Caffeine Alters $A_{2A}$ Adenosine Receptors and Their Function in Human Platelets

Katia Varani, PhD; Francesco Portaluppi, MD; Stefania Merighi, MSc; Ennio Ongini, PhD; Luiz Belardinelli, MD; Pier Andrea Borea, PhD

**Background**—Caffeine acts mainly via blockade of adenosine receptors, which have been classified into $A_1$, $A_{2A}$, $A_{2B}$, and $A_3$ subtypes. We determined whether repeated caffeine administration (750 mg/d for 1 week) upregulates the human platelet $A_{2A}$ adenosine receptor and is accompanied by sensitization of platelet responses (increase in cAMP accumulation and decrease in platelet aggregation) to selective stimulation of the $A_{2A}$ receptors.

**Methods and Results**—Platelets were obtained from peripheral venous blood of 9 human volunteers at the end of 1 week of caffeine abstinence (control) and at 12 and 60 hours after the last dose of caffeine (withdrawal). The $A_{2A}$ receptor radioligand $[^3H]SCH 58261$ ([5-amino-7(phenylethyl)-2-(2-furyl)-pyrazolo[4,3-[$e$]-1,2,4-triazolo[1,5-$c$]-pyrimidine] bound to a single affinity class of sites in platelet membranes from controls with a $B_{max}$ of 98±2 fmol/mg protein and a $K_D$ of 1.29±0.05 nmol/L. At 12 and 60 hours after caffeine withdrawal, the radioligand bound with similar affinity ($K_D$=1.36±0.06 and 1.21±0.05 nmol/L, respectively), but the $B_{max}$ was increased ($P<0.01$) to 128±3 and 132±2 fmol/mg protein. The $A_{2A}$ receptor agonist 2-hexynyl-$N$-ethylcarboxamidoadenosine (HE-NECA) increased cAMP accumulation ($EC_{50}=59.3$ nmol/L) and inhibited ($IC_{50}=90.6$ nmol/L) aggregation of control platelets. The $EC_{50}$ values for HE-NECA to increase cAMP accumulation of platelets were reduced ($P<0.01$) at 12 and 60 hours after caffeine withdrawal (31±3 and 21±2 nmol/L, respectively). The $IC_{50}$ values for HE-NECA to inhibit ADP-induced platelet aggregation were 50±5 and 30±2 nmol/L at 12 and 60 hours after caffeine withdrawal, respectively.

**Conclusions**—Chronic caffeine intake leads to upregulation of $A_{2A}$ receptors and is accompanied by sensitization to the actions of the agonist HE-NECA. 

**Key Words:** adenosine receptor, caffeine, platelets, platelet aggregation inhibitors

The majority of adult humans consume a daily amount of caffeine averaging between 170 and 200 mg, the most important sources being coffee and tea. Caffeine produces a variety of effects through the blockade of adenosine receptors in various parts of the body, including blood vessels, platelets, and polymorphonuclear leukocytes. Blockade by caffeine of adenosine receptor subtype, $A_1$ and $A_{3A}$ receptor subtypes, inhibits the action of endogenous adenosine on a variety of physiological processes. Platelets express only 1 adenosine receptor subtype, ie, $A_{2A}$ receptors. Activation of $A_{2A}$ receptors in platelets causes an increase in cAMP accumulation and a decrease in platelet aggregation. Recently, in $A_{2A}$ receptor–knockout mice, it was reported that platelet aggregation was increased, indicating the importance of this receptor subtype in platelet function. Biaggioni et al found that a repeated dosing regimen with caffeine in human volunteers leads to significant changes in the functional response of platelets to the adenosine receptor agonist 2'-N'-ethylcarboxamidoadenosine (NECA). Caffeine withdrawal caused a significant leftward shift of the concentration-response curve of NECA-induced inhibition of aggregation. Owing to the lack of measurement of $A_{2A}$ receptor density by radioligand binding techniques, Biaggioni et al could not directly determine whether chronic caffeine intake increases the number of $A_{2A}$ receptors or increases the affinity of the receptor for the ligand. Given the recent availability of the selective $A_{2A}$ adenosine receptor antagonist radioligand $[^3H]SCH 58261$ ([5-amino-7(phenylethyl)-2-(2-furyl)-pyrazolo[4,3-[$e$]-1,2,4-triazolo[1,5-$c$]-pyrimidine}], we extended the findings of Biaggioni et al by directly measuring $A_{2A}$ adenosine receptor changes (density and affinity) and their function (ie, increase), by the $A_{2A}$-selective agonist 2-hexynyl-NECA (HE-NECA), of cAMP accumulation and inhibition of platelet aggregation.

Our findings provide further evidence that repeated intake of caffeine alters the response of platelets to adenosine. After chronic caffeine consumption, platelet aggregability may be reduced owing to upregulation of $A_{2A}$ receptors present on the platelet surface. The antiaggregatory effects are associated...
Caffeine Alters A2A Adenosine Receptors

with a rise in intracellular cAMP levels due to activation, by a selective A2A agonist, of adenylate cyclase.

**Methods**

Nine healthy, nonsmoking subjects, 25 to 45 years of age, of both sexes were studied. After written informed consent was obtained, the subjects were asked to abstain from dietary methylxanthines for at least 1 week. They were then given 250 mg caffeine orally 3 times a day for 7 days. Subjects were studied before starting caffeine (day 0) and at 12 and 60 hours after the last dose of caffeine (in the morning of days 8 and 10). For further in vitro experiments, platelet-rich plasma (PRP) from 3 additional subjects was incubated at 37°C in a thermostatic bath for 6 or 12 hours in the absence or presence of caffeine (20 μmol/L) or SCH 58261 (100 nmol/L).

**[3H]SCH 58261 Binding Assay in Platelet Membranes**

Binding assays were carried out according to Dionisotti et al. A weighted nonlinear least-squares curve-fitting program, LIGAND, was used for computer analysis of saturation experiments.

**Measurement of cAMP Levels in Human Platelets**

Washed human platelets obtained from the peripheral blood of the 9 volunteers were prepared for cAMP assays as described by Varani et al.

**Platelet Aggregation Assay**

Citrated human blood was centrifuged to obtain PRP and platelet-poor plasma for the platelet aggregation assay as described by Dionisotti et al.

**Plasma Concentrations of Caffeine**

Plasma caffeine concentration was measured by high-performance liquid chromatography separation and UV detection as described previously.

**Statistical Analysis**

Analysis of data was done by 1-way ANOVA. Analysis of difference between caffeine-treated groups (12 and 60 hours) and control subjects was done with Student’s t test (unpaired analysis). Differences were considered significant at a value of P<0.01. All data are reported as mean±SEM.

**Results**

Platelets from the subjects were harvested before the administration of caffeine was begun (day 0, control) and at 12 and 60 hours after the last dose (caffeine withdrawal). As shown in the Figure (panel A), [3H]SCH 58261 bound to a single affinity class of sites in platelet membranes from controls with a Bmax of 98±2 fmol/mg protein and a Kd of 1.29±0.05 nmol/L. In membranes from platelets harvested at 12 and 60 hours after caffeine withdrawal, the radioligand bound with the same affinity (Kd =1.36±0.06 and 1.21±0.05 nmol/L, respectively), but the number of binding sites (Bmax) was increased significantly (P<0.01), to 128±3 and 132±2 fmol/mg protein, respectively. In parallel studies, the functional response of platelets to the A2A receptor agonist HE-NECA was determined. In control platelets, HE-NECA increased cAMP levels with an EC50 of 59±3 nmol/L and inhibited ADP-induced platelet aggregation with an IC50 of 90±6 nmol/L. The same experiments carried out at 12 and 60 hours after caffeine withdrawal revealed a significant increase in the overall functional responsiveness of the platelets to HE-NECA. The EC50 of HE-NECA in increasing cAMP

Effects of caffeine withdrawal after 7-day treatment with caffeine, 250 mg TID PO. A, Specific binding of [3H]SCH 58261 to membranes prepared from platelets obtained from subjects before caffeine administration (●) and 12 (◆) and 60 (▲) hours after caffeine withdrawal (postcaffeine). Inset, Scatchard plot of specific binding. B and F denote bound and free ligand, respectively. Points represent mean of results of 9 experiments. B and C, HE-NECA concentration-effect curves to stimulate accumulation of platelet cAMP (B) and to inhibit platelet aggregation (C). Platelets were obtained from human subjects before caffeine administration (control, ●) and at 12 (◆) and 60 (▲) hours postcaffeine after a week of daily oral administration of 750 mg of caffeine. Basal cAMP levels (pmol/10^6 cells) were 8.4±0.9 (control), 8.2±0.8 (12 hours postcaffeine), and 8.5±0.6 (60 hours postcaffeine). Each data point is mean of results of 9 experiments.
Binding Parameters of the \(A_{2A}\) Adenosine Receptor Antagonist \([3H]\)SCH 58261 in Platelet Membranes and Potency of the \(A_{2A}\) Adenosine Receptor Agonist HE-NECA to Increase Platelet cAMP and to Inhibit Platelet Aggregation

<table>
<thead>
<tr>
<th>Subject</th>
<th>(K_v), nmol/L</th>
<th>(B_{max}), fmol/mg Protein</th>
<th>(EC_{50}), cAMP, nmol/L</th>
<th>(IC_{50}), Aggregation, nmol/L</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>1.29±0.05</td>
<td>98±2</td>
<td>59±3</td>
<td>90±6</td>
</tr>
<tr>
<td>12 h after caffeine</td>
<td>1.36±0.06</td>
<td>128±3*</td>
<td>31±3*</td>
<td>50±5*</td>
</tr>
<tr>
<td>60 h after caffeine</td>
<td>1.21±0.05</td>
<td>132±2*</td>
<td>21±3*</td>
<td>30±2*</td>
</tr>
</tbody>
</table>

\(*P<0.01\) vs control. Analysis was by ANOVA followed by Student’s t test.

Discussion

The present study describes, for the first time, the changes in the density and affinity of \(A_{2A}\) adenosine receptors in human platelet membranes of control (before caffeine administration) and caffeine-treated (12 and 60 hours after the last dose of caffeine) subjects. In control platelet membranes, the radioligand \([3H]\)SCH 58261 labeled a single class of binding sites with an affinity (\(K_v=1.29\) nmol/L) on the same order of magnitude as that determined during caffeine withdrawal. After caffeine treatment, however, a significant increase (upregulation) of \(A_{2A}\) adenosine binding sites was observed. This increase in \(A_{2A}\) receptor density is unlikely to have been due to the synthesis of new \(A_{2A}\) receptors of mature platelets; more likely, it occurred during differentiation of precursor cells. To clarify the mechanism of the upregulation of platelet \(A_{2A}\) receptors, we tested whether caffeine or the \(A_{2A}\) antagonist SCH 58261 could increase the number and/ or affinity of \(A_{2A}\) receptors during incubation of PRP from control subjects for a period of 6 or 12 hours with these antagonists. The lack of upregulation of \(A_{2A}\) receptors under these conditions strongly suggests that translocation of receptors to the membrane surface of mature platelets is not responsible for the upregulation of \(A_{2A}\) receptors observed in subjects treated with caffeine. To assess whether the changes in receptor density accompanied changes in the functional response of platelets to \(A_{2A}\) receptor activation, we investigated the regulation of adenylate cyclase activity and platelet aggregation. The EC\(_{50}\) values for accumulation of cAMP in platelets caused by the \(A_{2A}\) receptor agonist HE-NECA in control and caffeine-treated subjects were determined. There was a 2- to 3-fold increase in the potency of HE-NECA to cause cAMP accumulation. Likewise, the potency of HE-NECA to inhibit platelet aggregation at 12 and 60 hours after caffeine withdrawal was significantly increased. Thus, cessation of repeated administration of high doses of caffeine leads to significant changes in the number of \(A_{2A}\) receptors on the platelet surface associated with enhanced responsiveness to receptor stimulation.

Caffeine and other methylxanthines are nonselective adenosine receptor blockers. Many of the effects of caffeine appear to be due to blockade of the actions of endogenous adenosine. The effects of adenosine on platelet aggregation are coupled to adenylate cyclase activation. During ischemia and/or hypoxia, extracellular levels of adenosine increase markedly, and plasma levels of this nucleoside may rise sufficiently to cause a decrease in platelet aggregability. In fact, in dogs, adenosine released during myocardial ischemia inhibits platelet aggregation, an effect that is antagonized by 8-phenyltheophylline. If one assumes that endogenous released adenosine during episodes of ischemia inhibits platelet aggregation and thromboembolization, it is conceivable that mild chronic caffeine consumption may lead to a paradoxical reduction in platelet aggregability.

Over the past few years, it has become apparent that the effects of acute and chronic treatment with caffeine, as well as of other adenosine receptor antagonists, are qualitatively different. Thus, long-term treatment with adenosine receptor antagonists can have effects that resemble those of acute administration of adenosine receptor agonists, and vice versa.

Our findings not only confirm those of Biagioni et al but also extend them to provide an explanation for the functional changes in platelet responsiveness to activation of \(A_{2A}\) receptors. The results of the present study support the hypothesis that chronic caffeine consumption results in sensitization and/or upregulation of endogenous adenosine receptors in normal subjects. The upregulation of adenosine \(A_{2A}\) receptors caused by chronic intake of caffeine could be interpreted to indicate that endogenous adenosine has a “tonic influence” on human platelets, and the presence of the antagonist is counterbalanced by the upregulation of \(A_{2A}\) receptors. Consistent with this interpretation is the finding that platelet aggregation is more efficient in mice lacking the \(A_{2A}\) receptor; which also supports the conclusion that the classic \(A_{2A}\) receptor is responsible for the antiaggregate properties of adenosine and its analogues.

In summary, the data provide further evidence that chronic intake of caffeine alters the response of platelets to the actions...
of adenosine. Chronic caffeine consumption may lead to a reduction in platelet aggregability as a result of upregulation of the A2A receptors located on the platelet surface. The results reported here should serve as an impetus for further investigation of the changes in platelet function produced by chronic caffeine consumption and sudden withdrawal.

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References
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