Caffeine, found in different sources such as coffee, tea, chocolate, and cola drinks, is the most widely active substance in the world. Average caffeine consumption by adult humans varies among different cultures and nations from 80 to 400 mg per person per day. Caffeine elicits a diverse number of pharmacological responses, including increased vigilance, decreased psychomotor reaction time, and increased sleep latency and wakening time and may also influence intellectual performance. Moreover, caffeine causes relaxation of smooth muscles, enhances the secretion of gastric acid and the release of catecholamines, and increases metabolic activity.

The precise mechanism(s) underlying the actions of caffeine remain poorly defined. Although the inhibition of phosphodiesterases may contribute to the actions of caffeine, there is growing evidence that most pharmacological effects of this xanthine result from antagonism of adenosine receptors designated as A₁, A₂A, A₂B, and A₃ subtypes. Caffeine acts most potently at A₂A receptors, followed closely by A₁ receptors, then A₂B receptors, and as a weak antagonist at human A₃ receptors. Blockade by caffeine of adenosine receptors, namely the A₁ and the A₂A receptor types, inhibits the action of endogenous adenosine on a variety of physiological processes. Under normal conditions, blood levels of adenosine appear to be sufficient to tonically activate A₂A receptors in platelets. Recently, in A₂A receptor–knockout mice, it was reported that platelet aggregation was increased, indicating the importance of this receptor subtype in platelet function. It is therefore conceivable that caffeine could block these tonically activated A₂A receptors in platelets and alter their functions modulated by adenosine.

For many years, an association has been suspected between coffee drinking and cardiovascular diseases, in particular coronary heart disease, but recently it has been demonstrated that coffee or caffeine consumption does not increase the risk of coronary heart diseases or stroke. Numerous epidemiological studies considering myocardial infarction have found no deleterious effect of ≤5 cups of coffee per day, whereas results are controversial at higher intake levels. In patients with hypertension, no adverse outcome risk was observed at any level of caffeine intake.

A study by Biaggioni et al found that a repeated dosing regimen of caffeine leads to significant changes in human
platelets in the functional responses to the adenosine receptor agonist 5'-N-ethylcarboxamidoadenosine (NECA). Caffeine withdrawal produced a significant leftward shift of the NECA-induced inhibition of aggregation. Consistent with this, we recently demonstrated, in subjects treated with 750 mg/d for 1 week, that chronic intake of caffeine alters the platelet aggregability as a result of upregulation of the A<sub>2A</sub> receptors located on the platelet surface.

In the present article, we provide further evidence that a similar response is found in subjects treated with caffeine at different doses, such as 600 mg/d for 1 week or 400 mg/d, administered for a longer period of time, such as 2 weeks. In the present study, this was done by directly measuring adenosine A<sub>2A</sub> receptor changes (density and affinity) and their function by determining the effect of the A<sub>2A</sub> selective agonist 2-hexynyl-NECA (HE-NECA) to (1) increase cAMP accumulation, (2) inhibit platelet aggregation, and (3) decrease intracellular calcium levels. After chronic consumption (600 mg/d for 1 week or 400 mg/d for 2 weeks), upregulation of platelet adenosine A<sub>2A</sub> receptors was found, which was highly correlated with antiaggregatory effects, a rise in cAMP accumulation, and a decrease in intracellular calcium levels. No differences were revealed on binding and functional parameters from subjects treated with 400 mg/d for 1 week.

Methods
Forty-five healthy, nonsmoking subjects, 25 to 45 years of age, of both sexes, were studied. After having written informed consent, subjects were asked to abstain from dietary methylxanthines for ≥2 weeks. They were divided into 3 groups (15 subjects each) according to the caffeine administration regimen (ie, dose and duration of administration): 200 mg orally 2 times a day for a period of 7 days (group 1); 200 mg orally 1 time a day for a period of 14 days (group 2); and 200 mg orally 3 times a day for a period of 7 days (group 3). Platelets from these subjects were studied before they started caffeine (day 0) and at 1, 12, 60, and 108 hours after the last dose of caffeine. In particular, the 1-hour time point was studied only in the group receiving the maximum dosage of caffeine (600 mg/d).

\[ ^{3}P \text{HSCH 58261 Binding Assay in Human Platelet Membranes} \]

Membranes from human platelets were prepared as previously described and used for radioligand binding assays with \[^{3}P \text{HSCH 58261} \] according to Varani et al. In saturation studies, human platelet membranes were incubated with 8 to 10 different concentrations of \[^{3}P \text{HSCH 58261} \] ranging from 0.01 to 10 nmol/L. Nonspecific binding was determined in the presence of NECA 10 µmol/L. After 60 minutes of incubation at 4°C, samples were filtered through Whatman GF/B filters with a Micro-Mate 196 Cell Harvester (Packard Instrument Co.). A weighted nonlinear least-squares curve-fitting program, LIGAND, was used for computer analysis of the data from the saturation experiments.

Measurement of cAMP Levels in Human Platelets
Washed human platelets obtained from the peripheral blood of healthy volunteers were prepared as described previously. Platelets (6×10<sup>9</sup> to 8×10<sup>10</sup> cells) were incubated with 1.0 U of adenosine deaminase/mL, 0.5 mmol/L 4-(3-butoxy-4-methoxybenzyl)-2-imidazolidinone (Ro 20-1724) as phosphodiesterase inhibitor, and 6 to 8 different concentrations of HE-NECA. EC<sub>50</sub> values were obtained from concentration-response curves after log-logit transform.

Statistical Analysis
Analysis of data was carried out by 1-way ANOVA. Analysis of difference between caffeine-treated groups (12, 60, and 108 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 3 groups of subjects were harvested before the administration of caffeine was begun (day 0, control) and at 1, 12, 60, and 108 hours after the last dose (caffeine withdrawal). The Table summarizes the results of binding and functional experiments from the 3 groups of subjects.

Group 1 (400 mg/d for 1 Week)
Binding parameters revealed a control B<sub>max</sub> value of 105±6 fmol/mg protein and a K<sub>D</sub> value of 1.28±0.08 nmol/L. In control platelets, HE-NECA increased cAMP levels with an EC<sub>50</sub> of 60±5 nmol/L and inhibited (1) aggregation with an IC<sub>50</sub> of 86±10 nmol/L and (2) calcium levels with an IC<sub>50</sub> of 104±8 nmol/L. No statistically significant differences were found in binding and functional parameters at 12, 60, and 108 hours after caffeine withdrawal (Table).

Group 2 (400 mg/d for 2 Weeks)
Binding parameters revealed a control B<sub>max</sub> value of 110±3 fmol/mg protein and a K<sub>D</sub> value of 1.21±0.09 nmol/L. At 12 and 60 hours after caffeine withdrawal, the receptor densities, B<sub>max</sub> increased at both time points by some 20%, but the K<sub>D</sub> values were unchanged. Functional experiments revealed that the adenosine A<sub>2A</sub> receptor agonist HE-NECA was significantly more potent in increasing platelet cAMP (ie, the EC<sub>50</sub> values were 45% and 65% smaller at 12 and 60 hours after withdrawal).
Binding Parameters of the \( A_{2A} \) Adenosine Receptor Antagonist \(^{[3]H}\)SCH 58261 in Platelet Membranes and Potency of the \( A_{2A} \) Adenosine Receptor Agonist HE-NECA in Human Platelets to Increase cAMP, Inhibit Aggregation, and Decrease Calcium Levels

<table>
<thead>
<tr>
<th>Group, Dose of Caffeine/d, Time</th>
<th>( K_c ), nmol/L</th>
<th>( B_{max} ), fmol/mg protein</th>
<th>( IC_{50} ), cAMP, nmol/L</th>
<th>( IC_{50} ), Aggregation, nmol/L</th>
<th>( IC_{50} ), Ca(^{2+} ), nmol/L</th>
</tr>
</thead>
<tbody>
<tr>
<td>1, 400 mg, 1 wk</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>1.28±0.08</td>
<td>105±6</td>
<td>60±5</td>
<td>86±10</td>
<td>104±8</td>
</tr>
<tr>
<td>12 h after caffeine</td>
<td>1.29±0.04</td>
<td>108±6</td>
<td>62±6</td>
<td>88±10</td>
<td>100±11</td>
</tr>
<tr>
<td>60 h after caffeine</td>
<td>1.32±0.05</td>
<td>107±4</td>
<td>64±4</td>
<td>85±8</td>
<td>95±9</td>
</tr>
<tr>
<td>108 h after caffeine</td>
<td>1.31±0.09</td>
<td>105±5</td>
<td>63±8</td>
<td>86±9</td>
<td>103±6</td>
</tr>
<tr>
<td>2, 400 mg, 2 wks</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>1.21±0.09</td>
<td>110±3</td>
<td>60±6</td>
<td>92±10</td>
<td>97±4</td>
</tr>
<tr>
<td>12 h after caffeine</td>
<td>1.32±0.06</td>
<td>131±8*</td>
<td>33±8*</td>
<td>45±7*</td>
<td>62±3*</td>
</tr>
<tr>
<td>60 h after caffeine</td>
<td>1.34±0.08</td>
<td>135±5*</td>
<td>22±6*</td>
<td>34±5*</td>
<td>46±5*</td>
</tr>
<tr>
<td>3, 600 mg, 1 wk</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>1.27±0.09</td>
<td>100±4</td>
<td>60±5</td>
<td>86±11</td>
<td>97±9</td>
</tr>
<tr>
<td>1 h after caffeine</td>
<td>1.29±0.07</td>
<td>132±4*</td>
<td>...</td>
<td>...</td>
<td>...</td>
</tr>
<tr>
<td>12 h after caffeine</td>
<td>1.28±0.08</td>
<td>134±5*</td>
<td>38±6*</td>
<td>48±4*</td>
<td>62±7*</td>
</tr>
<tr>
<td>60 h after caffeine</td>
<td>1.30±0.06</td>
<td>132±6*</td>
<td>30±6*</td>
<td>36±8*</td>
<td>48±5*</td>
</tr>
<tr>
<td>108 h after caffeine</td>
<td>1.28±0.09</td>
<td>133±3*</td>
<td>32±4*</td>
<td>34±6*</td>
<td>46±6*</td>
</tr>
<tr>
<td>750 mg, 1 wk†</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>1.29±0.05</td>
<td>98±2</td>
<td>59±3</td>
<td>90±6</td>
<td></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>
<td></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>
<td></td>
</tr>
</tbody>
</table>

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

†From reference 15.

Caffeine withdrawal than control values, respectively). A similar trend was observed in the \( IC_{50} \) values obtained in aggregation experiments and in cytoplasmic free calcium concentration measurements (Table).

**Group 3 (600 mg/d for 1 Week)**

Overall, we obtained data similar to those of group 2. \(^{[3]H}\)SCH 58261 bound to a single affinity class of sites in platelet membranes from controls with a \( B_{max} \) of 100±4 fmol/mg protein and a \( K_c \) of 1.27±0.09 nmol/L. As shown in Figure 1A, in membranes from platelets harvested at 1, 12, 60, and 108 hours after caffeine withdrawal, the radioligand bound with the same affinity, but the number of binding sites (\( B_{max} \)) was significantly \((P<0.01)\) increased. In parallel studies, the functional responses of platelets to the \( A_{2A} \) receptor agonist HE-NECA were determined. As summarized in the Table, the potency of HE-NECA to (1) increase cAMP formation, (2) inhibit ADP-induced platelet aggregation, and (3) decrease calcium levels was significantly increased in platelets obtained at 12, 60, and 108 hours after caffeine withdrawal (Figure 1B, 1C, and 1D). Experiments were also carried out to determine whether the increase in the density of \( A_{2A} \) receptors would be accompanied by a decrease in potency and/or efficiency of ADP to induce aggregation. The \( EC_{50} \) values of ADP to stimulate platelet aggregation at 12, 60, and 108 hours after caffeine withdrawal were 0.7±0.2, 0.9±0.1, and 0.8±0.1 μmol/L, respectively, values not significantly different from the 0.9±0.2 μmol/L obtained in platelets from controls (Figure 2).

**Discussion**

The effects of long-term administration of caffeine in humans and animals and its role in their tolerance to the actions of caffeine are controversial. Some studies showing an increase of \( A_1 \) receptors in the mouse brain found evidence for a dose-dependent upregulation of \( A_1 \) receptors by caffeine. Other studies revealed an upregulation of \( A_{2A} \) receptors, suggesting an adaptive effect of caffeine intake. Moreover, chronic caffeine consumption may lead to a reduction in platelet aggregability as a result of upregulation of the \( A_{2A} \) receptors located on the platelet surface playing a potential role in pathophysiological processes, such as aggregation and thrombogenesis. Although such changes may contribute to alterations in platelet function, tolerance to caffeine does not modify the potency of this xanthine as a competitive antagonist of the effects of adenosine. Other changes, such as shifting of receptor affinity to a high-affinity state, alterations in G protein levels or the coupling of these proteins to adenosine receptors, or long-term receptor occupancy may be involved in the phenomenon of tolerance. Withdrawal symptoms to caffeine occur in humans; typically, they are headache, fatigue, apathy, and drowsiness. In particular, caffeine increases plasma adenosine concentration, and its reduction after antagonist withdrawal suggests receptor-mediated regulation of the plasma adenosine concentration. During ischemia and/or hypoxia, adenosine also has neuroprotective actions. In the adult brain, chronic caffeine treatment, which leads to upregulation of adenosine receptors,
reduces ischemic damage, whereas acute exposure (receptor antagonistic effect) increases ischemic damage.\textsuperscript{28}

It has been demonstrated that chronic intake of caffeine alters the response of platelets to the actions of adenosine.\textsuperscript{14} Repeated administration of caffeine (750 mg/d for 1 week) revealed an increase in $A_{2A}$ receptor density, accompanied by sensitization of platelet responses, such as an increase in cAMP accumulation and decrease in platelet aggregation.\textsuperscript{15}

The aim of the present study was to determine the effect of caffeine dosage and of the duration of administration on binding and functional parameters. Hence, we studied the changes in the density and affinity of adenosine $A_{2A}$ receptors in human platelet membranes of subjects treated with different doses (400 or 600 mg/d) for different periods of caffeine intake (1 or 2 weeks). Specifically, we studied control (before caffeine administration) and caffeine-treated (1, 12, 60, and 108 hours after the last dose of caffeine) subjects.

The treatment with 400 mg/d caffeine for 1 week did not modify $A_{2A}$ receptor binding and functional parameters. However, treatment with 400 mg/d for 2 weeks or 600 mg/d for 1 week resulted in (1) a significant increase (upregulation) of adenosine $A_{2A}$ binding sites, (2) a rise in cAMP accumulation, (3) an increase of antiaggregatory effects, and (4) a decrease in calcium levels elicited by the $A_{2A}$ receptor agonist HE-NECA.

The upregulation of $A_{2A}$ receptors can probably be ascribed to the synthesis of new receptors during differentiation of precursor cells. This interpretation is based on the results of in vitro experiments showing that the incubation of platelet-rich plasma from control subjects for a period of 6 or 12 hours with caffeine or SCH 58261 did not affect the binding parameters.\textsuperscript{15} 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. In adults, caffeine is adsorbed efficiently from the gastrointestinal tract; peak plasma concentrations occur 15 to 120 minutes after ingestion, and the half-life of caffeine is 2.5 to 4.5 hours.\textsuperscript{7} The increase of adenosine $A_{2A}$ receptors found at 1 hour after the last dose of caffeine treatment was similar to that obtained at 12 or 60 hours after caffeine withdrawal, showing that the withdrawal was not necessary for the upregulation of $A_{2A}$ receptors.

Another aim of the present study was to determine whether the changes in binding parameters correlated with changes in functional response(s). It was found that platelet aggregation was associated with activation of adenylate cyclase and with a rise in intracellular calcium concentrations. The potency of HE-NECA at 12, 60, and 108 hours after caffeine withdrawal was significantly increased compared with the control group. This finding indicates that repeated administration of different doses of caffeine leads to significant changes in the number of $A_{2A}$ receptors on the platelet surface, accompanied...
by an enhanced responsiveness to receptor stimulation. The classic adenosine A2A receptor is responsible for the anti-aggregatory properties of adenosine and its analogues, which is consistent with the observation that aggregation is more efficient in mice lacking the A2A receptors. However, platelet aggregation induced by the observation that aggregation is more efficient in mice lacking the A2A receptors was not significantly different between control and caffeine-treated subjects. One possible explanation for this latter observation is that there is not enough adenosine in the assay to produce a response. Alternatively, in the caffeine-treated subjects, the magnitude of receptor upregulation (ie, number of receptors) was not sufficient to produce a shift of the ADP-induced aggregation concentration-response curve. However, when the levels of endogenous adenosine increase, such as during myocardial ischemia, the extracellular concentration of adenosine rises rapidly to a level sufficient to act on upregulated receptors and may have greater platelet-inhibitory effects than control. Thus, it is possible that chronic caffeine consumption in doses not far from the average dietary intake may lead to a paradoxical reduction in platelet aggregability during ischemia.

In conclusion, all the data together provide further evidence that chronic intake of caffeine alters the response of platelets to the actions of adenosine. The major finding of the present study is that the effects of chronic caffeine consumption on platelet functions are dependent on both the dose and the duration of the treatment and underlie a reduction in platelet aggregability due to upregulation of the adenosine A2A receptors.

References

Dose and Time Effects of Caffeine Intake on Human Platelet Adenosine A2A Receptors: Functional and Biochemical Aspects

Katia Varani, Francesco Portaluppi, Stefania Gessi, Stefania Merighi, Ennio Ongini, Luiz Belardinelli and Pier Andrea Borea

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