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Caffeic acid treatment alters the extracellular adenine nucleotide hydrolysis in platelets and lymphocytes of adult rats

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ABSTRACT

This study evaluated the effects of caffeic acid on ectonucleotidase activities such as NTPDase (nucleoside triphosphate diphosphohydrolase), Ecto-NPP (nucleotide pyrophosphatase/phosphodiesterase), 5′-nucleotidase and adenosine deaminase (ADA) in platelets and lymphocytes of rats, as well as in the profile of platelet aggregation. Animals were divided into five groups: I (control); II (oil); III (caffeic acid 10 mg/kg); IV (caffeic acid 50 mg/kg); and V (caffeic acid 100 mg/kg). Animals were treated with caffeic acid diluted in oil for 30 days. In platelets, caffeic acid decreased the ATP hydrolysis and increased ADP hydrolysis in groups III, IV and V when compared to control ($P < 0.05$). The 5′-nucleotidase activity was decreased, while E-NPP and ADA activities were increased in platelets of rats of groups III, IV and V ($P < 0.05$). Caffeic acid reduced significantly the platelet aggregation in the animals of groups III, IV and V in relation to group I ($P < 0.05$). In lymphocytes, the NTPDase and ADA activities were increased in all groups treated with caffeic acid when compared to control ($P < 0.05$). These findings demonstrated that the enzymes were altered in tissues by caffeic acid and this compound decreased the platelet aggregation suggesting that caffeic acid should be considered a potentially therapeutic agent in disorders related to the purinergic system.

1. Introduction

Adenine nucleotides (ATP, ADP) and nucleoside adenosine represent an important class of extracellular molecules involved in the modulation of signaling pathways crucial for the normal functioning of vascular and immune systems (Yegutkin, 2008). In the vascular system, ADP and adenosine modulate the processes linked to vascular inflammation and thrombosis exerting various effects in platelets (Soslau and Youngprapakorn, 1997; Gachet, 2001). In addition, it is well established that ATP acts through specific cell receptors and is involved in pro-inflammatory functions such as stimulation and proliferation of lymphocytes and cytokine release, while adenosine exhibits potent anti-inflammatory and immunosuppressive actions (Dwyer et al., 2007; Gessi et al., 2007).

Signaling events induced by extracellular adenine nucleotides and nucleosides are tightly regulated by cell surface ectoenzymes known as ectonucleotidases. The most relevant ecto-enzymes involved in adenine nucleotide extracellular hydrolysis are NTPDase (ecto-nucleoside triphosphate diphosphohydrolase), ecto-NPPs (ecto-nucleotide pyrophosphatase/phosphodiesterase), ecto-5′-nucleotidase, and adenosine deaminase (ADA) (Robson et al., 2006). NTPDase hydrolyzes ATP and ADP to AMP, while E-NPP enzymes are responsible for hydrolyzing 5′-phosphodiester bonds in nucleotides and their derivatives, resulting in the production of monophosphate nucleotide. AMP resulting from the action of NTPDase and E-NPP is subsequently hydrolyzed to adenosine by ecto-5′-nucleotidase (Colgan et al., 2006; Strater, 2006). The resultant adenosine can be inactivated through the action of ADA, which catalyzes the irreversible deamination of adenosine to inosine.

Together, these enzymes constitute a highly refined system for the regulation of nucleotide mediated signaling, controlling the rate, degradation and nucleoside formation. As a consequence of their key physiological role, ectonucleotidases have been studied

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in different pathological and experimental conditions (Leal et al., 2005a, 2005b; Spanevello et al., 2009; Thomé et al., 2012). Of particular importance, studies from our laboratory have also demonstrated that natural substances such as resveratrol and curcumin altered ectonucleotidase activities in different tissues, demonstrating that phenolic compounds may interfere in the purinergic signaling (Schmatz et al., 2009; Jaques et al., 2011).

Caffeic acid (3,4-dihydroxy cinnamic acid) is a phenolic compound naturally found in several fruits, vegetables and herbs, such as coffee, artichoke, pear, basil, thyme, oregano, and apple (Clifford, 1999). Caffeic acid is known to have a broad spectrum of pharmacological activities including anti-inflammatory, antioxidant, immunomodulatory and neuroprotective (Chan and Ho, 1997; Chung et al., 2004; Tanaka et al., 1993; Ban et al., 2006). The mechanisms involved in these beneficial properties of caffeic acid have not yet been fully understood. In addition, there is no evidence on the effects promoted by caffeic acid on purinergic signaling parameters.

Therefore, considering the beneficial activities of caffeic acid on human health and the importance of ectonucleotidases, the aim of this study was to evaluate changes in the adenine nucleotide hydrolysis promoted by ectonucleotidases in platelets and lymphocytes of rats after treatment with caffeic acid, as well as to investigate the effects of this compound on the profile platelet aggregation.

2. Materials and methods

2.1. Materials

Nucleotides, Trizma Base, Ficcoll, Adenosine and caffeic acid were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Clopidogrel was obtained from Germed Farmacêutica LTDA (Hortolôndia, SP, Brazil). All other reagents used in the experiments were of analytical grade and of the highest purity.

2.2. Animals

Adult male Wistar rats (70–90 days, 220–300 g) were obtained from the Central Animal House of Federal University of Santa Maria (UFSM). Animals were maintained at a constant temperature (23 ± 1 °C) on a 12 h light/dark cycle with free access to food and water. Animals were used according to the guidelines of the Committee on Care and Use of Experimental Animal Resources, (COBEA) and are in accordance with international guidelines.

2.3. Treatment with caffeic acid

Fifty rats were used in this study. Animals were divided into five groups ($n = 10$): group I (control saline); group II (canola oil); group III (caffeic acid 10 mg/kg); group IV (caffeic acid 50 mg/kg); and group V (caffeic acid 100 mg/kg). The caffeic acid was diluted in canola oil and administered via gavage to the animals of groups III, IV and V, while animals of group I received saline and group II canola oil. Caffeic acid was freshly prepared and administered once a day for 30 days. After this time, animals were anesthetized and submitted to euthanasia. The total blood was collected by cardiac puncture for lymphocytes, platelet separation and platelet profile aggregation.

2.4. Platelet preparation

Platelet-Rich Plasma (PRP) was prepared by the method of Lunkes et al. (2004). Total blood was collected by cardiac puncture with 0.120 M sodium citrate as anticoagulant and centrifuged at 160g for 15 min. Next, PRP was centrifuged at 1400g for 30 min and washed twice with 3.5 mM HEPES buffer, pH 7.0. The washed platelets were resuspended in HEPES isosmolar buffer and adjusted to 0.4–0.6 mg of protein per milliliter.

2.5. Isolation of lymphocytes

Lymphocytes were isolated from blood collected with EDTA as anticoagulant and separated on Ficoll–Histopaque density gradients as described by Böyum (1968). Then, lymphocytes were suspended in saline solution and the final protein concentration was adjusted to 0.1–0.2 mg/mL.

2.6. NTPDase and 5′-nucleotidase activity assays

In platelets, the reaction medium for NTPDase activity containing 5 mM $CaCl_2$, 100 mM NaCl, 4 mM KCl, 5 mM glucose and 50 mM Tris–HCl buffer, pH 7.4, at a final volume of 200 μL was carried out as described by Lunkes et al. (2004). For 5′-nucleotidase, the reaction medium was used as previously described, except that 5 mM $CaCl_2$ was replaced by 10 mM $MgCl_2$.

In lymphocytes, the NTPDase activity was determined as described by Leal et al. (2005a,b). The reaction medium contained 0.5 mM $CaCl_2$, 120 mM NaCl, 5 mM KCl, 6 mM glicose and 50 mM Tris HCl buffer pH 8.0, at a final volume of 200 μL.

In both cases, 20 μL of the enzyme preparation (8–12 μg of protein) was added to the reaction mixture and pre-incubated at 37 °C. The reaction was initiated by the addition of ATP, ADP or AMP. Reactions were stopped by the addition of 10% trichloroacetic acid (TCA). Released inorganic phosphate (Pi) was assayed by the method of Chan et al. (1986) using malachite green as the colorimetric reagent and KH_2PO_4 as standard. Controls were carried out to correct for non-enzymatic hydrolyses of nucleotides by adding platelets after TCA addition. All samples were run in triplicate. Enzyme specific activities were reported as nmol Pi released/min/mg of protein.

2.7. E-NPP activity assay

The E-NPP activity from platelets was assessed using p-nitrophenyl 5′-thymidine monophosphate (p-Nph-5′-TMP) as substrate as described by Fürstenau et al. (2006). The reaction medium containing 50 mM Tris–HCl buffer, (pH 8.9), 120 mM NaCl, 5.0 mM KCl, 60 mM glucose and 5.0 mM CaCl2 was pre-incubated with approximately 20 μg per tube of platelet protein for 10 min at 37 °C to a final volume of 200 μL. The enzyme reaction was started by the addition of p-Nph-5′-TMP at a final concentration of 0.5 mM. After 80 min of incubation, 200 μL NaOH 0.2 N was added to the medium to stop the reaction. The amount of p-nitrophenol released from the substrate was measured at 400 nm using a molar extinction coefficient of 18.8×10^{-3}/M/cm. The enzyme activity was expressed as nmol p-nitrophenol released/min/mg protein.

2.8. ADA activity assay

The adenosine deaminase activity was measured in lymphocytes and platelets using the method of Giusti (1974). The reaction was started by the addition of the substrate (adenosine) to a final concentration of 21 mmol/L and incubations were carried out for 1 h at 37 °C. The pH of enzymatic assay of ADA was 6.5. The reaction was stopped by adding 106 mmol/L/0.16 mmol/L phenol–nitroprusside/ml solution. The reaction mixtures were immediately mixed to 125 mmol/L/11 mmol/L alkaline hypochlorite (sodium hypochlorite) and vortexed. Ammonium sulfate of 75 μmol/l was used as ammonium standard. The ammonia concentration is directly proportional to the absorption of indophenol at 620 nm. The specific activity is reported as U/L. One unit (1 U) of ADA is defined as the amount of enzyme required to release 1 mmol of ammonia per minute from adenosine at standard assay conditions.

2.9. Protein determination

Protein was measured by the method of Bradford (1976) using bovine serum albumin as standard.

2.10. Effects of the caffeic acid on platelet aggregation of rats

After 30 days of treatment with caffeic acid, the platelet aggregation profile was evaluated in the groups I, II, III, IV and V using the method of Born and Cross (1963) by measuring turbidity with a Chrono-log optical aggregomete (AGGRO/LINK® Model 810-CA software for Windows version 5.1) and using ADP at a concentration of 5 μM as agonist. Results are expressed as percentage of aggregation assuming that platelet-poor plasma (PPP) represented 100% light transmission and that PRP represented 0% light transmission. After the addition of the agonist, the platelet shape changed from discoid to irregular and small aggregate form. The intensity of the transmitted light increase moderately. As the platelets secrete granule contents, larger aggregates form, more light cross and the tracing moves toward 100% light transmittance constituting 100% of platelet aggregation.

2.11. Effect of caffeic acid associated with clopidogrel in the profile of the platelet aggregation of rats

Clopidogrel is the most popular antiplatelet drug with high efficacy. In this line, this experiment was carried out to compare the effects of caffeic acid and clopidogrel *in vivo* in the platelet aggregation of rats. Twenty rats were used in this study. Animals were divided into four groups ($n = 5$): group I (control saline); group II (20 mg/kg clopidogrel); group III (caffeic acid 50 mg/kg); and group IV (clopidogrel 20 mg/kg and caffeic acid 50 mg/kg). Clopidogrel was suspended in saline and administered by gavage in the animals of groups II and IV. Caffeic acid was diluted in canola oil and administered by gavage in the animals of groups III and IV. The

control group (I) received only saline. All groups were treated once a day for 13 days consecutively. After this time, animals were anesthetized and submitted to euthanasia. The total blood was collected by cardiac puncture and the platelet aggregation was evaluated as described above using collagen at concentration of 5 and 7.5 μg/ml as agonist.

We also evaluated the time for whole blood visual coagulation in all the groups of this experiment. Blood was incubated at 30 °C and the whole blood coagulation was monitored carefully. Results are expressed as time (s) spent in coagulations.

2.12. In vitro effect of caffeic acid in platelet aggregation of human samples

The sample consisted of 10 healthy subjects (age 20–30 years). All subjects gave written informed consent to participate in this study. The Human Ethics and the project were approved by the Ethic Committee of the Federal University of Santa Maria. Twelve milliliters of blood was collected from each subject and used to evaluate the *in vitro* effect of the caffeic acid on the platelet aggregation. Caffeic acid was diluted in ethanol 30% and tested in the platelet aggregation at the final concentrations of 0, 0.1, 0.25, 0.50, 0.75 and 1.0 mM, using ADP (5 and 7.5 μM) as agonist.

2.13. Statistical analysis

The statistical analysis used was one-way ANOVA followed by Duncan's multiple range tests. $P < 0.05$ was considered to represent a significant difference between groups. All data were expressed as mean ± SEM.

3. Results

The findings of the present study demonstrated that caffeic acid altered the ectonucleotidase activities in platelets after treatment for 30 days. As can be observed in Fig. 1, caffeic acid decreased the ATP hydrolysis in the animals treated with 10 mg/kg (39.30%), 50 mg/kg (31%) and 100 mg/kg (33%) when compared with the control group ($P < 0.05$, Fig. 1A). On the other hand, this compound caused an increase in the NTPDase activity for ADP hydrolysis in platelets of rats treated with 10 mg/kg (22%), 50 mg/kg (34%) and 100 mg/kg (30%) in relation to the control group ($P < 0.05$, Fig. 1B). Similar to the results obtained for ATP hydrolysis, the 5′-nucleotidase activity was also reduced in the groups exposed to caffeic acid (10 mg/kg (39%), 50 mg/kg (44%) and 100 mg/kg (40%) ($P < 0.05$, Fig. 1C).

Fig. 1 also shows the effects of the caffeic acid on the E-NPP and ADA activities in platelets. Results demonstrated that this compound increased the E-NPP (10 mg/kg (72%), 50 mg/kg (138%) and 100 mg/kg (179%)) and ADA activities (10 mg/kg (71%), 50 mg/kg (105%) and 100 mg/kg (148%)) in a dose-dependent manner in all the animals treated in relation to the control group ($P < 0.05$, Fig. 1D and E, respectively).

In addition, the findings obtained regarding the aggregation profile revealed that the treatment with caffeic acid with 10 mg/kg, 50 mg/kg and 100 mg/kg for 30 days decreased platelet aggregation by 39%, 18% and 18%, respectively, using ADP as agonist, when compared with the control group ($P < 0.05$) (Fig. 2).

In the other set of experiments, we compared the effects of clopidogrel and caffeic acid in the profile of the platelet aggregation of rats. Fig. 3 shows the *in vivo* effect of caffeic acid and clopidogrel alone or in association on platelet aggregation using collagen as agonist. When 5 μg/ml collagen was used, we observed a decrease in the platelet aggregation in the group treated with clopidogrel (20 mg/kg, 70.68%), caffeic acid (50 mg/kg, 67.813%) and clopidogrel together with caffeic acid (20 mg/kg and 50 mg/kg, respectively, 79.31%) when compared to the control group ($P < 0.05$). When 7.5 μg/ml collagen was used as agonist a decrease was also observed in the platelet aggregation in all groups evaluated. These findings were similar to results obtained with 5 μg/ml of collagen (Fig. 3, $P < 0.05$).

Fig. 4 demonstrates the whole blood visual coagulation time. We observed that the coagulation time significantly increased with clopidogrel (20 mg/kg, 33.02%) caffeic acid (50 mg, 41.59% and clopidogrel together with caffeic acid (20 mg/kg and 50 mg/kg, 38.650%) when compared to the control group ($P < 0.05$).

We also evaluated the *in vitro* effects of caffeic acid on the platelet aggregation of healthy subjects. When ADP (5 μM) was used as agonist, caffeic acid inhibited platelet aggregation at the concentrations of 0.25 mM (43%), 0.50 mM (44.04%), 0.75 mM (48%), and 1.0 mM (50%). The same pattern was observed when ADP (7.5 μM) was utilized as agonist. Caffeic acid also inhibited platelet aggregation at the concentrations of 0.50 mM (26.5%), 0.75 mM (32.91%), and 1.0 mM (43.12%) when compared to control groups ($P < 0.05$, Fig. 5).

Treatment for 30 days with caffeic acid also altered the NTPDase and ADA activities in lymphocytes of rats. The ATP and ADP hydrolysis was increased in the animals treated with 10 mg/kg, 50 mg/kg and 100 mg/kg of caffeic acid (102%, 118%, 135% and 35%, 18%, 71%), respectively, when compared with the control group ($P < 0.05$) (Fig. 6A and B). Similarly, the ADA activity also showed an increase in the lymphocytes of animals treated with 10 mg/kg (53%), 50 mg/kg (77%) and 100 mg/kg (103%) when compared with the control group ($P < 0.05$) (Fig. 6C).

In addition, it is also important to note that controls were performed to correct for vehicle (canola oil or ethanol) interference. No differences between vehicle group and control were observed (Fig. 5: ethanol group 30%, and Figs. 1 and 6: oil group).

4. Discussion

The present study was carried out to evaluate the effects of caffeic acid treatment on the ectonucleotidase activities in different cells such as platelets and lymphocytes of rats. To the best of our knowledge, there are no studies in literature evaluating the effects of caffeic acid on purinergic signaling until now.

Our results showed that ATP and AMP hydrolysis in platelets was decreased, while ADP hydrolysis was increased in all groups treated with caffeic acid for 30 days (Fig. 1A–C). In addition, E-NPP and ADA activity also increased in the platelets of animals treated with this compound (Fig. 1D and E). Corroborating these results, previous studies from our laboratory demonstrated that other phenolic compounds such as resveratrol also altered the ectonucleotidase activities from platelets (Schmatz et al., 2009). Taken together, these findings indicated that phenolic compounds play an important role in the purinergic signaling of platelets.

It has been established that ATP, ADP and adenosine influence vascular tone, cardiac function and platelet aggregation (Burnstock, 2004). ADP acts upon platelets regulating their aggregation and modifying their shape, while ATP has been postulated to be a competitive inhibitor of ADP platelet aggregation (Birk et al., 2002; Remijin et al., 2002). Furthermore, adenosine produced by nucleotide catabolism is recognized as a vasodilator and inhibitor of platelet aggregation (Atkinson et al., 2006; Robson et al., 2006). The metabolism of these extracellular nucleotides in platelets occurs by a multienzymatic system on their surface (Zimmermann, 2001). In fact, in the vascular system the NTPDase is accepted to be a potent antithrombotic agent because this enzyme rapidly metabolizes ADP, terminating further platelet recruitment and aggregation (Anfossi et al., 2002; Lunkes et al., 2004).

Studies performed with caffeic and 5-caffeoylquinic acids have reported a decrease in the risk of inflammation and cardiovascular diseases (Bonita et al., 2007). In addition, caffeic acid from *Salvia miltiorrhiza*, a traditional Chinese herbal medicine, (Hirose et al., 1998) has been used extensively for the treatment of coronary artery diseases and myocardial infarct (Beyer and Melzig, 2003). Our findings suggest that the beneficial effects of caffeic acid in the cardiovascular diseases could be associated with the modulation of adenine nucleotide hydrolysis in platelets. Firstly, we showed that

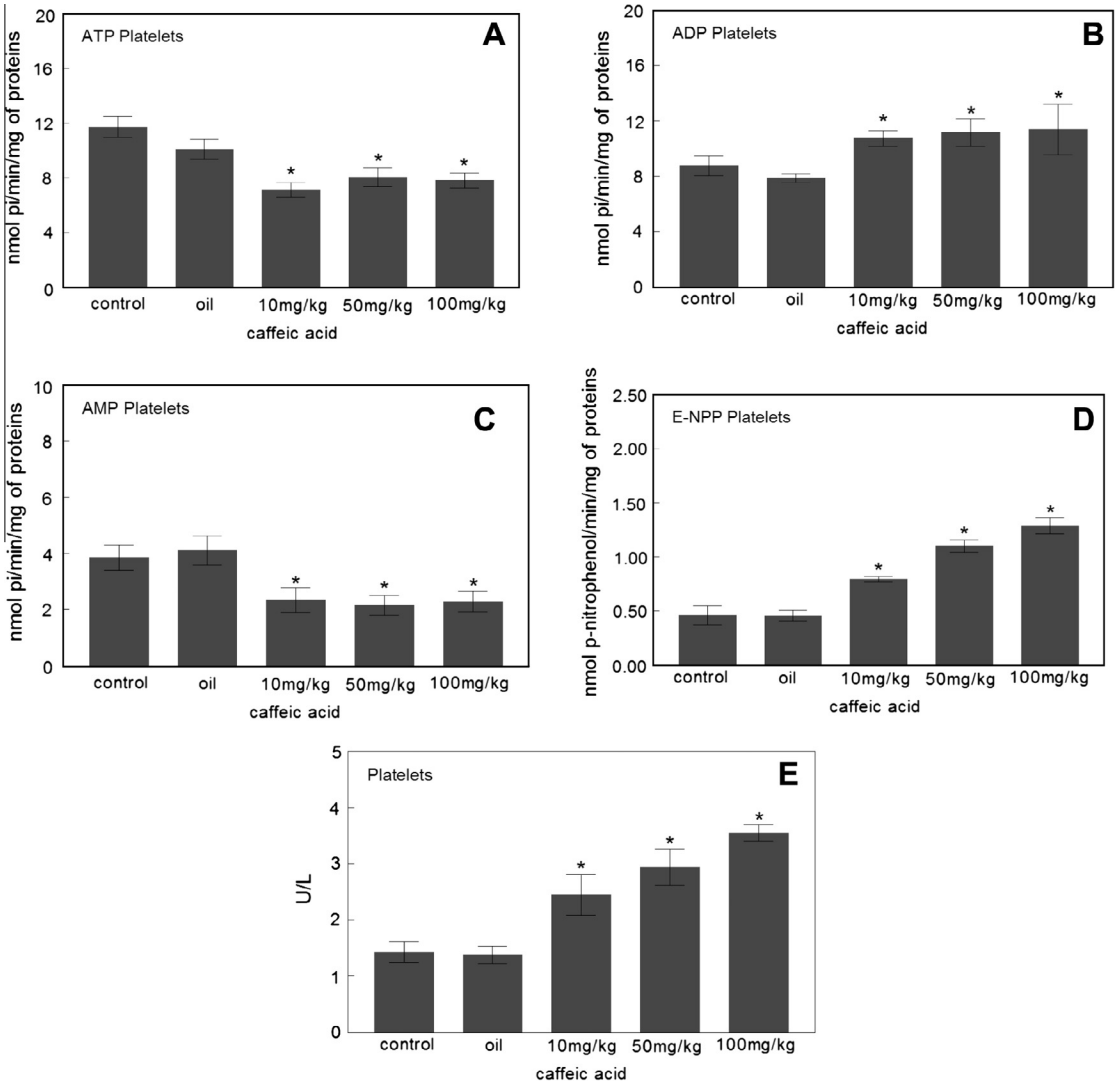

Fig. 1. Effects of the treatment for 30 days with caffeic acid (10, 50 and 100 mg/kg) on NTPDase (A) and (B), 5′-nucleotidase (C), E-NPP (D) and ADA (E) activities in platelets of rats. Bars represent mean ± SEM. Results are expressed in nmol Pi/min/mg of protein. * Different from control ($P < 0.05$, with $n = 10$).

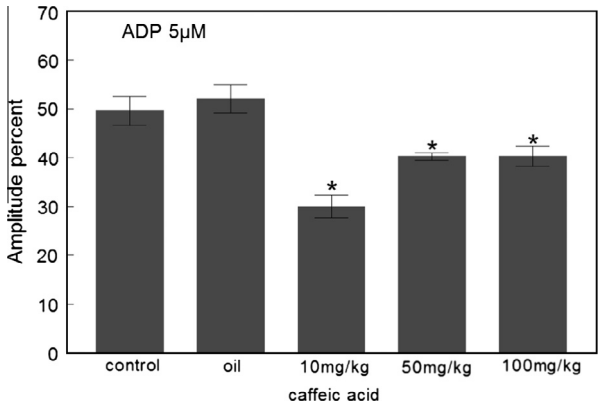

Fig. 2. Percentage of platelet aggregation of rats treated with caffeic acid (10, 50 and 100 mg/kg) by 30 days, using ADP (5 μM) as agonist. Bars represent mean ± SEM. * Indicates statistical differences in relation to the control groups for $P < 0.05$; with $n = 5$.

caffeic acid has heterogeneously effects on the ectonucleotidase activities in platelets. Caffeic acid decreased AMP hydrolysis and increased the ADA activity (Fig. 1C and E). This alteration could decrease the adenosine levels in the circulation causing vascular complications, since adenosine has an important role in preventing the thrombotic process. On the other hand, the inhibition of the ATP hydrolysis could be important to decrease the ADP extracellular levels. Moreover, the enhancement in the ADP hydrolysis in platelets due to caffeic acid plays a crucial role in controlling the platelet coagulant status by removing the extracellular ADP, which is the main agonist of platelet aggregation.

Reinforcing this hypothesis, we have also observed that treatment with caffeic acid for 30 days induced a significant reduction in platelet aggregation in rats (Fig. 2). Similar results were obtained *in vitro*, which showed that this compound also reduced the platelet aggregation in humans, using ADP as agonist (Fig. 5). These findings support the opinion that this phenolic compound can be associated with a reduction and prevention of the risk of cardiovascular diseases by interfering with mechanisms of platelet aggregation induced by ADP.

Caffeic acid is metabolized after absorption; however, the percentage metabolized or absorbed has not been established in literature. Omar et al. (2012) for instance showed that 3% of caffeic acid can be detected in plasma after 1 h, while Olthof et al. (2001) demonstrated that a maximum of 95% of the ingested caffeic acid (2.8 mmol) was absorbed from the small intestine in humans.

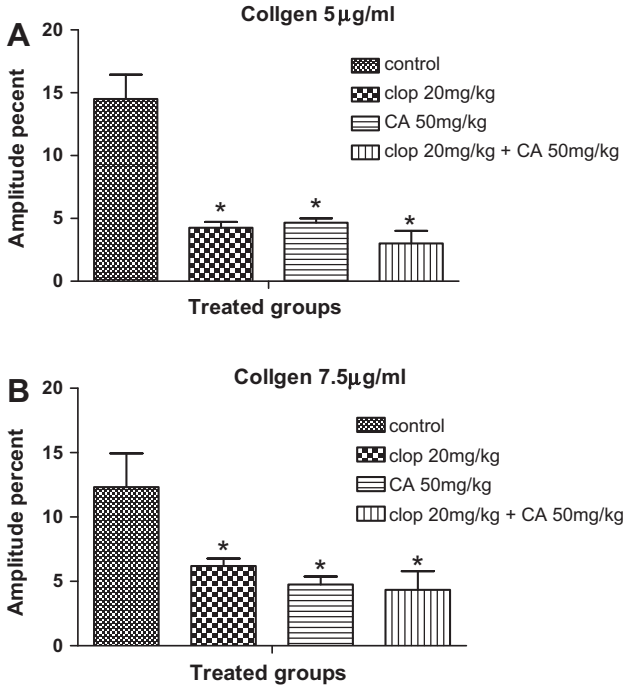

Fig. 3. Percentage of platelet aggregation of rats treated with caffeic acid and clopidogrel for 13 days, using collagen at the concentrations of 5 and 7.5 µg/ml as agonist. Groups: Control, Clopidogrel 20 mg/kg (Clop 20 mg/kg), caffeic acid 50 mg/kg (CA 50 mg/kg), and clopidogrel (20 mg/kg) + caffeic acid (50 mg/kg) (clop 20 mg/kg + CA 50 mg/kg). Bars represent mean ± SEM. * Indicates statistical differences in relation to the control group ($P < 0.05$; with $n = 5$).

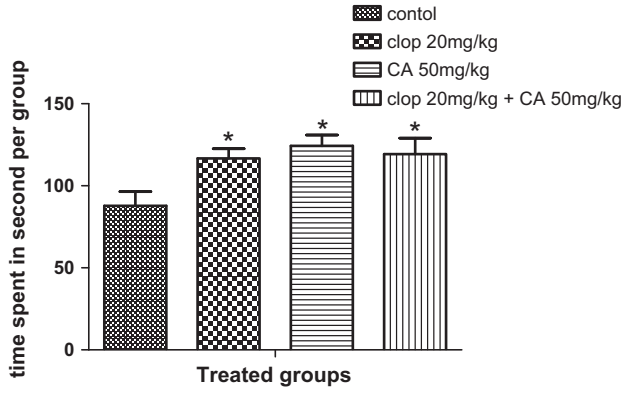

Fig. 4. Time of blood visual coagulation of rats treated with caffeic acid and clopidogrel for 13 days. Groups: Control, Clopidogrel 20 mg/kg (Clop 20 mg/kg), caffeic acid 50 mg/kg (CA 50 mg/kg), and clopidogrel (20 mg/kg) + caffeic acid (50 mg/kg) (clop 20 mg/kg + CA 50 mg/kg). Bars represent mean ± SEM. Results were expressed in time spent in second per group. * Indicates statistical differences in relation to the control group ($P < 0.05$; with $n = 5$).

Azuma et al. (2000) showed that ingeste caffeic acid (700 µmol/kg caffeic acid) was absorbed from the alimentary tract and was present in the rat blood circulation in the form of various metabolites. They also showed that caffeic acid metabolites found in rat plasma were in the form of glucuronide, sulfate, and sulfate/glucuronide conjugates of caffeic acid or its methylated compound, ferulic acid.

The absorbed fraction of caffeic acid and its metabolic may induce biological effects in blood circulation. In relation to our results, it is possible that the platelet aggregation or ectonucleotidase activities *in vivo* could be influenced by caffeic acid metabolites. In this line, previous studies demonstrated that p-coumaric, caffeic and ferulic acid inhibit collagen-induced aggregation in human platelets

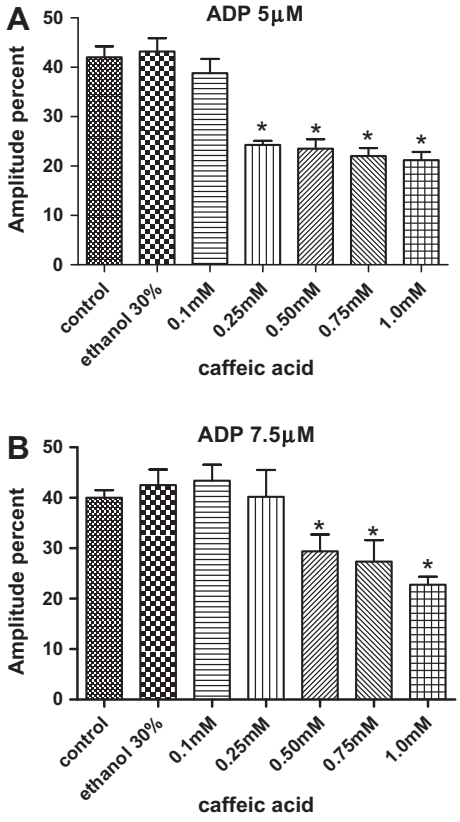

Fig. 5. *In vitro* effects of caffeic acid on platelet aggregation of healthy subjects using ADP 5 µM and 7.5 µM as agonist of the aggregation. Bars represent mean ± SEM. Results were expressed in percentage of the aggregation. * Indicates statistical differences in relation to the control group ($P < 0.05$; with $n = 10$).

by 50% at concentrations between 478 and 816 µmol (Hubbard et al., 2003). In addition, the incubation of whole blood with concentrations of 100 µmol/L p-coumaric acid, caffeic acid, ferulic acid, 4-hydroxyphenylpropiony, 5-methoxysalicylic acid, and catechol inhibited significantly the surface of P-selectin expression (marker platelet activation) (Ostertag et al., 2011). Comparing to the study of Ostertag et al. (2011), our results suggest that high concentrations of caffeic acid are necessary to inhibit the platelet aggregation induced by ADP *in vitro* (Fig. 5). As concentrations of 0.5–1 mM are unlikely to be reached in the circulation, further studies are needed to evaluate whether the consumption of caffeic acid at the concentration nutritionally attainable may change the profile of platelet aggregation in humans.

Another important aspect to be discussed is that platelets express receptors on their surfaces which play a crucial role in the thrombus formation (Hourani, 1996; Hechler et al., 2005). Extracellular nucleotides P2 receptors consist of two classes: P2X, ligand-gated cation channels, classified in 7 subtypes ($P2X_1$–$P2X_7$) and P2Y, receptors G protein coupled, classified in 8 subtypes ($P2Y_1$, $P2Y_2$, $P2Y_4$, $P2Y_6$, $P2Y_{11}$, $P2Y_{12}$, $P2Y_{13}$, $P2Y_{14}$) (Moheimani and Jackon, 2012). Among these receptors, only $P2X_1$, $P2Y_1$, $P2Y_{12}$, are expressed at significant levels in platelets, with the $P2Y_{12}$ expressed at the highest extent followed by $P2X_1$ and $P2Y_1$, respectively (Moheimani and Jackon, 2012). In relation to platelet thrombus formation, two of the P2Y receptors play an essential role: the $P2Y_1$ initiates platelet activation in response to ADP and participates in platelet aggregation mediated by collagen, while stimulation of the $P2Y_{12}$ receptor by ADP completes and amplifies platelet activation and aggregation (Dorsam and Kunapuli, 2004; Moheimani and Jackon, 2012).

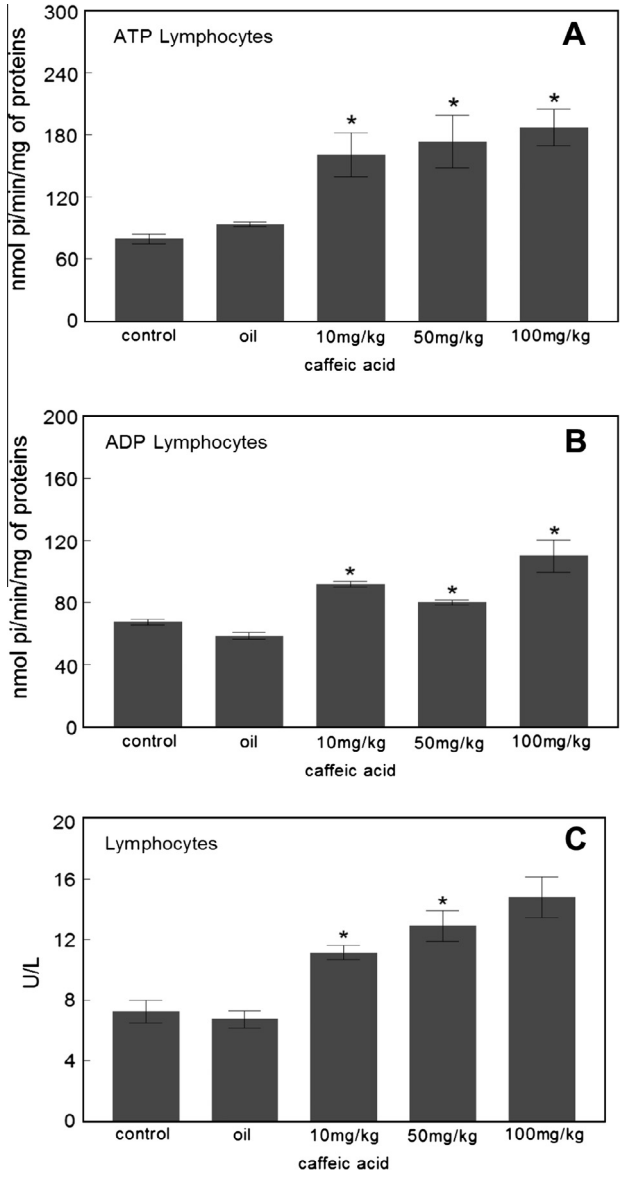

Fig. 6. Effects of caffeic acid on NTPDase (A and B) and adenosine deaminase (C) activity on lymphocytes of adult rats treated for 30 days with caffeic acid (10, 50 and 100 mg/kg). Bars represent mean ± SEM. * Different from control ($P < 0.05$; with $n = 10$).

In this line, clopidogrel is an antiplatelet compound whose active metabolites are known as platelet ADP receptor blockers $P2Y_{12}$ (Savi et al., 2006). Clopidogrel is the most commonly prescribed $P2Y_{12}$ blocker and has been shown to be effective in the prevention of cardiovascular complications (Pereillo et al., 2002). In this study, we compared the *in vivo* effects of caffeic acid and clopidogrel in platelet aggregation induced by collagen as well as the time of coagulation in rats. As shown in Figs. 3 and 4, caffeic acid and clopidogrel have similar effects in platelet aggregation and in the time of coagulation. Based on this, it is plausible to consider that caffeic acid also interferes with the purinoreceptores $P2Y_1$ and $P2Y_{12}$. This novel finding can provide additional mechanisms by which the antiaggregant effects of caffeic acid could be mediated.

The following set of experiments was performed in order to evaluate the effects of caffeic acid on the ectonucleotidase activity from lymphocytes. Our results showed that after 30 days of exposure, the NTPDase and ADA activities were increased in the animals treated with 10, 50 and 100 mg/kg of caffeic acid (Fig. 6A–C), demonstrating that this compound may also be able to induce alterations in the purinergic signaling in immune cells.

Extracellular ATP is involved in proinflammatory functions such as stimulation and proliferation of lymphocytes and cytokine release. These effects of ATP are controlled by the action of ectonucleotidases, including NTPDase, which is expressed in numerous types of immune cells (Dwyer et al., 2007). Studies have demonstrated that the expression and activity of NTPDase is up-regulated in the activated lymphocytes (Zimmermann et al., 1998; Pulte et al., 2007). It has been also reported that besides the catalytic property, NTPDase has also an important role in cell adhesion and in the control of lymphocyte functions including antigen recognition and/or the activation of effector activities of cytotoxic T cells (Dombrowski et al., 1995). The treatment with caffeic acid was able to increase the NTPDase activity in rat lymphocytes. These effects suggest a modulatory role of caffeic acid on the nucleotidase pathway, which possibly induces a decrease of extracellular ATP, representing a mechanism that may help to reduce inflammation. In fact, literature describes that caffeic acid has anti-inflammatory properties, and based on this study it is plausible to suggest that this effect can be explained, at least in part, by the alterations in the NTPDase activity in lymphocytes.

On the other hand, caffeic acid has also increased the ADA activity in lymphocytes. ADA is considered essential for the differentiation, normal growth and proliferation of lymphocytes (Aldrich et al., 2000). This enzyme catalyzes the deamination of adenosine to inosine and closely regulates extracellular concentrations of this nucleoside (Franco et al., 1997). Adenosine is considered the anti-inflammatory molecule that inhibits the lymphocyte activation and decreases the cytokine secretion through A_{2A} receptors (Dombrowski et al., 1998; Hershfield, 2005). In this context, the increase in the ADA activity by caffeic acid may lead to a rapid deamination of adenosine causing a decrease in the extracellular levels of this molecule, which can affect the sensitivity of receptors (A_{2A} and A_{2B}) and alter the inflammatory responses.

The changes promoted by caffeic acid on the nucleotide hydrolysis may be possible due to distinct mechanisms. Caffeic acid is effective as a free radical scavenging (Gulcin, 2006) and chelation of metal ions (Psotova et al., 2003). Some studies have reported that an antioxidant may become a pro-oxidant to accelerate lipid peroxidation and/or induce DNA damage under special conditions (Yamanaka et al., 1997). Polyphenolic antioxidants, such as resveratrol may induce lipid peroxidation and/or DNA damage in the presence of cupric ions (Azmi et al., 2005). In this line, caffeic acid has been reported to induce lipid peroxidation and DNA damage either alone or in the presence of copper ions (Yamanaka et al., 1997). Bhat et al. (2007) showed that concentrations of 200 and 400 μM of caffeic acid are capable of promoting DNA breakage in lymphocytes. In light of these reports, the effect of the caffeic acid associated with lipid peroxidation is able to alter membrane fluidity. This phenomenon could partially contribute to explain the alterations in the ectonucleotidases observed in this study.

Another important aspect to be discussed is that caffeic acid has also been reported to chelate metal (Yamanaka et al., 1997). This effect may be attributed to the presence of a catechol group that has two hydroxyl groups attached to its main ring that may produce a site for chelation. Based on these observations, we can suppose that this chelating property of caffeic acid may alter the ectonucleotidase activities because divalent cations are required for the catalytic function of these enzymes. Reinforcing this hypothesis, Da Silva et al. (2006) also suggests that the chelating property of flavonoids may diminish NTPDase, 5′- nucleotidase and Na^+/K^+-ATPase activities in cortical membrane preparation.

5. Conclusion

In conclusion, our results demonstrated that caffeic acid alters the ectonucleotidase pathway, modulating the balance in the purine levels which can induce relevant effects in the lymphocytes and platelets. Furthermore, caffeic acids also diminished aggregation platelet in human and animals. These findings suggest that caffeic acid is a natural and promising compound and further studies have to be carried out in order to determine the therapeutic potential of the caffeic acid in the purinergic signaling associated with immune and vascular disorders.

Conflict of Interest

The authors declare that there are not conflict of interest.

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