

Caffedymine from Cocoa Has COX Inhibitory Activity Suppressing the Expression of a Platelet Activation Marker, P-Selectin

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Caffedymine (*N*-caffeoyldopamine) is a clovamide-type phenylpropenoic acid amide found in plants. Previous studies indicate that caffedymine inhibits P-selectin expression via increasing cAMP through beta-2 adrenoceptors, but the inhibition was only partially repressed by beta-2 adrenoceptor antagonists, suggesting additional mechanisms underlying the inhibitory effect. Therefore, in this study, the effect of caffedymine and its analogues (*N*-caffeoyltyramine, *N*-feruloyltyramine, *N*-coumaroyltyramine, *N*-cinnamoyltyramine) on COX enzymes (I and II) was investigated, because COX enzymes are deeply involved in regulating P-selectin expression on human platelets. The decreasing order of COX-I inhibitory activity was caffedymine > *N*-caffeoyltyramine > *N*-feruloyltyramine > *N*-coumaroyltyramine > *N*-cinnamoyltyramine. Caffedymine was the most potent compound tested, able to inhibit COX-I enzyme activity by 43% ($P < 0.013$) at the concentration of 0.01 μM . At the same concentration, caffedymine was also able to inhibit COX-II enzyme activity by 36% ($P < 0.015$), and the decreasing order of COX-II inhibitory activity was similar as that of COX-I. As a result of the COX inhibition, the production of thromboxane B2 (thromboxane A2 derivative) also decreased significantly in mouse blood treated with caffedymine and its analogues (0.05 μM). Caffedymine and *N*-caffeoyltyramine, both with potent COX inhibitory activity, were also able to inhibit P-selectin expression and platelet-leukocyte interactions. These data indicate that COX inhibition is likely to be another mechanism for caffedymine to inhibit P-selectin expression on platelets.

KEYWORDS: Caffedymine; COX inhibitor; thromboxane B2; P-selectin; platelet-leukocyte interactions

INTRODUCTION

Caffedymine (*N*-caffeoyldopamine) and its analogues (*N*-caffeoyltyramine, *N*-feruloyltyramine, *N*-coumaroyltyramine, *N*-cinnamoyltyramine) are phytochemicals found in plants such as *Capsicum annuum*, *Theobroma cacao*, and *Lycium chinense* (1–4). In my laboratory, caffedymine and its analogues were synthesized, and their biological activities have been investigated for the last 10 years. Lately, caffedymine was reported to suppress platelet-leukocyte interactions via inhibiting P-selectin expression on platelets (5). The inhibition of P-selectin expression was partially blocked by beta 2-adrenoceptor antagonists, but the inhibition could not be completely repressed even at relatively high concentrations of beta 2-adrenoceptor antagonists, suggesting there may be additional mechanisms for caffedymine to inhibit P-selectin expression on platelets (5).

Cyclooxygenases (Prostaglandin H Synthase or PGHS) are enzymes with both cyclooxygenase and peroxidase activities (6–8), and there are two forms of cyclooxygenases (COX-I and COX-II). COX-I is constitutively expressed in numerous cell types, meanwhile the expression of COX-II is transiently

induced by a variety of stimuli such as phorbol esters, lipopolysaccharides, and cytokines (8–10). Cyclooxygenases are known to catalyze the conversion of arachidonic acid to prostaglandin H₂, the intermediate molecule for prostacyclin and thromboxane A₂, which are involved in regulating the P-selectin expression on platelets (9–13). Therefore, in this paper, the effects of caffedymine and its analogues on COX-I and COX-II were investigated in a way to elucidate another underlying mechanism of the P-selectin inhibition by caffedymine and its analogues. Also, potential beneficial effects of caffedymine on reported adverse effects of COX inhibitors were discussed in this paper.

MATERIALS AND METHODS

Materials. COX-I and II enzymes and other chemicals were purchased from Sigma Chemical Co. (St. Louis, MO). Collagen was obtained from Chrono-Log Corp. (Hampton, PA). *N*-Caffeoyldopamine (caffedymine), *N*-caffeoyltyramine, *N*-feruloyltyramine, *N*-coumaroyltyramine, and *N*-cinnamoyltyramine were synthesized and purified (more than 96% purity) as described previously (14, 15).

COX-I and II Inhibition Assay. COX-I and II activities were measured in a 96-well plate using a chemiluminescent cy-

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cloxygenase kit (Assay Designs Inc., Ann Arbor, MI). In short, 50 μL Tris-phenol buffer (100 microM Tris, 0.5 microM phenol buffer, pH 7.3) was added into the wells. A 50 μL sample of hematin solution (hematin was dissolved in DMSO at 0.380 mg/mL, and diluted 5000-fold with 100 mM phosphate buffer, pH 7.5) and 50 μL COX-I (700 units) or COX-II (700 units) were added into the wells. The samples were incubated at room temperature for 5 min. After the incubation, caffeodymine, its analogues, or COX inhibitors were added. For additional 10 min, the samples were incubated at room temperature (in the dark). Following the incubation, COX activity was measured using a luminometer, by injecting 50 μL of chemiluminescent COX substrate (4 °C) and arachidonic acid, respectively. Relative light units (RLU) output was measured to determine COX activity.

Blood Samples. 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 caffeodymine and its analogues (*N*-caffeoyltyramine, *N*-feruloyltyramine, *N*-coumaroyltyramine, and *N*-cinnamoyltyramine) tested in the study; the diet was analyzed by HPLC for confirming that caffeodymine or its analogues were not in the diet. Blood was collected from mice once a week, via tail bleeding technique. Blood samples were used for thromboxane B₂, P-selectin, and platelet-leukocyte interaction assays.

Thromboxane B₂ Assay. The measurement of thromboxane B₂ was performed using Correlate-CLIA Thromboxane B₂ Immunoassay kit (Assay Designs Inc., Ann Arbor, MI), which uses a polyclonal antibody binding to thromboxane B₂ in a competitive manner. All measurements were performed according to the kit's protocol. Briefly, samples were treated for 10 min with caffeodymine or its analogues. Thromboxane B₂ in the samples and covalently attached to an alkaline phosphatase molecule was simultaneously incubated in the well coated with the antibody, the excess reagents were washed away, and chemiluminescent substrate was added. The substrate reacts with the bound alkaline phosphatase conjugate to produce light emission at approximately 530 nm. Chemiluminescence was measured to calculate the concentration of thromboxane B₂, according to the protocol in the Thromboxane B₂ Immunoassay kit.

Measurement of P-Selectin Expression. Blood was collected 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 microliters. From the diluted samples, aliquots were placed in 12 × 75 polypropylene tubes along with the appropriate antibody, and the modified Tyrodes buffer in a final volume of 200 microliters. Caffeodymine and *N*-caffeoyltyramine 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 (16, 17). 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)-

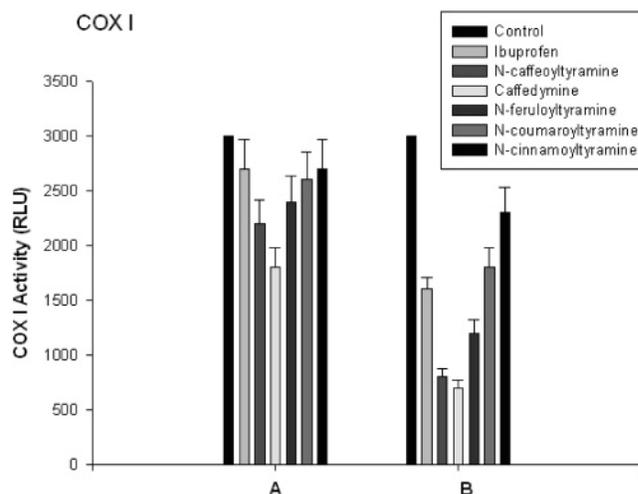


Figure 1. Effects of caffeodymine and its analogues on COX-I. Caffeodymine, its analogues, and inhibitors (A group, 0.01 μM ; B group, 0.1 μM) were added to the samples, and the reaction mixtures were incubated at room temperature (in the dark) for 10 min. Following the incubation, COX-I activity was measured according to the kit's protocol using a luminometer.

conjugated rat anti-mouse CD62p monoclonal antibody and the isotype control were obtained from BD Biosciences (Camarillo, CA) (17, 18).

Platelet-leukocyte Interactions in Whole Blood. Blood samples were collected 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 caffeodymine or *N*-caffeoyltyramine 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 IgG_{1 κ} (BD Biosciences) for platelets, and fluorescein isothiocyanate (FITC) rat anti mouse CD45 and isotype control FITC conjugated rat IgG_{2b} (Serotec) for leukocytes. Platelet-leukocyte interactions were determined by flow cytometry as described (19).

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 \pm SD of three or more samples.

RESULTS

Effects of Caffeodymine and Its Analogues on COX-I. COX-I is constitutively expressed in a variety of cell types and involved in prostaglandin homeostasis. The inhibition of COX-I is known to prevent platelet activation via numerous mechanisms, including the inhibition of P-selectin expression. Ibuprofen and some COX-I inhibitors are able to inhibit platelet cyclooxygenase (COX)-I, the enzyme converting arachidonic acid (AA) to the potent platelet agonist thromboxane A₂ (TXA₂). Therefore, the effects of caffeodymine (*N*-caffeoyltyramine) and its analogues (*N*-caffeoyltyramine, *N*-feruloyltyramine, *N*-coumaroyltyramine and *N*-cinnamoyltyramine) on COX-I were investigated to elucidate the mechanism underlying the P-selectin inhibition. As shown in **Figure 1**, caffeodymine was the most potent compound able to inhibit COX-I enzyme by 43% ($P < 0.013$) at the concentration of 0.01 μM . The decreasing order of the inhibitory activity was caffeodymine > *N*-caffeoyltyramine > *N*-feruloyltyramine > *N*-coumaroyl-

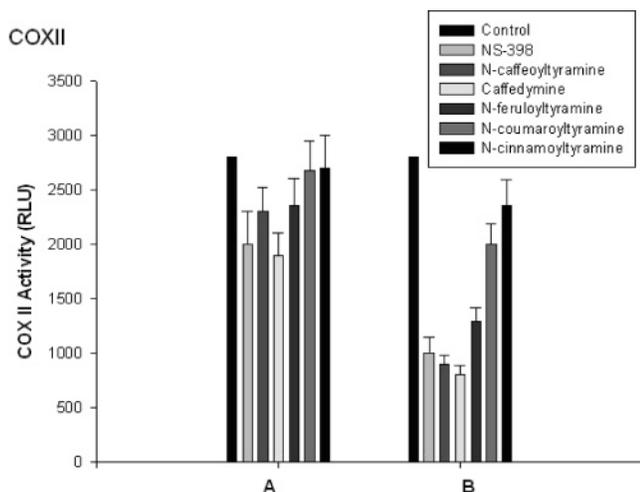


Figure 2. Effects of caffedyimine and its analogues on COX-II. Caffedyimine, its analogues, and inhibitors (A group, 0.01 μM ; B group, 0.1 μM) were added to the samples, and the reaction mixtures were incubated at room temperature (in the dark) for 10 min. Following the incubation, COX-II activity was measured according to the kit's protocol using a luminometer.

tyramine > *N*-cinnamoyltyramine. On comparison with a well-known COX-I inhibitor (ibuprofen), caffedyimine and *N*-caffeoyltyramine were able to inhibit COX-I to a greater extent than ibuprofen, even though caffedyimine, *N*-caffeoyltyramine, and *N*-feruloyltyramine were a relatively good COX-I inhibitors. As shown in **Figure 1**, *N*-caffeoyltyramine is a potent COX-I inhibitor, but *N*-cinnamoyltyramine is not as potent as *N*-caffeoyltyramine. The structural difference between the two compounds is that *N*-caffeoyltyramine has an additional hydroxyl group at the 3-position of the phenylpropenoic acid moiety. This indicates that hydroxyl groups at the 3- and 4-positions of the phenylpropenoic acid may be critical in the COX-I inhibition. This finding is also supported by the data that *N*-caffeoyltyramine and *N*-coumaroyltyramine were more potent COX-I inhibitors than *N*-feruloyltyramine and *N*-cinnamoyltyramine, respectively (**Figure 1**).

Effects of Caffedyimine and Its Analogues on COX-II.

Although COX-I inhibitors are primarily involved in inhibiting platelet COX-I enzyme, the effects of caffedyimine and its analogues on COX-II were also investigated in this paper, because COX-II is likely to be involved in many important physiological processes. As shown in **Figure 2**, the decreasing order of the inhibitory activity was caffedyimine > *N*-caffeoyltyramine > *N*-feruloyltyramine > *N*-coumaroyltyramine > *N*-cinnamoyltyramine. Like the COX I inhibition, caffedyimine was also the most potent compound able to inhibit COX-II enzyme by 36% ($P < 0.015$) at the concentration of 0.01 μM . Compared to NS-398 (a COX-II specific inhibitor), caffedyimine was able to inhibit COX-II better than NS-398 (**Figure 2**). The data suggest that caffedyimine is a potent compound with COX-I and II inhibiting activity.

Effects of Caffedyimine and Its Analogues on Thromboxane B2. Thromboxane A2 (TXA2) produced from COX enzymes is involved in platelet aggregation, vasoconstriction, and other functions (20, 21). Therefore, thromboxane A2 is measured and used as an inflammatory and/or cardiovascular marker determining platelet activation and others. However a half-life of TXA2 is very short under physiological conditions. Therefore, the production of TXA2 is typically monitored by measuring TXB2, because thromboxane B2 (TXB2) produced by the nonenzymatic hydration of TXA2, is shown to be stable.

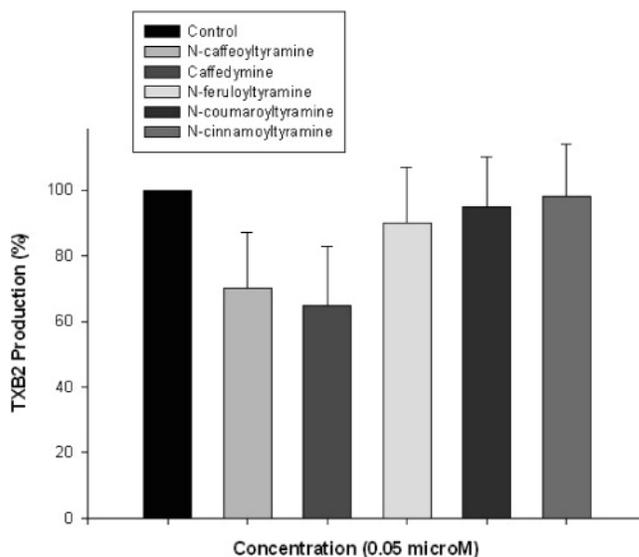


Figure 3. Effects of caffedyimine and its analogues on thromboxane B2. Caffedyimine and its analogues (0.05 μM) were added to the samples, and the reaction mixtures were incubated at room temperature for 10 min. Following the incubation, Amount of thromboxane B2 in the samples was measured according to the kit's protocol.

In this study, thromboxane B2 was measured as another way to gauge the level of the COX inhibition by caffedyimine and its analogues. As shown in **Figure 3**, caffedyimine and its analogues were able to inhibit the production of TXB2 in mouse blood samples, and the decreasing order of the inhibitory activity was caffedyimine > *N*-caffeoyltyramine > *N*-feruloyltyramine > *N*-coumaroyltyramine > *N*-cinnamoyltyramine. As expected, caffedyimine was the most potent compound able to inhibit the production of thromboxane B2 by 32% ($P < 0.015$) at the concentration of 0.05 μM . All these data are in line with the data of the COX inhibition, indicating that the inhibition of COX enzymes may be accountable for the reduction of thromboxane B2 production.

Effects of Caffedyimine and Its Analogues on P-Selectin Expression.

P-selectin (CD62p) protein is a transmembrane glycoprotein, and the protein has been commonly used as a good marker for platelet activation (19–21). The expression of the protein is reported to be greatly modulated by COX enzyme activity and cAMP. This and previous studies indicate that caffedyimine and *N*-caffeoyltyramine contain both COX inhibitory and cAMP producing activities. Therefore, the effects of the two potent compounds (caffedyimine and *N*-caffeoyltyramine) on P-selectin expression on platelets were determined as a way to evaluate the inhibition of platelet activation. As shown in **Figure 4**, they are able to suppress P-selectin expression on platelets by 33% ($P < 0.010$) and 31% ($P < 0.013$), respectively, at the concentration of 0.05 μM . P-selectin inhibitory activity was correlated positively to COX inhibiting activity, suggesting that the inhibition of COX enzymes may be a main contributing factor to suppressing P-selectin expression. This deduction was also confirmed by the data that *N*-caffeoylphenylethylamine with potent COX inhibitory activity, but little cAMP producing activity, was still quite potent in suppressing P-selectin expression on platelets by 30% ($P < 0.011$) at the concentration of 0.05 μM . These data also indicate that COX inhibition may be a main contributing factor in suppressing P-selectin expression on platelets.

Effects of Caffedyimine and Its Analogues on Platelet-Leukocyte Interactions. As demonstrated above, caffedyimine

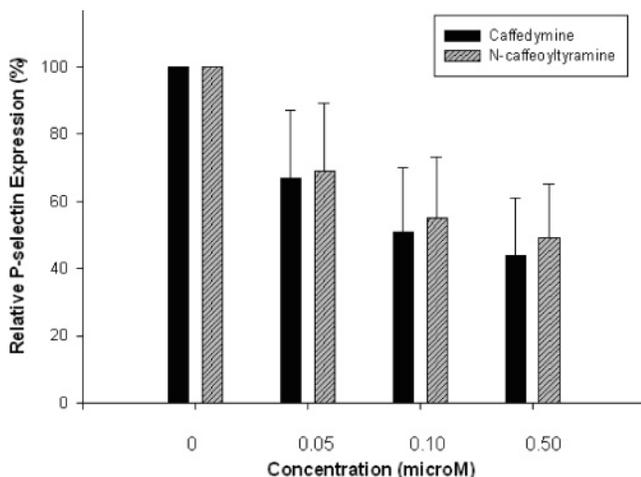


Figure 4. Effects of caffedymine and *N*-caffeoyltyramine on P-selectin expression on platelets. Platelets were incubated with caffedymine and *N*-caffeoyltyramine for 10 min. P-selectin expression was determined as described in "Materials and Methods." Data points represent the mean \pm SD of five samples.

and *N*-caffeoyltyramine were able to suppress P-selectin expression on platelets. Therefore, their effects on platelet-leukocyte interactions were also investigated, because P-selectin expression leads to platelet-leukocyte interactions, inducing a series of pathophysiological processes of platelets implicated in cardiovascular diseases (5, 15). As shown in **Figure 5**, caffedymine and *N*-caffeoyltyramine were very potent in inhibiting platelet-leukocyte interactions by 36% ($P < 0.012$) and 31% ($P < 0.013$), respectively, at the concentration of 0.05 μ M. Likewise, the inhibition of platelet-leukocyte interactions was correlated to the inhibition of COX enzymes, suggesting that the inhibition of platelet-leukocyte interactions may be attributed from the suppression of P-selectin expression via inhibiting COX enzymes in platelets. All these data indicate clearly that the COX inhibition is likely to be another mechanism for caffedymine and *N*-caffeoyltyramine to inhibit P-selectin expression on platelets and the inhibition is mainly accountable for suppressing platelet-leukocyte interactions.

DISCUSSION

P-selectin is a 140 kDa type-1 transmembrane glycoprotein belonging to the selectin family of cell adhesion receptors. P-selectin is commonly used as a biomarker for platelet activation (22, 23), because the protein is involved in platelet-leukocyte interactions and platelet-endothelium interactions via binding to P-selectin ligand (PSGL-1) on leukocytes and endothelium. Previously, caffedymine was reported to inhibit the P-selectin expression via producing cAMP via beta 2-adrenoceptors (5). However, additional mechanisms for caffedymine to inhibit P-selectin expression on platelets have been speculated, because the inhibition of P-selectin expression was only partially blocked by beta 2-adrenoceptor antagonists.

In fact, P-selectin expression on platelets is regulated by prostaglandins (e.g., thromboxane B2, prostacyclin I) synthesized by cyclooxygenases. Cyclooxygenases (COX-I and COX-II) are proteins involved in many important physiological processes (24, 25). In platelets, COX-I is known to catalyze the conversion of arachidonic acid to prostaglandin H2, the intermediate molecule in the formation of both prostacyclin and thromboxane A2. They are critically involved in regulating the P-selectin expression on platelets (25, 26). In this study, caffedymine was

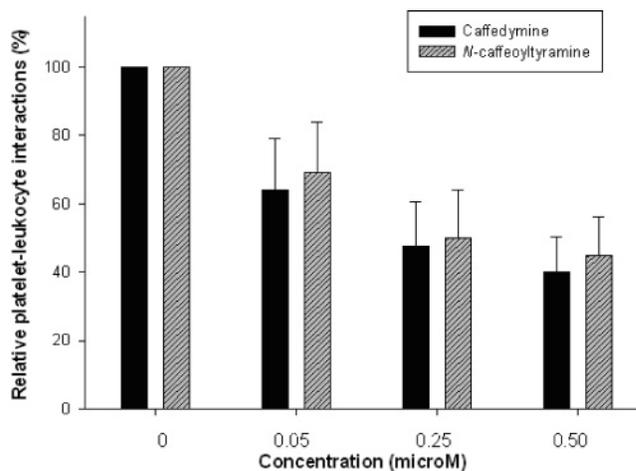


Figure 5. Effects of caffedymine and *N*-caffeoyltyramine on platelet-leukocyte interactions in whole blood. Blood samples were prepared from tail blood collections, and the samples were incubated with antibodies to identify platelets and leukocytes; R-phycoerythrin (PE) rat anti-mouse CD41 (the antibody for platelets) and fluorescein isothiocyanate (FITC) rat anti-mouse CD 45 (the antibody for leukocytes) (BD Biosciences). Platelet-leukocyte interactions were determined by flow cytometry as described in "Materials and Methods." Data points represent the mean \pm SD of four samples.

demonstrated to have COX inhibitory activity, thereby inhibiting P-selectin expression on human platelets. Interestingly, recent studies suggest that the balance between thromboxane and prostacyclin by cyclooxygenases is a critical factor in cardiovascular homeostasis (26, 27). The inhibition of the prostacyclin signaling pathway by COX inhibitors is reported to be involved in increasing atherosclerosis and narrowing coronary artery in animal models, thereby producing adverse cardiovascular outcomes (27, 28). In cell culture models, prostacyclin was demonstrated to increase cAMP via IP (prostacyclin) receptors. The process is likely to participate in the inhibition of platelet activation induced by thromboxane A2. Several reports suggest that the phosphorylation of G α by protein kinase A is accountable for the inhibition of the thromboxane A2-mediated signaling pathway in platelets (26, 27). By activating the cAMP/PKA pathway, prostacyclin is also believed to inhibit agonist-induced Ca²⁺ increases in platelets. However, general COX inhibitors (COX I and II) inhibit an early stage between arachidonic acid and PGH2, a precursor molecule for both thromboxanes and prostacyclins. This type of COX inhibition seems unfortunately responsible for the reported adverse effects of COX inhibitors (27, 28). Hypothetically, the adverse effects may be modulated, if the COX inhibitors are able to activate the cAMP/PKA pathway via increasing cAMP. Currently, such COX inhibitors have not been reported and the efficacy of those inhibitors not yet investigated, related to the reported adverse effects of COX-2 inhibitors.

In this study, caffedymine was found to contain both COX inhibitory and cAMP producing activities. Due to the dual activities of caffedymine, the compound may be a potent agent to treat human chronic diseases treated typically with COX inhibitors with less adverse effects. Caffedymine should be further investigated in the future, in order to find out whether the compound has better efficacy and its use results in less adverse effects in treating human diseases such as cardiovascular and inflammatory diseases.

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