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Research Article

Caffeine intake induces an alteration in human neutrophil A_{2A} adenosine receptors

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Abstract. Caffeine is the most widely used drug in the world and acts mainly through antagonism of the effects mediated by the adenosine receptor subtypes A_1, A_{2A}, A_{2B} and A_3. We determined whether repeated caffeine administration at different doses and for different periods of time (400 or 600 mg/day for 1 week and 400 mg/day for 2 weeks) alters human neutrophil A_{2A} adenosine receptor density and function. Saturation binding assays showed an increase in affinity (K_D) and density (B_{max}) of A_{2A} adenosine receptors after caffeine intake. These changes were accompanied by increases in cAMP accumulation and decreases in superoxide anion production after stimulation of the A_{2A} receptor subtype using the agonist 5'-N-ethylcarboxamidoadenosine (NECA). Binding and functional changes of A_{2A} receptors returned to baseline after 48 h of caffeine withdrawal. The findings are consistent with a potential anti-inflammatory effect of caffeine mediated by neutrophil A_{2A} receptors.

Key words. Caffeine; adenosine receptors; human neutrophils; binding assays; cyclic AMP; superoxide anion production.

Caffeine (1,3,7-trimethylxanthine) is the most widely used drug in the world: 80–90% of adults report regular consumption of caffeine-containing beverages and foods such as coffee, tea, chocolate and cola drinks [1]. Caffeine intake from all sources is estimated to be 200–250 mg/person per day in the USA and Canada, and about 300–400 mg/person per day in Nordic countries and in Great Britain [1, 2]. Caffeine is readily absorbed from the gastrointestinal tract, distributed rapidly in all body fluids and metabolised via a complex set of reactions, reaching a peak plasma concentration between 15 and 120 min after oral ingestion. Although many potential mechanisms exist, considerable evidence indicates that physiologic concentrations of caffeine antagonise adenosine receptors named A_1, A_{2A}, A_{2B} and A_3 subtypes [3–5] both in the central nervous [6–8] and in the cardiovascular systems [9–11]. While the A_1 and A_3 receptors have been associated with inhibition of adenylyl cyclase activity, activation of the A_{2A} and A_{2B} receptors invariably leads to stimulation of this effector system and thereby an increase in cellular cyclic AMP production [3]. In the peripheral system, different experimental approaches have revealed the existence and function of A_{2A} adenosine receptors in various cell types such as monocytes, lymphocytes, neutrophils, basophils, platelets, mast cells and vascular smooth muscle [12, 13]. For example, the activation of A_{2A} adenosine receptors results in coronary and peripheral vasodilation [14, 15], neo-angiogenesis [16] and inhibition of the production of some cytokines

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from immune and inflammatory cells [17]. In human neutrophils, the anti-inflammatory effects of adenosine and adenosine analogues are known to be mediated via the increase of cellular cAMP levels, due to a reduction in superoxide anion generation and degranulation [12]. In human subjects, chronic caffeine consumption leads to antiaggregatory and potentially antithrombotic effects due to upregulation of A_{2A} adenosine receptors in platelets [18–20]. The effects of chronic caffeine intake on platelet functions are dependent on both the amount and the duration of the consumption, and underlie a reduction in platelet aggregability due to upregulation of the A_{2A} adenosine receptors [19, 20]. Controversial results have been reported on the link between caffeine and inflammation. In this respect, selective agonists led to better protocols of anti-inflammatory treatments and consequently antagonists could enhance inflammation and tissue damage [21–23]. Recently, caffeine has been found to suppress in vitro the production of the proinflammatory cytokine tumour necrosis factor (TNF)-α in human blood [24]. Of interest and rationale for the present study is the evidence that caffeine can have anti-inflammatory activity in different human substrates [24, 25].

From this background, the present study was designed to evaluate the effect of caffeine at different doses, such as 600 mg/day for 1 week or 400 mg/day administered for 1 or 2 weeks on A_{2A} adenosine receptors in human neutrophils. The following biochemical parameters were evaluated: (i) mean plasma or whole-blood concentrations of caffeine and its metabolites theophylline and theobromine; (ii) A_{2A} adenosine receptor density and affinity determined using [^{3}H]-4-(2-[7-amino-2-[furyl] [1,2,4] triazolo [2,3-a][1,3,5]triazin-5-ylamino]ethyl] phenol ([^{3}H]-ZM 241385) saturation binding assays (iii) accumulation of mRNA encoding the A_{2A} subtype using a quantitative real-time RT-PCR; (iv) A_{2A} adenosine receptor-mediated effect of the agonist 5 -N-ethylcarboxamidoadenosine (NECA) to increase cAMP levels and to inhibit superoxide anion production.

Materials and methods

Material. [^{3}H]-ZM 241385 (specific activity 17 Ci/mmol) was obtained from Tocris Cookson (Northpoint Fourth Way, Bristol, UK). 1,3-Dipropyl-8-cyclopentyl-xanthine (DPCPX), cytochrome c, N-formyl-L-methionyl-L-lencyl-L-phenylalanine (fMLP), Ro-201724, cyclic AMP, bovine serum albumin, cytochalasin B, adenosine deaminase, caffeine, theophylline, theobromine and 7-propylxanthine were obtained from Sigma-RBI (St. Louis, M.). Dextran and Ficoll-Paque were purchased from Pharmacia (Uppsala, Sweden). [^{3}H]-cyclic AMP (specific activity 21 Ci/mmol) was obtained from NEN Research Products (Boston, Mass.)

MRE 2029F20 {N-benzo [1,3]dioxol-5-yl-2-[5-(2,6-dioxo-1,3-dipropyl-2,3,6,7-tetrahydro-1H-purin-8-yl)-1-methyl-1H-pyrazol-3-yloxy] acetamide} and MRE 3008F20, {-5-n-(4-methoxy-phenylcarbamoyl)amino-8-propyl-2-(2-furyl) pyrazolo [4,3-e]-1,2,4-triazolo [1,5-c]pyrimidine} were synthesized by Prof. P. G. Baraldi (Department of Pharmaceutical Sciences, University of Ferrara, Italy).

All other reagents were of analytical grade and obtained from commercial sources.

Subjects. Potential subjects were screened by a physician to confirm no history of disease, or drug or alcohol abuse. Thirty-three, non-smoking subjects (15 men and 18 women, mean age 34±3 years) were enrolled for the study, according to the recommendations of the Declaration of Helsinki. The study protocol was approved by the local Ethics Committee of the University of Ferrara and the subjects provided written consent after receiving detailed verbal and written explanations of the study. Before the caffeine treatment, the subjects were asked to abstain from dietary methylxanthines for 2 weeks. They were divided into three groups according to the dose and duration of caffeine administration: (i) 200 mg orally twice a day for a period of 7 days (group 1, 11 subjects); (ii) 200 mg orally twice a day for a period of 14 days (group 2, 11 subjects); (iii) 200 mg orally three times a day for a period of 7 days (group 3, 11 subjects). Neutrophils from these subjects were studied prior to caffeine intake (day 0) and at 1, 12, 24, 36, 48 and 60 h after the last dose of caffeine. The 600-mg caffeine-treated group was also compared with a control group of seven age-matched subjects from whom blood was drawn without caffeine treatment. None of the subjects consumed caffeine or its derivatives in their during the study period.

Preparation of human neutrophils and membrane suspensions. Human neutrophils isolated from the peripheral blood of healthy volunteers and were prepared as previously described [12]. Briefly, the isolation of neutrophils started no later than 1–2 h after drawing the blood samples. Blood was supplemented with 20 ml of a solution consisting of 6% (by weight) Dextran T500. After gentle mixing, erythrocytes were allowed to settle down at 20 °C for 60 min. The turbid upper layer containing leukocytes was carefully removed with a polyethylene transfer pipette and placed into a 50-ml polypropylene centrifuge tube. Leukocytes were pelleted by centrifugation at 20 °C for 12 min at 100 g and remaining erythrocytes were lysed by suspending the cell pellet in 10 ml of distilled water at 4 °C under gentle agitation. After 30 s, isotonicity was restored by adding 0.6 mM NaCl. Cells were pelleted by centrifugation at 20 °C for 5 min at 250 g, suspended in Krebs-Ringer

phosphate buffer and layered onto 10 ml Fycoll-Hypaque. Neutrophils were sedimented by centrifugation at 20 °C for 20 min at 250 g. This procedure resulted in approximately 97% neutrophils and the cell viability was more than 95% as detected by a trypan blue exclusion test. Neutrophils were used to measure A_{2A} mRNA transcript and cyclic AMP and superoxide anion production. To obtain membrane suspensions, neutrophils were suspended in 50 mM Tris HCl, 10 mM $MgCl_2$, adenosine deaminase (2 IU/ml), pH 7.4. After centrifugation, the resulting pellet was resuspended in the same buffer at a concentration of 100 μg protein/100 μl and this homogenate was used for the binding assays. The protein concentration was determined according to a Bio-Rad method using bovine albumin as reference standard [26].

Methylxanthine plasma or whole-blood levels. Blood samples (1 ml) were obtained prior to caffeine intake (day 0, control condition) and at 1, 12, 24, 36, 48 and 60 h after administration of caffeine (600 mg/day for 1 week). After centrifugation of the plasma or whole-blood samples, the supernatant was utilised to measure the levels of methylxanthines using 7-propylxanthine as internal standard by HPLC (model 1100 series pump and diode array detector set at 272 nm; Agilent, Waldbroon, Germany). Chromatography was performed at room temperature on a reversed phase coloumn (LUNA C18, 0.46 x 15 cm 3 μm; Phenomenex, Torrance, Calif.). The mobile phase consisted of a mixture of 0.1% trifluoracetic acid and acetonitrile (85:15 v/v). The flow rate was 0.6 ml/min and the retention times of caffeine, theophylline, theobromine and 7-propylxanthine were 6.7, 4.6, 4.0 and 5.5 min, respectively.

Real-time RT-PCR experiments. Total cytoplasmic RNA was extracted by the acid guanidinium thiocyanate phenol method. Quantitative real-time RT-PCR assay [27] of A_{2A} mRNA transcript was carried out using a gene-specific double fluorescent labelled TaqMan MGB probe (minor groove binder) in a ABI Prism 7700 Sequence Detection System (Applied Biosystems, Warrington, UK). For the real-time RT-PCR of the A_{2A} gene, the Assay On Demand Gene Expression Products RefSeq NM 000675 was used in which the fluorescent reporter FAM and the quencher TAMRA are 6-carboxy fluorescein and 6-carboxy-N,N,N,N -tetramethylrhodamine, respectively. For the real-time RT-PCR of the reference gene the endogenous control human β-actin kit was used, and the probe used for • fluorescently-labeled • was VIC (Applied Biosystems, Monza, Italy).

[^{3}H]-ZM 241385 binding assay in the neutrophil membranes. Saturation binding assays were carried out by incubating membranes obtained from isolated neutrophils using eight to ten different concentrations of [^{3}H]-ZM 241385 ranging from 0.05 to 10 nM at 4 °C for 60 min [17]. Non-specific binding was determined in the presence of 10 μM NECA. Bound and free radioactivity were separated by filtering the assay mixture through Whatman GF/B glass fiber filters using a Brandel cell harvester. The filter-bound radioactivity was counted using an LS-1800 Beckman liquid scintillation counter with an efficiency of 57%.

Measurement of cyclic AMP levels in human neutrophils. Human neutrophils (10^6 cells/ml) were suspended in the Krebs-Ringer phosphate buffer containing 2 IU of adenosine deaminase, 0.5 mM of 4-(3-butoxy-4-methoxybenzyl)-2-imidazolidinone (Ro 20-1724) as phosphodiesterase inhibitor and preincubated for 10 min at 37 °C. Then, six to eight different concentrations of NECA were added for 10 min and the reaction was terminated by the addition of cold 6% trichloroacetic acid (TCA). Some experiments were also carried out in human neutrophils from control subjects who had not been treated with caffeine. In these cells, the potency of caffeine was determined by antagonism of NECA (200 nM)-induced stimulation of cyclic AMP levels (n = 3 experiments). To study the contribution of the different adenosine subtypes, the stimulatory effect of NECA on cAMP levels was evaluated in the absence and in the presence of typical A_1, A_{2B} and A_3 adenosine antagonists such as DPCPX, MRE 2029F20 and MRE 3008F20, respectively, at 100 nM (n = 3 experiments). The TCA suspension was centrifuged at 2000 g for 10 min at 4 °C and the supernatant was extracted four times with water-saturated diethyl ether. The final aqueous solution was used for determinations of cAMP levels by a competition protein-binding assay [12]. The binding protein, previously prepared from beef adrenals, was added to the samples previously incubated at 4 °C for 150 min and, after the addition of charcoal, these were centrifuged at 2000 g for 10 min. The clear supernatant (0.2 ml) was mixed with 4 ml Atomlight and counted in an LS-1800 Beckman liquid scintillation counter with an efficiency of 57%.

Superoxide anion production. O_2^- release was monitored continuously using a temperature-controlled spectrophotometer by the reduction of ferricytochrome c inhibited by superoxide dismutase (SOD), as described elsewhere [12]. The mixture was incubated with either control or different concentrations of NECA for 5 min at 37 °C in the presence of the peptide fMLP. Neutrophils were also incubated with 5 μg/ml cytochalasin at 550 nm for cytochrome c reduction. The amount of O_2^- produced was calculated by the differences in absorbance of the samples using a 15.5 mmol/l extinction coefficient at 550 nm for cytochrome c reduction. The net nanomoles of

O_2^- release were calculated using the nanomoles released by stimulated neutrophils minus nanomoles released by resting neutrophils.

Data and statistical analysis. A weighted non-linear least-squares curve-fitting program Ligand (Kell; Biosoft, Ferguson, Mo.) was used for computer analysis of the data from the saturation experiments [28]. EC_{50} and IC_{50} values (representing the concentration of agonist that cause 50% stimulation or inhibition of the examined effect, respectively) were calculated using the non-linear least-squares curve-fitting program Prism (Graph PAD version 3.03; San Diego, Calif.). Analysis of data was carried out by one-way analysis of variance (ANOVA). Analysis of differences between caffeine-treated groups (1, 12, 24, 36, 48 and 60 h) and subjects in the control condition was done using Dunnett's test. Differences were considered significant at a value of $p < 0.01$. All data are reported as mean ± SE.

Results

Neutrophils or neutrophil membranes from the examined subjects were analysed for specific binding and for intracellular cAMP and superoxide anion production prior to caffeine intake (day 0, control condition) and at 1, 12, 24, 36, 48 and 60 h after the last dose of caffeine (caffeine withdrawal). Table 1 summarizes the results of binding and functional studies in the three groups of subjects treated with different doses and durations of caffeine intake. In in vitro experiments, caffeine is able to inhibit cAMP accumulation in human neutrophils from control subjects induced by 200 nM-NECA with an IC_{50} of 87±9 μM. Caffeine (1 μM) reveals no significant change in cAMP levels, mediating a slight reduction of 2±1% of cAMP accumulation. Moreover, in our experimental conditions, the potency of NECA evaluated in the absence (EC_{50}= 124 ±12) and in the presence of A_1, A_{2B} and A_3 adenosine antagonists (EC_{50}= 117 ±11) revealed no significant change in cAMP levels suggesting a predominant A_{2A}-mediated response.

Group 1 (400 mg/day for 1 week). In control condition subjects, the affinity (K_D value) was 1.54±0.12 nM and the receptor density (B_{max} value) was 94±10 fmol/mg protein. In the same control condition, NECA increased cAMP levels with a potency (EC_{50}) of 124±16 nM and inhibited superoxide anion generation with a potency (IC_{50}) of 120±13 nM. No statistically significant differences were found in binding and functional parameters from 1 to 60 h after caffeine withdrawal (table 1).

Group 2 (400 mg/day for 2 weeks). Examined subjects in the control condition showed a K_D value of 1.48±0.10 nM and B_{max} value of 86±9 fmol/mg protein. In control condition subjects, NECA increased cAMP levels with an EC_{50} of 129±13 nM and inhibited superoxide anion generation with an IC_{50} of 131±14 nM. After caffeine withdrawal at 1 until 36 h, the K_D values gradually decreased, that is, indicating an increase in affinity, at different time points, by 30–44% and a gradual increase in receptor densities, at different time points, that varied from 172 to 213% ($p < 0.01$, vs control condition). Functional experiments revealed that the A_{2A} agonist NECA was significantly more potent in increasing cAMP intracellular levels: the EC_{50} values were smaller (from 40–69%) at different time points than control condition values ($p < 0.01$). A similar trend was observed in the IC_{50} values of NECA to reduce superoxide anion production, which from 54–68% with respect to control values ($p < 0.01$). Binding and functional parameters returned to baseline at 48 and 60 h after caffeine withdrawal (table 1).

Group 3 (600 mg/day for 1 week). The K_D value in the control condition was 1.51±0.11 nM and the B_{max} value was 92 ± 10 fmol/mg protein. In control condition subjects, NECA increased cAMP levels with an EC_{50} of 121±13 nM and inhibited superoxide anion generation with an IC_{50} of 116±12 nM (figs. 1, 2, table 1). After caffeine withdrawal from 1 until 36 h, [^{3}H]-ZM 241385 bound to a single class of binding sites with significantly increased K_D and B_{max} values at different time points by 28 to 50% and by 168 to 193%, respectively ($p < 0.01$, vs control condition; fig. 1, table 1). To determine whether these changes in binding parameters can alter A_{2A} receptor function, the effect of the A_{2A} agonist NECA to stimulate cAMP was measured. In caffeine-treated subjects, agonist concentration-response curves were significantly shifted to the left with respect to subjects in the control condition, indicating an increased potency of NECA to stimulate cAMP formation ($p < 0.01$). Hence, the EC_{50} values for NECA were significantly lower (i.e. greater potency), varying from 45–70% at different time points with respect to control values (fig. 2A). A similar trend was observed in the IC_{50} values obtained for the NECA-induced reduction in superoxide anion production, resulting in a decrease in IC_{50} values (i.e. greater potency) of 51–62% at different time points relative to control values ($p < 0.01$) (fig. 2B). Binding and functional parameters showed a gradual return to baseline at 48 and 60 h after caffeine withdrawal (fig. 1, 2, table 1). This group was also compared with a control group of seven age-matched subjects without caffeine treatment. Binding parameters were highly similar to the control condition of the examined subjects revealing a K_D value of 1.49±0.10 nM and a B_{max} value of 90±11 fmol/mg protein. Similarly, functional parameters did not change with respect to the control condition, showing an EC_{50} value of 120±10 nM in the cAMP assay and an EC_{50}

Table 1. Binding parameters (K_D and B_{max}) of the A_{2A} adenosine receptors in neutrophil membranes and potency of the agonist NECA to increase cAMP (EC_{50}) and to inhibit superoxide anion production (IC_{50}) in neutrophils.

	Binding assays	cAMP assays	O_2^- assays	
Group, daily dose of caffeine, duration, time after last dose	K_D (nM)	B_{max} (fmol/mg protein)	EC_{50} (nM)	IC_{50} (nM)
1, 400 mg, 1 week				
Control condition	1.54±0.12	94±10	124±16	120±13
1 h after caffeine	1.48±0.12	92±7	136±12	128±15
12 h after caffeine	1.45±0.09	90±6	134±11	109±11
24 h after caffeine	1.44±0.10	97±5	125±15	116±14
36 h after caffeine	1.37±0.12	95±9	115±14	130±15
48 h after caffeine	1.58±0.09	88±8	118±13	124±11
60 h after caffeine	1.52±0.14	86±6	138±10	135±12
2, 400 mg, 2 weeks				
Control condition	1.48±0.10	86±9	129±13	131±14
1 h after caffeine	1.03±0.09*	148±16*	77±8*	60±5*
12 h after caffeine	0.90±0.05*	157±14*	56±9*	52±6*
24 h after caffeine	0.95±0.07*	174±15*	54±7*	49±5*
36 h after caffeine	0.83±0.07*	183±19*	40±6*	42±7*
48 h after caffeine	1.50±0.12	88±13	141±16	121±11
60 h after caffeine	1.45±0.14	92±10	132±12	138±13
3, 600 mg, 1 week				
control	1.49±0.10	90±11	120±10	114±13
Control condition	1.51±0.11	92±10	121±13	116±12
1 h after caffeine	1.08±0.09*	155±14*	66±8*	57±6*
12 h after caffeine	1.02±0.10*	167±13*	42±5*	50±5*
24 h after caffeine	0.92±0.08*	170±15*	40±7*	48±6*
36 h after caffeine	0.76±0.09*	178±18*	36±6*	44±5*
48 h after caffeine	1.45±0.14	99±11	107±10	122±9
60 h after caffeine	1.52±0.14	95±8	114±15	115±11

K_D, affinity value or dissociation constant: represents the concentration of the drug able to bind 50% of the receptors. B_{max}, maximal binding capacity or the receptor density: represents the number of binding sites. EC_{50}, IC_{50} values, concentrations of NECA that cause 50% stimulation or inhibition, respectively. * $p < 0.01$ vs control condition. Analysis was by ANOVA followed by Dunnett's test.
Values are the mean ± SE, obtained at various times (h) after the last dose of caffeine, i.e. times after caffeine withdrawal.

value of 114±13 nM in the superoxide anion production assay.

To determine whether the increase in A_{2A} receptor density was caused by an increase in A_{2A} gene expression, the mRNA content of human neutrophils was measured using quantitative real-time RT-PCR. The expression level of A_{2A} adenosine receptors was normalized to the expression level of the endogenous reference (β-actin) in each sample. Our results demonstrate that subjects after caffeine treatment did not reveal a statistically significant increase in A_{2A} mRNA: the fold increase was 1.12±0.11, 1.27±0.16, 1.19±0.15, 1.31±0.19, 1.28±0.17 and 1.13±0.12 after 1, 12, 24, 36, 48 and 60 h of caffeine withdrawal, respectively in comparison to the control condition (1.00±0.11).

Blood and plasma levels of methylxanthines revealed no significant concentrations of caffeine (0.3±0.1 μM), theophylline (0.2±0.1 μM) and theobromine (0.3±0.1 μM) after caffeine abstinence. On the other hand, after the last dose of caffeine, its mean plasma concentration ranged from 63±7 to 0.3±0.1 μM at 1 and 60 h postdosing, respectively. Concentrations higher than 1 μM were still found 24 h postdosing (fig. 3). The caffeine concen-

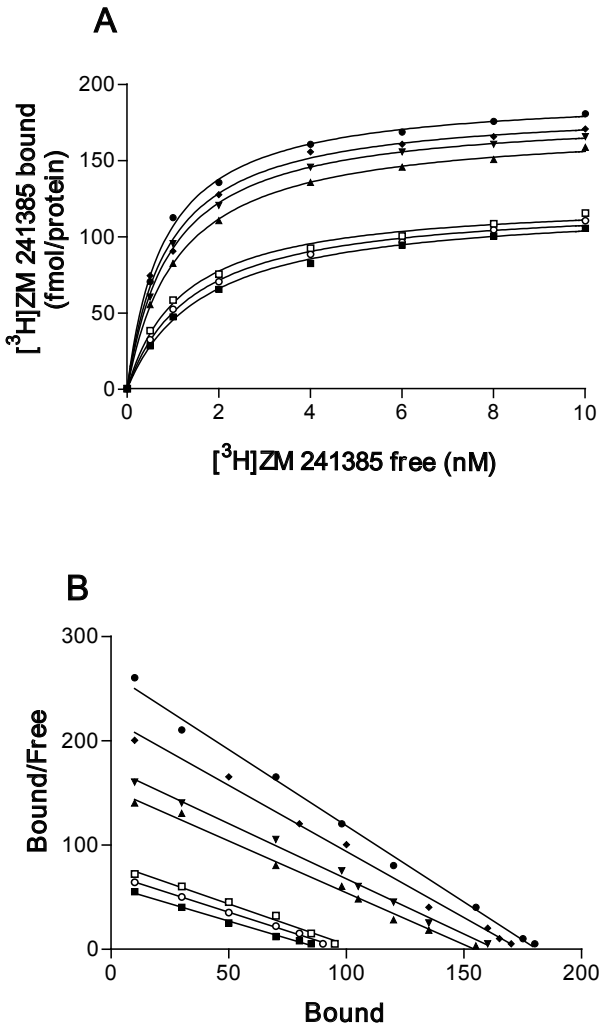

Figure 1. Effect of caffeine in 11 subjects treated with 600 mg/day for 1 week before caffeine (control condition ■,) and at 1 (▲), 12 (▼), 24 (◆), 36 (•), 48 (□) and 60 (○) h after caffeine withdrawal. Points represent the mean of the results (shown in table 1). (A) Specific binding of [^{3}H]ZM 241385 to neutrophil membranes from subjects before and after caffeine administration. (B) Scatchard plot of specific binding.

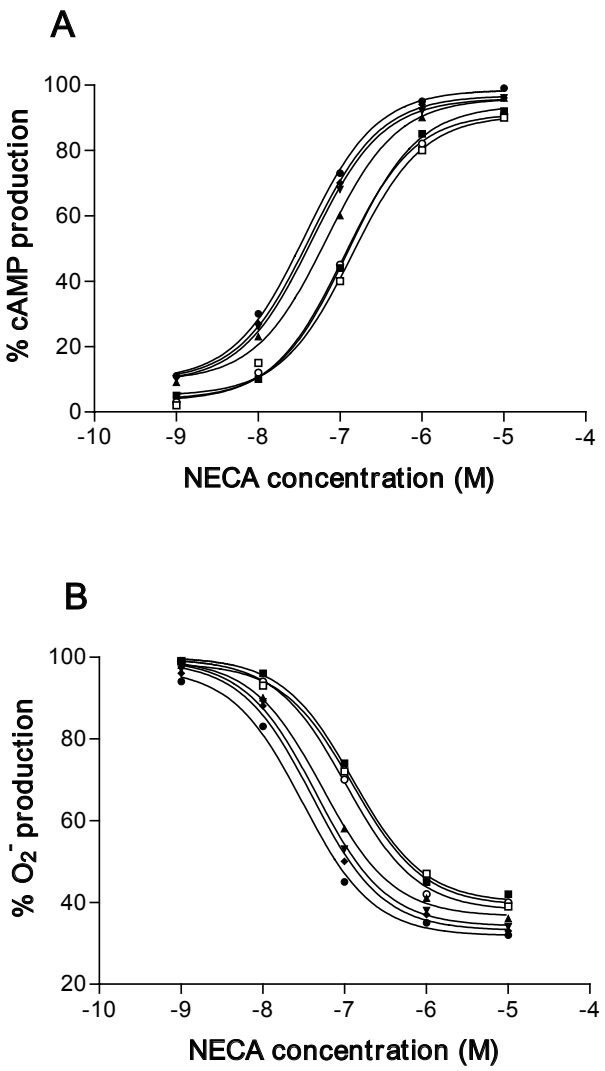

Figure 2. Effect of caffeine in 11 subjects treated with 600 mg/day for 1 week before caffeine (control condition) and at 1, 12, 24, 36, 48 and 60 h after caffeine withdrawal (symbols as in fig. 1). NECA concentration-effect curves to stimulate cAMP levels (A) and to inhibit superoxide anion production (B). Points represent the mean of the results (shown in table 1).

trations in whole blood were about half those in plasma, indicating the presence of the drug in the aqueous compartment of the blood. In plasma, the levels of theophylline and theobromine were, respectively, from 18±2 to 0.5±0.1 μM and from 5.1±0.4 to 0.3±0.1 μM (1 and 60 h after caffeine withdrawal, respectively). Concentrations of theophylline and theobromine were found to be higher than 1 μM up to 36 h after dosing of caffeine.

Discussion

The biochemical mechanism underlying the actions of caffeine is largely the antagonism of adenosine receptor-mediated responses [1, 29]. There is considerable evidence that antagonism of the A_{2A} adenosine subtype plays an important role in mediating the various effects of caffeine and that an upregulation of A_{2A} receptors represents an adaptive effect to chronic caffeine intake [30, 31]. For example, chronic intake of caffeine has been demonstrated to alter the response of human platelets to the actions of adenosine [19, 20]. Repeated administration of caffeine results in an increase in A_{2A} receptor density accompanied by sensitisation of platelet responses, such as an increase in cAMP accumulation and a decrease in platelet aggregation and calcium levels [19, 20]. Similarly, a recent paper on microglia reported that A_1 adenosine receptor activation, after

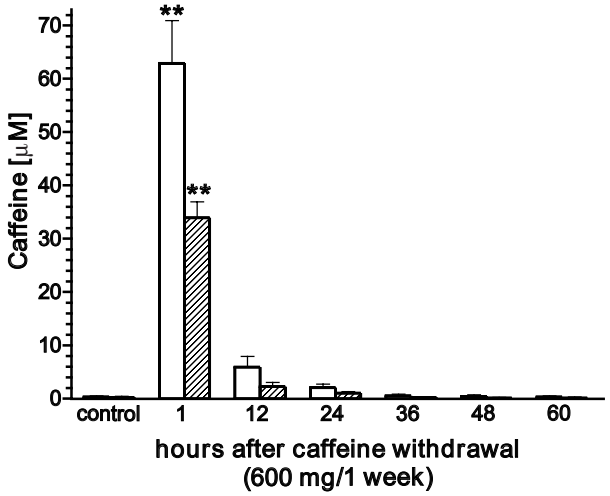

Figure 3. Plasma (open bars) and blood (solid bars) concentration of caffeine in 11 subjects treated with 600 mg/day for 1 week before caffeine (control condition) and at 1, 12, 24, 36, 48 and 60 h after caffeine withdrawal. Values are the mean ± SE.
$^{**}p < 0.05$ vs control condition. Analysis was by ANOVA followed by Dunnett's test.

its upregulation caused by chronic caffeine treatment, enhances related downstream signaling, suppressing proinflammatory cytokine production and augmenting anti-inflammatory responses [32]. Controversial results have been reported on the link between caffeine and inflammation. In this respect, targeting of adenosine receptors by selective agonists led to better protocols of anti-inflammatory treatment [22, 33]. Finally, inhibition of the protein coupled-mediated signaling with antagonists has been suggested as an approache to enhance inflammation and tissue damage [21, 34]. In vitro caffeine has recently been found to suppress the production of the pro-inflammatory cytokine TNF-α in human blood, and this effect was mediated by the cyclic AMP protein kinase A pathway [24]. In addition, caffeine is also frequently used in combination with non-steroidal anti-inflammatory drugs, revealing a possible interaction of adenosine systems in the modulation of inflammation processes. The combination of caffeine with indomethacin, an inhibitor of prostaglandin synthesis, mediated the effect of caffeine on alveolar macrophages by increasing cellular viability and lowering superoxide anion production [25]. Caffeine has also been suggested to have a beneficial effect in such inflammatory diseases as rheumatoid arthritis, multiple sclerosis and Alzheimer's disease [35, 36]. Caffeine intake over a period of 20 years was associated with a significantly lower risk for Alzheimer's disease [37]. Pentoxifylline (400 mg, three times daily for 1 month) also suppressed plasma TNF-α in untreated patients with severe heart failure associated with elevated plasma cytokine concentrations [38].

Starting from this complex background, the present study focused on the effects of caffeine, after different doses (400 or 600 mg/day) and duration of administration (1 or 2 weeks) on binding and functional parameters on human neutrophils. The concentration of caffeine appears to decrease in plasma or whole blood with a half-life of approximately 4 h, and becomes negligible after 24 h of withdrawal. It is important to note that in the present study, human volunteers used pure caffeine rather than caffeine-containing beverages such as coffee or chocolate to eliminate the potential involvement of a myriad of compounds that constitute the coffee's chemical profile of coffee [39]. Interestingly, the increase in density (i.e. B_{max}) of A_{2A} adenosine receptors found at 1 h after caffeine withdrawal was similar to that observed at 12, 24 and 36 h after the last dose of caffeine. On the other hand, at 48 and 60 h after the last dose of caffeine, the binding parameters were not different from the control condition or from the control group. Furthermore, the upregulation of A_{2A} receptors does not appear to be ascribable to the synthesis of new receptors because there were no changes in mRNA levels at different times after the last dose of caffeine. As a consequence, the increase in density of A_{2A} receptors may likely be due to translocation of the receptors from the cytoplasm to the membrane surface. On the other hand, the changes in A_{2A} receptors following long-term caffeine treatment could be due to the inhibition of downregulation and the desensitization induced by the endogenous adenosine. In addition, the increase in A_{2A} affinity and receptor density could be linked to a compensatory mechanism due to the presence of plasma adenosine and could represent an adaptive effect to caffeine intake. Consistent with our results, literature data have shown that a long-term caffeine treatment for 14 days in mouse brain caused an increase in A_1 and A_{2A} receptor binding [40]. Regarding the mechanisms involved in A_{2A} receptor changes (B_{max} and K_D), caffeine treatment interferes may plausibly with the kinetics of the binding of endogenous adenosine to the A_{2A} receptors which may influence the conformation of the ligand recognition site.

Another objective of the present study was to determine whether the changes in binding parameters correlated with changes in functional responses. The potency of NECA to stimulate cyclic AMP production at 1, 12, 24 and 36 h after caffeine withdrawal was significantly increased when compared with the subjects in the control condition. A good concordance was found between cyclic AMP accumulation data and inhibition of superoxide anion production by NECA, suggesting that cyclic AMP is likely to be involved in the action of A_{2A} receptor inhibition of superoxide anion formation. No changes in basal and forskolin-stimulated adenylate cyclase activity were found in the control condition or in the control group and caffeine-treated subjects, suggesting

that the observed effects were related to changes in A_{2A} receptor signalling. To further clarify the involvement of the A_{2A} adenosine receptors, we also tested in human neutrophils from control subjects, the potency of caffeine on NECA-stimulated cAMP levels, suggesting its role as an adenosine antagonist. Caffeine is a non-selective adenosine receptor antagonist with affinity values in the micromolar range. The coexpression of all adenosine subtypes in human neutrophils makes it difficult to study the contribution of each receptor. The selectivity of NECA was investigated using the blockade of A_1, A_{2B} and A_3 adenosine receptors. Interestingly, in our experimental conditions, the use of 100 nM DPCPX, MRE 2029F20 and MRE 3008F20 did not modify the potency of NECA, suggesting the dominant A_{2A} stimulatory component. In addition, the good agreement between binding and functional experiments suggests a pivotal role of A_{2A} receptors. On the other hand, further studies are necessary to evaluate the potential anti-inflammatory role played by other adenosine receptor subtypes after chronic caffeine ingestion.

Our binding and functional data show an increase in B_{max} after caffeine treatment strictly associated with an over-stimulation of A_{2A} adenosine receptor-mediated cAMP production. In fact, due to the ratio between extracellular receptors and transductional G proteins in cellular membranes, an increase in B_{max} mediates an increased coupling with G proteins and consequently an increase in function. The present study also revealed that the effects of chronic caffeine consumption on neutrophil functions are dependent on both the dose and the duration of the treatment. In our experimental conditions, the treatment with 400 mg/day for 2 weeks or 600 mg/day for 1 week corresponding approximately to two to three or four to five cups of brewed coffee, respectively, resulted in a significant increase and functionality of A_{2A} adenosine receptors.

In summary, caffeine intake and subsequent withdrawal mediates a temporary upregulation of A_{2A} adenosine receptors and alters their functional responses in human neutrophils. As a consequence, A_{2A} upregulation and sensitization, due to the effect of caffeine intake, might represent the mechanism responsible for the observed anti-inflammatory effects.

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