CLOVAMIDE-TYPE PHENYLPROPENOIC ACID AMIDES, N-COUMAROYL-DOPAMINE AND N-CAFFEEOYL-DOPAMINE, INHIBIT PLATELET-LEUKOCYTE INTERACTIONS VIA SUPPRESSING P-SELECTIN EXPRESSION

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A recommended section assignment: Cardiovascular
Abstract

N-Coumaroyldopamine and N-caffeoyldopamine are clovamide-type phenylpropenoic acid amides found in Theobroma cacao. In this paper, 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 beta 2-adrenoceptor antagonists, suggesting that beta-2 receptors are likely 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 animal study, mice administrated orally with N-caffeoyldopamine (50 and 100 µg per 35 g body weight) also showed great reduction in the P-selectin expression and platelet-leukocyte interactions by 31-45 % ($P < 0.011$) and 34-43% ($P < 0.014$) respectively. These data suggest that the clovamide-type phenylpropenoic acid amides are able to suppress platelet-leukocyte interactions via inhibiting P-selectin expression.
Introduction

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 (Andrews et al., 2004; Schror, 2003; Huo and Ley, 2004; Bouchard and Tracy, 2001; Krieglstein and Granger, 2001). 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 liganding to P-selectin ligand (PSGL-1) on leukocytes and endothelium (Blann et al., 2003; Merten and Thiagarajan, 2004). P-selectin is stored in the alpha-granules of platelets. By the exposure of platelets to ADP (adenosine diphosphate), collagen, thrombin, thromboxane A2 (TXA2), 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). Due to 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 (Back et al., 2001; Alemanno et al. 2003; Schmidt et al., 1996; Yamamoto et al., 1991; Wu et al., 2003; Cutillo et al., 2003). Clovamide-type phenylpropenoic acid amides were originally discovered as phytoalexins.
accumulated in response to wounding and pathogen attacks (Yamamoto et al., 1991; Wu et al., 2003; Cutillo 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 (Park, 2005; Rein et al., 2000; Visioli et al., 2000). In this study, two clovamide-type phenylpropenoic acid amides (N-caffeoyldopamine and N-coumaroyldopamine; Figure 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, 2005; Park and Schoene, 2002). Collagen was obtained from Chrono-Log Corp. (Hampton, PA). U937 cells were purchased from ATCC (Manassas, VA). Swiss Webster mice 7-9 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 microfuge tubes containing 15 % EDTA. The modified Tyrodes buffer (134 mM NaCl, 0.34 mM Na2HPO4, 2.9 mM KCl, 12 mM NaHCO3, 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 x 75 polypropylene tubes along with the appropriate antibody, collagen (2.5 µg/mL) and the modified Tyrodes 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 one hour of
the collection by flow cytometry (Nieswandt et al., 2005; Chen et al., 2003). 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-selection) monoclonal antibody and the isotype control were obtained from BD Biosciences (Camarillo, CA) (Tárno et al., 1999; Ahn et al., 2005).

**Platelet-Leukocyte Interactions in Whole Blood.**

Blood samples were collected from mice tail in microfuge tubes containing 3.8% sodium citrate (10 µL) and immediately adjusted to 100 µL with the modified Tyrodes buffer. The samples were treated with N-caffeoyldopamine or N-coumaroyldopamine prior to 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 IgG1κ (BD Biosciences) for platelets, and fluorescein isothiocyanate (FITC) rat anti mouse CD45 and isotype control FITC conjugated rat IgG2b; (Serotec) for leukocytes. Platelet-leukocyte interactions were determined by flow cytometry as described (Tárno et al., 1999).

**Animal experiments.**

Swiss Webster mice 3-4 weeks old were purchased from Charles River (Wilmington, MA). 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 HPLC for confirming that $N$-caffeoyldopamine was not in the diet. After 8 weeks, mice were assigned and remained to 3 groups (n=5) for 10 weeks. Mice in the first group (control) were administrated orally using a dosing needle with distilled water (100 µL), 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, following 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 ANOVA or ANOVA by ranks as appropriate. Differences were considered to be significant when $p < 0.05$. Data points represent the mean ± SD 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 in “Materials and Methods”. Then, blood samples were analyzed for CD62p exposure on platelet membranes by flow cytometry. As shown in Figure 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 interactions 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 (Figure 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 beta-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 cAMP (Ryningen et al., 1999; Libersan et al., 2003). Previously, N-caffeoyldopamine and N-coumaroyldopamine were reported to produce cAMP via beta-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 due to its better efficacy in inhibiting P-selectin expression on platelets, and three different types of beta-blockers (non-selective beta-adrenoceptor antagonists, beta-1 specific antagonists, and beta-2 specific antagonists) were used to block effects of N-caffeoyldopamine on P-selectin expression. For blocking beta-adrenoceptors on the platelets, non-selective beta-adrenoceptor antagonists (alprenolol and propranolol), beta-1 specific antagonists (atenolol and metoprolol) and beta-2 specific antagonists (butoxamine and ICI 118551) were pretreated prior to the addition of N-caffeoyldopamine to blood samples. As shown in Figure 4A and 4C, the pretreatment of non-selective beta-adrenoceptor antagonists (alprenolol and propranolol) and beta-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 beta-1 specific antagonists (atenolol and metoprolol) did not block the inhibitory effects of N-caffeoyldopamine (Figure 4B). The similar experiments were performed using platelets without the treatment of collagens, and similar data were obtained that the beta-adrenoceptor antagonists and the beta-2 specific antagonists partially blocked the inhibitory effects of N-caffeoyldopamine on the P-selectin expression, but not the beta-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 beta 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 beta 2-adrenoceptors, because beta-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 beta-2 specific antagonists used in the experiments (Table 1).

Effects of cilostazol on P-selectin expression.

The data suggest that the cAMP production is involved in the inhibition of P-selection. 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 Figure 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-selection 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 in “Materials and Methods”. As shown in Figure 6A, both N-caffeoyldopamine and N-coumaroyldopamine 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 were not as significant as when using blood samples treated with beta-2 antagonists (butoxamine and ICI 118551) (Figure 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.**

Due to 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 due to its greater potency than N-coumaroyldopamine. For the experiments, mice were assigned to 3 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 µg or 100 µg / 35 g body weight). Blood samples were
collected and analyzed as described in “Materials and Method”. As expected, mice administrated orally with N-caffeoyldopamine (both 50 and 100µg) showed great reduction in both the P-selectin expression and platelet-leukocyte interactions by 31-45% (P < 0.011) and 34-43% (P < 0.014) respectively, compared to the control mice (Figure 7A and 7B). To verify the presence of N-caffeoyldopamine in the plasma of the mice administrated orally with N-caffeoyldopamine (both 50 and 100µg), the quantity of N-caffeoyldopamine was determined using the HPLC method, and the plasma concentrations of N-caffeoyldopamine were found between 0.020-0.060 µM. In vitro, approximately 30% P-selectin inhibition was observed at the concentrations of 0.020-0.060 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 beta-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 leukocyte rolling on stimulated endothelial cells and heterotypic aggregation of activated platelets onto leukocytes. The significance of P-selectin-mediated cell adhesive interactions in the pathogenesis of coronary heart disease including the acute coronary syndrome (ACS) has been well documented, and compounds able to modulate P-selectin-mediated cell adhesive interactions have been searched and/or developed for many years (Vandendries et al., 2004; Geng et al., 2004; Kappelmayer 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 due to its potential effects on coronary heart disease. However, the studies are still inconclusive, and more studies are required (Sanbongi et al., 1997; Weisburger, 2001; Kondo et al., 1996; Kris-Etherton et al., 2000). 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, ADP activation, meanwhile P-selectin expression can be down-regulated by TXI2 producing cAMP in the cells. Intracellular cyclic AMP (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 (PKA) (Libersan et al., 2003; Fisch et al., 1997). Therefore, cellular events leading to producing cAMP are probably able to modulate 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 beta 2-adrenoceptor antagonists (butoxamine and ICI 118551). These data suggest clearly that the cAMP production is involved in the inhibition of P-selection. The involvement of cAMP was also demonstrated by cilostazol, a phosphodiesterase III inhibitor, modulating levels of cAMP. However the effect of cilostazol was less potently 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 beta-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 as 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 very significant. Therefore, it is rational approach to investigate 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 paper, \textit{N}-caffeoyldopamine and \textit{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 \textit{in vitro} effects \textit{in vivo}. As described in “Materials and Method”, the duration of the animal study was 10 weeks. During the period, no sign abnormality of behavioral, body-weight, or food consumption pattern has been observed at the concentrations provided in this study, indicating no acute effects of \textit{N}-caffeoyldopamine on mice. As demonstrated in the animal study, the potent inhibitory effects of a clovamide-type phenylpropenoic acid amide, \textit{N}-caffeoyldopamine, on P-selectin expression and platelet-leukocyte interactions were confirmed in mice administrated orally with \textit{N}-caffeoyldopamine (both 50 and 100 µg). Currently, several sets of animal studies are being conducted to assess long-term effects (6 months, one 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-terms, and there is no significant abnormality of behavior, body weight, or food consumption pattern. In summary, all the data indicate that the intake of \textit{N}-caffeoyldopamine and \textit{N}-coumaroyldopamine may provide potentially beneficial effects on P-selectin expression and platelet-leukocyte interactions (Ito et al., 2004; Vandendries et al., 2004).
References


and leukocyte activation in patients with venous thromboembolism.

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Figure Legends

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

Figure 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 in “Materials and Methods”. Data points represent the mean ± SD of three or more samples. All treatment levels resulted in a significant reduction in the relative expression of P-selection on platelets compared to control samples (p<0.015).

Figure 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 in “Materials and Methods”. Data points represent the mean ± SD of three or more samples. All treatment levels resulted in a significant reduction in the relative expression of P-selection on platelets compared to control samples (p<0.017).

Figure 4. The effects of beta-blockers on P-selectin expression inhibited by N-caffeoyldopamine. Platelets were pretreated for 5 min with beta adrenoceptor antagonists prior to the incubation with N-caffeoyldopamine, and treated with collagen (2.5 µg/mL). Control was not pretreated with the antagonists. 3A) Platelets were treated with 1 microM non-selective beta adrenoceptor antagonists (alprenolol and propranolol). 3B) Platelets were treated with 1 microM beta-1 specific antagonists (atenolol and...
metoprolol). 3C) Platelets were treated with 1microM beta-2 selective beta adrenoceptor antagonists (butoxamine and ICI118551). Data points represent the mean ± SD of three or more samples. All treatment levels resulted in a significant reduction in the relative expression of P-selection on platelets compared to control samples (p<0.05).

**Figure 5.** Effects of cilostazol on P-selectin expression. Platelets were incubated with cilostazol for 10 min. P-selectin expression was determined as described in “Materials and Methods”. Data points represent the mean ± SD of three or more samples. All treatment levels resulted in a reduction in the relative expression of P-selection on platelets compared to control samples (p<0.01).

**Figure 6.** The effects of N-coumaroyldopamine and N-cafeoyldopamine 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-phycoerythrin (PE) rat anti-mouse CD41 (the antibody for platelets) and fluorescein isothiocyanate (FITC) rat anti mouse CD 45 (the antibody for leukocytes) (BD Biosciences). 6A) Blood samples were pretreated for 10 min with N-coumaroyldopamine or N-cafeoyldopamine. Platelet-leukocyte interactions were determined by flow cytometry as described in “Materials and Methods”. 6B) Blood samples were pretreated for 5 min with 1microM beta adrenoceptor antagonists (butoxamine and ICI 118551), then treated for 10 min with N-cafeoyldopamine (0.50 µM); (I) N-cafeoyldopamine, (II) N-cafeoyldopamine+ butoxamine, (III) N-cafeoyldopamine+ ICI 118551. Data points represent the mean ± SD of three or more samples. All treatment levels produced a significant reduction in the relative percentage of platelet-leukocyte interactions compared to control samples (p<0.01).
Figure 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 3 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 were significant for both T1 and T2 groups compared to the control group (p<0.01).
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-selection on platelets compared to control 
samples (p<0.05).

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<th>CD62p (%)</th>
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<th>CD62p + B (%)</th>
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Fig. 1

(I)

(II)
Fig. 2

Relative P-selectin Expression (%)

Concentrations (microM)

N-caffeoyldopamine
N-coumaroyldopamine
Fig. 3

[Bar chart showing relative P-selectin expression (%) at different concentrations (microM) of N-caffeoyldopamine and N-coumaroyldopamine.]
Fig. 4B

The graph shows the relative P-selectin expression (%) on the y-axis against the concentration of N-caffeoyldopamine (micromolar) on the x-axis. Three conditions are compared:

- **N-caffeoyldopamine**
- **N-caffeoyldopamine + Atenolol**
- **N-caffeoyldopamine + Metoprolol**

The graph indicates a decrease in relative P-selectin expression as the concentration of N-caffeoyldopamine increases, with the different treatments showing slight variations.
Fig. 4C

![Graph showing relative P-selectin expression (%) in relation to N-caffeoyldopamine concentration (microM). The graph compares different concentrations of N-caffeoyldopamine, with and without additional treatments such as Butoxamine and HCl118551.](image-url)
Fig. 5

The graph shows the relative P-selectin expression (%) for different concentrations (microM) of a substance. The concentrations tested are 0, 0.05, 0.25, and 0.50 microM. The expression decreases as the concentration increases.
Fig. 6A

Relative platelet-leukocyte interactions (%)

Concentration (microM)

0

0.05

0.25

0.50

N-caffeoyldopamine

N-coumaroyldopamine
Fig. 6B

Relative Platelet-Leukocyte Interactions (%)

- Control
- I
- II
- III
Fig. 7B

Relative platelet-leukocyte interactions (%)

Control  T1  T2

0  20  40  60  80  100  120