watanabe K, and Yoshimura H (1991) Analysis and pharmacotoxicity of feruloyltyramine as a new constituent and p-coumaroyltyramine in Cannabis sativa L. *Pharmacol Biochem Behav* **40:**465–469.

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