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Chronic intake of caffeine during gestation down regulates metabotropic glutamate receptors in maternal and fetal rat heart

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Summary. Caffeine is the most widely consumed substance in the world which antagonizes adenosine effects. Adenosine acting through A_1 receptors inhibits glutamate release which binds to metabotropic glutamate receptors (mGluRs). Recently, we have shown that maternal caffeine intake during gestation causes down-regulation of A_1 and metabotropic glutamate receptors in the brain of both rat mothers and fetuses. In the present work we provide evidence that caffeine also affects receptors in hearts, causing a decrease in mGluRs from both maternal and fetal hearts. A decrease in $G_{q/11}$ and PLC β_1 proteins level was also observed in both tissues. However, phospholipase C activity was only affected in fetal heart, being significantly decreased. These results suggest an *in vivo* cross-talk mechanism between adenosine and glutamate receptors in peripheral tissues. Therefore, special attention should be paid to caffeine ingestion during gestation.

Keywords: Heart – Metabotropic glutamate receptor – Caffeine – Pregnancy

Introduction

Glutamate is the main excitatory neurotransmitter in the CNS that at physiological concentrations mediates learning and memory processes. However, at higher concentrations it causes degeneration and neuronal death. Different actions of glutamate are mediated by specific receptors classified into ionotropic and metabotropic receptors. Ionotropic glutamate receptors are ion channel classified on the basis of the potency order of specific agonists into NMDA, kainic and AMPA receptors. Metabotropic glutamate receptors are G-protein coupled receptors classified into mGluR I, II and III groups. mGlu I receptors are coupled to phospholipase C stimulation through $G_{q/11}$ protein whereas mGlu II and III receptors are coupled to adenylyl cyclase activity through $G_{i/0}$ protein (Pin and Acher, 2002; Kew and Kemp, 2005).

The presence and localization of metabotropic glutamate receptors in the rat heart have been described by Gill and coworkers using immunohistochemistry (Gill et al., 1999). Experimental data show that $mGluR_{1\alpha}$, $mGluR_{2/3}$ and $mGluR_5$ are present in the rat heart and their preferential localization includes nerve terminals, ganglion cells, and elements of the conduction system. This suggests that they play a role in the physiology of the heart (Gill et al., 1999), although it is not clear what the role is. However, glutamate has an important role in cellular physiology, regulating energy metabolism, and its uptake increases during hypoxic or ischemic conditions. Moreover, it seems to be beneficial to this tissue, because glutamate supplementation provides cardioprotection during hypoxic or ischemic conditions (Pisarenko, 1996; Arsenian, 1998; Us et al., 2001).

Caffeine is the stimulatory substance most widely consumed in the world. It is present in coffee, tea and cola beverages. At doses usually consumed by humans, the caffeine effect is produced by antagonizing the adenosine effect. Caffeine is metabolized by the liver in theophylline and theobromine which also activate adenosine receptors (Fredholm et al., 1999).

Adenosine is a nucleoside widely distributed in both the central and peripheral nervous systems that acts through G-protein coupled receptors which have been classified into four types: A_1, A_{2A}, A_{2B} and A_3 receptors. A_1 and A_3 receptors mediate inhibition of adenylyl cyclase activity through Gi protein, whereas A_{2A} and A_{2B} receptors stimulate adenylyl cyclase activity through Gs protein. As A_1 and A_{2A}

receptors have higher affinity with adenosine, these receptors are blockaded by caffeine (Linden et al., 2001; Ralevic and Burnstock, 2003). One of the effects of adenosine acting through A_1 receptors is the inhibition of glutamate release, thereby playing a role as a neuromodulator and neuroprotector (Dunwiddie and Masino, 2001).

We have previously described how caffeine or theophylline chronically administered to pregnant rats during gestation causes a down-regulation of adenosine A_1 receptors in both maternal and fetal rat brain, related to the observed increase in endogenous adenosine levels (León et al., 2002). The caffeine or theophylline effect is not only at the receptor level but at the post-receptor level as well, affecting coupled Gi-protein and inhibiting the functionality of adenosine A_1 receptors through adenylyl cyclase inhibition in mothers (León et al., 2005a). Moreover, caffeine also causes a down-regulation of mGluRs in maternal and fetal rat brain (León et al., 2005b). Therefore, the aim of this work was to study the effect of caffeine intake during pregnancy in mGluRs present in both maternal and fetal rat heart. The results show that caffeine causes the down-regulation of these receptors in maternal and fetal rat heart, suggesting *in vivo* cross-talk between adenosine and mGlu receptors.

Materials and methods

Materials

L-[^{3}H]Glutamic acid (48.1 Ci/mmol) and phosphatidylinositol 4,5-bisphosphate (myo-inositol-2-^{3}H(N)) ([^{3}H]PIP_2) (8 Ci/mmol), were obtained from PerkinElmer (Madrid, Spain). L-Glutamate, N-methyl-D aspartic acid (NMDA), (RS)-α-Amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA), kainate and DL-threo-β-hydroxyaspartic acid (TBHA) came from Tocris Cookson (London, UK), and phosphatidylinositol 4,5-bisphosphate (tri-ammonium salt) was purchased from Avanti Polar Lipids (Alabaster, AL). Anti-PLC β_1 monoclonal antibody and anti-rat polyclonal antibodies against $mGlu_1$ and Anti $\alpha G_{q/11}$ protein were from Upstate (Reactiva, Madrid, Spain). All other reagents were of analytical grade.

Animals

Pregnant Wistar rats, kept on a 12 h light/12 h dark cycle (lights on at 7:00 am) and with free access to food and drinking water, were treated with caffeine (1 g/l) in the drinking water from gestational day 2 (GD 2) onwards during the entire gestational period. The day when sperm was observed in the vaginal smear was designated day 1 of pregnancy. Control pregnant rats received drug-free tap water. At the end of this period, rats were killed and fetuses were delivered surgically. Maternal and fetal hearts were then removed, frozen in liquid N_2 and stored at −70 °C until experiments were performed. Experiments were performed in groups of four pregnant animals (two controls and two caffeine-treated). A total of twelve animals were used. All experiments followed the European Community regulations for the care and use of laboratory animals.

Rat heart membrane isolation

Membranes were isolated using the method described by Matherne et al. (1997). Hearts from pregnant rats were homogenized in 10 vol of ice–cold buffer (10 mM EDTA/10 mM HEPES/0.1 mM benzamidine, pH 7.4). Homogenate was centrifuged at 48,000 × g for 10 min. The pellet was resuspended in 30 ml of buffer with 1 mM EDTA, centrifuged again and washed twice more by resuspension/centrifugation. The final pellet was resuspended in 1 vol of the appropriate buffer (50 mM Tris-HCl plus 10 mM $MgCl_2$) for assays. Membrane suspensions were stored at −70 °C until use. Protein was determined by the Lowry method using BSA for standards.

L-[^{3}H]Glutamate binding assays to plasma membranes

Metabotropic glutamate receptors in rat hearts were determined by binding assays using L-[^{3}H]Glutamate as radioligand as described previously (Albasanz et al., 2002; León et al., 2005b). Briefly, 60 to 100 μg of protein was incubated for 60 min at 25 °C in the presence of 100 μM α-amino-3-hydroxy-5-methyl-isoxazole-4 propionic acid (AMPA), 100 μM kainate, and 100 μM N-methyl-D-aspartic acid (NMDA), in order to block ionotropic glutamate binding, and different L-[^{3}H]Glutamate concentrations (40 nM–1500 nM) with 10 mM potassium phosphate buffer pH 7.4, in the presence or absence of unlabeled L-glutamate, to obtain non-specific binding. All assays were performed in the presence of 1 mM DL-threo-β-hydroxyaspartic acid (THBA), an L-glutamate uptake inhibitor. After incubation at 25 °C, free and bound ligands were separated by centrifugation for 10 min at 12,000 g in a Hettich Mikrolitter microfuge. Supernatant was discharged and the pellet was washed with 1 ml of ice cold buffer. After washing, 100 μl of 0.01% SDS was added and radioactivity was measured with 3 ml scintillation liquid in a LS1701 Beckman counter.

Immunodetection of $mGluR_1$, $\alpha G_{q/11}$ and the phospholipase C β_1 isoform

One hundred micrograms of protein was subjected to 7.5% polyacrilamide gel electrophoresis in the presence of SDS. Western blotting was performed as described earlier (León et al., 2005b). Immunodetection was carried out by incubating the nitrocellulose membranes with specific polyclonal antibody (anti-mGlu1 and anti-$\alpha G_{q/11}$) diluted 1:1000 and isoenzyme-specific monoclonal antibody (anti-PLCβ_1) diluted 1:400. After washing, blots were incubated with horseradish peroxidase-coupled goat anti-rabbit or -mouse IgG diluted 1:3000. Antigen was visualized using the ECL chemiluminescent detection kit from Amersham and specific bands were quantified by scanning densitometry in a GS-690 imaging densitometer from Bio-Rad (Madrid, Spain).

RT-PCR analysis

Total RNA was isolated by guanidium thiocyanate/phenol/chloroform extraction following the method of Chomczynski and Sacchi (1987). RT-PCR assays were performed as described previously (León et al., 2002, 2005b) using the primers 5′-AAA TCT ACA GCA ATG CTG GCG A-3′ and 5′-CTT CGA TGA CTT CAT CTC TGT-3′ for $mGluR_1$, 5′-CGG CAA GTC TGT GTC ATG GT-3′ and 5′-CAG GGT GGA AGA GCT TTG TC-3′ for $mGluR_{1a}$, 5′-TGA AAG AAG ATG TAC GAG GGA GTG-3′ and 5′-TTT CTT GGA GCG GAA GGA AGA AG-3′ for $mGluR_5$, 5′-GAG AAC CGA ATG GAG GAG AGC AA-3′ and 5′-GTC CAC GAA CAT CTT CAG GAT GAA-3′ for $\alpha G_{q/11}$ and 5′-TTT TCG GCA GAC CGG AAG CGA-3′ and 5′-TGC TGT TGG GCT CGT ACT TCT-3′ for PLCβ_1. PCR products were analyzed by electrophoresis in 2% agarose gels and stained with ethidium bromide. The PCR product sizes expected for $mGluR_1$, $mGluR_{1a}$, $mGluR_5$ $\alpha G_{q/11}$ and PLCβ_1 were 206, 930, 385, 212 and 315 bp, respectively. In all cases, amplification of a fragment corresponding to the β-actin sequence was carried out in parallel

using the same cDNA samples in order to correct possible variations in the amount of cDNA used for the process. The primers used for β-actin were 5′-GGT ATG GAA TCC TGT CGC ATC CAT GAA A-3′ and 5′-GTG TAA AAC GCA GCT CAG TAA CAG TCC G-3′. The size of the PCR product for the β-actin was 320 bp. Bands corresponding to PCR products were quantified by densitometry in a BioRad GS-690 densitometer using MultiAnalyst 1.0 software from Bio-Rad (Madrid, Spain).

Phospholipase C assay

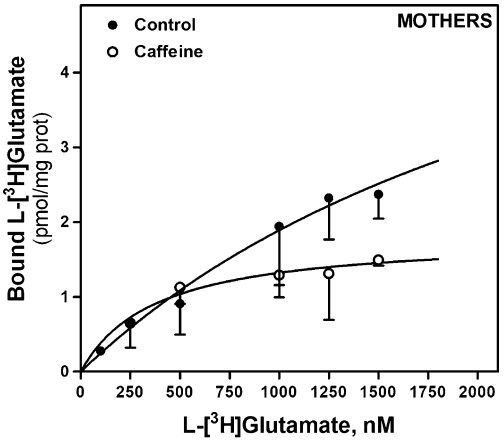
Phospholipase C activity in plasma membranes was assayed in the presence of exogenous [^{3}H]PIP_2 as described earlier (Albasanz et al., 2002; León et al., 2005b). [^{3}H]PIP_2 was dried under an N_2 stream, dissolved in 2 mM sodium deoxicholate, 50 mM Tris-HCl pH 6.5 and sonicated using an Ultrasonic Processor UP 200 S. Phospholipase C assay was carried out for 10 min at 37 °C, incubating [^{3}H]PIP_2 (17000 dpm) with or without 20 μg of plasma membrane protein in 100 μl of buffer (100 mM NaCl, 1 mM sodium deoxicholate, 1 mM EGTA, 25 μM Cl_2Ca, 40 mM ClLi and 50 mM Tris-HCl pH 6.8) The incubation was terminated by adding 360 μl of chloroform/methanol/HCl (1:2:0.2 v/v) and putting the tubes on ice. After the addition of 120 μl 2 M KCl and 160 μl of chloroform, the tubes were centrifuged for 5 min at 3,500 g. Upper aqueous phase (250 μl) containing [^{3}H]inositol phosphates was mixed with 3.5 ml of scintillation liquid.

Results

Pregnant rats were treated daily with caffeine (1 g/l) in the drinking water. The water intake was measured daily in the control- and caffeine-treated groups. Caffeine intake was 83.2 ± 5.3 mg/kg day whereas the tap water intake of the control group was 84.41 ± 5.5 ml/kg day. Caffeine did not significantly alter the weight of mothers at the end of the gestational period (data not shown). The daily caffeine consumption average was in a dose range previously reported (Johansson et al., 1993, 1997; Svenningsson et al., 1999; León et al., 2002, 2004). If we assume that there is approximately 80–180 mg caffeine in a cup of coffee, caffeine intake in our study could correspond to one cup of coffee.

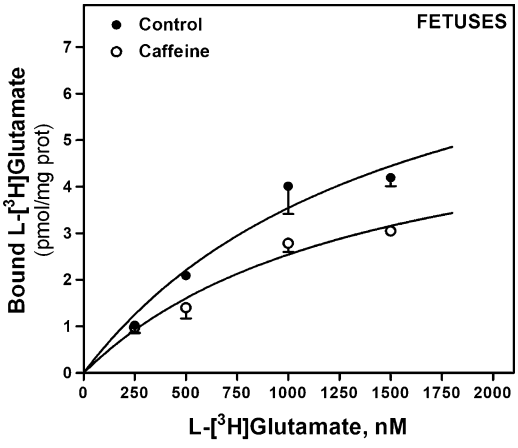
Effect of caffeine on mGluRs from both maternal and fetal heart

Firstly, the effect of caffeine intake was analyzed in plasma membranes from mothers' heart by radioligand binding assays using L-[^{3}H]Glutamate as radioligand and under conditions in which binding to ionotropic glutamate receptors was blocked. As can be observed in Fig. 1, caffeine intake causes a significant decrease in total mGluRs numbers, without significant alteration of the receptor affinity. The same analysis showed a significant and similar decrease in total mGluRs in fetus heart from caffeine-treated mothers (Fig. 2). Again, these changes were not associated with variations in the receptor affinity.



	Bmax (pmol/mg prot)	K_D (nM)
CONTROL	4.2 ± 1.0 (5)	1062.8 ± 393.5 (5)
CAFFEINE	1.8 ± 0.4 (4) *	447.7 ± 167.5 (4)

Fig. 1. Saturation curves of L-[^{3}H]Glutamate binding to plasma membranes from maternal heart. 100 μg of plasma membranes from control and caffeine-treated pregnant rat heart were incubated with different concentrations of L-[^{3}H]Glutamate, as described in Materials and methods, after pretreatment with 0.04% Triton X-100, in order to remove endogenous glutamate. Total receptor numbers (Bmax) and receptor affinity (K_D) were determined by Scatchard analysis of saturation curves. Data are mean ± SEM of number of experiments in parentheses, each one performed using different membrane isolations. *$p < 0.05$ significantly different from control values



	Bmax (pmol/mg prot)	K_D (nM)
CONTROL	7.9 ± 1.4 (4)	1868.6 ± 793.8 (4)
CAFFEINE	5.6 ± 0.8 (4) *	1592.3 ± 430.3 (4)

Fig. 2. Saturation curves of L-[^{3}H]Glutamate binding to plasma membranes from fetal heart. Binding of L-[^{3}H]Glutamate to fetal heart plasma membranes from control and caffeine-treated rats was performed as described in Materials and methods and in the legend of Fig. 1. Kinetic parameters are shown in Table inset and were determined by Scatchard analysis. Data are mean ± SEM of number of experiments in parentheses, each one performed using different membrane isolations. *$p < 0.05$ significantly different from control values

$MGluR_1$ and $mGluR_5$ subtypes of group I mGluRs have been immunohistochemically detected in rat heart (Gill et al., 1999). In order to determine whether changes observed in mGluRs were due to mGluI group, we measured the steady-state level of $mGluR_1$ and $mGluR_5$ subtypes in both maternal and fetal heart by Western-blotting assays using specific anti-$mGluR_1$ and $mGluR_5$ antibodies. As can be seen in Fig. 3, the immunodetection of $mGluR_1$ and $mGluR_5$ subtypes did not show significant variations in heart plasma membranes from rats treated with caffeine. With respect to fetal heart, $mGluR_1$ immunodetection was lower in the caffeine-treated group (Fig. 4).

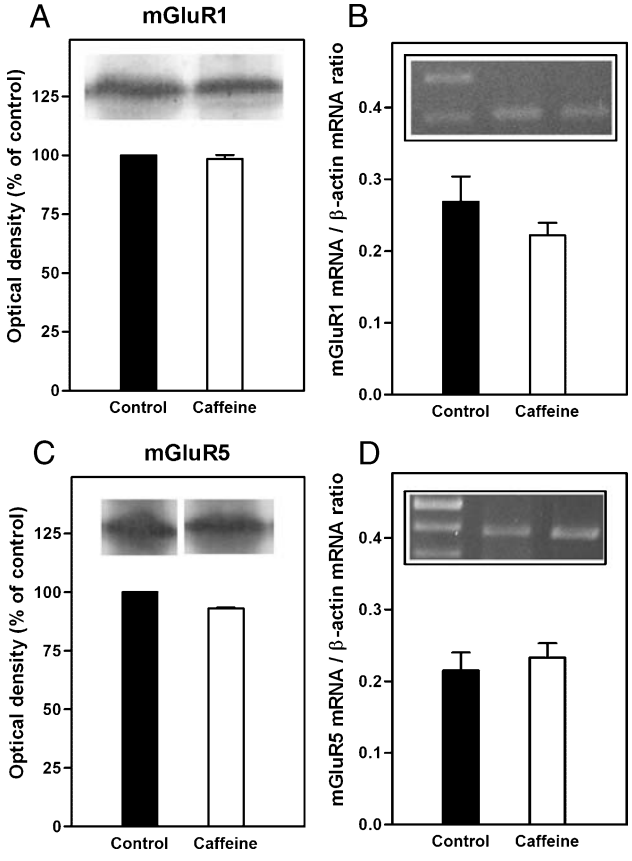


Fig. 3. A, **C** Immunodetection of $mGluR_1$ and $mGluR_5$ in plasma membranes from maternal heart. 100 µg of plasma membranes from control and caffeine-treated rat heart were subjected to SDS/PAGE, transferred electrophoretically to nitrocellulose membrane and incubated with specific anti-$mGluR_1$ and $mGluR_5$ polyclonal antibodies, as described in Materials and methods. Data, expressed as percentage of control values, are mean ± SEM of at least three experiments performed using different membranes isolations. **B**, **D** $mGluR_1$ and $mGluR_5$ expression detected by RT-PCR. RT-PCR assays were performed using specific oligonucleotides to $mGluR_1$ and $mGluR_5$ subtypes as described in Materials and methods. The histograms show the ratio between amplification of $mGluR_1$ or $mGluR_5$ and amplification of β-actin using the same RNA samples. Data are mean ± SEM of at least three experiments performed with different RNA isolations. Inset shows representative experiments

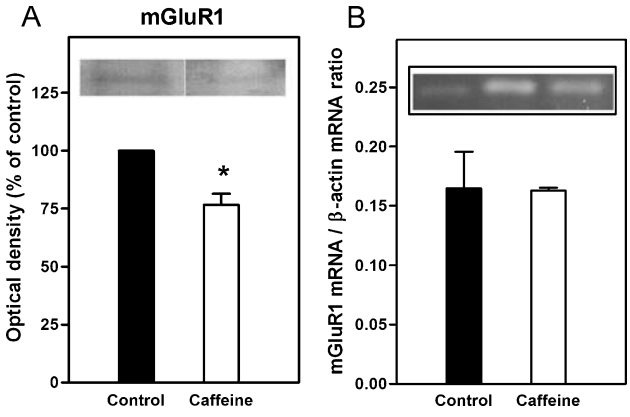


Fig. 4. A Immunodetection of $mGluR_1$ in plasma membranes from fetal heart. 100 µg of plasma membranes of fetus heart from control and caffeine-treated mothers was subjected to SDS/PAGE, transferred electrophoretically to nitrocellulose membrane and incubated with specific anti-$mGluR_1$ polyclonal antibody, as described in Materials and methods. Data, expressed as percentage of control values, are mean ± SEM of at least three experiments performed using different membrane isolations. **B** $mGluR_1$ expression detected by RT-PCR. RT-PCR assays were performed using specific oligonucleotides to $mGluR_1$ subtype as described in Materials and methods. The histograms show the ratio between amplification of $mGluR_1$ and amplification of β-actin using the same RNA samples. Data are mean ± SEM of at least three experiments performed with different RNA isolations. Inset shows representative experiments. $^*p < 0.05$ significantly different from control values

The possibility that chronic caffeine could down-regulate $mGluR_1$ by decreased $mGluR_1$ gene transcription was analyzed by reverse transcription polymerase chain reaction (RT-PCR) following total RNA isolation from control and treated heart, using specific primers corresponding to this receptor and β-actin, which was used as control. Caffeine intake did not produce significant changes in $mGluR_1$ nor in $mGluR_5$ mRNAs levels present in both maternal (Fig. 3) and fetal heart (Fig. 4). These results point to a post-transcriptional mechanism as being responsible for the down-regulation of $mGluR_1$ in fetal heart.

Effect of caffeine on $G_{q/11}$ protein from both maternal and fetal heart

To investigate whether chronic caffeine intake affected $G_{q/11}$ protein level, we performed Western-blotting assays using a specific antibody against α subunit of this protein. These experiments were carried out using membranes from both maternal and fetal heart. The densitometric analysis of the band specifically recognized by the antibody in maternal heart showed a significant decrease in membranes from caffeine-treated versus control mothers

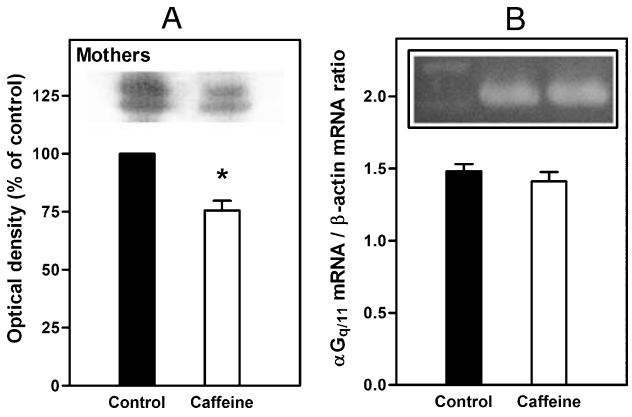


Fig. 5. **A** Immunodetection of $\alpha G_{q/11}$ in maternal heart by Western-blot. Nitrocellulose membranes with controls and caffeine-treated samples were tested with anti-$\alpha G_{q/11}$ antisera, as described in Materials and methods. Data, expressed as percentage of control values, are mean ± SEM of four experiments performed using different membrane isolations. Inset shows $\alpha G_{q/11}$ bands corresponding to a representative experiment. $^*p < 0.05$ significantly different from control. **B** Detection mRNA encoding alpha subunit of $G_{q/11}$ protein by RT-PCR. RT-PCR assays were performed using RNA isolated from control and caffeine-treated rat heart and oligonucleotides specific to $\alpha G_{q/11}$. Results are expressed as the ratio between amplification of $\alpha G_{q/11}$ and amplification of β-actin mRNAs. Data are mean ± SEM of three experiments performed with different RNA isolations. Inset shows the amplification of mRNA encoding $\alpha G_{q/11}$ protein of a representative experiment

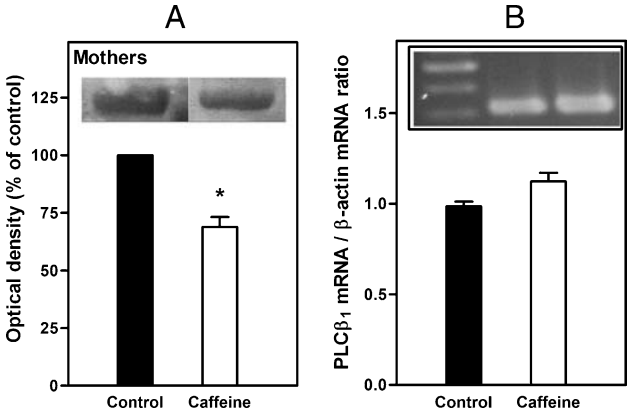


Fig. 7. **A** Immunoblotting analysis of $PLC\beta_1$ in membranes from maternal heart. One hundred micrograms of membranes from control and caffeine treated rat heart was tested with anti-$PLC\beta_1$ antisera. Data, expressed as percentage of control values, are mean ± SEM of four experiments performed using different membrane isolations. Inset shows $PLC\beta_1$ bands corresponding to a representative experiment. $^*p < 0.05$ significantly different from control value. **B** Analysis of the expression of $PLC\beta_1$ in maternal heart by RT-PCR. RT-PCR assays were performed using 5 µg of total RNA isolated from control and caffeine-treated rat heart and oligonucleotides specific to $PLC\beta_1$. Data show the ratio between amplification of $PLC\beta_1$ and amplification of β-actin mRNAs using the same RNA samples. Data are mean ± SEM of three experiments performed with different RNA isolations. Inset shows the amplification of mRNA encoding $PLC\beta_1$ protein of a representative experiment

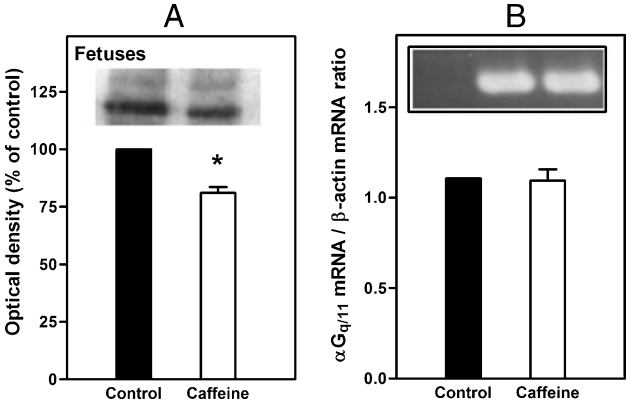


Fig. 6. **A** Immunodetection of $\alpha G_{q/11}$ in fetal heart by Western-blot. Nitrocellulose membranes with controls and caffeine-treated samples were incubated with anti-$\alpha G_{q/11}$ antisera. Data, expressed as percentage of control values, are mean ± SEM of four experiments performed using different membrane isolations. Inset shows $\alpha G_{q/11}$ bands corresponding to a representative experiment. $^*p < 0.05$ significantly different from control. **B** Detection mRNA encoding alpha subunit of $G_{q/11}$ protein by RT-PCR. RT-PCR assays were performed using RNA isolated from control and caffeine-treated rat heart and oligonucleotides specific to $\alpha G_{q/11}$. Results are expressed as the ratio between amplification of $\alpha G_{q/11}$ and amplification of β-actin mRNAs. Data are mean ± SEM of three experiments performed with different RNA isolations. Inset shows the amplification of mRNA encoding $\alpha G_{q/11}$ protein of a representative experiment

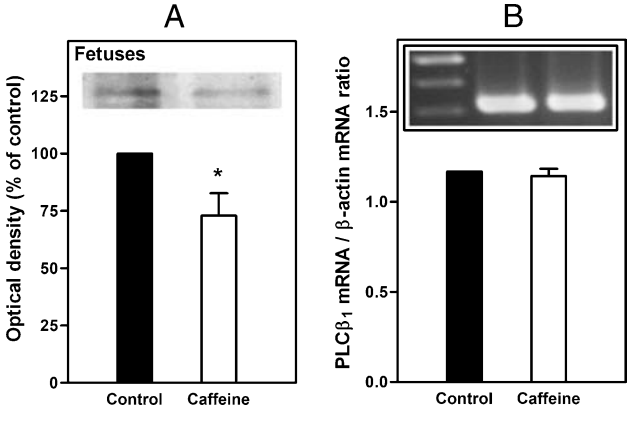


Fig. 8. **A** Immunoblotting analysis of $PLC\beta_1$ in membranes from fetal heart. One hundred micrograms of membranes from control and caffeine-treated rat heart was tested with anti-$PLC\beta_1$ antisera. Data, expressed as percentage of control values, are mean ± SEM of four experiments performed using different membrane isolations. Inset shows $PLC\beta_1$ bands corresponding to a representative experiment. $^*p < 0.05$ significantly different from control value. **B** Analysis of the expression of $PLC\beta_1$ in maternal heart by RT-PCR. RT-PCR assays were performed using 5 µg of total RNA isolated from control and caffeine-treated rat heart and oligonucleotides specific to $PLC\beta_1$. Data show the ratio between amplification of $PLC\beta_1$ and amplification of β-actin mRNAs using the same RNA samples. Data are mean ± SEM of three experiments performed with different RNA isolations. Inset shows the amplification of mRNA encoding $PLC\beta_1$ protein of a representative experiment

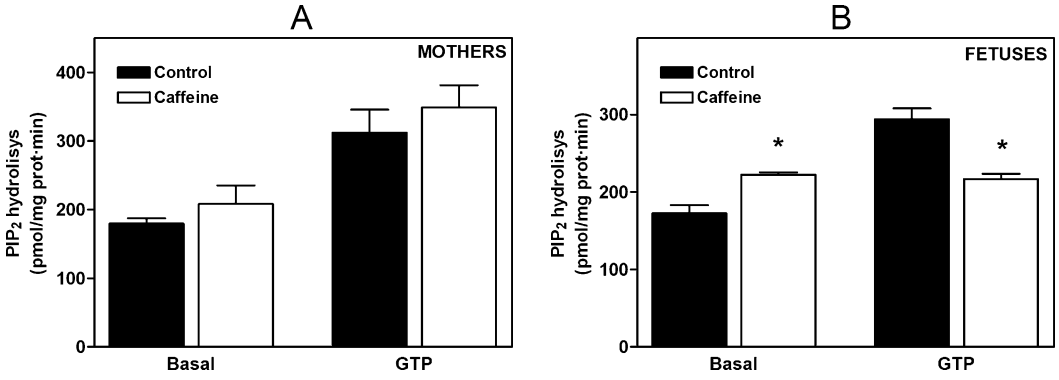


Fig. 9. Phospholipase C activity in plasma membranes from maternal and fetal heart. PLC activity was measured as described in Materials and methods using [^{3}H]PIP_2 as exogenous substrate. Sixty-eighty micrograms of membranes was incubated in basal conditions and in the presence of 100 μM GTPγS (GTP). Data are mean ± SEM of four experiments performed in duplicate using different plasma membrane isolations from mothers (**A**) or fetuses (**B**). $^*p < 0.05$ significantly different from respective control values

(Fig. 5A). Similar results were observed when assays were performed with membranes from fetus heart (Fig. 6A).

Level of mRNA coding $\alpha G_{q/11}$ was also studied by RT-PCR. Results obtained after analyzing the corresponding band showed that caffeine intake did not modify the mRNA level, either in mothers (Fig. 5B) or fetuses (Fig. 6B), again suggesting the involvement of post-transcriptional modifications in the down-regulation of $\alpha G_{q/11}$ protein detected after caffeine treatment.

Effect of caffeine on phospholipase C from both maternal and fetal heart

After investigating the components $mGluR_1$ and $\alpha G_{q/11}$ of Group I-mGluR transduction pathway, we decided to complete the study analyzing the effect of caffeine intake on phosphoinositide-specific PLC, the main effector system coupled to Group I mGluRs. Firstly, we analyzed the $PLC\beta_1$ isoform by Western-blotting using a specific antibody that recognizes this protein, which migrates as 150 kDa peptides on SDS polyacrilamide gels. Densitometric analysis of the immunoblots showed that chronic treatment of pregnant rats with caffeine significantly reduced the intensity of $PLC\beta_1$ immunoreactivity in maternal (Fig. 7A) and fetal heart membranes (Fig. 8A). The reduction in $PLC\beta_1$ protein content clearly contrasted with the absence of variations in the steady-state level of $PLC\beta_1$ mRNA transcript in both analyzed tissues (Figs. 7B and 8B).

To determine whether variations observed in $PLC\beta_1$ and $G_{q/11}$ protein levels were associated with alteration of the enzyme activity, we evaluated the effect of chronic caffeine treatment on basal PLC activity in membranes from mothers' heart, using [^{3}H]PIP_2 as an exogenous substrate. No significant difference was detected (Fig. 9A), although a significant variation in basal and GTP-stimulated PLC activity was observed in fetuses from caffeine-treated mothers (Fig. 9B). Finally, we assayed the functional consequences of variation observed in different components of mGluR/PLC pathway by determination of phospholipase C activity stimulated with L-Glutamate and Quisqualate. When we performed these assays we did not detect any significant differences in mothers' heart (Fig. 10). However, an important and significant decrease in the stimulatory effect exhibited by Glutamate and Quisqualate in the presence and absence of GTP

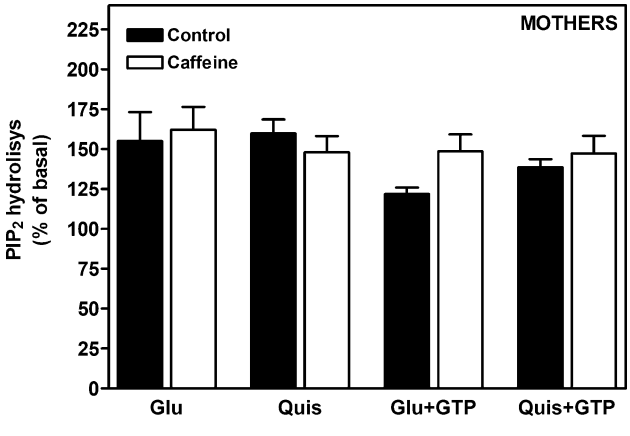


Fig. 10. Metabotropic glutamate receptor/phospholipase C activity coupling in plasma membranes from maternal heart. PLC activity was measured in the presence of 100 μM Glutamate (Glu) or 100 μM Quisqualate (Quis) with or without 100 μM GTPγS (GTP) using [^{3}H]PIP_2 as exogenous substrate as described in Materials and methods. Data are mean ± SEM of four experiments performed in duplicate using different plasma membrane isolations from maternal heart

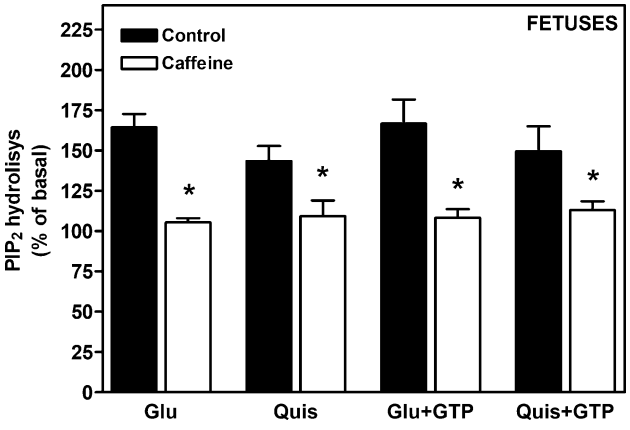


Fig. 11. Metabotropic glutamate receptor/phospholipase C activity coupling in plasma membranes from fetal heart. PLC activity was measured in the presence of 100 μM Glutamate (Glu) or 100 μM Quisqualate (Quis) with or without 100 μM GTPγS (GTP) using [^{3}H]PIP_2 as exogenous substrate as described in Materials and methods. Data are mean ± SEM of four experiments performed in duplicate using different plasma membrane isolations from fetal heart. $^*p < 0.05$ significantly different from control value

was observed in fetal heart from caffeine-treated mothers (Fig. 11).

Discussion

We have previously described how caffeine consumption during gestation causes the down-regulation of adenosine A_1 receptors in both maternal and fetal brain (León et al., 2002) and inhibits A_1 receptor function in the maternal rat brain (León et al., 2005a). Moreover, caffeine also affects metabotropic glutamate receptors, causing a down-regulation of these receptors in brain from both mothers and fetuses (León et al., 2005b). In the present paper we have provided evidence that maternal caffeine intake during pregnancy also causes a significant decrease in mGluRs present in hearts from mothers and their pups, associated with the loss of receptor functionality only in the immature heart.

Caffeine is the most widely-consumed psychoactive substance in the world (more than 80% of the population) due to its presence in coffee, tea and caffeinated soft drinks (including "energy" drinks). For most people, the first exposure to caffeine is *in utero*, because most women consume caffeine while pregnant and caffeine readily crosses the placenta (Dumas et al., 1982). There is considerable evidence concerning the widespread use of caffeine during pregnancy (Nawrot et al., 2003). Following oral ingestion, caffeine is rapidly absorbed from the gastrointestinal tract into the bloodstream and exerts central and peripheral actions. In this sense, chronic coffee consumption exerts a detrimental effect on aortic stiffness and wave reflections, having been reported as a cardiovascular risk factor in women and men (Hartley et al., 2004; James, 2004; James and Gregg, 2004; Vlachopoulos et al., 2005). Furthermore, caffeine has been described as inducing arrhythmias related to changes in cellular calcium homeostasis (Balasubramaniam et al., 2005). Moreover, caffeine brings about an increase in blood pressure (Dunwiddie and Masino, 2001); an extensive study has shown that each increase of 1 mmHg in population blood pressure is associated with 3.5% increased mortality from coronary heart disease and 5% from stroke (Prospective Studies Collaboration, 2002). However, as several authors have suggested, it is not clear that this risk is from caffeine consumption, especially as habitual moderate coffee intake has been associated with beneficial effects on cardiovascular health (Sudano et al., 2005).

The main mechanism of caffeine's action is antagonism of the potent endogenous neuromodulator adenosine (Smits et al., 1987; Dunwiddie and Masino, 2001). The antagonism is mediated by blocking of its regulatory actions mainly through adenosine A_1 and A_{2A} receptors, which modulate blood pressure and heart rate (Schindler et al., 2005). Adenosine has been shown to be a cardioprotective substance in both *in vitro* and *in vivo* systems, and it prevents mitochondrial oxidant damage in rat cardiomyocytes (Xu et al., 2005). Adenosine is mainly released from the heart when the oxygen supply is insufficient for the oxygen requirement such as during ischemia, hypoxia and other times of enhanced oxygen consumption. Conversely, adenosine release is decreased when excess oxygen is supplied by overperfusion. Endogenous adenosine protects against vascular dysfunction via A_1 receptors while intrinsic A_{2A} activation apparently worsens contractile dysfunction (Flood et al., 2002).

Adenosine concentrations are low during resting conditions but may be raised substantially in hypoxia, ischemia and increased mechanical or biochemical work and after administration of drugs that inhibit its uptake, such as dipyridamole (Fredholm and Sollevi, 1986). It has been noted that plasma adenosine concentrations increase in chronic heart failure, and the extent of increases in adenosine levels correlated well with the severity of heart failure (Funaya et al., 1997). Moreover, the increase in plasma adenosine levels may attenuate the severity of chronic heart failure (Kitakaze et al., 1998), suggesting that this increase is an endogenous compensatory mechanism for heart failure. The mechanism underlying this increase could be related to activation of ecto-5′-nucleotidase, an enzyme responsible for production of

adenosine which is activated by endogenous norepinephrine increases due to the progressive severity of chronic heart failure (Kitakaze et al., 1994, 1995).

We have previously shown that caffeine significantly increased plasma adenosine levels in mothers (León et al., 2005), in agreement with previous reports (Conlay et al., 1997; Andresen et al., 1999). This could suggest that increased adenosine levels after caffeine treatment may be beneficial to protect hearts from the harmful effects of caffeine. However, caffeine consumption by mothers during pregnancy causes a significant loss of adenosine A_1 receptors from mothers' brain (León et al., 2002) associated with an increased functionality of the receptor mediated by adenylyl cyclase activity inhibition (León et al., 2005) produced by overexposure to increased endogenous adenosine levels. In the same manner increased levels of adenosine could affect adenosine A_1 receptors in the heart. Along these lines, it has been suggested that the blockade of the cellular uptake of adenosine, or indirect enhancement of its release and subsequent activation of adenosine A_1 receptors, may be responsible for the inhibitory effect of antidepressants on glutamate and aspartate release (Golembiowska and Dziubina, 2001). In agreement with this, in a previous study rats were given two weeks of drinking water containing caffeine in doses similar to those in our study, and changes in resting heart rate and blood pressure were observed concurrent with changes in adenosine A_1 receptor function (While and Nguyen, 2002).

During development A_1 adenosine receptors are especially important; they are among the earliest receptors expressed in the embryonic brain and heart. Additionally, in the developing heart, the adenosinergic system is the dominant regulator of fetal cardiac function, and A_1R activation inhibits cardiac cell division, leading to cardiac hypoplasia. In the forming central nervous system, A_1R activation potently inhibits the development of axons and can lead to leukomalacia. These recent data suggest that adenosine is an important modulator of mammalian development (Rivkees et al., 2001).

In brain, adenosine acting through A_1 receptor activation inhibits glutamate release (Dunwiddie and Masino, 2001). Therefore, it is possible that loss of A_1R detected after chronic gestational treatment with caffeine or theophylline (León et al., 2002) in maternal and fetal brain and the decreased A_1R function in the maternal brain (León et al., 2005) may modify the A_1-mediated inhibition of glutamate release (Dunwiddie and Masino, 2001). At any rate, the antagonism of adenosine A_1R produced by short-term exposure to caffeine as well as the down-regulation of this receptor after chronic caffeine or theophylline treatment could result in a loss of A_1R responsiveness, which at the presynaptic level would produce an increase in glutamate release that would in turn bring about the down-regulation of mGluRs. In addition, it has been shown that caffeine induces glutamate release in the shell of the nucleus accumbens mediated by adenosine A_1 receptor blockade (Solinas et al., 2002). In this sense, the down-regulation of mGluR reported here could be a response to the receptor overstimulation produced by an excessive glutamate release. In agreement with our results, it has been shown that there is a down-regulation of $mGluR_1$ in C6 glioma cells (Albasanz et al., 2002a) and $mGluR_5$ in astrocytes (Balazs et al., 1997) after prolonged treatment with mGluR agonists. Moreover, rats treated with adenosine A_1 receptor agonists show an up-regulation of metabotropic glutamate receptors (Albasanz et al., 2002b). A similar mechanism could also mediate the effects of caffeine in heart. On the other hand, a molecular interaction between metabotropic glutamate receptor type 1a and the adenosine A_1 receptor has been reported, suggesting that both receptors may form part of a signaling complex *in vivo*. $MGluR_{1a}$ and adenosine A_1 receptors show a high degree of co-localization in neurons from rat cerebelum and from rat cortex, forming functional heteromers. Moreover, activation of $mGluR_1$ and A_1 in the same neuron results in synergism (Ciruela et al., 2001). Therefore, down-regulation of glutamate receptors after caffeine intake could also be related to the metabotropic glutamate and adenosine A_1 receptor–receptor interaction.

The immunodetection of $mGluR_1$ carried out in this study has shown that this receptor subtype contributes to the mGluR down-regulation detected by kinetic experiments in both maternal and fetal heart. However, after comparing the results obtained in radioligand binding and Western-blotting assays, we cannot rule out the regulation of other mGluR subtypes after treatment. Additionally, the lack of variation in $mGluR_1$ and the splicing variant $mGluR_{1a}$ mRNA level in both tissues points to a post-transcriptional mechanism as being responsible for the down-regulation. In support of this hypothesis, it has been demonstrated that there is endocytosis of mGluRs through clathrin-coated vesicles following agonist exposure (Mundell et al., 2001; Albasanz et al., 2002a).

The presence of glutamate receptors and their effector system in the heart has been demonstrated using immunochemical and molecular techniques (Gill et al., 1998, 2000; Hansen et al., 1995; Renard and Poggioli, 1990). Although their role is still unknown, the mGluRs may be

involved in the mediation of excitatory effects in the heart, as in the brain (Mueller et al., 2003). Furthermore, Glutamate appears to be cardioprotective (King et al., 2003; Danbolt, 2001). Moreover, in the myocardium, stimulation of the generation of the two second messengers, IP_3 and DG, by receptors such as α-adrenoceptors is an important mechanism in the mediation of positive inotropic effects (Terzic et al., 1993). Therefore, the down-regulation of different components of mGluR I/PLC pathway in maternal and fetal heart and the loss of receptor responsiveness in fetuses can alter the physiological function of heart, especially in this fetal tissue. Results presented herein suggest that caffeine consumption during gestation should be restricted.

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References

Albasanz JL, Fernandez M, Martin M (2002a) Internalization of metabotropic glutamate receptor in C6 cells through clathrin-coated vesicles. Brain Res Mol Brain Res 99: 54–66

Albasanz JL, Leon D, Ruiz MA, Fernandez M, Martin M (2002b) Adenosine A1 receptor agonist treatment up-regulates rat brain metabotropic glutamate receptors. Biochim Biophys Acta 1593: 69–75

Andresen BT, Gillespie DG, Mi Z, Dubey RK, Jackson EK (1999) Role of adenosine A(1) receptors in modulating extracellular adenosine levels. J Pharmacol Exp Ther 291: 76–80

Arsenian M (1998) Potential cardiovascular applications of glutamate, aspartate, and other amino acids. Clin Cardiol 21: 620–624

Balasubramaniam R, Chawla S, Grace AA, Huang CL (2005) Caffeine-induced arrhythmias in murine hearts parallel changes in cellular Ca(2+) homeostasis. Am J Physiol Heart Circ Physiol 289: H1584–H1593

Balazs R, Miller S, Romano C, de Vries A, Chun Y, Cotman CW (1997) Metabotropic glutamate receptor mGluR5 in astrocytes: pharmacological properties and agonist regulation. J Neurochem 69: 151–163

Chomczynski P, Sacchi N (1987) Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. Anal Biochem 162: 156–169

Ciruela F, Escriche M, Burgueno J, Angulo E, Casado V, Soloviev MM, Canela EI, Mallol J, Chan WY, Lluis C, McIlhinney RA, Franco R (2001) Metabotropic glutamate 1alpha and adenosine A1 receptors assemble into functionally interacting complexes. J Biol Chem 276: 18345–18351

Conlay LA, Conant JA, deBros F, Wurtman R (1997) Caffeine alters plasma adenosine levels. Nature 389: 136

Danbolt NC (2001) Glutamate uptake. Prog Neurobiol 65: 1–105

Dumas M, Gouyon JB, Tenenbaum D, Michiels Y, Escousse A, Alison M (1982) Systematic determination of caffeine plasma concentrations at birth in preterm and full-term infants. Dev Pharmacol Ther 4 [Suppl]: 182–186

Dunwiddie TV, Masino SA (2001) The role and regulation of adenosine in the central nervous system. Annu Rev Neurosci 4: 31–55

Flood AJ, Willems L, Headrick JP (2002) Coronary function and adenosine receptor-mediated responses in ischemic-reperfused mouse heart. Cardiovasc Res 55: 161–170

Fredholm BB, Battig K, Holmen J, Nehlig A, Zvartau EE (1999) Actions of caffeine in the brain with special reference to factors that contribute to its widespread use. Pharmacol Rev 51: 83–133

Fredholm BB, Sollevi A (1986) Cardiovascular effects of adenosine. Clin Physiol 6: 1–21

Funaya H, Kitakaze M, Node K, Minamino T, Komamura K, Hori M (1997) Plasma adenosine levels increase in patients with chronic heart failure. Circulation 95: 1363–1365

Gill SS, Mueller RW, McGuire PF, Pulido OM (2000) Potential target sites in peripheral tissues for excitatory neurotransmission and excitotoxicity. Toxicol Pathol 28: 277–284

Gill SS, Pulido OM, Mueller RW, McGuire PF (1998) Molecular and immunochemical characterization of the ionotropic glutamate receptors in the rat heart. Brain Res Bull 46: 429–434

Gill SS, Pulido OM, Mueller RW, McGuire PF (1999) Immunochemical localization of the metabotropic glutamate receptors in the rat heart. Brain Res Bull 48: 143–146

Golembiowska K, Dziubina A (2001) Involvement of adenosine in the effect of antidepressants on glutamate and aspartate release in the rat prefrontal cortex. Naunyn Schmiedebergs Arch Pharmacol 363: 663–670

Hansen CA, Schroering AG, Robishaw JD (1995) Subunit expression of signal transducing G proteins in cardiac tissue: implications for phospholipase C-beta regulation. J Mol Cell Cardiol 27: 471–484

Hartley TR, Lovallo WR, Whitsett TL (2004) Cardiovascular effects of caffeine in men and women. Am J Cardiol 93: 1022–1026

James JE, Gregg ME (2004) Hemodynamic effects of dietary caffeine, sleep restriction, and laboratory stress. Psychophysiology 41: 914–923

Johansson B, Ahlberg S, van der Ploeg I, Brene S, Lindefors N, Persson H, Fredholm BB (1993) Effect of long term caffeine treatment on A1 and A2 adenosine receptor binding and on mRNA levels in rat brain. Naunyn Schmiedebergs Arch Pharmacol 347: 407–414

Johansson B, Georgiev V, Lindstrom K, Fredholm BB (1997) A1 and A2A adenosine receptors and A1 mRNA in mouse brain: effect of long-term caffeine treatment. Brain Res 762: 153–164

Kew JN, Kemp JA (2005) Ionotropic and metabotropic glutamate receptor structure and pharmacology. Psychopharmacology (Berl) 179: 4–29

King N, McGivan JD, Griffiths EJ, Halestrap AP, Suleiman MS (2003) Glutamate loading protects freshly isolated and perfused adult cardiomyocytes against intracellular ROS generation. J Mol Cell Cardiol 35: 975–984

Kitakaze M, Hori M, Morioka T, Minamino T, Takashima S, Okazaki Y, Node K, Komamura K, Iwakura K, Itoh T, et al. (1995) Alpha 1-adrenoceptor activation increases ecto-5′-nucleotidase activity and adenosine release in rat cardiomyocytes by activating protein kinase C. Circulation 91: 2226–2234

Kitakaze M, Hori M, Morioka T, Minamino T, Takashima S, Sato H, Shinozaki Y, Chujo M, Mori H, Inoue M, et al. (1994) Infarct size-limiting effect of ischemic preconditioning is blunted by inhibition of 5′-nucleotidase activity and attenuation of adenosine release. Circulation 89: 1237–1246

Kitakaze M, Minamino T, Node K, Koretsune Y, Komamura K, Funaya H, Kuzuya T, Hori M (1998) Elevation of plasma adenosine levels may attenuate the severity of chronic heart failure. Cardiovasc Drugs Ther 12: 307–309

Leon D, Albasanz JL, Ruiz MA, Fernandez M, Martin M (2002) Adenosine A1 receptor down-regulation in mothers and fetal brain after caffeine and theophylline treatments to pregnant rats. J Neurochem 82: 625–634

Leon D, Albasanz JL, Ruiz MA, Iglesias I, Martin M (2005b) Effect of chronic gestational treatment with caffeine or theophylline on Group I metabotropic glutamate receptors in maternal and fetal brain. J Neurochem 94: 440–451

Leon D, Albasanz JL, Ruiz MA, Martin M (2005a) Chronic caffeine or theophylline intake during pregnancy inhibits A1 receptor function in the rat brain. Neuroscience 131: 481–489

Linden J (2001) Molecular approach to adenosine receptors: receptor-mediated mechanisms of tissue protection. Annu Rev Pharmacol Toxicol 41: 775–787

Matherne GP, Linden J, Byford AM, Gauthier NS, Headrick JP (1997) Transgenic A1 adenosine receptor overexpression increases myocardial resistance to ischemia. Proc Natl Acad Sci USA 94: 6541–6546

Mueller RW, Gill SS, Pulido OM (2003) The monkey (Macaca fascicularis) heart neural structures and conducting system: an immunochemical study of selected neural biomarkers and glutamate receptors. Toxicol Pathol 31: 227–234

Mundell SJ, Matharu AL, Pula G, Roberts PJ, Kelly E (2001) Agonist-induced internalization of the metabotropic glutamate receptor 1a is arrestin- and dynamin-dependent. J Neurochem 78: 546–551

Nawrot P, Jordan S, Eastwood J, Rotstein J, Hugenholtz A, Feeley M (2003) Effects of caffeine on human health. Food Addit Contam 20: 1–30

Pin JP, Acher F (2002) The metabotropic glutamate receptors: structure, activation mechanism and pharmacology. Curr Drug Targets CNS Neurol Disord 1: 297–317

Pisarenko OI (1996) Mechanisms of myocardial protection by amino acids: facts and hypotheses. Clin Exp Pharmacol Physiol 23: 627–633

Prospective Studies Collaboration (2002) Age-specific relevance of usual blood pressure to vascular mortality: a meta-analysis of individual data for one million adults in 61 prospective studies. Lancet 360: 1903–1913

Ralevic V, Burnstock G (2003) Involvement of purinergic signaling in cardiovascular diseases. Drug News Perspect 16: 133–140

Renard D, Poggioli J (1990) Mediation by GTP gamma S and Ca^{2+} of inositol trisphosphate generation in rat heart membranes. J Mol Cell Cardiol 22: 13–22

Rivkees SA, Zhao Z, Porter G, Turner C (2001) Influences of adenosine on the fetus and newborn. Mol Genet Metab 74: 160–171

Schindler CW, Karcz-Kubicha M, Thorndike EB, Muller CE, Tella SR, Ferre S, Goldberg SR (2005) Role of central and peripheral adenosine receptors in the cardiovascular responses to intraperitoneal injections of adenosine A1 and A2A subtype receptor agonists. Br J Pharmacol 144: 642–650

Smits P, Boekema P, De Abreu R, Thien T, van't Laar A (1987) Evidence for an antagonism between caffeine and adenosine in the human cardiovascular system. J Cardiovasc Pharmacol 10: 136–143

Solinas M, Ferre S, You ZB, Karcz-Kubicha M, Popoli P, Goldberg SR (2002) Caffeine induces dopamine and glutamate release in the shell of the nucleus accumbens. J Neurosci 22: 6321–6324

Sudano I, Binggeli C, Spieker L, Luscher TF, Ruschitzka F, Noll G, Corti R (2005) Cardiovascular effects of coffee: is it a risk factor? Prog Cardiovasc Nurs Spring 20: 65–69

Svenningsson P, Nomikos GG, Fredholm BB (1999) The stimulatory action and the development of tolerance to caffeine is associated with alterations in gene expression in specific brain regions. J Neurosci 19: 4011–4022

Terzic A, Puceat M, Vassort G, Vogel SM (1993) Cardiac alpha 1-adrenoceptors: an overview. Pharmacol Rev 45: 147–175

Us MH, Ozkan S, Ogus T, Acar HV, Ege T, Cakir O, Gokben M, Ozturk OY (2001) Efficacy of topically applied glutamate-aspartate and pentoxifylline solutions in decreasing myocardial damage during open-heart surgery in rats. J Int Med Res 29: 497–502

Vlachopoulos C, Panagiotakos D, Ioakeimidis N, Dima I, Stefanadis C (2005) Chronic coffee consumption has a detrimental effect on aortic stiffness and wave reflections. Am J Clin Nutr 81: 1307–1312

White PJ, Nguyen TT (2002) Chronic caffeine treatment causes changes in cardiac adenosine receptor function in rats. Pharmacology 65: 129–135

Xu Z, Park SS, Mueller RA, Bagnell RC, Patterson C, Boysen PG (2005) Adenosine produces nitric oxide and prevents mitochondrial oxidant damage in rat cardiomyocytes. Cardiovasc Res 65: 803–812

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