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THE EFFECTS OF CAFFEIC ACID AND CAFFEIC ACID PHENETHYL ESTER ON THE ACTIVITIES OF ACETYLCHOLINESTERASE AND ECTO-NUCLEOTIDASES IN RATS

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Santa Maria, RS, Brasil.

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THE EFFECTS OF CAFFEIC ACID AND CAFFEIC ACID PHENETHYL ESTER ON THE ACTIVITIES OF ACETYLCHOLINESTERASE AND ECTO-NUCLEOTIDASES IN RATS

Javed anwar

A thesis submitted to, **PPGBQ – CCNE – UFSM,** in partial fulfillment of the requirements for the Degree of Doctor of Philosophy (PhD) in Biochemical Toxicology by the Post Graduate Program in Biochemical Toxicology Center of Exact and Natural Sciences Federal University of Santa Maria Santa Maria, RS, Brazil

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By
Javed Anwar

In partial fulfillment of the requirements for the Degree of Doctor of Philosophy (PhD) in Biochemical Toxicology by the Post Graduate Program in Biochemical Toxicology Center of Exact and Natural Sciences Federal University of Santa Maria Santa Maria, RS, Brazil

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RESUMO

Em cumprimento parcial dos requisitos para obtenção do grau de Doutor em Filosofia (PhD) em Toxicologia Bioquímica do Programa de Pós-Graduação em Ciências Biológicas: Bioquímica Toxicológica
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Os efeitos do ácido cafeico e do éster fenetil do ácido cafeico sobre as atividades da acetilcolinesterase e das ecto-nucleotidases em ratos

Os compostos fenólicos e seus derivados constituem uma importante família de compostos naturais. O ácido cafeico (AC) e o éster fenetil do ácido cafeico (CAPE) são membros importantes dessa família e compartilham algumas aplicações biológicas, tais como: antioxidante, neuroprotetor, antiinflamatório, antiproliferativo, antibacteriano, antiviral, antiaterosclerótico e anticancerígeno. Entretanto, a literatura relata algumas atividades pró-oxidantes, dependendo do ambiente celular. Devido a estas propriedades patofisiológicas, aumentou o interesse com o objetivo de avaliar o efeito de CA e CAPE sob as atividades das enzimas purinérgicas e da acetilcolinesterase (AChE), tanto no Sistema Nervoso Periférico (SNP) como no Sistema Nervoso Central (SNC). Previamente, nosso grupo de pesquisa relatou que o composto fenólico tem a capacidade de alterar as atividades dessas enzimas. A AChE rapidamente hidrolisa a acetilcolina (ACh) em tecidos neuronais e não neuronais, mediando algumas doenças neurodegenerativas. Ao lado da ACh, o ATP (como co-neurotransmissor) e adenosina são importantes moléculas sinalizadoras, comunicando as células em ambos os SNP e do SNC. Nas vias de sinalização extracelulares, os nucleotídeos de adenina e seus derivados podem ser acoplados a receptores específicos e desse modo ter um papel crucial no sistema nervoso, sistema vascular e imune. Uma vez liberadas, estas moléculas são hidrolisadas por uma cascata de enzimas incluindo a ectonucleosídeo trifosfato difosfoidrolase (NTPDase; EC 3.6.1.5, CD39), 5'-nucleotidase (EC 3.1.3.5, CD73), ectonucleotideo pirofosfatase/fosfodiesterase (E-NPP), modulando definitivamente as vias de sinalização do funcionamento normal do sistema nervoso, sistema vascular e imune. Além disso, a adenosina

deaminase (ADA) e a xantina oxidase (XO) degradam a adenosina e a xantina, respectivamente, as quais controlam o funcionamento de mecanismos em eventos celulares. As enzimas encontradas em tecidos neuronais e não neuronais como a AChE, a NTPDase, a 5'-nucleotidase, a E-NPP e a ADA regulam eventos celulares incluindo a neurotransmissão, inflamação e processos trombogênicos. Com essas informações, nós introduzimos a hipótese de avaliar primeiramente os efeitos *in vitro* de CA na atividade da AChE periférica e no sistema central colinérgico de ratos. Os resultados demonstraram que o CA modula significativamente o sistema colinérgico no estudo *in vitro*. Essa modulação demonstra aparentemente que o CA (estrutura fenólica) possui propriedades de ação que altera a neurotransmissão. Portanto, a hipótese de se avaliar os efeitos *in vivo* de CA na atividade da AChE, NTPDase, E-NPP, 5'-nucleotidase, ADA e da agregação de plaquetas em diferentes tecidos de ratos tornou-se evidente. Para esse estudo, os animais foram tratados durante 30 dias e sacrificados após o teste comportamental. Os resultados do experimento demonstraram que o CA aumentou significativamente a atividade da AChE em hipocampo, hipotálamo, ponte e nos linfócitos, enquanto que no córtex cerebral, cerebelo e estriado a AChE foi inibida. No teste comportamental o CA teve evolução de melhora na latência de passos da esquiva inibitória. A investigação dos efeitos *in vivo* do CA no sistema purinérgico demonstrou aumento na hidrólise de ATP e AMP em sinaptossomas. Entretanto, não foram observadas alterações significativas na atividade da ADA em sinaptossomas dos grupos avaliados neste estudo. Em plaquetas, o CA aumentou significativamente a hidrólise de ATP e AMP, enquanto que a hidrólise de ADP foi diminuída nesse tecido. No presente estudo o CA reduziu significativamente a agregação de plaquetas induzida pelo agonista ADP. Além disso, o tratamento com CA aumentou significativamente as atividades da NTPDase e da ADA em linfócitos de ratos. Considerando a dupla função de CA, *in vitro* e *in vivo*, o presente estudo foi estendido para CAPE seguindo o modelo de tratamento agudo pela via intraperitoneal (ip) com o objetivo de elucidar o efeito de uma segunda estrutura fenólica sobre os mesmos parâmetros. Nesta linha de pesquisa, os animais foram tratados ip com CAPE e eutanasiados após 40 minutos. Em plaquetas, os resultados demonstraram que o CAPE aumentou significativamente a atividade da NTPDase, E-NPP e 5'-nucleotidase, enquanto que a atividade da ADA não foi alterada significativamente. Em sinaptossomas, o CAPE inibiu significativamente a atividade da NTPDase e da 5'-nucleotidase. O CAPE não induziu alterações significativas na atividade da ADA em sinaptossomas, mas reduziu significativamente a atividade da XO em todo o cérebro. Finalmente, nós investigamos a atividade da AChE no córtex cerebral, cerebelo, estriado, hipocampo, hipotálamo, ponte, linfócitos e músculos de ratos tratados com CAPE. Os resultados demonstraram que CAPE diminuiu significativamente a atividade da AChE em córtex cerebral, cerebelo e estriado. O CAPE aumentou significativamente a atividade da AChE em hipotálamo, hipocampo, ponte, músculo e linfócitos. No sistema colinérgico, nossos resultados demonstram claramente que ambos os compostos possuem dupla função. Estes resultados demonstram que as atividades da AChE e da cascata das ecto-enzimas foram alteradas em diferentes tecidos após o tratamento com CA ou CAPE em ratos, sugerindo que estes compostos devem ser considerados agentes com

potencial terapêutico em doenças imunes, vasculares e neurológicas relacionadas com o sistema colinérgico e purinérgico.

Palavras-chaves: Acetilcolinesterase; Acetilcolina; Nucleotídeo de Adenina; Ectonucleotidases; Purinoreceptores; Ácido Cafeico; Éster fenetil do ácido cafeico.

SUMMARY

In partial fulfillment of the requirements for the Degree of Doctor of Philosophy (PhD) in Biochemical Toxicology the Post Graduate Program in Biochemical Toxicology
Centre for Exact and Natural Sciences Federal University of Santa Maria
Santa Maria, RS, Brazil

THE EFFECTS OF CAFFEIC ACID AND CAFFEIC ACID PHENETHYL ESTER ON THE ACTIVITIES OF ACETYLCHOLINESTERASE AND ECTO-NUCLEOTIDASES IN RATS

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Place and Date of Defense: Santa Maria, January 21th, 2013

Phenolic compouds and their derivatives constitute a leading family of natural compounds. Caffeic acid (CA) and caffeic acid phenethyl ester (CAPE) are the important members of phenolic compound, sharing several biological applications; antioxidant, neuroprotective, anti-inflammatory, antiproliferative, antibacterial, antiviral, antiatherosclerotic and anticancer properties. In spite of these, literature reportes some of its pro-oxidants activity depending on cellular environment. These pathophysiolocal properties increased the interest to evaluate the effect of CA and CAPE on the enzyme evolved in the purines salvage and the acetylcholine hydrolyzing enzyme the acetylcholineterase (AChE); in both PNS and CNS, since the essential constituent of our dietary items. Previously, our research group has reported that phenolic compound altered the activities of these enzymes. The AChE rapidly hydrolyzes the acetylcholine in neuronal and non neuronal tissues, mediating several neurodegenerative diseases. Beside the ACh, ATP (as co-neurotramittors) and adenosine are important signaling molecules, communicating cells in both PNS and CNS. In the extracellular signalling pathways; the adenine nucleotides, their derivative and the coupling of these molecules with specific receptor have a crucial role in nervous, vascular and immune systems. Once released, these molecules are hydrolyzed by a cascade of enzymes including ectonucleoside triphosphate diphosphohydrolase (NTPDase; E.C. 3.6.1.5, CD39), 5'-nucleotidase (E.C 3.1.3.5, CD73), ectonucleotide pyrophosphatase/phosphodiesterase (E-NPP), modulating crucially the signaling pathways in the normal functioning of nervous, vascular and immune systems. Next, adenosine deaminase (ADA) and xanthin oxidase (XO) degrade the adenosine and xanthine respectively which further control the functioning mechanisms in cellular events. Found in the neuronal and non neuronal (in both PNS and CNS) the AChE, NTPDase, 5'-nucleotidase, E-NPP, and ADA regulate several events including neurotransmission, inflammation, and thrombogenic process. We hypothesized to evaluate first the *in vitro* effects of CA on AChE activity in peripheral and central cholinergic system of rats. The results showed that CA significantly modulated the cholinergic system *in vitro*. By modulating the cholinergic system *in vitro*, apparently CA (e.g. phenolic structure) has proper role in neurotransmission. Therefore we hypothesizes to evaluate the *in vivo* effects of CA on AChE, NTPDase, E-NPP, 5'-nucleotidase, platelets aggregation and (ADA) in different tissues/cell from rats. The animals were treated during 30 days and killed after behavioral test. The results showed that caffeic acid increased significantly the AChE activity in hippocampus, hyphothalmus, pon and lymphocytes while that in cortex, cerebellum and striatum the AChE was inhibited. CA improves step-down latencies in the inhibitory avoidance. Investigating the *in vivo* effects of CA in purinergic system, caffecid acid increased the ATP and AMP hydrolysis in synaptosomes. However, in the synaptosomes no alterations were observed in the ADA activity in the groups evaluated in this study. CA increased the ATP and AMP hydrolysis, while the ADP hydrolysis was decreased in platelets. In the present findings caffeic acid decreased the platelets aggregation induced by ADP agonist. Treatment with CA also increased the NTPDase and ADA activities in lymphocytes of rats. Considering the dual function of caffeic acid *in vitro* and *in vivo*, the present study was extended to CAPE followed by acute treatment model (ip) in order to elucidate the effect of another phenolic structure on the same parameters. In this line the animals were treated (ip) with CAPE and killed after 40 minutes. In platelets, the results showed that the effect of CAPE increased the NTPDase, E-NPP, 5'-nucleotidase activities, while ADA activities did not change significantly. In synaptosomes CAPE significantly inhibited the NTPDase, and 5'-nucleotidase activity. CAPE induced no significant changes in ADA in synaptosomes but reduced XO in whole brain. Finaly we investigated the activity of AChE in cortex, cerebellum, striatum, hippocampus, hyphotalamus, pon, lymphocytes and muscules of rats treated with CAPE. The results showed that CAPE significantly decreased the AChE activity in cortex cerebellum and striatum. CAPE significantly increased the AChE activity in hippocampus hypothalamus, pons, muscle and lymphocytes. In cholinergic system our results clearly demonstrating that both compound with dual functions.These findings demonstrated that the AChE activities and the cascade of ecto-enzymes was alter in different tissues after treatment with CA and CAPE in rats, suggesting that these compound should be considered a potentially therapeutic agent in immune, vascular and neurological disorders related with the cholinergic purinergic system.

Key words: Actylcholinesterase. Actylcholine. Adenin nucleotides. Ecto-nucleotidases. Purinoreceptors. Caffeic acid. Caffeic acid phenethyl ester.

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ABBREVIATIONS

ACh -Acetylcholine

AChE - Acetylcholinesterase

ADA - Adenosine Deaminase

ADP - Adenosine diphosphate

AMP - Adenosine monophosphate

ATP - Adenosine triphosphate

cAMP - Cyclic AMP

CA - Caffeic acid

CAPE- Caffeic acid phenethyl ester

CNS - Central Nervous System

mAChRs - Muscrinic acetylcholine receptors

nAChR - Nicotinic acetylcholine receptors

PRP - Platelet Rich Plasma

PNS - Peripheral Nervous System

XO - Xanthine Oxidase

1 INTRODUCTION

1.1 Phenolic Acids and their derivatives

As the secondary plant metabolites (Jaganathan & Mandal 2009; BRAVO, 1998), phenolic compouds and their derivatives constitute a leading family of natural compounds. Among these some are reported as promising or classical antioxidants. One can trace these compounds in any parts of plants including leaves, fruits and roots. The quantity of these compouds in a plant species depends on different aspects, including origin, periods of development and parameters of processessing. There are some studies demonstrating the variation in levels of these compounds depending on the developmental stages of the plants and roots (MOSEL, & HERRMANN, 1974, STÖHR, & HERRMANN, 1975). These bioactive agents are classified as flavonoids, curcuminoids, stilbenes and hydroxycinnamic acids depending on chemical structures and phenolic groups (Bhat et al., 2006; Scalbert & Williamson, 2000; HARBORNE, 1989). Many studies demostrate that phenolic compound have many biological activities. Among these, the well known are, Antioxidant (FRIEDMAN, 1997), anti-inflammatory (MICHULART et al., 1999; SUBARNAS et al., 2000; WIDLANSKY et al., 2005), antibacterial (ALVESALO et al., 2000), antitumor (CHUNG et al., 2004), anticancer (FORMICA et al., 1995; LIU, 2003; LEE, et al., 2000), antimetastatic (CHUNG et al., 2004), vasodilatory (CALDERONE et al., 2004), and inhibit platelet aggregation and improve increased capillary permeability and fragility (HUBBARD, et al., 2004; VALENSI, 1996) and neuroprotective (ILHAN, 2004; YANG et al. 2008). The interactions with different tissues/structures followed by different biological functions including free-radical scavenging, modulation of enzymes, and inhibition of cellular proliferation, the potential role in antibiotic, antiallergic and antiinflammatory activities (BRAVO, 1998), stimulate a significant interest of research. Presently, convincing evidence suggests that regular consumption of fruit and vegetables significantly reduced the incidence of chronic diseases including cancer, cardiovascular disease, stroke, Alzheimer disease and functional declines associated with aging (LIU, 2003). Probably, common dietary components are being the direct sources of phenolic copounds and almost have biological activities in the line of pathological conditions. In this regards plant derived natural products always have been associated with reduced risks of many common important disease. (LIU, 2003)

Hydroxycinnamic acids constitute a major class of phenolic compounds. Caffeic acid (C.A.) (3-(3, 4-dihydroxyphenyl)-2-propenoic acid), is the most abundant representative of hydroxycinnamic acids with two hydroxyl groups and a simple cinnamic acid group (Figure 1).

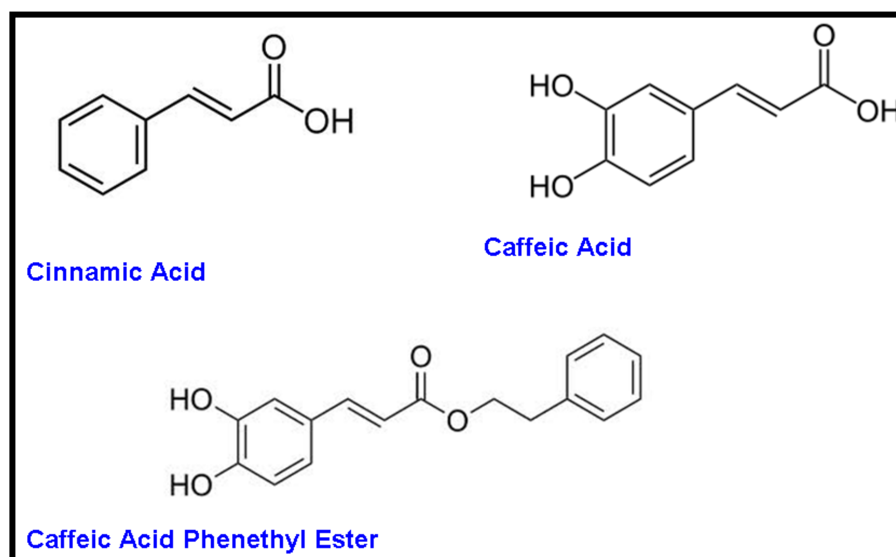

Figure 1- Chemical structures of cinnamic acid, caffeic acid and caffeic acid phenethyl ester.

Caffeic acid is found in various agricultural products such as coffee beans, potatoes, grains, and vegetables (FRIEDMAN, 1997). Other dietary sources of caffeic acid include apples, pears, berries, artichoke and aubergines (CLIFFORD, 1999). The presense of caffeic acid in the human dietary components demonstrates that the bioavailabilty and biological benefits of caffeic acid are originated from their absorption in digestive tract and then metabolised. In this context, the absorption of this compound has been reported in the small intestine both in human and animals (AZUMA et al., 2000; OLTHOF et al., 2001). It is absorbed by the intestine through an active transport system after oral administration (KONISHI et al., 2005), and followed by the antioxidant activities, the well-known biological property activities of caffeic acid (FRIEDMAN, 1997). The antioxidant activity of this compound always referred to Mg^{++} (Fe^{++}) ions chelating property due to (two hydroxyl group) catcholic group (KONO ET AL., 1998). In another study, caffeic acid has been reported to chelate Cu^{++} ions and scavenges free radicals (NARDINI et al., 1995), the main cause of oxidative stress.

In addition, studies also have demonstrated that this compound possesses anticarcinogenic, anti-inflammatory and neuroproctetive properties (FESEN et al., 1994). Caffeic acid (CA) and chlorogenic acid (esters of polyphenolic caffeic acid) exhibit

antioxidant activity *in vitro* and have been shown to have multiple pharmacological properties including anti-inflammatory (MICHULART et al., 1999), antimetastatic (CHUNG et al., 2004), and inhibition of HIV replication (KASHIWADA et al., 1995). Further, several studies have identified that caffeic acid to be an inducer of apoptosis in cancer cell lines and capable of tumor growth inhibition and regression in animals (CHUNG et al., 2004). The mechanisms of all these activities are poorly understood.

Phenethyl ester of caffeic acid (CAPE) is a structural derivative of flavonides and an active component of propolis from honey bee, which has been widely used as healthy food and folk medicine in many countries. On the hand, propolis is a plant derived honey bee product and recognized well for its many medicinal effects, including immunomodulatory, antiinflammatory, antioxidant, antibacterial, antiviral, antifungal and antiparasite activities, among others (SFORCIN & BANKOVA, 2011).

Propolis demonstrates a unique chemical composition. Its complex composition depending basically on the plant sources accessible to the bees, possessing a variety of biological and pharmacologic activities, increasing the interest of researchers. Beside, some studies revealed that propolis have also cytotoxic effects. In this line, naphthaliene derive from propolis showed cytotoxic effect *in vitro* against Chinese hamster ovary cancer cell lines (ROSS, 1990). In another *in vitro* study, the cytotoxic effects of Brazilian propolis extract from *Apis mellifera L* were related to quercetin, caffeic acid and phenyl ester constituents of propolis (MATSUNO, 1992). These effects could be related to large number of flavonoids contituents (VENNAT, 1995). Since propolis is a bees products and and considering the activity and behaviour of bees, different part of differrent plants should involved in its preparation. Therefore, the compostions of propolis involved may plants constituints. The compounds identified on structural similarities includes, alcohols, aldehydesaliphatic acid and aliphatic esters, amino acid, aromatic acid, aromatic ester, chalcones and dehydrochalcones, flavonones, flavones and flavonoides, Hydrocarbones ester, keto waxes, waxy acids, ketone, terpenoids and other compounds such as steroids and sugars (MARCUCCI, 1995).

Beside flavonoids and bioactive agents, propolis contains cinnamic derivatives such as caffeic, ferulic, cinnamic, chlorogenic acids and its esters (MIRZOEVA, 1996). Caffeic acid phenethyl ester (CAPE) is one of its abundant phenolic constituent (the ester of caffeic acid), which is a promising and well reported agent among the phenolic ingredients found in honey bee propolis.

CAPE is a well-known derivative of CA, reported by BANKOVA et al. (1987) and like caffeic acid, its pharmacological activities have related associated with its direct free radical scavenging ability due to its catechol ring configuration and these have been compared with other phenolic antioxidants (CHEN,& HO, 1997; SON & LEWIS, 2002; HSU et al., 2005). The others physiology properties of CAPE include antiviral, anti-inflammatory and immunomodulatory activities (HUANG et al., 1976; CHIAO et al., 1995; MIRZOEVA, CALDER, 1996; MICHALUART et al., 1999; FITZPATRICK et al., 2001). CAPE together with caffeic acid, ferulic acid and ethyl ferulate are constituents of propolis and structurally related, which allowed us to gather important information regarding the structure-activity relationships underlying the biological activity of such compounds.

1.2 Bioavailability and Chemistry

Either beneficial or non beneficial, the plant derived phenolic compounds “hydrocinnamic acids” are functional and chemically related; which help to facilitate the areas of research in food, beaverages and health sciences. Caffeic acid is receiving more attention since after oral administration this compound is absorbed from the gut and circulates in plasma (KONISHI et al., 2005). However, the mechanisms involved in beneficial effect of caffeic acid has not yet been fully understood. The presense in dietary items and its understanding simple (phenolic) structure make this compound interesting always for research. Beside the chemistry of caffeic acid also very interesting, perhaps the three active positions; substitution and chelation at the catechol group, addition at α, β- unsaturation (an acidic side chain) and at the most reactive carboxylic acid group and perhaps the same for CAPE. CA and CAPE inhibit certain enzyme activities such as lipoxygenases, cyclooxygenase, glutathione S-transferase, and xanthine oxidase (MICHALUART et al., 1999). Beside important nutritional components and health benefits, mostly these plants derived compound have been endowed with some degenerative activity *in vitro* and *in vivo*. In this context physiologicaly antioxidant, caffeic acid (YAMANAKA, 1997) has been reported to induce lipid peroxidation and DNA damage either alone or in the presence of copper ions. In consistency CA, CAPE (WANG, 2008) in the presence of Cu (II) ions: induce DNA damage and hence pro-oxidant.

1.3 Purinergic system

The extracellular adenine nucleotides/nucleosides, its hydrolyzing membrane bound ecto-enzymes and the site via adenine nucleotides/nucleosides are sensed to induced various cellular events, the purinoreceptors constitute a refined system; the Purinergic system.

The extracellular adenine nucleotides such as adenosine 5′-triphosphate (ATP), adenosine 5′-diphosphate (ADP), and adenosine 5′- monophoshate (AMP) and the nucleoside adenosine are essential constituents of mammalian cells. Either physiological or medical; the purines are release from their sources subsequently these molecules are degraded by a cascade of ectoenzymes (ROBSON et al., 2006; YEGUTKIN et al., 2008; COLGAN et al., 2006; STRATER, 2006).

1.3.1 Adenine Nucleotides and Nucleosides

Nucleosides are glycosylamines made by attaching a nucleobase (purine or pyrimidine) to a pentose sugar ring. Examples of these include cytidine, uridine, adenosine, guanosine, thymidine and inosine. Nucleosides are phosphorylated by specific kinases in the cell, producing nucleotides (ATKINSON et al., 2006). The important units/components of purinergic system are purine nucleotides/nucleosides; including ATP (adenosine 5′-triphosphate), ADP (adenosine 5′-diphosphate), AMP (adenosine 5′- monophoshate) and adenosine.

The role of ATP as an excitatory neurotransmitter, both centrally and in the periphery, is now well documented and accepted (BURNSTOCK, 2006, 2006, 2007 2009; RALEVIC, BURNSTOCK, 1998). In the nervous system, ATP acts a fast excitatory neurotransmitter (BURNSTOCK, 2006) and as a presynaptic neuromodulator (CUNHA, 2000), and it is also involved in the neuronal development and plasticity (RATHONE et al., 1999). In addition, ATP is co-released with other classical neurotransmitors including acethylcholine (ACh), norepinephrine, glutamate, γ-aminobutyric acid and neuropeptides (BURNSTOCK, 2004). Its breakdown product, adenosine, plays an important role in neuromodulation by regulating the release of several neurotransmitters, pre and post-synaptically (CUNHA 2001; DUNWIDDIE et al., 2001; RIBEIRO et al., 2003), which make them well suited in neuroprotection (ZIMMERMANN et al., 1998; DUNWIDDIE et al., 2001). Beside the neuromodulatory and neuroprotection actions of adenosine it has been recognized with many other physiological effects in CNS such as arousal, sleep, anxiety, cognition and memory (RIBEIRO et al., 2003).

Beside the neurotransmission, adenine nucleotides are also involved in the regulation of platelet aggregation and thrombus formation. In the vascular system, ADP and adenosine modulate the processes linked to vascular inflammation and thrombosis exerting various effects in the platelets (SOSLAU & YOUNGPRAPAKORN, 1997; GACHET, 2001). It has been well established in the literature that ATP, ADP and adenosine, at low concentrations influence vascular tone, cardiac function and platelet aggregation (BURNSTOCK, 2004).

The release of ADP has been considered the most important agent among the active substances that modifying and amplifying the platelet activation, followed by blood clothing and vasocontriction in circulation. ADP acts upon platelets, regulating their aggregation and modifying their shape and perhaps desensitization, while ATP has been postulated to be a competitive inhibitor of ADP platelet aggregation (SOSLAU & YOUNGPRAPAKORN, 1997; BIRK et al., 2002; REMIJIN et al., 2002). Furthermore adenosine produced by nucleotide catabolism is recognized as a vasodilator and inhibitor of platelet aggregation (ROBSON et al., 2006).

1.3.2 Purinergic Receptors

Once released the adenine nucleotides and nucleosides are sensed at the specific site on the cell surface; the purinegic receptors. In animals, these specific sites of the extracellular nucleotides and nucleosides are located on different cells (BURNSTOCK, 1993; RATHBONE et al., 1992). 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 ($P2Y_1$, $P2Y_2$, $P2Y_4$, $P2Y_6$, $P2Y_{11}$, $P2Y_{12}$, $P2Y_{13}$, $P2Y_{14}$). Adenosine is biologically active via other G protein-coupled P1 receptors (A_1, A_{2A}, A_{2B}, A_3) that modulate adenylate cyclase activity (YAAR et al., 2005).

The molecules ATP, ADP and adenosine act via these receptors signaling pathways; crucial for the normal functioning of vascular, nervous and immune systems (LECKA et al., 2010; HOURANI, 1996; ANSARI et al., 2007; BURNSTOCK & KNIGHT, 2004; BURNSTOCK, 2006).

1.3.3 Enzymatic cascade that degrade adenine nucleotides and nucleosides

Signaling events induced by extracellular adenine nucleotides and nucleosides are tightly regulated by cell surface ectoenzymes known as ectonucleotidases (YEGUTKIN et

al., 2008). 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; YEGUTKIN et al., 2008). 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. This enzymatic cascade controls the physiological level of nucleotides and their metabolites. The expression and activity of these enzymes have been elucidated in different tissues and cells (BUNSTOCK, 2010; ZIMMERMANN, 2001).

1.3.3.1 NTPDase family

NTPDase hydrolyze ATP and ADP to AMP (ROBSON et al., 2006), and on the basis of substrat specificity, chromosomal localization and tissues distribution and functional properties, eight different members constitute this family named NTPDase1-8 (ZIMMERMANN, 2001; ROBSON et al., 2006).

Four members (NTPDase1, 2, 3, 8) are the typical cell surface-located enzymes with an extracellularly facing catalytic site while the resting four NTPDase 4-7, are localized intracellulally (ROBSON et al., 2006; ZIMMERMANN, 2012). CD39/NTPDase1 hydrolyzes both tri- and diphosphonucleosides and blocks platelet aggregation responses to ADP. In contrast, CD39L1/NTPDase2, a preferential nucleoside triphosphatase, activates platelets by preferentially converting ATP to ADP, the major agonist of platelet P2 receptors (ROBSON et al., 2005). In the purines enzymatic cascade, the NTPDases are Ca^{++} and Mg^{++} dependent, being capable of hydrolyzing both ATP and ADP to AMP (ZIMMERMANN, 2001, 1999; ROBSON et al., 2006).

The NTPDases have been characterized in the neurons, platelets and lymphocytes and regulate a variety of physiological functions including neurotransmission, platelet activation and inflammatory process (MARCUS et al., 2003; LEAL et al., 2005; PILLA et al., 2006; DWYER et al., 2007). Recently, the findings in our laboratory confirmed that the alterations in these enzymes could be an important physiological and pathological factor

(SCHETINGER, et al., 2007; SPANEVELLO et al., 2010; PIMENTEL et al., 2011). For example, the neurological disorders, such as experimental model of epilepsy and cerebral ischemia (SCHETINGER et al., 1998; PIMENTEL. et al., 2011), myocardial infarction (BAGATINI et al., 2008). Moreover, NTPDase has an established role in the immune system including cytokine expression, cell–cell adhesion, cell proliferation and apoptosis via modulation of ATP levels (DWYER, 2007). In the context, the alterations in the activity of these enzymes can be very important in vascular, nervous and immune diseases (LEAL et al., 2005; SCHETINGER et al., 2007).

1.3.3.2 Ectonucleotide Pyrophosphatase/Phosphodiesterase (E-NPP) family

Like NTPDases, the E-NPP (GODING et al., 2003) also has a key contribution in nucleotide hydrolysis especially in vascular purine enzymatic cascade. Seven members constitute this family which is categorized on their order of cloning and discovery. The NPPs has a modular structure, except NPP2, which is secreted in the extracellular medium, the rest are single-span membrane proteins with type-II (NPP1 and NPP3) or type-I orientation (NPP4-7). The NPP1 and 3 have a type II transmembrane orientation with its aminoterminal portion facing the intracellular environment, while PPNs 4-7 have a type I orientation with its portion facing the aminoterminal extracellular medium. The nuclease-like domain and the two somatomedin B-like domains (SMB1 and SMB2) are found only in NPP1-3 (STEFAN et al., 2006).

The members of family have associated with large variety of functions. The modulation of purinergic receptor signaling via recycling of nucleotides, regulation of extracellular pyrophosphate levels, stimulation of cell motility, and possible roles in regulation of insulin receptor (IR) signalling and activity of ecto-kinases, highlight its broad substrate specificity and multiple physiological function (GODING et al., 2003). Extracellularly, this family of enzyme hydrolyzes pyrophosphate or phosphodiester bonds in compounds including nucleotides, (lyso) phospholipids and choline phosphate esters (STEFAN, 2005). Among the the seven members, only NPP1–3 have been implicated in purinergic signalling (ZIMMERMANN, 2000; BOLLEN et al., 2000; VOLLMAYER et al., 2003; STEFAN et al., 2006).

1.3.3.3 5’-nucleotidase enzyme

The enzyme 5'-nucleotidase (CD73) is another important members of purine enzymatic cascade. NTPDase and E-NPP convert extracellular adenine nucleotides to AMP which is subsequently hydrolysed by the action of CD73 (GPI-linked glycosylphosphatidylinositol) (SHETINGER et al., 2007). The CD73 exist in essentially every tissue cell system, platelets, lungs, kidney, liver, vascular endothelium, immune and CNS and its expression depend on high ion transport of the respective tissues. For example its expression varied in the order of; with the rank order of tissue activity as follows: Colon > kidney = brain > liver > lung > heart >> muscle (COLGAN et al., 2006).

The enzyme 5'-nucleotidase has been associated with many functions including vasodilatation, neuroprotection, immunomodulation and immunosuppression via production of adenosine. In fact the activity of this enzyme has been showed altered in many pathological condition including HIV infection (LEAL et al., 2005) inflammation (SPANEVELLO et al., 2006), diabetes (LUNKES et al., 2003), and myocardial infarction (BAGATINI et al., 2008) suggesting the 5’-nucleotidase can be the important therapeutic target in different diseases.

1.3.3.4 Adenosine Deaminase enzyme

The purines enzymatic cascade, continued by NTPDases, E-NPP and 5'-nucleotidase, is followed by two other important enzymes Adenosine deaminase (ADA) and xanthine oxidase (XO) are the other important enzymes, hydrolyzing adenosine and xanthine/hypoxanthine respectively. After the action of ecto-nucleotidases, ADA and XO are enzymes that catalyze the conversion of adenosine to inosine, deoxyadenosine to deoxyinosine, hypoxanthine to xanthine and xanthine to uric acid, respectively (SOGUT et al., 2002).

ADA is an enzyme which catalyzes the hydrolytic deamination of adenosine (or 2’-deoxyadenosine (dAdo) to inosine both in the cytosol and at the cell membrane (ROSEMBERG et al., 2008) which helps to maintain a strict control of adenosine levels, thus avoiding the cytotoxic actions of adenosine and especially deoxyadenosine in peripheral tissues as well as in brain. ADA is present in all cell types but the amount of enzyme differs widely among tissues. Two cell surface proteins are reported to be ecto-ADA anchor in the cell surface; CD26 and A_1R (FRANCO et al., 1997). The role of ecto-ADA in cell-to-cell

contacts is supposed to be impotant for the development of lymphoid tissues and for maturation of lymphocytes (FRANCO, et al, 1997).

Together with ecto-nucleotidases, ADA also is considered as a therapeutic targets since in the purines enzymatic cascade this enzyme also interfere with a variety of physiological and pathological functions mediated by nucleotides in CNS (ROBSON, 2006; SCHETINGER et al., 2007). ADA is considered essential for the differentiation, normal growth and proliferation of lymphocytes (ALDRICH et al., 2000). Elevated adenosine levels could trigger aberrant adenosine receptor signaling (ALDRICKH et al., 2000). NTPDase (CD39) and ADA have significant roles in immune response and alterations in their activities have been observed in many related health problems (LEAL et al. 2005). So the literature demonstrates that the alteration in ADA activities is good tool to trace medical condition involved in immunological disorders. As an important enzyme of the purine metabolism, XO catalyzes the oxidation of xanthine and hypoxanthine to uric acid (SOGUT et al., 2002). During this reaction XO produces high quantities of oxygen-derived free radicals that contribute to oxidative damage to living tissues that are involved in many pathological processes such as inflammation, atherosclerosis, cancer and aging (COS et al., 1998).

1.4. Cholinergic System

The cholinergic system exists in both neuronal and non neuronal tissues. This system consists of acetylcholine (ACh), cholinergic receptors (nicotinic and muscarinic), the enzymes choline acetyltransferase (ChAT), and acetylcholinesterases (AChE)

After its release, ACh binds to acetylcholine receptors across the postsynaptic nerve terminal. In pre-and post-synaptic, ACh specifically interacts with nicotinic (nAChRs) and muscarinic (mAChRs) cholinergic receptors present in membranes pre-and post-synaptic (DESCARRIES et al. 1997; KAWASHIMA & FUJII, 2003; FUJII et al. 2008). After binding with its receptors, ACh difused in to the postsynaptic membrane and stimulates a neuro impulse. At the end the action of the enzyme AChE terminates it actions by hydrolyzing in to to acetate and choline.

From the very beginning the study related to cholinergic system have been focused on synthesis, storage, release and the action of acetylcholine as neurotransmitter in the CNS. However, lymphocytes also possess cholinergic system comprising acetylcholine, choline acetyltransferase, muscarinic and nicotinic receptors and AChE enzyme (KAWASHIMA & FUJII, 2003). Acetylcholine synthesized and released from lymphocytes has been considered

immunomodulador acting via both muscarinic and nicotinic receptors present in these cells (WESSLER, et al., 2001).

1.4.1 Acetylcholinesterase

Acetylcholinesterase (E.C. 3.1.1.7, AChE) is a specific choline esterase that hydrolyzes predominantly choline esters. AChE molecular forms can be divided into: A. asymmetric and B. globular forms. The former preferentially localized at the neuromuscular junction while the globular (monomers (G1), dimmers (G3) or catalytic tetramers (G4) secreted as soluble forms, or anchored to the membrane by a hydrophobic domain (TALESA, 2001). Some molecular forms are membrane bound by a hydrophobic peptide (like; G4) and are preferentially expressed in mammalian central nervous system while other forms (like; G1) are linked to the membrane by a glycolipid anchor and are found in nerves, muscles, erythrocytes and lymphocytes (TAYLO & RADIC, 1994; TALESA, 2001).

In mammals, the AChE active center, which consists of the catalytic triad Ser203-Glu334-His447 (RACHINSKY et al., 1990), is located at the bottom of a deep and narrow gorge (SUSSMAN et al., 1991). The subunits contain a 12-stranded β -sheet surrounded by 14 α –helices (TAYLOR & RADIC. 1994) and gorge is lined with 14 aromatic residues. The two sub active site (a negatively charged or 'anionic' site, and an esteratic site) are also called the catalytic triad (SHAFFERMAN et al., 1992). In this line AChE presents its catalytics domain in the arrangement of β sheets and α helices (α/β hydrolase fold). The three diamentional x-rays crystal structure of AChE (ABOU-DONIA, 2003) have a narrow and deep active site gorge with two sites of ligand binding, a catalytic site (an acylation site or A-site) at the base of the gorge, and a peripheral site (or P-site) near the gorge mouth (BOURNE et al., 2003; JOHNSON et al., 2003; TAYLOR & RADIC, 1994). Interestingly, the interaction of these two ligand sites (acylation or A-site and a peripheral binding or p-site) in cholinesterase can explain well its reaction mechanisms. The acylation or A-site is at the base of the active site gorge and the p-site is at its mouth (ROSENBERRY et al., 2005). These two sites are well coordinated because arrival of substrates at peripheral anionic site modulates activity at the catalytic site (JOHNSON et al., 2003). The P-site contributes to catalytic efficiency by transiently binding substrates on their way to the acylation site, a conformational interaction between the A- and P-sites can modulate ligand affinities via creating a short-lived acyl enzyme intermediate (JOHNSON et al., 2003). Since the there is

sheet of opposite charged residues in the enzyme and perhaps the electrostatic dipole of the residues can orientate the substrats to bind on the active site.

An acyl-enzyme intermediate is formed at the acylation site, and catalytic activity can be inhibited by ligand binding to a peripheral site (MALLENDER et al., 2000). On the other hand ligand binding to the P-site alters reaction rate constants for substrates bound to the A-site thus P-site can catalys AChE (JOHNSON et al., 2003).

AChE is one of the most efficient enzyme known that rapidly hydrolyses the neurotransmitter acetylcholine at cholinergic synapses as well as the neuromuscular junction. Apart from its catalytic function in hydrolyzing acetylcholine, the diverse localization of this enzyme in non cholinergic and non neural cells and tissues strongly suggest additional non classical functions for AChE. In fact, studies have demonstrated that AChE has potent effects in postsynaptic differentiation, cellular adhesion, neurite extension and hematopoietic differentiation (SUSSMAN et al., 1991). In addition, this enzyme emerges as a potential contributor in the pathways controlling inflammatory and immune responses in the blood. In fact, it has been demonstrated that inhibitors of AChE reduce lymphocyte proliferation and the secretion of pro-inflammatory cytokines and may attenuate inflammation by increasing the ACh concentration in the extracellular space (NIZRI et al., 2006).

As a consequence of its key physiological role, AChE is the target of many studies. The particular importance several studies have also demonstrated that natural substances and dietary components can affect the AChE activity in different tissues (REZG et al., 2008; AHMED and GILANI, 2009; SCHMATZ et al., 2009). In this line, the aim of this study was evaluate the effets of caffeic acid and acid caffeic phenethyl ester in acetylcholinesterase e ecto-nucleotidase activities, enzymes that have crucial roles in inflammation, neurotransmission and platelet aggregation. The findings of this study are very important for the identification of natural compounds that may be used for human consumption regarding health promotion and disease prevention.

2 OBJECTIVES

2.1 General Objectives

To evaluate the effect of two phenolic compounds, CA and CAPE on the activity of enzymes involved in the purinergic and cholinergic system in both neuronal and non neuronal tissues.

2.2 Specific Objectives

A) To investigate the effect of caffeic acid on the activities of:

- AChE in Cerebral Cortex, Cerebellum, Hippocampus, Striatum, Hipothalamus, Pon, Lymphocytes and Muscles from rats after treatment with CA.
- NTPDase on the hydrolysis of Adenine nucleotides in platelets and Lymphocytes of rats treated with CA.
- E-NPP and 5'-nucleotidase in Platelets of rats treated with CA.

B) To investigate the effect of CAPE on the activities of:

- AChE in Cerebral Cortex, Cerebellum, Hippocampus, Striatum, Hipothalamus Pon, Lymphocytes and Muscles from rats after treatment with CAPE.
- NTPDase, 5'-nucleotidase and ADA in Synaptosomes of rats treated with CAPE.
- NTPDase, E-NPP, 5'-nucleotidase and ADA in Platelets of rats treated with CAPE.
- XO in serum and whole brain from the rats treated with CAPE.

3 METHODS AND RESUTS

All related method and results to the thesis are metioned in the paper and written manuscripts.

3.1 Capter 1

First Article

Effects of caffeic acid on behavioral parameters and on the activity of acetylcholinesterase in different tissues from adult rats

Javed Anwar, Roselia Maria Spanevello, Gustavo Thomé, Naiara Stefanello, Roberta Schmatz, Jessié Gutierres, Juliano Vieira, Jucimara Baldissarelli, Fabiano Barbosa Carvalho, Michelle Melgarejo da Rosa, Maribel Antonello Rubin, Amanda Fiorenza, Vera Maria Morsch , Maria Rosa Chitolina Schetinger

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Effects of caffeic acid on behavioral parameters and on the activity of acetylcholinesterase in different tissues from adult rats

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ABSTRACT

Acetylcholinesterase (AChE) is distributed throughout the body in both neuronal and non-neuronal tissues and plays an important role in the regulation of physiological events. Caffeic acid is a phenolic compound that has anti-inflammatory and neuroprotective properties. The aim of this study was to investigate in vitro and in vivo whether caffeic acid alters the AChE activity and behavioral parameters in rats. In the in vitro study, the concentrations of 0, 0.1, 0.5, 1.0, 1.5, and 2 mM of caffeic acid were used. For the in vivo study, five groups were evaluated: group I (control); group II (canola oil), group III (10 mg/kg of caffeic acid); group IV (50 mg/kg of caffeic acid) and group V (100 mg/kg of caffeic acid). Caffeic acid was diluted in canola oil and administered for 30 days. In vitro, the caffeic acid increased the AChE activity in the cerebral cortex, cerebellum, hypothalamus, whole blood, and lymphocytes at different concentrations. In muscle, this compound caused an inhibition in the AChE activity at concentrations of 0.5, 1.0, 1.5, and 2 mM when compared to the control ($P<0.05$). In vivo, 50 and 100 mg/kg of caffeic acid decreased the AChE activity in the cerebral cortex and striatum and increased the activity of this enzyme in the cerebellum, hippocampus, hypothalamus, pons, lymphocytes, and muscles when compared to the control group ($P<0.05$). The amount of 100 mg/kg of caffeic acid improved the step-down latencies in the inhibitory avoidance. Our results demonstrated that caffeic acid improved memory and interfered with the cholinergic signaling. As a natural and promising compound caffeic acid should be considered potentially therapeutic in disorders that involve the cholinergic system.

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1. Introduction

Cholinesterases belong to a family of proteins that is widely distributed throughout the body in both neuronal and non-neuronal tissues and is classified as either acetylcholinesterase (AChE) or butyrylcholinesterase (BuChE) based on their substrate and inhibitor specificity (Anglister et al., 2008).

Acetylcholinesterase (E.C. 3.1.1.7, AChE) is a specific choline esterase that hydrolyzes predominantly choline esters. This enzyme is present in the brain, erythrocytes, lymphocytes and neuromuscular junction and plays an essential role in the regulation of physiological events involving the turnover of acetylcholine (Anglister et al., 2008; Szelenki et al., 1982; Gaspersic et al., 1999). AChE is one of the most efficient enzymes that rapidly hydrolyzes the neurotransmitter acetylcholine at cholinergic synapses as well as the neuromuscular junction (Anglister et al., 2008; Gaspersic et al., 1999). Besides its catalytic properties, AChE has potent effects in cellular adhesion, neurite extension and postsynaptic differentiation and has been accepted as the most important biochemical indicator of cholinergic signaling in the central nervous system (CNS) (Silman and Sussman, 2005). This enzyme is also widely accepted as having roles in non-neuronal tissues including hematopoietic differentiation (Deutsch et al., 2002) and regulation of the immune function (Kawashima and Fujii, 2003).

As a consequence of its key physiological role, AChE is the target of natural toxins (Senanayake and Román, 1992) and the therapeutic drugs designed to combat neuromuscular disorders and to alleviate the cholinergic deficiency associated with neurodegenerative diseases (Brenner et al., 2003). Furthermore, several studies have also demonstrated that natural substances and dietary components can affect the

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or triplicate, and the enzyme activity was expressed in micromoles ASCh/h/mg of protein.

2.7. AChE assay in whole blood

Whole blood AChE activity was determined by the method of Ellman et al. (1961) modified by Worek et al. (1999). The blood was collected in vaccutainer tubes using EDTA as anticoagulant. Samples were hemolyzed with phosphate buffer, pH 7.4 containing Triton X-100 and stored at −30 °C for 1 week. The specific activity of whole blood AChE was calculated from the quotient between the AChE activity and hemoglobin content and the results are expressed as mU/μmol Hb.

2.8. AChE assay in lymphocytes

The blood was collected in vaccutainer tubes using EDTA as anticoagulant. The peripheral lymphocytes were isolated using Ficoll Hypaque density gradient as described by Böyum (1968). Lymphocyte viability and integrity were confirmed by determining the percentage of cells, excluding 0.1% trypan blue and measuring lactate dehydrogenase (LDH) activity (Bergmeyer, 1983).

After the isolation of the lymphocytes, the AChE activity was determined according to the method described by Ellman et al. (1961) modified by Fitzgerald and Costa (1993). Briefly, proteins of all samples were adjusted to 0.1–0.2 mg/ml. The amount of 0.2 ml of intact cells was added to a solution containing 1.0 mM acetylthiocholine, 0.1 mM of DTNB, and 0.1 M phosphate buffer (pH 8.0). Immediately before and after incubation for 30 min at 27 °C, the absorbance was read on a spectrophotometer at 412 nm. AChE was calculated from the quotient between lymphocyte AChE activity and protein content and the results are expressed as μmol/h/mg of protein.

2.9. AChE assay in muscle

The gastrocnemius muscles was homogenized in a glass potter in Tris–HCl (pH 7.2) solution and centrifuged for 1000 *g* for 15 min. Aliquots of the resulting centrifugation were used to determine the AChE activity. The AChE enzymatic assay in muscle was determined by a modification of the spectrophotometric method of Ellman et al. (1961). The reaction mixture (2 ml final volume) was composed of 24 mM K^+-phosphate buffer, pH 7.2 and 10 mM of DTNB. The reaction was initiated by adding 0.8 mM acetylcholine iodide. All samples were run in duplicate or triplicate and enzyme activity was expressed in micromoles ASCh/h/mg of protein.

2.10. Protein determination

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

2.11. Statistical analysis

Because the distributions of data of inhibitory avoidance test latencies were not normally distributed, the statistical analysis of test step-down latencies was carried out by Kruskal–Wallis followed by post hoc analyses (nonparametric Dunn's test) and the results were expressed as the median and interquartile range. Crossing, rearing, latency to training and biochemical analysis were normally distributed and were analyzed by one-way ANOVA followed by post hoc Duncan's multiple range tests and the results were expressed as mean ± SEM. $P<0.05$ was considered to represent a significant difference between groups.

3. Results

3.1. In vitro experiments with caffeic acid

Results demonstrated that in vitro caffeic acid altered the AChE activity in different brain structures. It can be observed in Fig. 1 that caffeic acid significantly increased the AChE activity in the cerebral cortex at concentrations of 1.0 mM (18%), 1.5 mM (27%), and 2.0 mM (37%); in the cerebellum at concentrations of 1.5 mM (20%) and 2.0 mM (22%); and in the hypothalamus at concentrations of 1.5 mM (42%) and 2.0 mM (42%) when compared to the control group ($P<0.05$). In the striatum, hippocampus and pons caffeic acid did not affect in vitro the AChE activity at any concentration evaluated.

Fig. 2 shows the effects of caffeic acid on the AChE activity in whole blood. It can be observed that caffeic acid significantly increased the AChE activity in whole blood only at a high concentration (2 mM (−32%)); whereas in the lymphocytes, caffeic acid significantly increased the enzyme activity at concentrations of 0.5 mM (26%), 1.0 mM (56%), 1.5 mM (68%), and 2.0 mM (68%) when compared with the control group ($P<0.05$) (Fig. 3). On the other hand, this compound in vitro caused a significant inhibition in the AChE activity in muscle at concentrations of 0.5 mM (28%), 1.0 mM (33%), 1.5 mM (37%), and 2.0 mM (42%) when compared to the control group ($P<0.05$) (Fig. 4).

3.2. In vivo experiments with caffeic acid

3.2.1. AChE activity

When rats were treated with caffeic acid, the AChE activity was altered in different brain structures. Fig. 5 shows that in the cerebral cortex ,the AChE activity was significantly decreased in the groups treated with 10 mg/kg (23%), 50 mg/kg (25%) and 100 mg/kg (28%) of caffeic acid when compared to the control group ($P<0.05$). Similarly, in the striatum the AChE activity was also significantly inhibited in the groups treated with 10 mg/kg (36%), 50 mg/kg (36%), and 100 mg/kg (37%) of caffeic acid in relation to the control group ($P<0.05$). On the other hand, in the hippocampus the AChE activity was significantly increased in the animals treated with 10 mg/kg (29%), 50 mg/kg (40%) and 100 mg/kg (40%) of caffeic acid as well as in the pons at dosages of 10 mg/kg (22%), 50 mg/kg (24%), and 100 mg/kg (22%) of caffeic acid in comparison with the control group ($P<0.05$) (Fig. 5). In addition, a significant increase was also observed in the AChE activity in the hypothalamus of animals treated with 50 mg/kg (53%) and 100 mg/kg (40%) of caffeic acid, while in the cerebellum this increase was observed only in rats treated with 10 mg/kg (27%) and 50 mg/kg (33%) of caffeic acid ($P<0.05$) (Fig. 5).

Fig. 6 shows the effect of caffeic acid on the AChE activity in lymphocytes. This compound significantly increased the AChE activity in lymphocytes only in animals treated with 100 mg/kg (73%) ($P<0.05$). In relation to the AChE from muscle, it can be observed that caffeic acid significantly increased the enzyme activity in all animals treated with this compound (10 mg/kg (59%), 50 mg/kg (65%) and 100 mg/kg (37%)) when compared to the control group ($P<0.05$).

In addition, it is also important to note that controls were performed in vitro and in vivo to correct the vehicle (methanol or canola oil) interference and no difference between vehicle and control enzyme was observed (Figs. 1–7, groups methanol and oil).

3.2.2. Behavioral tests

Fig. 8 shows the effect of oral administration of caffeic acid (10, 50, 100 mg/kg) once a day during 30 days on test step-down latencies of rats subjected to the inhibitory avoidance task. Statistical analysis (Kruskal–Wallis) revealed a significant effect of treatment [$F_{(4,35)}=11.54$; $P<0.05$, Fig. 8]. Post hoc analysis showed that caffeic acid (100 mg/kg) significantly increased step-down latencies compared to the respective control group, suggesting that oral administration of caffeic acid improves memory in the inhibitory avoidance task.

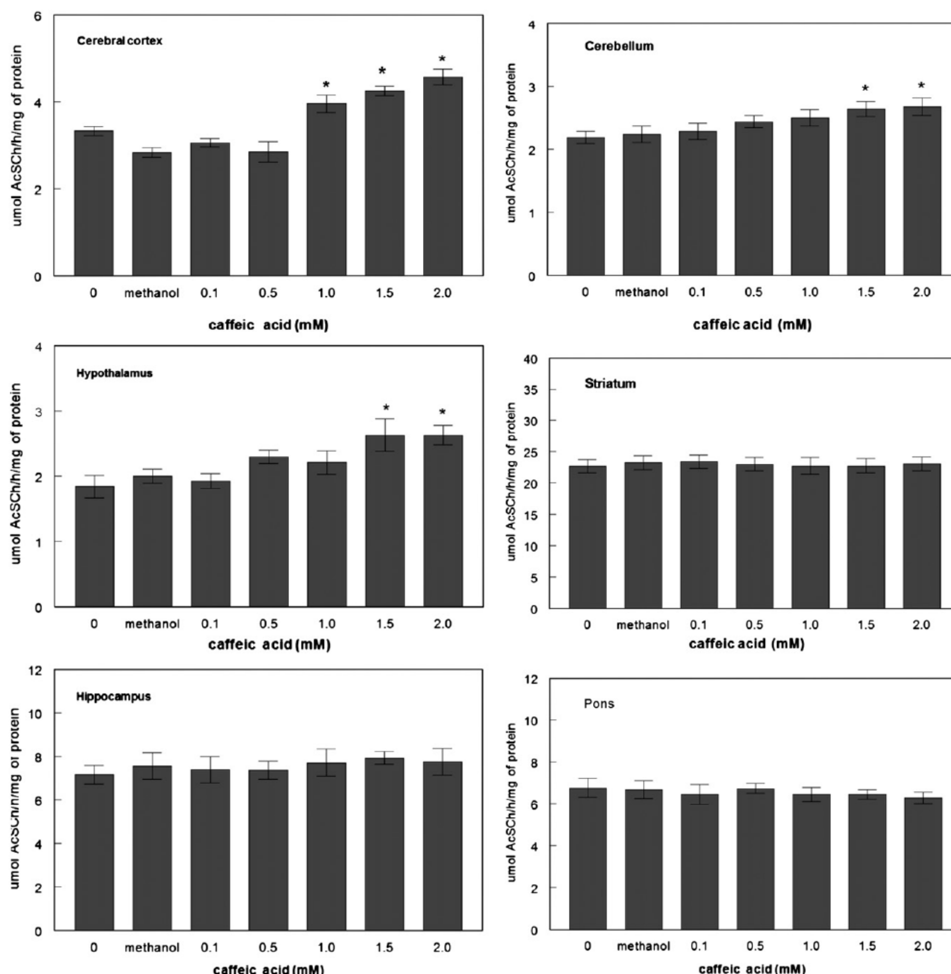

Fig. 1. In vitro effects of caffeic acid on the AChE activity in the cerebral cortex, cerebellum, striatum, hippocampus, hypothalamus, and pons of rats. Each bar represents mean ± SEM. AChE activity is expressed as μmol of acetylthiocholine/h/mg of protein. *Different from control ($P<0.05$ n = 5). ANOVA–Duncan's test.

Table 1 shows the effect of oral administration of caffeic acid on training step-down latencies in the inhibitory avoidance task and exploratory behavior in the open field immediately after the inhibitory avoidance testing session. Statistical analysis (one-way ANOVA) revealed that oral administration of caffeic acid did not alter the training step-down latencies and the number of crossing or rearing responses in a subsequent open-field testing session (F values shown in Table 1), suggesting that caffeic acid once a day during 30 days did not cause gross motor disabilities during testing.

4. Discussion

Caffeic acid, due to its therapeutic properties, represents a promising candidate for the treatment of many diseases. Although in literature it is established that caffeic acid has many effects on human heath, the mechanisms involved in these beneficial properties have not yet been fully understood. In this study we evaluated in vitro and in vivo the effects the caffeic acid on the AChE activity in the brain, whole blood, lymphocytes and muscle of rats as well as the effect of this compound on memory.

Our results in the brain showed that caffeic acid altered in vitro and in vivo the AChE activity, but these alterations were not homogeneous in all evaluated brain structures. In the cerebral cortex, cerebellum and hypothalamus, caffeic acid significantly increased the AChE activity at concentrations varying from 1.0 to 2.0 mM, while in the striatum, hippocampus and pons this compound did not alter the enzyme activity (Fig. 1). On the other hand, treatment for 30 days with caffeic acid at the dosage of 10 mg/kg, 50 mg/kg and 100 mg/kg inhibited significantly the AChE activity in cerebral cortex and striatum and increased the enzyme activity in the hippocampus, hypothalamus and pons (Fig. 5).

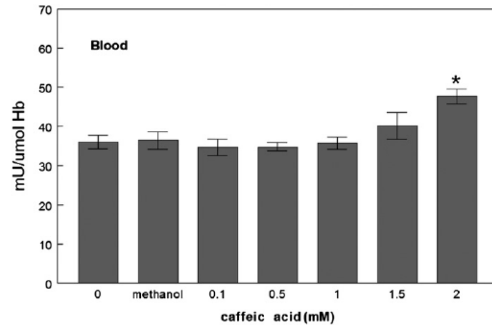

Fig. 2. In vitro effect of caffeic acid on the AChE activity in the whole blood of rats. Each bar represents mean ± SEM. AChE activity is expressed as mU/μmol Hb. *Different from control ($P<0.05$ n = 5). ANOVA–Duncan's test.

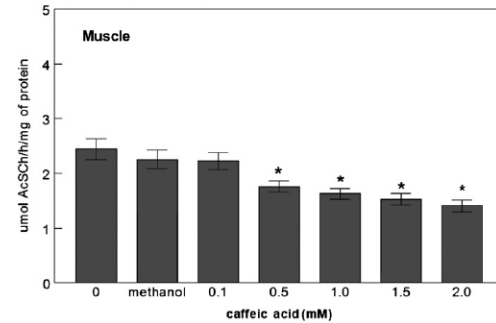

Fig. 4. In vitro effect of caffeic acid on the AChE activity in the muscle of rats. AChE activity is expressed as μmol of acetylthiocholine/h/mg of protein. Each bar represents mean ± SEM. *Different from control $P<0.05$. ANOVA–Duncan's test.

A possible explanation for the differences in relation to the AChE activity in brain regions is the fact that AChE exists in a variety of molecular forms that differ in solubility and type of membrane attachment rather than in catalytic activity. In the brain, AChE occurs mainly as tetrameric G4 forms (membrane bound) together with the monomeric G1 form (cytosolic) (Das et al., 2001). Literature shows that many drugs, including those of therapeutic use, did not affect any form of AChE equally well, most importantly, sometimes these drugs behaved differently, with the same isoform from different brain areas (Zhao and Tang, 2002). Based on this, we can suggest that caffeic acid also may have the form specific selectivity in relation to the AChE from brain regions. Although this hypothesis must be confirmed with further studies, these findings open doors to the discovery of mechanisms about more specific targets of the caffeic acid in the CNS.

The AChE activity measured in CNS has been extensively studied not only because it is involved in the cholinergic neurotransmission (Silman and Sussman, 2005; Soreq and Seidman, 2001) but also because the deleterious consequences of its inhibition (Lotti, 1995) and its action as therapeutic target in neurodegenerative diseases (Rakonczay, 2003). AChE is one of the most efficient enzymes known, and its presence leads to rapid deactivation of acetylcholine at the cholinergic synapse (Silman and Sussman, 2005). Previous studies have showed that natural substances and dietary components such as resveratrol (Schmatz et al., 2009), quercetin (Tota et al., 2010), curcuminoids (Ahmed and Gilani, 2009) and chlorogenic acid (Know et al., 2010) altered the AChE activity in CNS. Although it has been demonstrated that caffeic acid has neuroprotective effects in many experimental conditions (Yang et al., 2007; Kalonia et al., 2009; Kart et al., 2009; Sul et al., 2009) our findings led us to propose that alterations in the AChE activity induced by caffeic acid could alter the acetylcholine level interfering in the cholinergic neurotransmission.

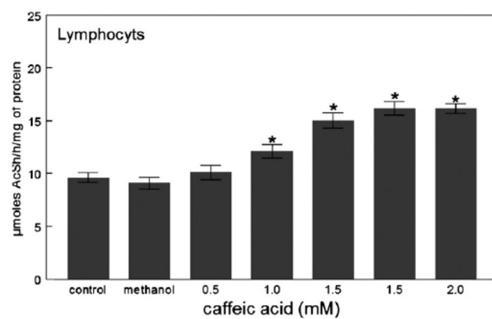

Fig. 3. In vitro effect of caffeic acid on the AChE activity in lymphocytes of rats. AChE activity is expressed as μmol of acetylthiocholine/h/mg of protein. Each bar represents mean ± SEM. *Different from control $P<0.05$. ANOVA–Duncan's test.

We also observed in this study that animals treated with caffeic acid only at the dose of 100 mg/kg increased the step-down latencies in relation to the control group, suggesting that caffeic acid improved the learning and memory of rats in the inhibitory avoidance task (Fig. 8), although the exact mechanism involved in this effect is still unknown. The inhibitory avoidance task is commonly used as a parameter to evaluate the learning and memory in rats and mice (Izquierdo et al., 2000a,b, 2002). This task involves the formation of memories through an aversive stimulus (electric shock). Although this aversive stimulus can induce emotional components of fear and anxiety in the animal, this task was performed in animals treated with caffeic acid in order to evaluate only the process of learning and memory formation under these conditions. Some other behavioral parameters to evaluate other types of memory, such as object recognition, could be used to investigate if the caffeic acid could improve the memory in other conditions (non-aversive). These results are important to complement our findings related to the caffeic acid effect with the improvement of learning and memory.

Research regarding caffeic acid on behavioral parameters including memory tests is scarce in the literature, although some studies have shown that the acute administration of caffeic acid (1 mg/kg) increased the number of entries and the time spent in the open arms of a plus maze suggested an anxiolytic-like effect. The treatment for 15 days (10 and 30 mg/kg) reverted the alterations in memory and learning induced by aluminum (Yang et al., 2008) but it remains unknown if caffeic acid could be interfering in such behavior. Another important aspect to be discussed is that studies have demonstrated that caffeic acid phenethyl ester (CAPE), a derivate of caffeic acid, protects blood brain barrier integrity (Aladag et al., 2006; Zhao et al., 2012) and has relaxing properties on arteries (Cicala et al., 2003; Long et al., 2009). Moreover, it has also been reported that caffeic acid and CAPE are able to ameliorate glucose metabolism and uptake (Huang et al., 2009; Lee et al., 2007; Tsuda et al., 2012). Taken together, the vasodilator effect and the ability to improve the cellular energetic metabolism in the brain can explain, in part, the alterations in memory observed in the animals treated with caffeic acid with 100 mg/kg.

On the other hand, it is known that AChE inhibitors are used by improving the memory and other cognitive deficits by increasing the acetylcholine concentration in the extracellular medium. In this

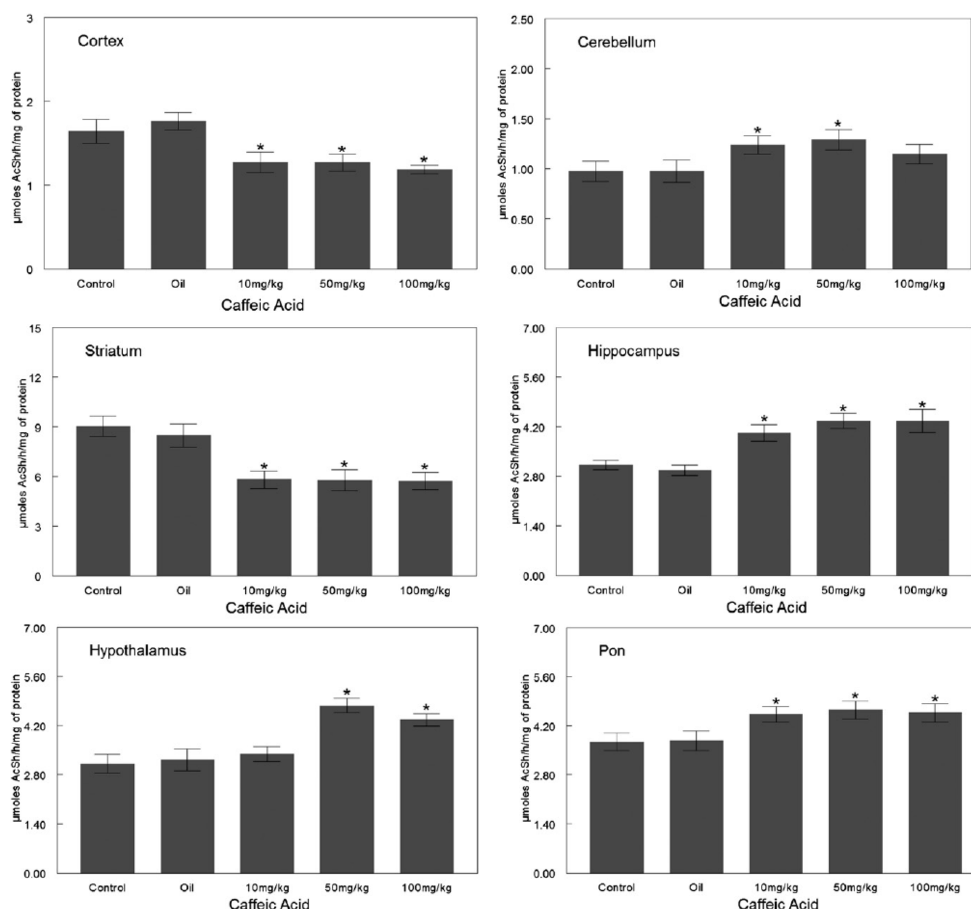

Fig. 5. In vivo effects of caffeic acid on the AChE activity in the cerebral cortex, cerebellum, hippocampus, hypothalamus, and pons of rats. Each bar represents mean ± SEM. AChE activity is expressed as μmol of acetylthiocholine/h/mg of protein. *Different from control ($P<0.05$). ANOVA–Duncan's test.

study, in vivo treatment with caffeic acid decreased the AChE activity in the cortex and striatum. This effect can be important and may be associated with the improvement of the memory observed. Furthermore, the cholinergic system has synaptic and extra-synaptic functions (Soreq and Seidman, 2001). Studies have demonstrated that the cholinergic system is involved in brain circulation (Wahl and Schilling, 1993; Bertrand et al., 1996; Moser et al., 2003). Based on these findings, caffeic acid can modulate AChE activity, and depending on the tissue involved it contributes to the cerebral circulation and improvement of cognitive performance.

In the present investigation, we also demonstrated that caffeic acid in vitro and in vivo increased the AChE activity in whole blood and lymphocytes from rats, demonstrating that this compound also affected the cholinergic signaling in non neural cells. In blood cells, AChE showed a similar structure to the enzyme that occurs in neurons, and the catalytic subunits resemble which was found in synaptic AChE (Thiermann et al., 2005). AChE is expressed in several types of hematopoietic lineages and can contribute to cell regulation (Wessler and Kirkpatrick, 2001). Studies showed that AChE is related to progenitor blood cells mainly for cell expansion of megakaryocytics and erythrocyte lineages (Soreq and Seidman, 2001; Soreq et al., 1994; Grisaru et al., 1999). Moreover, the activity of this enzyme has been used as an indicator of human cell aging with lowered levels commonly associated with older human red blood cells (Prall et al., 1998).

In addition, it has been known that lymphocytes possess a complete cholinergic system including acetylcholine, choline acetyltransferase, muscarinic and nicotinic receptors and AChE enzyme (Kawashima and Fujii, 2000, 2003). Acetylcholine synthesized and released from lymphocytes has been considered an immunomodulator acting via both muscarinic and nicotinic receptors present in these cells (Wessler and Kirkpatrick, 2001). In this context, AChE emerges as a potential contributor to the pathway controlling inflammatory and immune response in the blood. In fact, it has been demonstrated that inhibitors of the AChE reduce lymphocyte proliferation and the secretion of pro-inflammatory

In vivo Results

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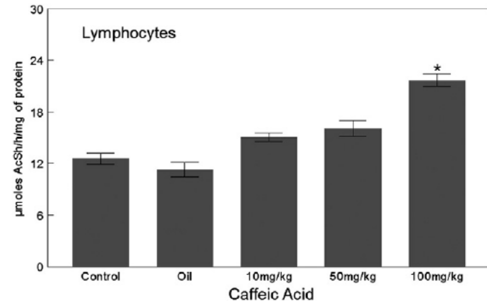

Fig. 6. In vivo effect of caffeic acid on the AChE activity in lymphocytes of rats. AChE activity is expressed as µmol of acetylthiocholine/h/mg of protein. Each bar represents mean ± SEM. *Different from control $P<0.05$. ANOVA–Duncan's test.

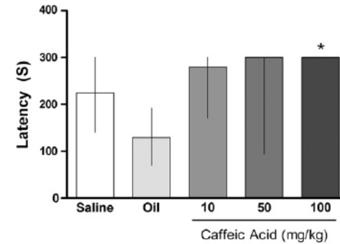

Fig. 8. Oral administration of caffeic acid (10, 50 or 100 mg/kg) once a day for 30 days in the training and test of inhibitory avoidance task. Caffeic acid, 100 mg/kg, improves memory in inhibitory avoidance test in adult rats. Data are the median ± interquartile range for 8 animals in each group. *$P<0.05$ compared with the oil group.

cytokines and may attenuate inflammation by increasing the acetylcholine concentration in the extracellular space (Nizri and Hamra-Amitay, 2006).

Although studies revealed that caffeic acid exhibits radioprotective effects in human lymphocytes (Devipriya et al., 2008) and that CAPE has anti-inflammatory properties by inhibiting (IL-2) gene transcription and IL-2 synthesis in stimulated T cells (Márquez et al., 2004), our results suggest that the increase in the AChE activity in lymphocytes by caffeic acid will lead to a decrease in the acetylcholine levels that could contribute to the onset of low-grade inflammation. These findings suggest that caffeic acid may exert negative effects that demand further investigations. In this line, other studies described that this compound has pro-oxidant activity in the presence of transition metal ions such as iron and copper causing oxidative DNA damage in lymphocytes (Ahmad et al., 1992; Yamanaka et al., 1997; Galati and Brien, 2004; Bhat et al., 2007). In addition, Wu et al. (2001) also observed that caffeic acid exhibits proglycative effects causing elevation of oxidative stress and inflammation in monocytes, macrophages and vascular endothelial cells.

AChE is also one of the key functional proteins in neuromuscular transmission by rapidly hydrolyzing acetylcholine molecules after its binding to receptors (Gaspersic et al., 1999). It has been demonstrated that the absence of AChE leads to marked alterations in muscle function including the contractile properties and the lack of resistance to fatigue (Mouisel et al., 2006; Vignaud et al., 2008). In addition, many diseases such as myasthenia gravis have been associated with disturbances in the acetylcholine homeostasis in the neuromuscular junction and in some cases inhibitors of AChE are used for the treatment of the symptoms (Kokontis and Gutmann, 2000; Shleton, 2002).

In the present study we demonstrated that caffeic acid was capable of inhibiting in vitro the AChE activity from the muscle at concentrations varying from 0.5 to 2 mM (Fig. 4), while this enzyme activity was increased when rats were treated with 10, 50 and 100 mg/kg of caffeic acid for 30 days (Fig. 7). Considering the results obtained in vivo the increase in the AChE activity caused by the ingestion of caffeic acid may increase the hydrolysis of acetylcholine at the neuromuscular junction, decreasing the levels of acetylcholine molecules for interaction with receptors. This activation of AChE by caffeic acid may modify acetylcholine homeostasis that may trigger cholinergic deficits in neuromuscular junction.

Another important aspect is the discrepancy in relation to the results obtained in vivo and in vitro, mainly in the brain and muscle (Figs. 1, 4, 5 and 7). The lack of uniformity in the profile of AChE may be a reflection of the route of administration, dose and time of exposure, absorption and metabolism of caffeic acid in the organism. Taken together, these associations can contribute to the differences observed between AChE activity in vivo and in vitro induced by caffeic acid.

Our laboratory has demonstrated that AChE activity is altered in CNS and lymphocytes in many pathological and experimental conditions (Battisti et al., 2009; Schmatz et al., 2009; Mazzanti et al., 2009; Kaizer et al., 2009). These findings reinforce the hypothesis that AChE may be considered an important therapeutic target in different tissues. In this line, compounds that may interfere with the activity of this enzyme may be important research targets regarding the treatment of inflammatory, cognitive and neurochemical dysfunctions. In the present study, based on the compiled results we demonstrated that caffeic acid exerts an effect in the cholinergic system by altering the AChE activity in different tissues.

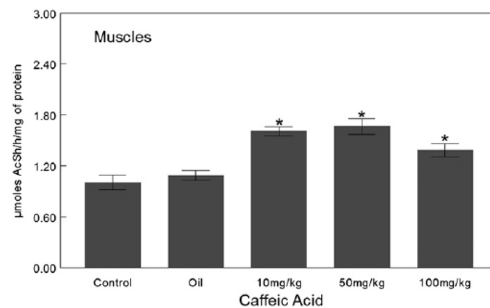

Fig. 7. In vivo effect of caffeic acid on the AChE activity in the muscle of rats. AChE activity is expressed as µmol of acetylthiocholine/h/mg of protein. Each bar represents mean ± SEM. *Different from control $P<0.05$, ANOVA–Duncan's test.

Table 1
Oral administration of caffeic acid (10,50 or 100 mg/Kg) once a day for 30 days on the latency of training (inhibitory avoidance task) and behavior of rats (number of crossing and rearing responses) in the open field immediately after the inhibitory avoidance testing session. Data are means ± SEM for 8 animals in each group.

Group	Training	Crossing	Rearing
Saline	6.37 ± 1.54	23.38 ± 4.32	11.13 ± 2.41
Oil	7.127 ± 0.97	17.38 ± 1.11	7.00 ± 0.96
10 mg/kg	5.37 ± 1.11	18.88 ± 3.30	8.75 ± 1.88
50 mg/kg	15.25 ± 7.06	17.50 ± 3.02	8.87 ± 1.69
100 mg/kg	10.75 ± 2.27	17.88 ± 2.78	11.63 ± 2.87
Statistical analysis	$F_{(4,35)}=1.377$; $P>0.05$	$F_{(4,35)}=0.662$; $P>0.05$	$F_{(4,35)}=0.835$; $P>0.05$

Data are means ± SEM for 8 animals in each group.

5. Conclusion

In conclusion, we have shown in this study that caffeic acid in vitro and in vivo altered the AChE activity in different structures and cells and improved memory. Further studies are necessary to elucidate the exact mechanism by which this compound affects the AChE activity and thus provide important information regarding the use of caffeic acid as therapeutic option in disorders associated with the cholinergic system.

Conflict of interest

The authors declare that there is no conflict of interest.

Acknowledgments

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3.2 Capter 2

First Manuscript

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.

Key words: lymphocytes, platelets, ectonucleotidases, caffeic acid, platelet aggregation

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 (Yegutkin et al., 2008). 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; Yegutkin et al., 2008). 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 in different pathological and experimental conditions (Leal et al., 2005; 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 a, b; 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 et al., 1999). Caffeic acid is known to have a broad spectrum of pharmacological activities including anti-inflammatory, antioxidant, immunomodulatory and neuroprotective (Chan 1997; Chung et al., 2004; Tanaka et al., 1993; Ban et al., 2006; Koo 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 of platelet aggregation.

1. Materials and Methods

2.1. Materials

Nucleotides, Trizma Base, Ficcoll, Adenosine and caffeic acid were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Clopidogel was obtained from Germed

Farmacêutica LTDA (Hortolândia, 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 12h 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 profile of platelet 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 160×g for 15 min. Next, PRP was centrifuged at 1400×g 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 $CaCl_2$, 100mM NaCl, 4mM KCl, 5 mM glucose and 50 mM Tris–HCl buffer, pH 7.4, at a final volume of 200µL 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. (2005). 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 37ºC. 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 et al. (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 sulphate of 75 umol/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 increased moderately. As the platelets secrete granule contents, larger aggregates form, more light passes, and the tracing moves toward 100% light transmittance constituting 100% of platelet aggregation**.**

2.11. Effect of caffeic acid associated with clopidogel 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 clopidogel *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 ºC 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 7.5μM) 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 Figure 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).

Figure 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. Figure 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 clopidrogel (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).

Figure 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 0.25mM (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. 6B).

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 Fig.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.1 A, B, C). In addition, E-NPP and ADA activity also increased in the platelets of animals treated with this compound (Fig. 1 D, 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., 2009a). 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 et al., 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 caffeic acid has heterogeneously effects on the ectonucleotidase activities in platelets. Caffeic acid decreased AMP hydrolysis and increased the ADA activity (Fig 1. C, 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% can be detected in plasma after 1 h, while Otthof 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. Azuma et al., (2000) showed that ingested 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 by 50% at concentrations between 478 and 816 μmol (Hubbart 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 0.5-1mM 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 (Hounari, 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 ($P2Y_1$, $P2Y_2$, $P2Y_4$, $P2Y_6$, $P2Y_{11}$, $P2Y_{12}$, $P2Y_{13}$, $P2Y_{14}$) (Di Virgilio et al., 2008; 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).

In this line, clopidogrel is an antiplatelet compound whose active metabolites are known as platelet ADP receptor blockers $P2Y_{12}$ (Savi et al., 2006). Clopidrogrel is the most commonly prescribed $P2Y_{12}$ blocker and has been shown to be effective in the prevention of cardiovascular complications (Pereilo et al., 2002). In this study, we compared the *in vivo* effects of caffeic acid and clopidrogel in platelet aggregation induced by collagen as well as the time of coagulation in rats. As shown in Fig 3 and 4, caffeic acid and clopidrogrel 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 6, A, B, 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, 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, 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, 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.

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.

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Legends of figures

Figure 1 - Effects of the treatment for 30 days with caffeic acid (10, 50 and 100mg/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).

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

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

Figure 4 – Time of blood visual coagulation of rats treated with caffeic acid and clopidrogel for 13 days. Groups: Control, Clopidrogel 20mg/kg (Clop 20mg/kg), caffeic acid 50 mg/kg (CA 50mg/kg), and clopidrogel (20mg/kg) + caffeic acid (50mg/kg) (clop 20mg/kg + CA 50mg/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).

Figure 5 – *In vitro* effects of caffeic acid on platelet aggregation of healthy subjects using ADP 5μM 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).

Figure 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 100mg/kg). Bars represent mean ± SEM. *Different from control (P<0.05; with n=10).

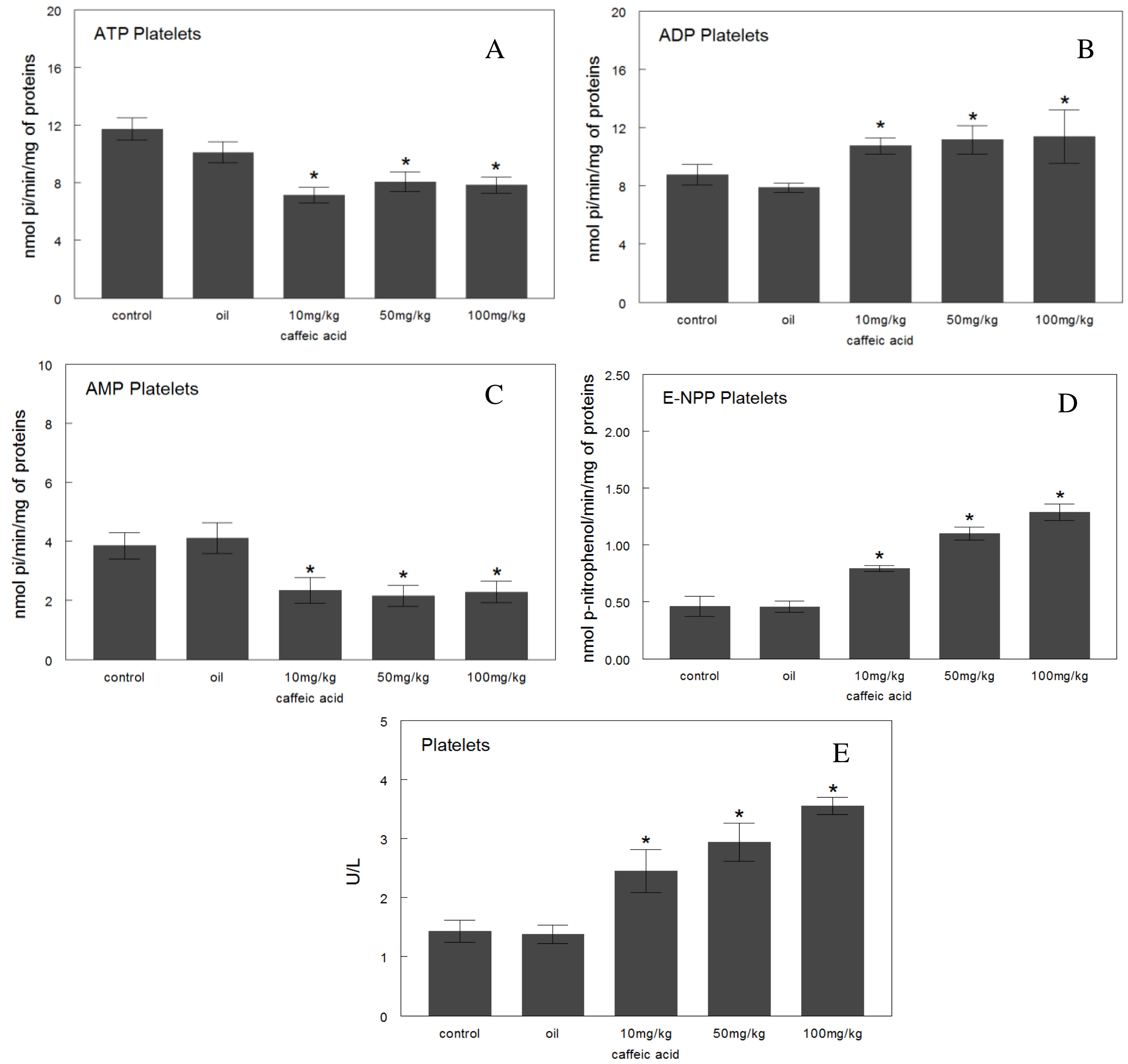

Figure 1

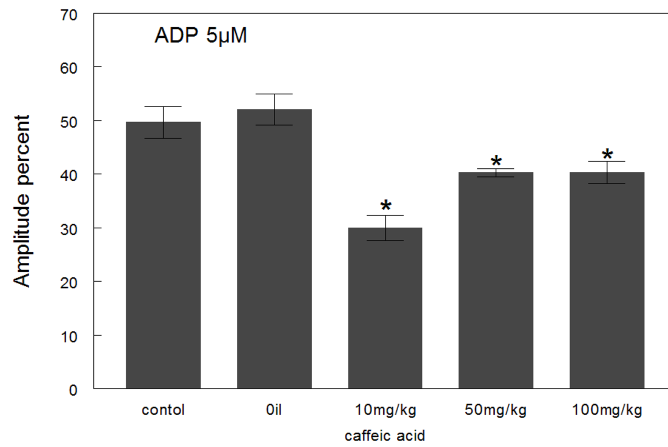

Figure 2

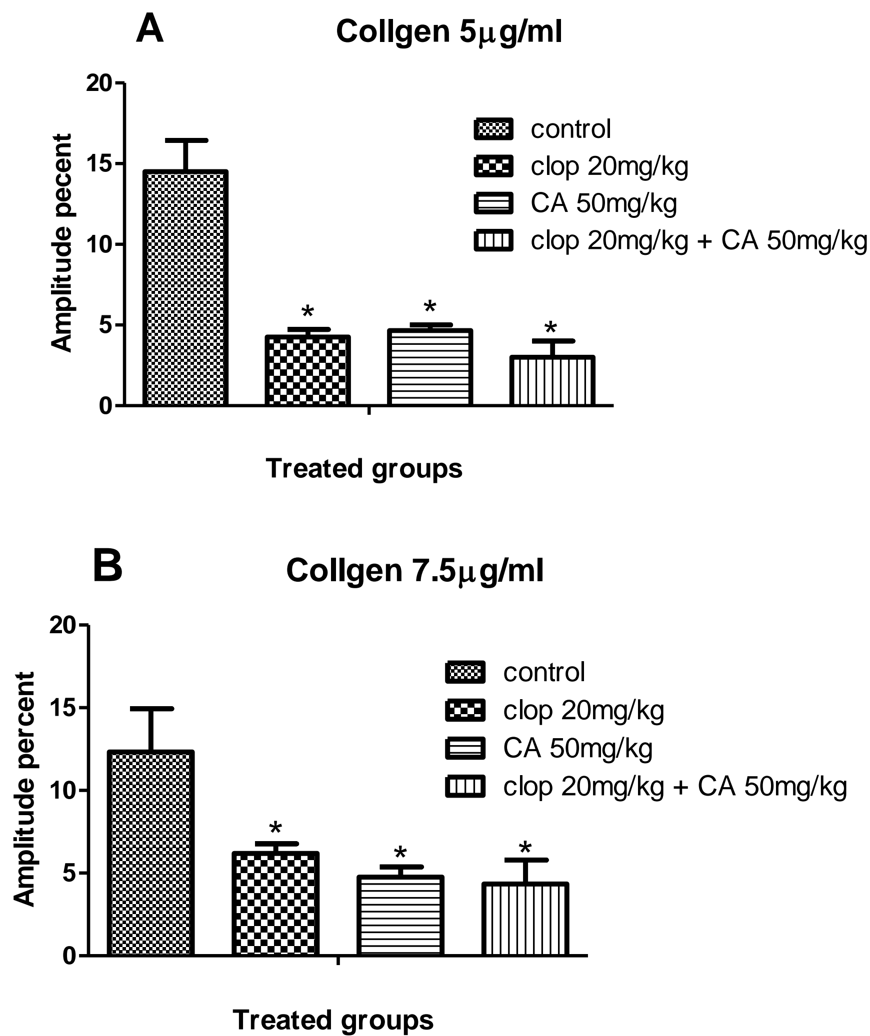

Figure 3

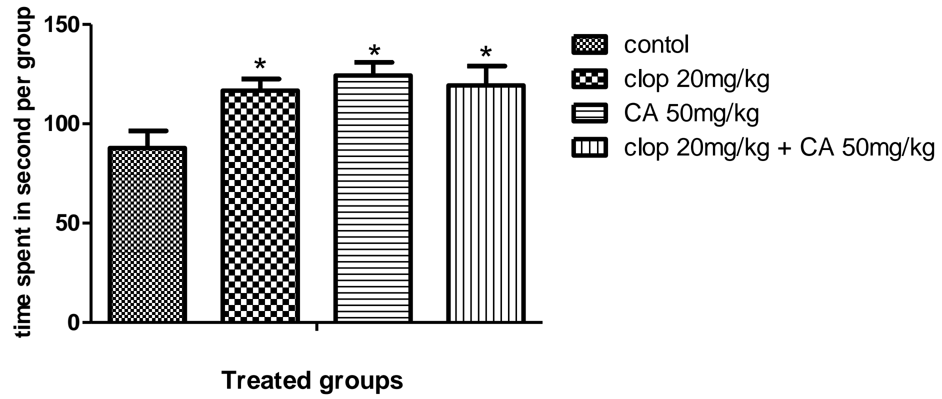

Figure 4

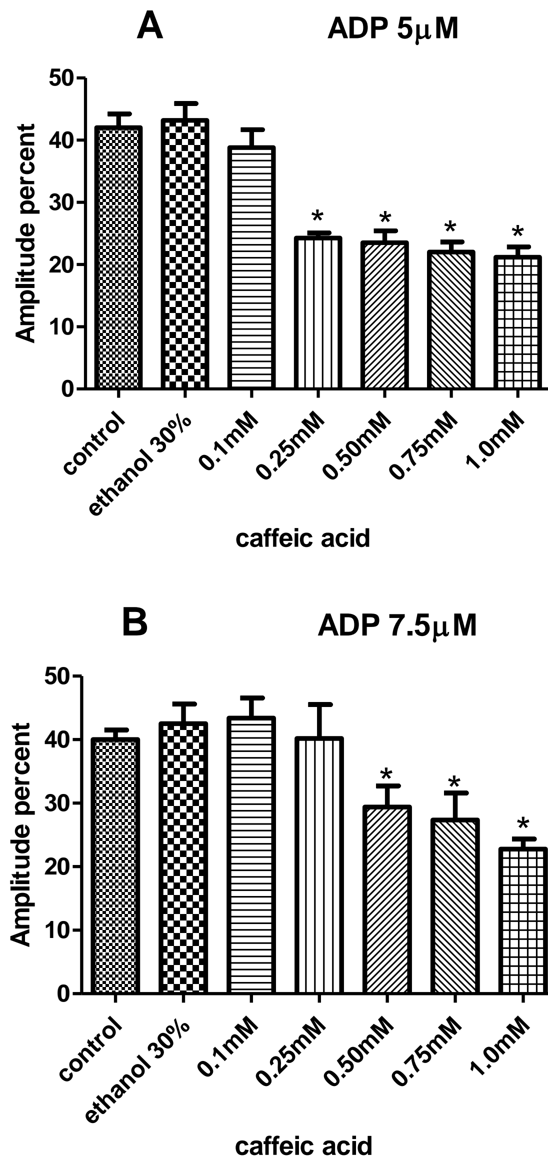

Figure 5

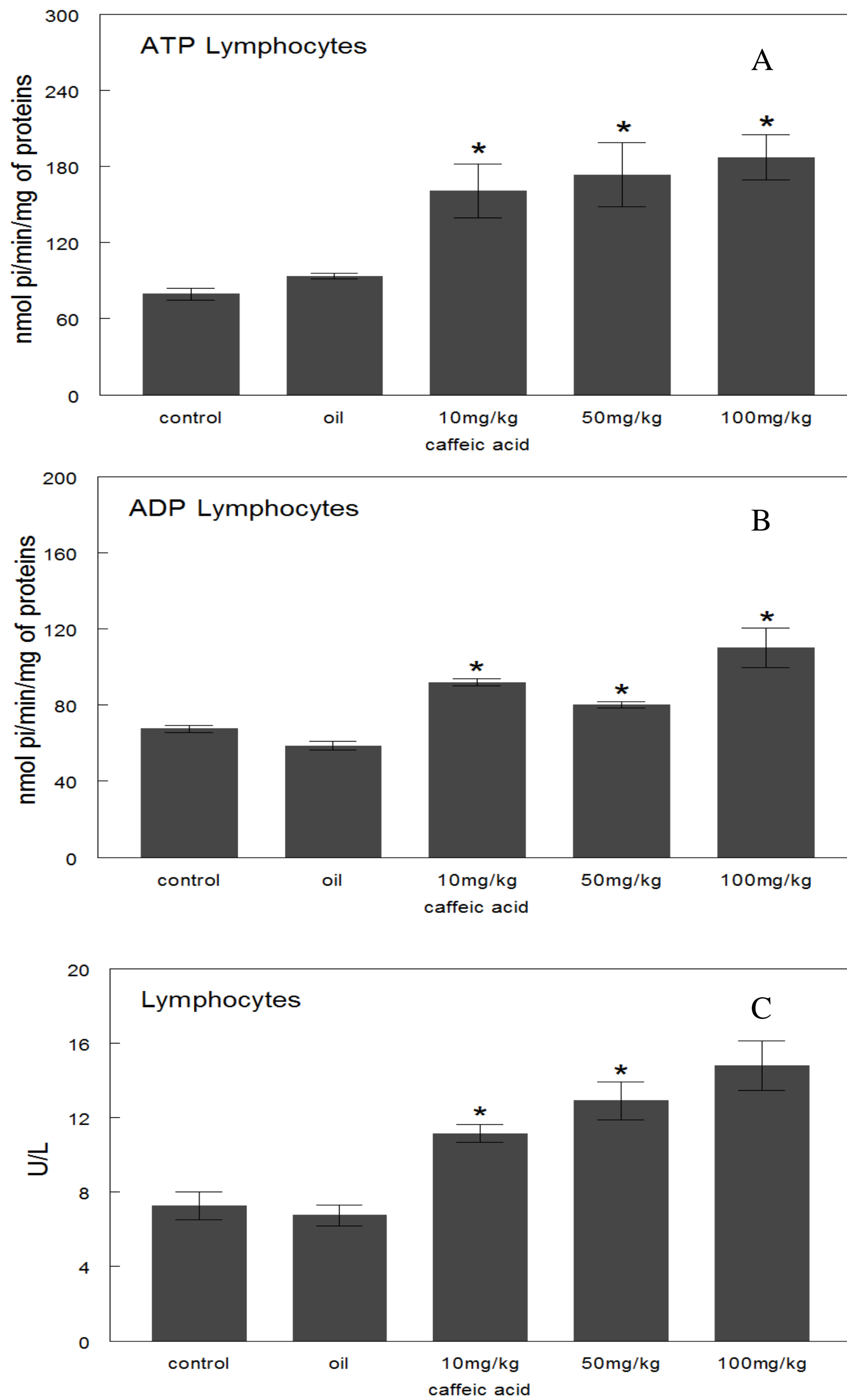

Figure 6

3.3 Chapter 3

Second manuscript

Acute effects of caffeic acid phenethyl ester on the acetylcholinesterase activity in the brain, lymphocytes and muscles of rats

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Acute effects of caffeic acid phenethyl ester (CAPE) on the acetylcholinesterase activity in the brain, lymphocytes and muscles of rats

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ABSTRACT

The present study was designed to investigate whether the acute treatment with caffeic acid phenthyl ester (CAPE) alters the acetylcholinesterase (AChE) activity in different tissues

of rats. Animals were divided into five groups (n=10): group I (control); group II (oil), group III (treated with 0.5 mg/kg the CAPE); group IV (treated with 2.0 mg/kg the CAPE) and group V (treated with 5.0 mg/kg of CAPE). CAPE was dissolved in oil and administered intraperitoneally in the animals. After 40 min, animals were submitted to euthanasia and the AChE activity was measured in the brain, lymphocytes and muscles. Results showed that CAPE decreased the AChE activity in cerebral cortex of the animals of groups IV (28%) and V (29%) in relation to the control group ($P<0.05$). Similarly, CAPE also decreased the AChE activity in the cerebellum of the animals of groups IV (36%) and V (34%), and in the striatum in the groups III (34%), IV (47%) and V (40%) when compared to control ($P<0.05$). The acute administration of CAPE increased the AChE activity in the hippocampus of animals of groups III (63%), IV (47%), and V (50%); in hypothalamus in the groups IV (34%); and pons in the group V (44%). In muscle, an increase in the AChE activity was observed in the group III (58%) and in lymphocytes only in the group IV (163%) in relation to the controls ($P<0.05$).Our results suggest CAPE may provide benefits in the treatement of disorders involving the cholinergic system.

KEY WORDS: Acetylcolinesterase, brain, lymphocytes, muscles, CAPE.

INTRODUCTION

Acetylcholinesterase (AChE) is one of the most prominent constituents of central cholinergic pathways (1). AChE is an enzyme that has an important role in the synaptic transmission by rapidly hydrolyzing the neurotransmitter acetylcholine at cholinergic synapses as well as at neuromuscular junctions (1-3). Apart from its catalytic function in hydrolyzing acetylcholine (ACh), the diverse localization of this enzyme in non cholinergic

and non neural cells and tissues strongly suggest additional non classical functions for AChE. In fact, studies have demonstrated that AChE has potent effects in postsynaptic differentiation, cellular adhesion, neurite extension and hematopoietic differentiation (1, 4, 5). In addition, this enzyme emerges as a potential contributor in the pathways controlling inflammatory and immune responses in the blood (6, 7). Therefore, alterations in the activity of this enzyme may have important consequences in the brain and other organs. As a consequence of its key physiological role, AChE activity has been studied in different pathological and experimental conditions (8-11). In a pharmacology context, the controlled application of AChE inhibitors is used to increase synaptic levels of ACh in diseases that impair cholinergic neurotransmission, such as Alzheimer's disease and myasthenia gravis (12-14). In this line, in recent years considerable attention has been directed towards the identification of natural compounds that may be used for human consumption regarding health promotion and disease prevention. Data from literature have demonstrated that natural substances and dietary components can affect the AChE activity (15, 16). Of particular importance, studies from our laboratory have also demonstrated that natural substances such as resveratrol and curcumin alter the AChE activity in the brain suggesting that phenolic compounds should be considered potentially therapeutic in disorders that involve the cholinergic system. (11, 17)

Caffeic acid phenethyl ester [CAPE] is the active phenolic compound present in propolis secreted by honeybees. The biological activities of CAPE have been extensively studied and it has been shown that CAPE has antioxidant (18), anti-inflammatory (19) and neuroprotective actions (20-22). Based on these findings, CAPE has become attractive as a therapeutic agent in the treatment of a variety of pathologies including inflammatory, autoimmune and neurodegenerative diseases (22-24). However, the mechanisms underlying the benefic effects of CAPE have not been totally elucidated yet. To the best of our

knowledge, there are few studies about the effects promoted by CAPE on the cholinergic signaling parameters. Considering the role of the AChE in inflammation and neurotransmission in the brain and neuromuscular junction, it is relevant to evaluate the effects of this compound on this enzyme activity in different tissues. Based on this, the present work aimed to investigate whether the acute treatment with CAPE affects the AChE activity in the brain, lymphocytes and muscles of rats.

MATERIALS AND METHODS

Chemicals

Acetylthiocholine iodide (ASCh), 5,5′-dithio-bis-2-nitrobenzoic acid (DTNB), Triton X-100 and CAPE were obtained from Sigma (Deisenhofen, Germany). Ethopropazine hydrochloride was purchased from Aldrich (Steinheim, Germany) and Ficoll Hypaque (Lymphoprep™) from Nycomed Pharma (Oslo, Norway). All the other reagents used in the experiments were of analytical grade and of the highest purity.

Animals

Male Wistar rats (70 days, 280-300g) from the Central Animal House of the University Federal of Santa Maria (UFSM) were used. 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, (CONCEA) and are in accordance with international guidelines.

Treatment

After the acclimatization period, animals were divided into five groups (10 rats per group): group I (control), group II (canola oil), group III (0.5 mg/kg), group IV (2.0 mg/kg), and group V (5.0 mg/kg of CAPE). CAPE was dissolved in oil and administered intraperitoneally. Forty minutes after the CAPE administration, each animal was euthanized and brain structures, lymphocytes and muscle were separated for the enzyme assays.

AChE Assay in Brain Structures

Brain structures striatum (ST), hippocampus (HP), cerebral cortex (CC), cerebellum (CB), hypothalamus (HY) and pons (PN) were separated and placed in a solution of 10 mM Tris - HCl, pH 7.4, on ice. Brain structures were homogenized in a glass potter in Tris - HCl solution. Aliquots of resulting brain structure homogenates were stored at -30°C until utilization. Protein varied for each structure: ST (0.4 mg/mL), HP (0.8 mg/mL), CC (0.7 mg/mL), CB (0.6 mg/mL), PN (0.7mg/mlL), and HY (0.6 mg/mL).

The AChE enzymatic assay in brain regions was determined by a modification of the spectrophotometric method of Ellman et al (25), as previously described by Rocha et al (26). The reaction mixture (2 mL final volume) was composed of 50 mM K^+-phosphate buffer, pH 7.5 and 1 mM of DTNB. The method is based on the formation of yellow anion, 4.4 - dithio-bis-acid-nitrobenzoic measured by absorbance at 412 nm for 2 min incubation at 25°C. The reaction was initiated by adding 0.8 mM acetylcholine iodide. All samples were run in duplicate or triplicate, and the enzyme activity was expressed in micromole AcSCh/h/mg of protein.

AChE Assay in Lymphocytes

Blood was collected in vaccutainer tubes using EDTA as anticoagulant. Peripheral lymphocytes were isolated using Ficoll-Hystopaque density gradient as described by Böyum (27). Lymphocyte viability and integrity were confirmed by determining the percentage of cells, excluding 0.1% trypan blue and measuring lactate dehydrogenase (LDH) activity (28).

After the isolation of the lymphocytes, the AChE activity was determined according to the method described by Ellman et al (25).and modified by Fritzgerald and Costa (29).Briefly, proteins of all samples were adjusted to 0.1–0.2 mg/mL. The amount of 0.2 mL of intact cells was added to a solution containing 1.0mM acetylthiocholine, 0.1mM of DTNB, and 0.1 M phosphate buffer [pH 8.0]. Immediately before and after incubation for 30 min at 27 °C, the absorbance was read on a spectrophotometer at 412 nm. The AChE activity was expressed as micromoles AcSCh / h/mg of protein.

AChE Assay in Muscle

The gastrocnemium muscle was homogenized in a glass potter in Tris – HCl 7.2 solution and centrifuged for 1000 g for 15 min. Aliquots of resulting centrifugation were used to determine the AChE activity. AChE enzymatic assay in muscle was determined by a modification of the spectrophotometric method of Ellman et al (25).The reaction mixture (2 ml final volume) was composed of 24 mM K^+-phosphate buffer, pH 7.2 and 10 mM of DTNB. The enzyme was pre-incubated for 2 min. The reaction was initiated by adding 0.8 mM acetylcholine iodide. All samples were run in duplicate or triplicate, and the enzyme activity was expressed in micromoles AcSCh/h/mg of protein.

Protein Determination

Protein was measured by the method of Bradford (30) using bovine serum albumin as standard.

Statistical Analysis

All data were expressed as mean ± SEM. The comparison between groups was assessed by one-way analysis of variance (ANOVA) followed by Student–Newman–Keuls test. $P<0.05$ was considered to represent a significant difference between groups.

Results

After the acute treatment with CAPE results showed that it altered the AChE activity in different brain structures. Figure 1a, 1b and 1c show that CAPE significantly ($P<0.05$) decreased the AChE activity in cortex, cerebellum and striatum, respectively. In cortex, CAPE inhibited the AChE activity at 2.0 mg/kg (28%) and 5mg/kg (29%); in cerebellum at 2.0mg/kg (36%) and 5mg/kg (34%); and in striatum at 0.5mg/kg (34%), 2.0mg/kg (47%) and 5mg/kg (40%) when compared to the control groups ($P<0.05$). CAPE significantly ($P<0.05$) increased the AChE activity in hippocampus at 0.5mg/kg (63%), 2.0mg/kg (47%) and 5mg/kg (50%); in hypothalamus at the concentration of 2.0mg/kg (34%); and in pons at 5mg/kg (44%) of CAPE when compared to the control groups (Figs. 1d, 1e and 1f). Effects of CAPE on the AChE activity in lymphocytes and muscles were not similar. In lymphocytes, CAPE significantly ($P<0.05$) increased the AChE activity at the concentration of 2.0mg/kg (163%) when compared to the control group (Fig. 2). In relation to muscles, CAPE significantly ($P<0.05$) increased the AChE activity only at 0.5mg/kg (58%) when compared to the control group (Fig. 3).

DISCUSSION

Phenethyl ester of caffeic acid (CAPE) is an active component of propolis from honeybee, which has been widely used as healthy food and folk medicine in many countries. Studies with CAPE have shown a broad spectrum of patophysiological activities (19, 20, 31-33).Thus, we evaluated the effects of CAPE on the AChE activity to investigate whether CAPE modulates the cholinergic signaling. In the present study, CAPE significantly altered the AChE activity in the brain, lymphocytes and muscles.

In the brain, our results showed that after 40 min of CAPE administration the AChE activity was decreased in cerebral cortex, cerebellum and striatum (Figure 1, A,B and C). In contrast, CAPE significantly ($P<0.05$) increased the AChE activity in hippocampus, hypothalamus, pons when compared to the control groups (Figure 1, D, E, F). Similar results were obtained in previous studies from our laboratory when we demonstrated that caffeic acid also inhibited the AChE activity in cerebral cortex and striatum as well as increased the enzyme activity in the hippocampus, hypothalamus and pons (34).Taken together, these findings demonstrated that CAPE and caffeic acid have similar effects on the AChE activity in the CNS.

The lack of uniformity in the profile of AChE may be a reflection of the functional heterogeneity of the cholinergic system in the CNS AChE exists in a variety of molecular forms that differ in solubility and mode of membrane attachment rather than in catalytic activity. In the brain, for instance, AChE occurs mainly as tetrameric G_4 forms (membrane bound) together with the monomeric G_1 form (cytosolic) (35).In this line, literature has demonstrated that some compounds display selectivity for certain molecular forms, and most importantly, sometimes drugs behave differently with the same isoform from different brain areas (36).Investigations of this study elucidate for this time that CAPE has different effect on

the AChE activity depending on the brain region evaluated. A definitive answer for this type of selectivity of CAPE has now been elucidated yet. The microenvironment of the enzyme activity, the hydrophobicity of different molecular forms or the nature of the tissue lipids may play a role in determining different effects of these compounds in the AChE activity in specific brain regions (36).

Acetylcholine (ACh) is a neurotransmitter that has an important role in CNS and its implication in the behavioral as well as in learning and memory has been well documented (37).AChE controls the transmission of nerve impulses across cholinergic synapses by hydrolyzing the excitatory transmitter ACh (37). Lately, AChE has been considered an important therapeutic target in CNS. Reversible inhibitors of this enzyme have been used as cognitive enhancers in the treatment of Alzhmeir's diseases and other neurodegenerative disorders (1, 2, 29).In our experiments CAPE selectively inhibited the AChE activity in cerebral cortex, striatum and cerebellum suggesting that this mechanism could be important to increase the ACh concentration in the extracellular medium in these brain regions. On the other hand, the increase of the AChE activity in hippocampus, pons and hypothalamus by CAPE leads to a fast ACh degradation and a subsequent downstimulation of ACh receptors causing alterations in the cholinergic signaling in these brain structures. In this context, these findings support the notion that CAPE has different effects on the AChE activity in relation to the brain region. The pharmacological relevance of these effects needs to be confirmed in further experiments. Several studies have demonstrated that CAPE has neuroprotective actions. CAPE has been reported to protect the brain against traumatic injury in rats, (40) neonatal hypoxic ischaemic (21) and experimental allergic encephalomyelitis (39). In another study, the CAPE administration at a dose of 10 mg/kg was capable of protecting blood brain barrier integrity and cortical tissue loss but failed to offer significant improvements in motor or memory tasks in a model of brain traumatic injury (40).Although many of the protective

effects of CAPE have been attributed to its antioxidant and anti-inflammatory properties we found that CAPE can also interfere with the cholinergic pathways in the CNS. This novel finding can provide additional mechanisms by which neuroproctetive effects of CAPE could be mediated.

In the present investigation we also evaluated the effects of CAPE on the AChE activity of the gastrocnemium muscle of rats. Results showed that 40 min after CAPE administration, the AChE activity increased in muscles only at the dose of 0.5mg/kg (58%) (Fig. 3), demonstrating that this compound can also interfere with cholinergic signaling in the neuromuscular junction. An increase in the AChE activity in the muscle was also observed when rats were treated with 10, 50 and 100 mg/kg of caffeic acid for 30 days (34).We suggest that this increase in the AChE activity could reduce the level of ACh at the neuromuscular junction, which can affect the interaction with its receptors contributing to cholinergic signaling disrupts in the muscle.

AChE is an enzyme highly concentrated at the neuromuscular junction, which plays a crucial role by rapidly hydrolyzing ACh molecules after its binding to receptors (40).Impairment in the neuromuscular transmission has been associated with various diseases such as congenital myasthenias, myasthenia gravis and muscular dystrophy (41, 42). In several of these conditions ACh hemostasis is modified and the pharmacological inhibition of AChE is used to treat some of the symptoms (14).On the other hand, the inhibition of the AChE is assumed to be the most important mechanism in poisoning by organophosphorus compounds with the resulting accumulation of the acetylcholine leading the cholinergic disrupts (43). In this situation, therapy for the reactivation of the inhibited AChE is important especially to restore neuromuscular function (43). Of particular importance, Rezg et al (44) demonstrated that caffeic acid (100mg/kg) was capable of restoring the AChE activity in the animals treated with malathion (an organophosphorus). Based on the abovementioned, it is

plausible to consider that the AChE activity is also an important therapeutic target in muscle. In this line, further investigations should provide important information about dose and time of exposure as well as find the mechanism by which CAPE could be used as therapeutic strategy in the neuromuscular disorders.

ACh is also known to have anti-inflammatory actions and suppress the production of pro-inflammatory cytokines. Lymphocytes express a complete cholinergic system consisting of acetylcholine, muscarinic and nicotinic receptors, choline acetyltransferase and AChE (45).T-cells were found to contain about three times the amount of acetylcholine when compared to B-cells, and $CD4^{+}$ cells showed significantly high levels when compared to CD8+ (46). Corroborating these findings, previous studies have also demonstrated that the AChE activity is associated to the T cells among the human peripheral lymphocytes (47).In this context, moderate acetylcholine levels are important for controlling immune and inflammatory functions, and AChE enzyme is a key contributor towards sustaining these levels. In fact, it has been demonstrated that inhibitors of AChE reduce lymphocyte proliferation and the secretion of pro-inflammatory cytokines and may attenuate inflammation by increasing the ACh concentration in the extracellular space (48).

In the present investigation, we demonstrated that 40 min after the administration of CAPE, the AChE activity was increased in lymphocytes at the dose of 2.0 mg/kg (163%) (Fig. 2). These results agree with those obtained in previous studies from our laboratory which demonstrated that the treatment with caffeic acid (100mg/Kg) also increased the AChE activity in lymphocytes of rats (34).Taken together, these findings indicate that caffeic acid and CAPE (a derivate of caffeic acid) also have similar effects on the AChE activity in immune cells.

In recent years, it has been demonstrated that CAPE has anti-inflammatory properties. These anti-inflammatory actions of CAPE have been attributed to its ability to suppress the

activation of NF-κB (49), inhibition of the expression of pro-inflammatory cytokines such as TNF-α, IL-1α, IL-6 and increased the anti-inflammatory cytokines levels (50, 51).Another study has showed that CAPE inhibits antigen induced proliferation and IL-2 gene transcription and synthesis in human peripheral T-cells. Furthermore, CAPE also targets the NFAT (nuclear factor of activated cells) signaling pathway that is known to play a role in the immune response (51).

Considering the important role of the cholinergic system in the inflammatory and immune responses, it is interesting to discuss that this study was the first to evaluate the effects of CAPE on the AChE activity in lymphocytes. We demonstrated that CAPE increased AChE activity in these immune cells and this increase in the AChE activity may decrease the ACh levels in the extracellular medium contributing to the onset of low-grade inflammation. In line with this, the anti-inflammatory actions of CAPE described in the literature cannot be attributed to the AChE activity. These findings provide new insights into the molecular mechanisms involved in the immunomodulatory and anti-inflammatory activities of this natural compound.

Conclusion

In our findings, CAPE heterogeneously alters the AChE activity in different tissues, demonstrating its specificity in different structures and cells. Schemes 1 summarize the effects of CAPE on peripheral and central AChE activities. We suggest that these findings are more relevant clinically when considering the possible therapeutic use of CAPE in diseases that involve the cholinergic system. Further studies are necessary to elucidate the mechanism by which this compound affects the AChE activity in neuronal and non neuronal cells.

Conflict of interest

The authors declare no conflict of interest.

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Legends of figures

Figures 1: Effects of CAPE on the AChE activity in cerebral cortex (a), cerebellum (b), striatum (c), hippocampus (d), hypothalamus (e) and pons (f) of rats. Each bar represents mean ± SEM. AChE activity is expressed as μmol of acetylthiocholine/h/mg of protein. *Different from controls ($P<0.05$). ANOVA– Student–Newman–Keuls test.

Figure 2: Effects of CAPE on the AChE activity in lymphocytes of rats. AChE activity is expressed as μmol of acetylthiocholine/h/mg of protein. Each bar represents mean ± SEM. *Different from controls $P<0.05$. ANOVA– Student–Newman–Keuls test.

Figure 3: Effects of CAPE on AChE activity in the muscle of rats. AChE activity is expressed as μmol of acetylthiocholine/h/mg of protein. Each bar represents mean ± SEM. *Different from controls $P<0.05$, ANOVA– Student–Newman–Keuls test.

Scheme 1: Schematic representation of the dual function of CAPE in peripheral and central cholinergic system; ↑: increasing; ↓: decreasing. TNF-α (Tumour Necrosis Factor), IL (Interleukin), NO (Nitric Oxide), LP (Lipid Peroxides).

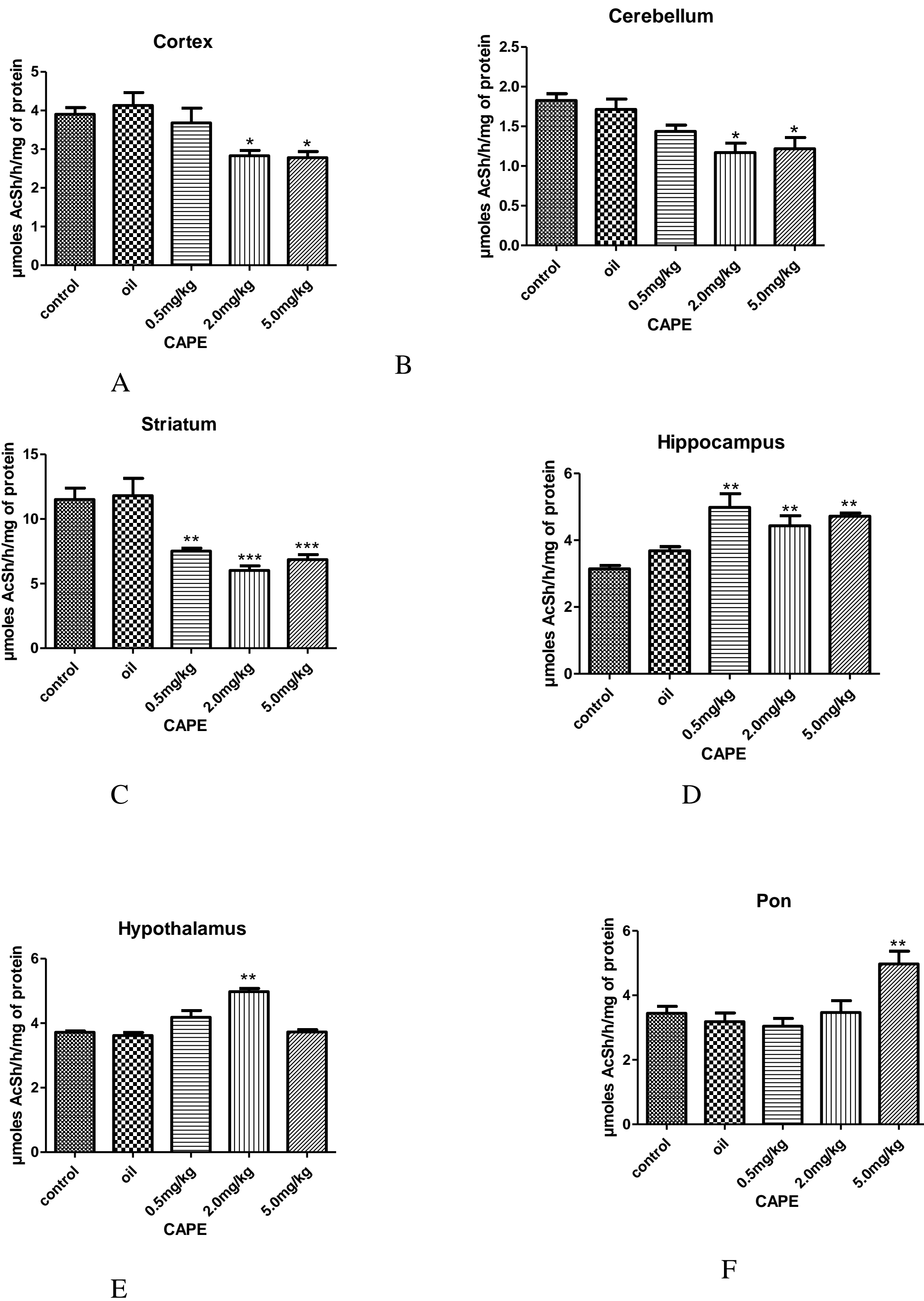


Figure 1

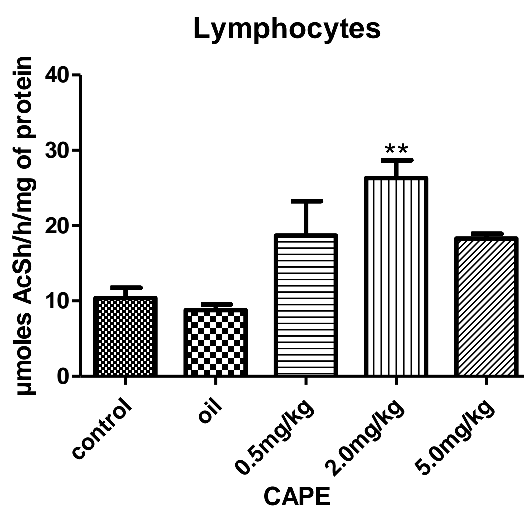


Figure 2

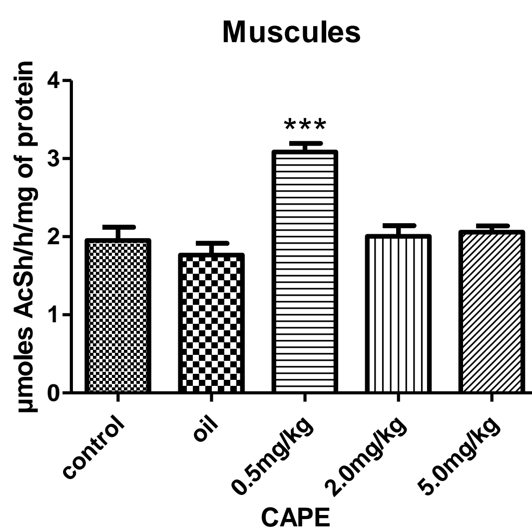
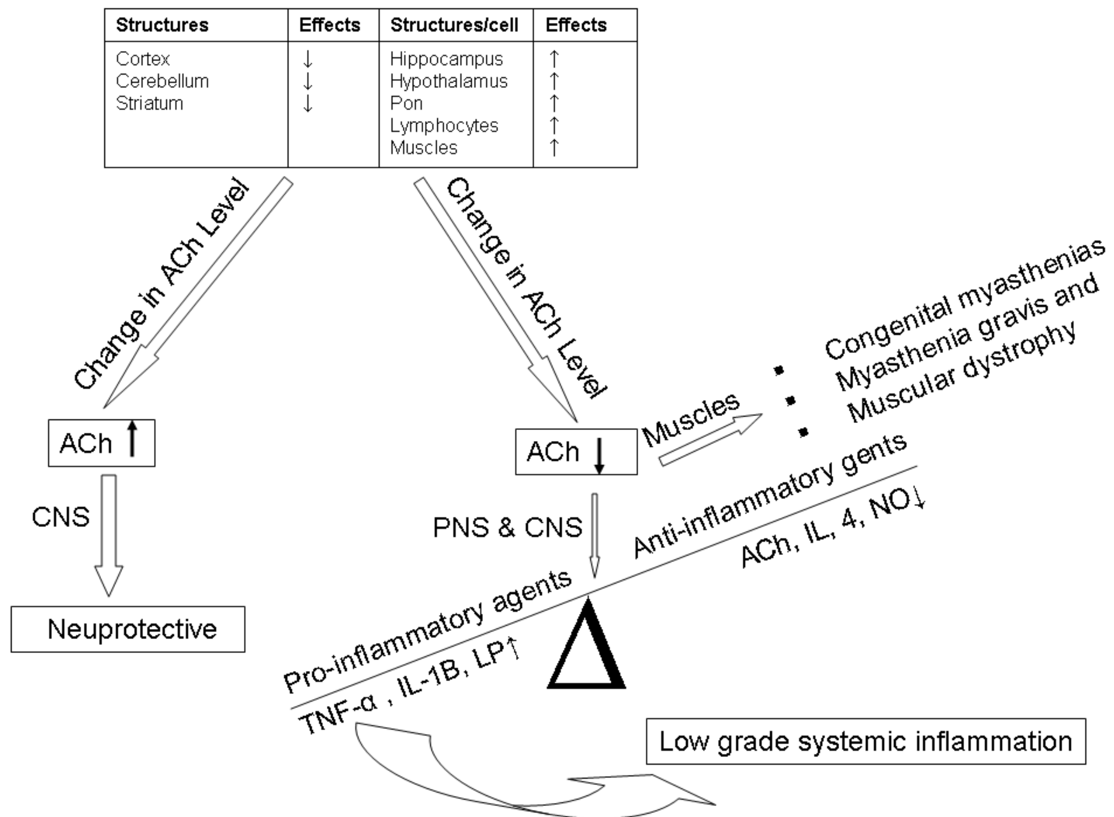


Figure 3

Scheme1



3.4 Chapter 4

Third Manuscript

(In Progress)

Enzymes that degrades adenine nucleotides in different tissues: acute effects of caffeic acid phenethyl ester

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Enzymes that degrades adenine nucleotides in different tissues: acute effects of caffeic acid phenethyl ester

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Abstract.

In pathological and physiological events, the participation of extracellular nucleotide/nucleoside and the enzymes that hydrolyzing these molecules play a crucial role. Caffeic acid phenethyl ester (CAPE), an active component of propolis, has several positive biological and pharmacological properties. CAPE, may functions in central nervous system (CNS), immunmodulation and in vascular circulation via purinegic signaling pathway, we randomly assigned 50 male Wistar rats and were divided into five groups: I control saline; II oil control; III (0.2 mg/kg); IV (2 mg/kg); and V (5 mg/kg of CAPE). CAPE was dissolved in oil and followed by acute treatment (intraperitoneally; i.p.). In platelets, the NTPDase activity on ATP hydrolysis increased at concentrations of 2.0 mg/kg 63.06% and at 5.0mg/kg 143.8%, while ADP hydrolysis increased at 2.0 mg/kg 65.205% and at 5.0mg/kg 135.9% of CAPE when compared to the control group at (P<0.05).The E-NPP activity was increased at 2.0 mg/kg 117% and 5.0mg/kg 118% of CAPE when compared to the control group at (P<0.05). The 5'-nucleotidase activity (platelets) on AMP hydrolysis was increased at 0.5 mg/kg 72%, at 2.0 mg/kg 67% and at 5.0mg/kg 111% in relation to the control groups, while Adenosine deaminase (ADA) in the platelets was inhibited at 0.5 mg/kg (67%.) and xanthine oxidase (serum) was also inhibited at higher concentration when compared to control (P<0.05). In synaptosomes, CAPE significantly inhibited the ATP hydrolysis at 0.5mg/k (25%), 2.0 mg/kg (28.%) and 5.0mg/kg (29%) while ADP hydrolysis inhibited at 0.5 mg/kg (64%), 2.0 mg/kg (75%) and 5.0 mg/kg (54%) respectively, when compared to control (P<0.05). The AMP hydrolysis in (synaptosomes) was decreased at 0.5 mg/kg 54%, 2.0 mg/kg (59%) and 5.0mg/kg at 75% when compared to the control group. CAPE induced no significant changes in Adenosine deamidnase in synaptosomes and while inhibited xanthine oxidase (32%) in whole brain in comparing to the control groups respectively (P<0.05). Mostly the enzymatic cascade of purine salvage pathway have been modulated in different cells suggest that CAPE is a promising compound and should be considered a potentially therapeutic agent in autoimmune, vascular and neurological disorders related to alteration in purinergic system.

Key words; nucleotides, ectonucleotidases, adenosine deaminase, platelets, synaptosomes, caffeic acid phenethyl ester.

INTRUDUCTION

Extracellular nucleotides and nucleosides act as signalling molecules modulating a wide spectrum of pathophysiological processes (Schetinger, et al., 2007) by different

mechanisms in various cells and system; essentially vascular (platelets and endothelial cells) and nervous system. The extracellular ATP (P2 receptor agoniist) recognized as neurotransmitter/co-transmitter and its product adenosine (P1 receptor agonist) as an important neuromodulator both in the peripheral and central nervous systems (Stojilkovic et al, 2010).

In CNS, extracellular ATP acts as a transient cellular signal and a fast excitatory neurotransmitter (Cunha and Ribeiro, 2000), co-transmitter in autonomic and sensory nerves and as a presynaptic modulator (Cunha and Ribeiro, 2000), develops neurons and its plasticity (Rathone et al., 1999). Its product adenosine acting at the P1-purinoreceptors and together with neuromodulatory and neuroprotection actions (Cunha and Ribeiro, 2000), play role in other physiological functions including arousal, sleep, anxiety, cognition and memory (Ribeiro et al., 2003).

The adenin nucleotids/nucleoside regulates the blood flow and vascular circulation together with the process of homeostasis and thrombus formation (Ralevic, 2000). ADP (the agonist of $P2Y_1$ and $P2Y_{12}$) promotes the platelet aggregation and process of thrombosis which complicate the vascular circulations (Gachet, 2001; Pinsky et al., 2002). In turn, ATP inhibits competitively the ADP induced platelet aggregation (Soslau and Youngprapakorn, 1997; Gachet, 2001) and adenosine inhibits platelets aggregation, which is essential for vasodilatation and cardioprotection (Soslau and Youngprapakorn, 1997). Further adenosine participates in apoptosis, necrosis and the proliferation of cells and plays a protective role under pathological conditions by modulating the release of the neurotransmitters and trophic factors (Rathbone et al., 1999).

The signaling events induced by adenine nucleotide depend on the timing and interaction with specific receptors. In this line in different organs the level of these molecule are regulated by cascade of enzymes, including ecto-nucleoside triphosphate

diphosphohydrolase (E-NTPDase), ectonucleotide pyrophosphatase phosphodiesterase (E-NPPs), and ecto-5'-nucleotidase/CD73 (Zimmermann, 2001; Robson et al., 2006; Yegutkin et al., 2008; Fürstenau et al., 2006). This enzymatic cascade controls the physiological level of nucleotides and their metabolites. NTPDase hydrolyze ATP and ADP to AMP (Robson et al, 2006), while Ectonucleotide Pyrophosphatase/Phosphodiesterase (E-NPP) hydrolyze 5′-phosphodiester bonds in nucleotides and their derivatives producing nucleotide monophosphate (Stefan et al., 2005). AMP resulting from the action of NTPDase and E-NPP is subsequently hydrolyzed to adenosine by ecto-5′-nucleotidase (Colgan et al., 2006).

The ecto-nucleotidases have been characterized in the neurons and platelets, regulating a variety of physiological functions including neurotransmission, platelet activation and immune response (Lunkes. al., 2004; Yegutkin, 2008). Recently, the findings in our laboratory confirmed that the alterations in these enzymes could be an important physiological and pathological parameter (Schetinger et al., 1998; Spanevello et al., 2010).For example, the neurological disorders, such as experimental model of epilepsy (Schetinger et al., 1998). Moreover, NTPDase has an established role in the immune system including cytokine expression, cell–cell adhesion and cell proliferation and apoptosis via modulation of ATP levels (Dwyer. 2007).

Adenosine deaminase (ADA) and xanthine oxidase (XO) are the other important enzymes, hydrolyzing (oxidatively) adenosine and xanthine/hypoxanthine respectively. Extracellular adenosine can be deaminated to inosine by adenosine deaminase (ADA). ADA (E.C.3.5.4.4); an enzyme which catalyzes the hydrolytic deamination of adenosine to inosine both in the cytosol and at the cell membrane (Rosemberg et al., 2008) which helps to maintain a strict control of adenosine levels, thus avoiding the cytotoxic actions of adenosine and especially deoxyadenosine in peripheral tissues as well as in brain. As an important enzyme of the purine metabolism, XO catalyzes the oxidation of xanthine and hypoxanthine to uric acid

(Sogut et al., 2002). During this reaction XO produce high quantities of oxygen-derived free radicals that contribute to oxidative damage to living tissues that are involved in many pathological processes such as inflammation, atherosclerosis, cancer and aging (Cos et al., 1998).

The phenethyl ester of caffeic acid (3, 4-dihydroxycinnamic acid) (CAPE), is an active component of propolis from honeybee which has been widely used as a health food and folk medicine in many countries. Studies with CAPE have shown a broad spectrum of biological activities including antioxidative, anti-inflammatory, antiproliferative, antibacterial, antiviral, antineoplastic, antiatherosclerotic, neuroprotective agent and antiviral action (Sud'ina et al., 1993; Nardini et al., 1995; Nagaoka etal., 2002; Kim and kim, 2000; Fesen et al., 1994). Following these physiological activities, the effects of CAPE has been evaluated in vascular circulation. Beside, CAPE has been reported to be a novel and potent antiplatelet agent for treatment of arterial thromboembolism (Chen. 2007). CAPE has been reported with vasorelaxant effect via inhibiting intracellular Ca^{++} movements (Cicala et al., 2003). In this context relating all these studies, Kart. et al., (2009) proposed that CAPE can prevent XO related O_2^{o-} production.

Though, CAPE has been reported with vasorelaxant activities (Cicala. et al., 2003; Chen. 2007). The mechanisms involved in the pathophysiological effects of CAPE are not yet clear. Since, it is widely accepted that beside to signaling functions, adenine nucleotides/nucleoside and the related enzymes contribute to several physiological and pathological conditions in CNS and vascular circulation. To contribute in understanding the mechanisms of the pathophysiological effects of CAPE, the present study aims to evaluate the effect of this compound on the enzymatic cascade involved in purine hydrolyzing enzymes vascular and central nervous system.

2. Materials and methods

2.1 Materials

Nucleotides, Trizma Base, Percoll, Ficcoll, HEPES, Adenosine, Pterin and Caffeic acid phenethyl ester were purchased from Sigma Chemical Co. (St. Louis, MO, USA). All other reagents used in the experiments were of analytical grade and of the highest purity.

2.2. Animals

Fifty male Wistar rats (70 days, 280-300g) from our breeding colony of the Federal University of Santa Maria (UFSM) were obtained and maintained healthy in plastic cages at a constant temperature (23 ± 1°C) on a 12 h light/dark cycle with free access to food and water. The 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

After the acclimatization period, the animals were divided into five groups (10 rats per group): Group I (control); Group II (canola oil), Group III (treated with 0.5 mg/kg of CAPE); Group IV (treated with 2.0 mg/kg of CAPE) and Group V (treated with 5.0 mg/kg of CAPE. CAPE was dissolved in oil and administrated intraperitoneally. After 40 minutes, each animal was euthanized and platelets, serum, synaptosomes and whole brain were carefully separated for estimating the enzymes activities.

2.4. Platelet preparation

Platelet-Rich Plasma (PRP) was prepared by the method of Lunkes et al., (2004) with the following minor modifications. Total blood was collected by cardiac puncture with 0.120 M sodium citrate as anticoagulant and was centrifuged at 160×g during 15 min. After this, the PRP was centrifuged at 1400×g for 30 min and washed twice with 3.5 mM HEPES buffer, pH 7.0, containing 142mM NaCl, 2.5mM KCl and 5.5mM glucose. The platelet pellets were re-suspended in HEPES buffer to assay enzymatic activities.

2.5. **Synaptosome preparation**

The synaptosomes from cerebral cortex of the rats were isolated as described by Nagy and Delgado-Escueta (1984) using a discontinuous Percoll gradient. The cerebral cortex was gently homogenized in 10 volumes of an ice-cold medium (medium I), consisting of 320 mM sucrose, 0.1 mM EDTA and 5 mM HEPES, with a pH of 7.5, in a motor driven Teflon–glass homogenizer, and then centrifuged at 1000 g for 10 min. An aliquot of 0.5 mL of the crude mitochondrial pellet was mixed with 4.0 mL of an 8.5% Percoll solution and layered onto an isoosmotic discontinuous Percoll/sucrose gradient (10%/16%). The synaptosomes that banded at the 10% and 16% Percoll interface were collected with a wide-tip disposable plastic transfer pipette. The synaptosomal fraction was washed twice with isoosmotic solution by centrifugation at 15,000 g for 20 min to remove any contaminating Percoll. The pellet from the second centrifugation was suspended in an isoosmotic solution and the final protein concentration was adjusted to 0.4 - 0.6 mg/mL.

2.6. NTPDase and ecto-5´-nucleotidase activities in platelets and synaptosomes

In platelets, the reaction medium for NTPDase activity containing 5 mM $CaCl_2$, 100mM NaCl, 4mM KCl, 5 mM glucose and 50 mM Tris–HCl buffer, pH 7.4, at a final

volume of 200µL was carried out as described by Lunkes. et al., (2004). For AMP hydrolysis, the reaction medium was used as previously described, except that the 5 mM $CaCl_2$ was replaced by 10 mM $MgCl_2$.

For the determination of NTPDase activity in synaptosomes the reaction medium containing 5mM KCl, 1.5 mM $CaCl_2$, 0.1 mM EDTA, 10 mM glucose, 225 mM sucrose and 45 mM Tris–HCl buffer, pH 8.0, in a final volume of 200 µL as described in a previous work from our laboratory (Schetinger et al., 2007). The 5'-nucleotidase activity was determined essentially by the method of Heymann et al. (1984) in a reaction medium containing 10mM $MgSO_4$ and 100mM Tris–HCl buffer, pH 7.5.

2.7. E-NPP activity determination in platelets

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, 120 mM NaCl, 5.0 mM KCl, 60 mM glucose and 5.0 mM CaCl2, pH 8.9, was preincubated with approximately 20µL per tube of platelet protein for 10 min at 37 °C to a final volume of 200µL. The enzyme reaction was started by the addition of p-Nph- 5′-TMP at a final concentration of 0.5mM. 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. Enzyme activity was expressed as nmol p-nitrophenol released/min/ mg protein.

2.8. ADA assay in platelets and synaptosomes

Adenosine deaminase activity was measured spectrophotometrically in platelets and synaptosomes by the method of Giusti et al. (1974). The reaction was started by 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 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/11mmol/l alkalinehypochlorite (sodium hypochlorite) and vortexed. Ammonium sulphate of 75µmol/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.

2.10. Xanthine Oxidase Assay in Serum and Whole Brain

To evaluate the activity of xanthine oxidase flurometically by the method of Zeinab et al. (1999), samples were homogenized in Tris-HCl buffer (50 mM, pH 7.4) containing EGTA 1mM, DTT 10mM and PMSF1 mM and the protein content was adjusted to 1mg/mL. Samples were then incubated with 20 µM pterin during 30 minutes at 37°C in a final volume of 1mL. After, 0.5 mL of acetate buffer (80 mM, pH 3.5) was added and the mixture was boiled during 10 minutes and centrifuged at 16000 xg for 10 minutes. The fluorescence developed was monitored at 347 nm excitation and 405 nm emission and the results were expressed as % of control group activity.

2.11. Protein determination

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

2.12. 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 results obtained demonstrated that CAPE altered the ecto-nucleotidase activities in platelets and synaptosomes from the cerebral cortex of rats. The results obtained for NTPDase activity in platelets are shown in Fig. 1. CAPE increased the NTPDase activity on ATP hydrolysis at concentrations of 2.0 mg/kg 63 % and at 5.0 mg/kg 144% while ADP hydrolysis increased at 2.0 mg/kg 65 % and at 5.0 mg/kg 136% of CAPE when compared to the control group ($P<0.05$).

In addition, the results obtained for 5′-nucleotidase activity were similar to those found for NTPDase (Fig. 1). It was observed that CAPE increased the AMP hydrolysis in platelets at 0.5 mg/kg 72 %, at 2.0 mg/kg 67 % and at 5.0 mg/kg 111 % of CAPE when compared to the control group ($P<0.05$).

(Fig. 2) shows the effect of CAPE on E-NPP activities in platelets. The increase in E-NPP activity was observed at 2.0 mg/kg 117 % and at 5.0mg/kg 118% of CAPE when compared to the control group ($P<0.05$).

The figur 3 shows the effects of CAPE on ADA in platelets. In platelets, ADA actictivity was inhibited significantly at 0.5 mg/kg 67 %. In serum, the XO activities inhibited at 0.5mg/kg 67% and at 5.0 mg/kg 46 % respectively when compared to control ($P <0.05$).

Fig. 4, show ecto-nucleotidases activities in synaptosomes. As can be seen in figure 4, CAPE significantly inhibited ATP at 0.5mg/k 25% at 2.0 mg/kg 28.% and at 5.0mg/kg 29%

while CAPE significantly inhibited ADP at 0.5 mg/kg 64%, at 2.0 mg/kg 75% and at 5.0mg/kg 54% respectively when compared to control (*P*<0.05).

In relation to AMP hydrolysis, a significant decrease was observed (Fig. 4) at 0.5 mg/kg 54%, at 2.0 mg/kg 59% and at 5.0mg/kg at 75% when compared to the control group (P<0.05).

The effects of CAPE on ADA in synaptosomes and XO activity from whole brain are shown in Figure 5 and 6. CAPE induced no significant alteration ADA activity in synaptosome and XO (32%) activity in whole brain at any concentration evaluated.

4. Discussion

Since many beneficial biological properties has been ascribed to CAPE in vitro and in vivo; however, its effect on purine catabolism enzymes in central/or peripheral system has not been yet studied. Several studies have used peripheral cells as models of nerve endings because they contain and release neurotransmitters and have receptors for neurotransmitters on their surface (Odagaki and Koyama. 2002; Rainesalo. et al., 2003). The alteration in activity of ecto-nucleotidase has been observed in various diseases, suggesting that this could be an important physiological and pathological parameter (Lunkes et al., 2004; Schetinger, et al., 2007) revealing the importance of these enzymes on the local events. Therefore, these studies in different tissues/cells on purine catabolism enzymes reinforced us to investigate and compared the effect of CAPE on these enzymes in different tissues and cells of rats.

The results showed significant increase in NTPDase, E-NPP and 5′-nucleotidase activities from platelets. CAPE inhibited significantly ADA activity in platelets and XO activity in serum. This investigation demonstrated a significant role of CAPE in vascular circulation, which regulates the nucleotide concentrations and nucleoside formation and hence the intensity of interaction with P2 and P1receptors. In our unpublished data, caffeic acid (a

phenolic compound) alters the ectonucleotidases activity. In addition the work of Jaques et al. (2011) with curcumin reported alteration in activities of ectonucliotidases. Taking all these results, emphasize the importance and relationship of ecto-nucleotidases with compounds having phenolic structure that can effects the catalytic site these enzymes in periphery.

In the vascular system, extracellular ATP is an important vasoconstrictor and ADP is the main promoter of platelet aggregation whereas adenosine is a potent inhibitor of this aggregation and an important modulator of vascular tone (Soslau and Youngprapakorn, 1997). The NTPDase and 5-nucleotidase interfere in the modulation of platelet activation and thrombus formation (Atkinson et al., 2006; Koszalka et al., 2004). On the concern that nucleotides in extracellular medium associated with platelet aggregation, the present effects demonstrating that CAPE as a modulating marker of the peripheral purinergic system and can play an excellent role against platelet activation and thrombus formation. Further, the increase in AMP hydrolysis, modulating adenosine level in the extracellular medium which possesses anti-inflammatory and analgesic properties (Fredholm et al., 1994). The present effects of CAPE, excellently favor the production of adenosine the anti-inflammatory agent. The removing of pro-aggregant agent ADP, demonstrating anti-thrombotic properties, which can further improve vasodilatation and facilitate blood flow.

Platelets are involved in the processes associated with vascular inflammation and thrombosis (Wagner et al., 2003). Extracellular adenine nucleotides and adenosine are known to regulate the vascular response to endothelial damage by exerting a variety of effects on platelets (Zimmermann 1999). After the action of ecto-nucleotidases, ADA and XO are enzymes that catalyze the conversion of adenosine to inosine, deoxyadenosine to deoxyinosine, hypoxanthine to xanthine and xanthine to uric acid, respectively (Sogut et al., 2002). ADA helps to maintain a strict control of adenosine levels, thus avoiding the cytotoxic

actions of adenosine and especially deoxyadenosine in peripheral tissues (Fox and Kelley, 1978) as well as in brain (Eells and Spector, 1983). On the other hand, in hypertensive patients the increased level of uric acid in serum has been related to increased cardiovascular risk (Alderman, 1999). We assessed the ADA activities in platelets and XO in serum. The effect of CAPE on ectonucleotidases is a convincing demonstration to have elevated ADA/XO activity. We observed an inhibitory tendency in ADA followed by the significant decrease XO activities. Since the enhanced levels of adenosine and xanthine, which are substrates for ADA and XO respectively, may amplify the activity of ADA and XO in the tissue (Uz et al., 2005) accompanied by inflammation and ROS production and hence medical conditions.

In this study, we next examined NTPDase, 5’-nucleotidase and ADA activity in synaptosomes while XO in whole brain. The results showed that the NTPDase and 5´-nucleotidase activities were modulated. CAPE showed no significant alterations in ADA synaptosomes and XO in whole brain. These investigations demonstrating the expected neurochemical effect of CAPE on CNS. Many herbals and insect productions including propolis and honey constitute a major class of flavonoids used as remedies. Presently, the use of flavonoids is increasing due to their established role to simulate hormones and neurotransmitters, to scavenge free radicals and inhibit specific enzymes (Havsteen. 2002). Beside to catalytic activity in platelets, the ecto-nucleotidase activities in synaptosome demonstrating that CAPE is also sensitive to the CNS.

Extracellular ATP can play an important role in synaptic transmission, acting as a neurotransmitter and/or a neuromodulator (Cunha and Ribeiro. 2000) whereas its breakdown product adenosine is an important, inhibitory, neuromodulator and a cytoprotective agent (Mendonça et al., 2000). NTPDase and 5’-nucleotidase activity control the physiological effects of extracellular adenine nucleotides. In the nervous system, purines are important

neuromodulators, acting at pre- and postsynaptic sites. The decreased activity of NTPDase on ATP hydrolysis can elevate the level of these neurotransmitters in the extracellular medium and perhaps could promote the sensitivity of P2 receptors.

The physiological importance of extracellular ATP in the CNS has been well established. Alterations in NTPDase and 5′-nucleotidase activities appear to be related to neurological disorders, such as experimental model of epilepsy and cerebral ischemia (Schetinger et al., 1998). We focused our present investigation on normal rats and perhaps the inhibition of ATP hydrolysis can elevate the level of this neurotransmitter in the CNS could up regulate the sensitivity of its respective receptors. In this line our present investigation reveals an improvement/benefits in excitatory neurotransmissions and hence purinergic signaling.

The action of 5’-nucleotidase catalyzes the hydrolysis of AMP, playing an important role in adenosine production, a neuromodulator (normally inhibitory) in the CNS. Adenosine acting at the P1-purinoreceptors plays an important regulatory role in neuronal activity and has neuroprotective actions (Cunha and Ribeiro, 2000) in CNS, reported as neuromodulatory and neuroprotection actions as well as by the regulation of physiological functions such as arousal, sleep, anxiety, cognition and memory (Ribeiro et al., 2003). CAPE inhibited the 5’-nucleotidase activity in synaptosomes reflecting a deficiency in adenosine (a cytoprotective agent) level which could be impairment in the purine hydrolyzing pathway and hence affect purine salvage ability. Therefore, the organism may predispose to brain damage because of the impairment in purine salvages ability and thus can desensitize P1 purinoreceptors.

Despite nucleotides regulating short-term and long-term cellular functions in the CNS (Zimmermann, 2006), ATP has been reported with various biological functions, such as neurotransmission both in the peripheral and CNS (Cunha and Ribeiro, 2000) and its breakdown product, adenosine, may modulate synaptic plasticity in rats (Mendonça and

Ribeiro, 1997). It seems in the present investigation that both purinergic system (CNS and periphery) are sensitive well to the effect of CAPE. We suppose that CAPE can alter all related signaling events by altering the extracellular adenine nucleotides in the neurotransmitting pathways.

Since the affects of CAPE on the ectonucleotidases activities are quite different in periphery and brain. For this discrepancy, a possible explanation would be the part from the fact of difference; (1) in purine salvage pathway of CNS and Periphery and the complements of specific receptors coupled to extracellular signalling pathways (2) in biological environment regarding the specificity in functions pattern and (3) the existence of these enzymes in the form of family and perhaps mode of membrane attachment rather than in catalytic activity. Here we suppose that CAPE have the capability to modulate the purinegic system selective and perhaps this compound may have the form-specific selectivity in relation to the ectonucleotidases in platelets and brain.

In the purine hydrolyzing pathway of CNS, the activities of ADA and XO have also an important role in the physiological level of adenosine and inosine. We assessed the activities of ADA in synaptosome and XO in whole brain. The results showed no significant effect on the activity of ADA in synaptosomes while inhibited the XO in whole Braine. The rapid hydrolysis of adenosine by ADA to inosine and subsequently the increased XO can enhance the production of free radical and reactive oxygen species which is a soul of inflammation and oxidative damage. The present effects of CAPE should be associated with its known protective effects against ischemia/reperfusion (I/R) injury, anti-oxidant, anti-inflammatory, free radical scavenging and perhaps their binding activity with specific receptors.

We do not know the mechanisms by which CAPE reduces ecto-nucleotidases activities in synaptosome, since in our unpublished data; caffeic acid also inhibited the ecto-nucleotidases activities *in vitro*, supposing a pro-inflammatory concern in CNS. Supporting

this hypothesis, in literature unlike most chemotherapeutic agent, some anti-oxidant and anti-inflammatory compounds have been reported as pro-oxidant and pro-inflammatory. In this context, caffeic acid has been reported to be able to induce lipid peroxidation and/or DNA damage in the presence of cupric ions (Yamanaka, 1997). Further, it is reported with proposed mechanisms that CAPE (Ting et al., 2008) in the presence of Cu (II) ions: induce DNA damage and hence pro-oxidant and with the similar proposed mechanism the affects of caffeic acid has been also reported. In this line, evidence demonstrated that copper can be found in CNS tightly bound for catalytic use by cuproproteins (Schlief and Gitlin, 2006). Copper may also be loosely bound to small molecules such as histidine and glutathione, representing an available pool for releasing and acting as a functional neuromodulator in CNS (Mathie et al., 2006).

CAPE is an ester of CA and both have a catechol group attached to its main ring that may produce a site for chelation. Ting et al. (2008) reported that CAPE could bind and form a complex with Cu (II) and also capable of binding to DNA. In this line, the chelating property of CAPE can diminish NTPDase and 5'-nucleotidase activities because divalent cations are required for the catalytic function of NTPDase and then perhaps 5'-nucleotidase activities too. Consequently, metals ions chelation properties of CAPE can modify metals ions homeostasis in the CNS that can trigger neuronal cell death. Reinforcing this hypothesis Da Silva et al. (2006) also suggests that chelating property of flavonoids can diminish NTPDase-like, 5'-nucleotidase and Na^+/K^+-ATPases activities in cortical membrane preparation.

Conclusion

In conclusion, mostly the activities of purine hydrolysing enzymes cascade altered in the PNS and CNS demonstrating that CAPE have the ability to modulate the purine salvage biochemistry. In the present investigation, unlike other chemotherapeutic agents, CAPE

showed physiological, anti-inflamatory and pro-inflammatory properties. Further research is necessary to understand its role both in pathological and physiological events mediated by purine hydrolyzing enzymes.

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Legends of figures

Figure 1 - In vivo acute effects of CAPE on NTPDase (A) and (B), 5´-nucleotidase (C) activities in platelets from rats treated with 0.2, 2 and 5mg of CAPE. Bars represent mean ± SEM. *Different from control (P<0.05).

Figure 2 - *In vivo* acute effects of CAPE on E-NPP activity activities in platelets from rats treated with 0.2, 2 and 5mg of CAPE. Bars represent mean ± SEM. *Different from control (P<0.05).

Figure 3 - *In vivo* acute effects of CAPE on ADA activity in platelets from rats treated with 0.2, 2 and 5mg of CAPE. Bars represent mean ± SEM. *Different from control (P<0.05).

Figure 4- *In vivo* acute effects of CAPE on NTPDase (A) and (B), 5´-nucleotidase (C) activities in synaptosomes from cerebral cortex the rats treated with 0.2, 2 and 5mg of CAPE. Bars represent mean ± SEM. *Different from control (P<0.05).

Figure 5 - *In vivo* acute effects of CAPE on ADA activity in synaptosomes from cerebral cortex the rats treated with 0.2, 2 and 5mg of CAPE. Bars represent mean ± SEM. *Different from control (P<0.05).

Figure 6- In vivo effects of CAPE on XO activity in brain from rats. Bars represent mean ± SEM. Each column represents mean ± S.E.M. *Different from control (P<0.05).

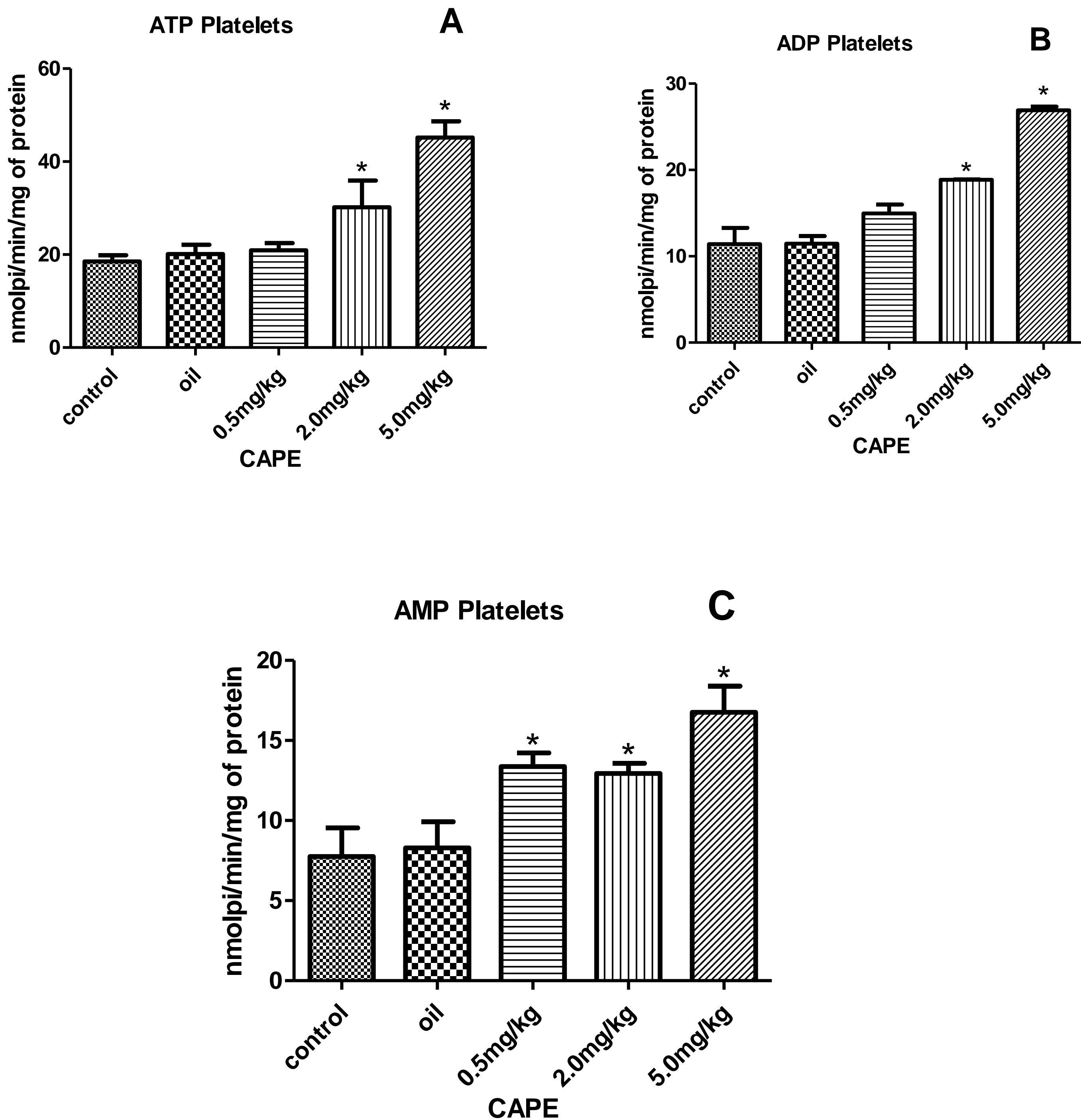

Figure 1

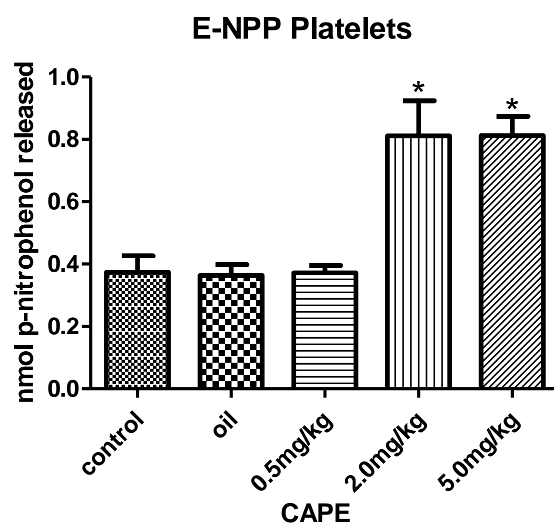

Figure 2

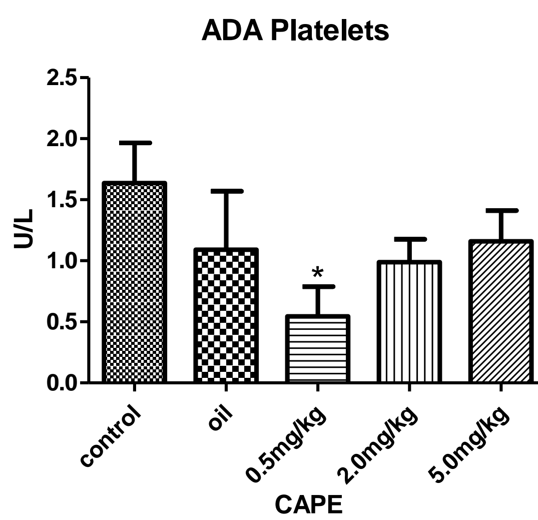

Figure 3

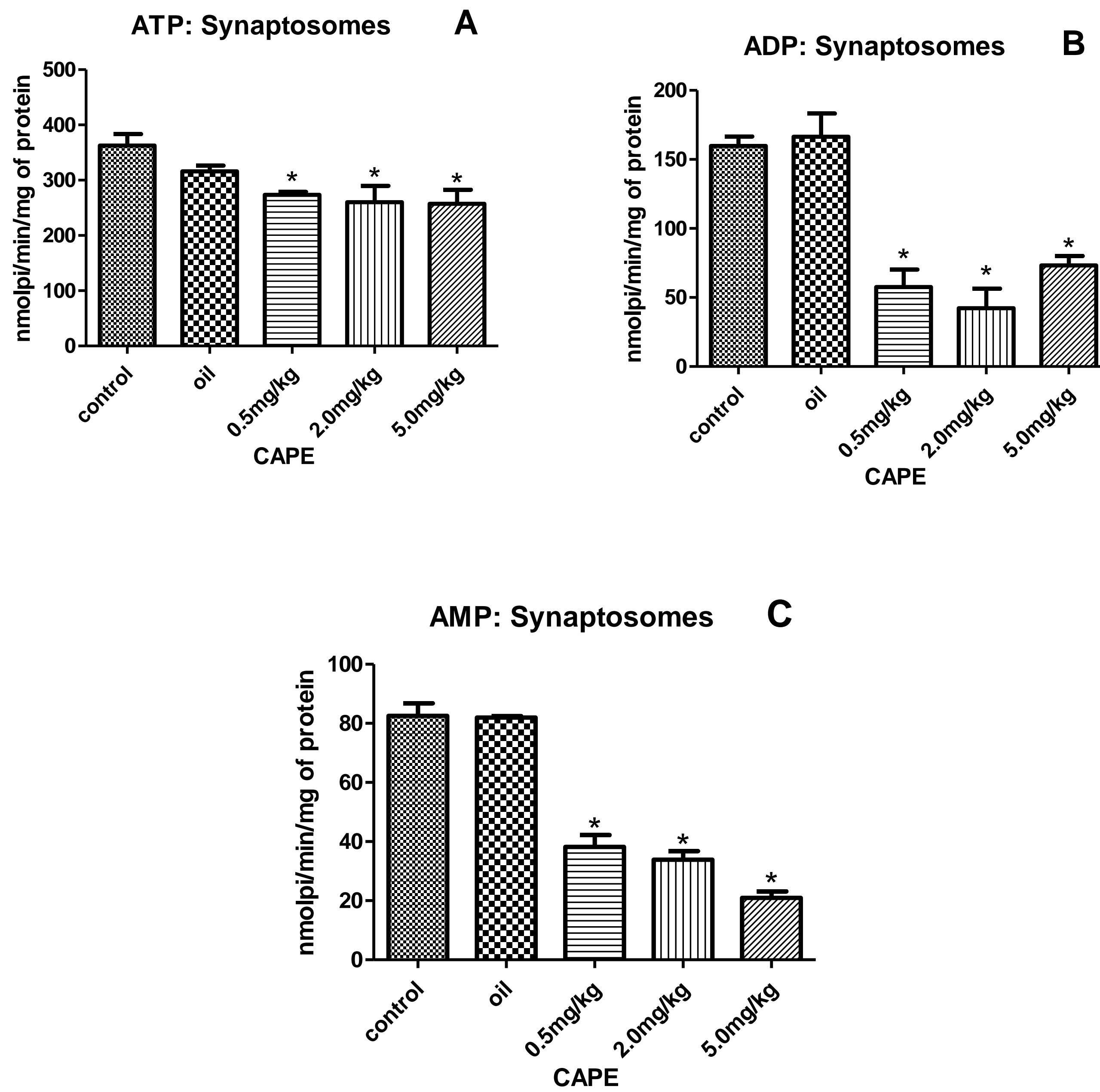

Figure 4

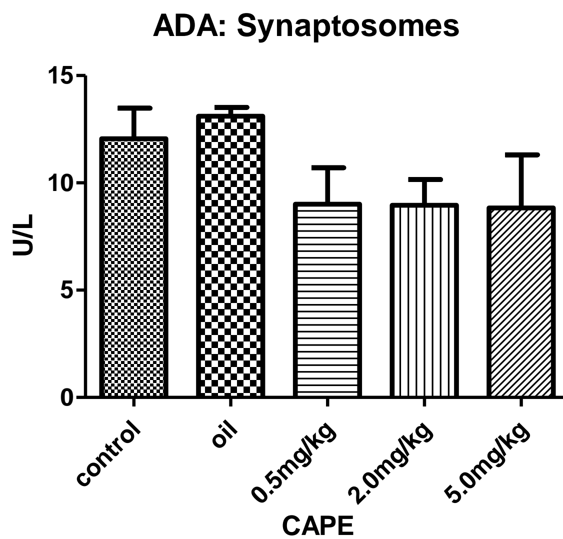

Figure 5

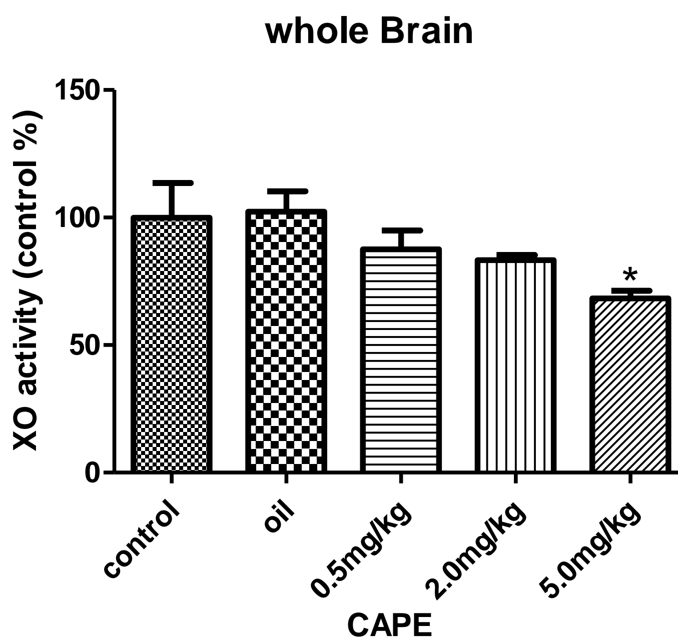

Figure 6

4 GENERAL DISCUSSION

Since ancient time plant derived dietary items are believed to be beneficial by mediating different variety of essential physiological effects. Dietary components like fruits, vegetables, and beverages are key source of these bioactive agents. Almost, the phenolic compounds are in majority among the plant derived natural compounds and most of physiologic effects of dietary items are referred to these compounds. With convincing evidence suggests that regular consumption of fruit and vegetables significantly reducing the incidence of chronic diseases including cancer, cardiovascular disease, stroke, Alzheimer disease, cataracts, and functional declines associated with aging (LIU 2003).

CA and CAPE are major representatives of phenolic compounds, receiving much attention. A number of studies also have demonstrated that natural substances and dietary components can affected the AChE activity in different tissues (REZG et al., 2008; AHMED & GILANI 2009; SCHMATZ et al., 2009). CA (3, 4 - dihydroxycinnamic acid) is a non-flavonoid catecholic compound abundantly present in many plants and occurs in diet as part of fruits, tea, coffee and wine (CLIFFORD, 1999). Pharmacology studies have shown that CA have antioxidant (CHAN & HO, 1997; GULCIN, 2006), neuroprotective (YANG et al. 2008) and antiinflamatory properties (CHALLIS & BARTLETT, 1995). The phenethyl ester of CA (3, 4-dihydroxycinnamic acid), is an active component of propolis from honeybee which has been widely used as a health food and folk medicine in many countries. It is an anti-inflammatory (MICHALUART et al., 1999), antiviral (FESEN et al., 1994), antioxidant (SUD'INA et al., 1993), and anticancer (LEE, et al., 2000) and neuroprotective agent (ILHAN, et al., 2004). The particular importance, REZG, et al., (2008) showed that CA increased the AChE activity in rats treated with malathion, demonstrating thus that this compound interfere with cholinergic signaling. CAPE also inhibited the acetylcholinesterase activity in an *in vitro* colorimetric assay (BORRELLI, et. al., 2005).

Regrarding this we evoluated we the effects of CA at concentrations varying from 0.1-2.0mM on the activities of AChE in brian stucture, lymphocytes and muscles. In another set of experiments, we used CA and its derivative CAPE together to investigate whether *in vivo* these two compounds modulate the cholinergic system.

For the first time, our results in brain showed that CA altered *in vitro* and *in vivo* the AChE activity. *In vitro* in cerebral cortex, cerebellum and hypothalamus, CA significantly increased the AChE activity at concentrations varying from 0.1 - 2.0 mM, while that in the

striatum, hippocampus and pons the enzyme activity was not altered at any concentration respectively. The AChE activity was inhibited significantly in cortex and striatum, while in cerebellum, hippocampus, hypothalamus and pon we observed significant increased in AChE activity over ACh degradation.

In a set of experiment, we investigated the effect of CAPE on AChE activities in brain structures. Our results in brain demonstrated that CAPE altered *in vivo* the AChE activity, but similar to CA (*in vitro* and *in vivo*) these alterations were not homogeneous in brain structures. We observed that in cortex, striatum and cerebellum the inhibitions were significant. In addition, CAPE enhanced significantly the AChE activity in hippocampus, hyphothalamus and pon respectively when compared to control.

These results indicate that there is a specific response of AChE to CA and CAPE. Because in the present study, CA (*in vitro* and *in vivo*) and CAPE (*in vivo*), significantly alter AChE activities on ACh degradation. Since the affects of both compounds on AChE activity in brain are not homogenous. For this discrepancy, a possible explanation would be the fact that acetylcholinesterase (AChE) exists in a variety of molecular profile that differ in solubility and mode of membrane attachment rather than in catalytic activity. For example in brain, AChE occurs mainly as tetrameric G_4 forms (membrane bound) together with the monomeric G_1 form (cytosolic) (DAS et al., 2001). Beside to this, it is well demonstrated from literature that many drugs, including those used for therapeutic, never affected all form of AChE equally well, most importantly, sometimes these drugs behaved differently with the same isoform from different brain areas (ZHAO & TANG, 2002). In fact, this phenomenon would be involved in the *in vivo* and *in vitro* different effects of CA and CAPE in brain structures. Based on this, we can suggest that CA and its derivative CAPE also may have the form-specific selectivity in relation to the AChE from brain regions.

In the present investigation, we also demonstrated that CA (*in vitro* and *in vivo*) and CAPE (*in vivo*) increased the AChE activity in lymphocytes from rats respectively demonstrating that these compounds also affect the cholinergic signaling in non neural cells. In blood cells AChE shows a similar structure to the enzyme that occurs in neurons and resembles the catalytic subunits which found in synaptic AChE (THIERMANN et al., 2005). AChE is expressed in several types of hematopoietic lineages and can contribute to cell regulation (WESSLER & KIRKPATRICK, 2001). Studies showed that their functions are related with progenitor's blood cells mainly for cell expansion of megakaryocytics and erythrocytes lineages (SOREQ et al., 1994; GRISARU et al., 1999; SOREQ & SEIDMAN, 2001). Besides, the activity of this enzyme has been used as an indicator of human cells aging

with lowered levels commonly associated with older human red blood cells (PRALL, et al., 1998).

Our results suggest that the increased in the AChE activity in whole blood and lymphocytes by these two compounds will decrease the acetylcholine levels that could contribute to the onset low-grade inflammation. These findings suggest that CA and CAPE might exert negative effects that need further investigations. In this line, others studies described that this compound has pro-oxidant activity in the presence of transition metal ions such as iron and copper (Ahmad et al., 1992). further causing oxidative DNA damage in lymphocytes (GALATI & O´BRIEN, 2004). In addition, WU et al. (2010) also observed that CA exhibits proglicative effects causing elevation of oxidative stress and inflammation in monocytes, macrophages and vascular endothelial cells. Further, CA has been reported to be able to induce lipid peroxidation and/or DNA damage in the presence of cupric ions (YAMANAKA, 1997). In addition, with proposed mechanism that CA phenyl ester (Wang et al., 2008) in the presence of Cu (II) ions: induce DNA damage and hence pro-oxidant and with the similar proposed mechanism and affects CA has been also reported.

AChE also is of one the key functional protein in neuromuscular transmission by rapidly hydrolysis acetylcholine molecules after its binding to receptors (GASPERSIC, et al., 1999). Recently reported that the absence of AChE leads to marked alterations in muscle function including the contractile properties and the lack of resistance to fatigue (MOUISEL, et al., 2006; VIGNAUD, et al., 2008). In addition, many diseases, as for example myasthenia gravis, have been associated with disturbances in the acetylcholine homeostasis in the neuromuscular junction and in some cases inhibitors of AChE enzyme are used for treating of the symptoms (KAKONTIS AND GUTMANN, 2001; SHELTON, 2002).

Next in the present investigation we evaluated the effects of CA and CAPE on AChE activity in muscles. In this set of experiment the *in vitro* effect of CA was definitely different than *in vivo* effects of CA and CAPE on the AChE activity in muscle from rats. The results demonstrated that CA was capable to inhibit *in vitro* the AChE activity from the muscle while *in vivo* CA and CAPE enhanced the AChE activity in muscles. *In vitro* the inhibition of AChE impedes the hydrolysis of acetylcholine at the neuromuscular junction, allowing more acetylcholine for interaction with receptors and thus restoration of the cholinergic balance in pathological conditions (KAKONTIS & GUTMANN, 2001). This finding provides evidence that CA might be an alternative therapeutic agent in cases of cholinergic deficits associated with neuromuscular junction. On the other hand, *in vivo* the enhanced AChE activity is an impairment of ACh in neuromuscular junctions. Since *in vivo*, CAPE activate the AChE in

neuromuscular junction which confirmed the pro-AChE property of CA in neuromuscular junctions. because mostly in the present study both these compounds showed the same pro-AChE pattern. Further studies are necessary to support this hypothesis and that will help in the discovery of mechanisms about most specific targets of the CA and CAPE in the CNS and neuromuscular junction.

Impairment in neuromuscular transmission has been associated with various diseases such as congenital myasthenias, myasthenia gravis, muscular dystrophy (ENGEL & SINE, 2005). In addition, myasthenia gravis have been associated with disturbances in the acetylcholine homeostasis in the neuromuscular junction and in some cases inhibitors of AChE enzyme are used for treating of the symptoms (KAKONTIS AND GUTMANN, 2001; SHELTON, 2002). Our present results in muscles suppose that the activation of AChE can modify ACh homeostasis at the neuromuscular junction that can trigger cholinergic deficits in neuromuscular junction.

Since anti-oxidant/pro-oxidant properties have been ascribed to these compounds. For example, LEE et al. (2008) showed that glutathione reductase activity decreased rapidly after a low-dose CAPE treatment in medulloblastoma (MB) cells and proposed that CAPE can enhance radiation induced apoptosis in Daoy cells. Moreover, LEE et al. (2008) proposed that CAPE possesses both apoptosis-inducing and radiosensitizing activities because CAPE depletes glutathione (GSH) in Daoy cells whose effect may be due to activation of reactive oxygen species (ROS) and inhibition of nuclear factor-kappa B (NF-κB) activity. In the present investigation mostly results demonstrated activated tendency. On the other hand CA reported as pro-oxidant (YAMANAKA, 1997). *in vitro*, the cell killing activity of plant polyphenols, have been related to their prooxidant activity (GALATI & O'BRIEN, 2004; YANG et al., 1998) which may be an important mechanism for their anticancer and apoptosis-inducing properties, as reactive oxygen species (ROS) can mediate apoptotic DNA fragmentation (HADI ET AL., 2000). In this line many anticancer drugs may trigger the generation of free radicals and hence act as pro-oxidants, such as ROS or reactive nitrogen species (SHEN & LIU, 2006), and promote apoptosis. Actually, ROS-induced oxidative stress and cell death play important roles in the efficacy of many antineoplastic agents (OZBEN, 2007). Probably, in our all experiment with both compounds AChE activities were enhanced and both compounds confirm and match the effects of each other in each structure/cell demonstrating cholinergic deficits. Since till to date, no study traced together the phytophysiological properties of these compounds. Therefore the reported pro-oxidant

properties of these compounds would be the possible and proposed mechanism which cause enhancement in AChE activity.

Since the present study demonstrated that both CA and CAPE potentially modulated the AChE activities in neuronal and nonneuronal tissue, apparently these compounds should modulate purinergic transmission. Considering the joint actions and co-release of neurotransmitors (ATP/ACh), we aimed to evoluate the efects of CA and CAPE PURINERGIC system. In the present study the effect of CA (Chronic) and CAPE (Acute) evaluated on the activities of ectonucleotidases and in platelets and synaptosome from rats. In addition, in the present study the effects of CAPE evaluated on XO activities in serum and whole brain. Both compound modulated the NTPDase, E-NPP, 5'-nucleotidase in platelets homogeneusly, except the effect of CA on NTPDase, 5'-nucleotidase where ATP and AMP hydrolysis in platelets was decreased.

Platelets are the important blood components in vascular circulation being loaded with extracellular nucleotides. It has been established that ATP, ADP and adenosine influence vascular tone, cardiac function and platelet aggregation (PILLA et al., 1996; BURNSTOCK, 2004). Beside other huge functions, platelets have important role in blood flow, since this component of blood contribute to thrombus formation by releasing active substances such as ADP (MARCUSE et al., 2003; SOSLAU & YOUNGPRAPAKORN, 1997). 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).

In this study the treatment for CA (30 days treatment) decreased the 5'-nucleotidase and increased the ADA activity in platelets possibly create the deficiency in the extracellular environment and consequently can triggers the problems in vascular circulation. On the other hand, the inhibition of the ATP hydrolysis could be important to decrease the ADP extracellular levels. Moreover, the enhancement in NTPDase activity on ADP hydrolysis and E-NPP in platelets due to CA can play a crucial role in controlling the platelet coagulant status by removing the extracellular ADP, which is the main agonist of platelet aggregation.

In this study CPAE also increased NTPDase, 5'-nucleotidase and E-NPP and decrease the ADA in platelets and XO activities in plasma (date not shown) which further demonstate the physiological effects of CAPE in vascular circulation.

NTPDase have a crucial role in thrombus formation platelets aggregation. In this line next in this study, CA induced significant reduction in platelet aggregation in rats. After this we further elucidated the effects of CA on platelet aggregation in human sample using ADP as agonist. We observed the effect similar to *in vivo.* The effect of CA on platelet aggregation profile is agree with existing study that phenolic comopoound including CA exhibited antipalte effects and which could help in reduce the incidence of chronic diseases including especially cardiovascular disease and stroke

Probably the proplatelets activities in vascular circulation are referred to the family of P2 receptors on platelet and its agonists ADP and ATP. Beside the particular importance of ADP in vascular circulations as a natural agonist of $P2Y_1$ receptor, another agonist is collagen which has been reported well to cause platelets activation via this receptor. GACHET (2005) confirms the role $P2Y_1$ receptor in aggregation induced by collagen. In addition, the coactivation of both $P2Y_1$ and $P2Y_{12}$ receptors is necessary for normal ADP-induced platelet aggregation and also play role in the procoagulant activity of platelets (GACHET, 2005).

The $P2Y_{12}$ receptor has also been shown to contribute to the procoagulant activity of platelets induced by collagen or thrombin (STOREY et al., 2000; DORSAM et al., 2004). Beside to ADP-induced platelet aggregation, the $P2Y_1$ and $P2Y_{12}$ receptors are differentially involved in the procoagulant activity of platelets (GACHET, 2005). In addition, collagen or thrombin can activate the platelets and induce procoagulant activity via $P2Y_{12}$ receptors (STOREY et al., 2000; DORSAM et al., 2004). The clopedogril has elucidated well as antithrombotic drug and cardioprotective compound via targeting the $P2Y_{12}$ receptor on platelets (PEREILO et al., 2002; SAVI et al., 2006).

In this context, to understand further the antiplatelets effects of CA, we treated the rats with clopidogrel, CA and clopidogrel plus CA for 13 days consecutively and compared the effects of CA and clopidogrel in platelet aggregation induced by collagen and also the time of blood coagulation in rats. The results demonstrated that both CA and clopidogrel inhibit the platelets aggregation and increased the time of blood coagulation. These results are further agreed with the rapid hydrolysis of ADP by CA and perhaps that CA also interfere with the purinoreceptores $P2Y_1$ and $P2Y_{12}$.

Extracellular ATP is involved in proinflammatory functions such as stimulation and proliferation of lymphocytes and cytokine release. In this regards the NTPDase activity have a

protective role controlling the level of extracellular ATP in cytolytic T lymphocytes. The NTPDase 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, 1998; PULTE et al., 2007). The increase in NTPDase activity by CA would result in the reduction of extracellular ATP, which can trigger anti-inflammatory responses.

On the other hand, CA 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 deamination 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 the A_{2A} receptors (DOMBROWSKI et al., 1998; HERSHFIELD, 2005). In this context, the increase in the ADA activity by CA can 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 CA on the nucleotide hydrolysis may be possible due to distinct mechanisms. CA is effective as a free radical scavenging (GULCIN, 2006) and chelation of metal ions (PSOTOVA et al., 2003). It has been supposed that an antioxidant may become a pro-oxidant to accelerate lipid peroxidation and/or induce DNA damage under special conditions (YAMANAKA, 1997). In this line, CA has been reported to induce lipid peroxidation and DNA damage either alone or in the presence of copper ions (YAMANAKA, 1997). BHAT et al. (2007) showed that concentrations of 200 and 400 μM of CA are capable of promoting DNA breakage in lymphocytes.

Next in this study, CAPE reduced the NTPDase, 5'-nucleotidase activity in synaptosomes. CAPE showed no significant alterations in ADA synaptosomes while inhibited XO in whole brain. These investigations demonstrating the expected neurochemical effect of CAPE on CNS. In the nervous system, purines are important neuromodulators, acting at pre- and postsynaptic sites. The decreased activity of NTPDase on ATP hydrolysis can elevate the level of these neurotransmitters in the extracellular medium and perhaps could promote the sensitivity of P2 receptors.

The physiological importance of extracellular ATP in the CNS has been well established. Alterations in NTPDase and 5′-nucleotidase activities appear to be related to neurological disorders, such as experimental model of epilepsy and cerebral ischemia

(Schetinger et al., 1998). We focused our present investigation on normal rats and perhaps the inhibition of ATP hydrolysis can elevate the level of this neurotransmitter in the CNS could up regulate the sensitivity of its respective receptors. In this line our present investigation reveals an improvement/benefits in excitatory neurotransmissions and hence purinergic signaling.

CAPE inhibited the 5'-nucleotidase activity in synaptosomes reflecting a deficiency in adenosine (a cytoprotective agent) level which could be impairment in the purine hydrolyzing pathway and hence affect purine salvage ability. Therefore, the organism may predispose to brain damage because the impairment in purine salvages ability and thus can desensitize P1 purinoreceptors. CAPE induced no significant effect on the activity of ADA while inhibited the XO in whole brain. The rapid hydrolysis of adenosine by ADA to inosine and subsequently the increased XO can enhance the production of free radical and reactive oxygen species which is a soul of inflammation and oxidative damage. The present effects of CAPE should be associated with its known protective effects against ischemia/reperfusion (I/R) injury, anti-oxidant, anti-inflammatory, free radical scavenging and perhaps their binding activity with specific receptors.

5 CONCLUSION

Caffeic acid (*in vitro* and *in vivo*) altered the AChE activity in different structures and cells. So facts of the matter, caffeic acid seemes to trigger cholinergic deficit. The increase in AChE activity may the consensus of the prooxidat activty of CA. and CAPE. While the decrease in AChE activity and the improvement in memory is its beneficial perspective. Further studies and debates are necessary to know the effect either neuroprotective/neurodegenartive and the precise mechanism underlying these effects.
The responses of ecto-nucleotidases to CA, demostrate a cardioproctive activity, since ATP, hydrlolysis decreased and ADP hydrolysis increased followed by the enhancemnt in E-NPP activity. The increase in NTPDase E-NPP, possiblely help to reduced the level of vasocontractive ADP.

Depend on the functions of 5'-nucleotidase and ADA in vascular circulation; the decrease of 5'-nucleotidase and increase of ADA demonstrates vascular complication.

The enhancement in NTPDase and ADA activtities in lymphocytes, demostartes that CA can alters the immune system too.

The inhibition of AChE in cortex, cerebellum and stritum, reflecting the neuroprotective effects of CAPE, while the enhancement of AChE in hippocampus, hypothalamus, pon, lymphocyte and muscles demonstrat the deficeint in ACh level, which clearly induce the cholinergic related problms

The increase in NTPDase E-NPP, demonstratesthates that CAPE helps to reduced the level of vasocontractive ADP.

The enhancement in 5'-nucleotidase, possibly increases the level of vasodialative agent; the adenosine and thus potently demostrate that CAPE can also play role in cardioprotection and facilitate the function of heart which is the peripheral excitatory organ.
The inhibition of ADA in platelets and XO in serum can protect the vascular system against oxidative damage.

In synaptosomes CAPE inhibits ATP hydrolysis possibly, could elevate its level and the inhibition of APM hydrolysis could reduce the level of adenosine which is the important neurolator. Since the present study focused on healthy rats, in this line the elevated level of ATP can improve the neurons while the reduction in adenosine can create related neurodegerative condition.

In turned the inhibition of XO can protect the brain from the oxidative related damage in purine hydrolysing pathway.

6 PERSPECTIVE

Nevertheless, the present study demonstrated that CA and CAPE can not be beneficial in every aspects, this work opened the gate and now need a more precise work to explore more either advantages related health via cholinegic and purinegic system.

Beside many aspects, in future the present study will be supported by

- To apply these compounds on experimental animals disease model like Alzheimer's disease.
- To measure the level of ATP, ADP, AMP and adenosine in PNS and CNS.
- Evaluating the effects CA and CAPE on the interaction of agonist and antagonist with relative receptors, both in Cholinergic and Purinergic system.
- To determine the absoption criteria of these two compound.
- To determine either these two compounds or its any active metabolite have effectively modulate the evaluated enzymes.

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