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Enhanced Cellular Adenosine Uptake Limits Adenosine Receptor Stimulation in Patients With Hyperhomocysteinemia

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Objective—Endogenous adenosine has several cardioprotective effects. We postulate that in patients with hyperhomocysteinemia increased intracellular formation of S-adenosylhomocysteine decreases free intracellular adenosine. Subsequently, facilitated diffusion of extracellular adenosine into cells through dipyridamole-sensitive transporters is enhanced, limiting adenosine receptor stimulation. We tested this hypothesis in patients with classical homocystinuria (n=9, plasma homocysteine 93.1±24.7 μmol/L) and matched controls (n=8, homocysteine 9.1±1.0).

Methods and Results—Infusion of adenosine (0.5, 1.5, 5.0, and 15.0 μg/min/dL forearm) into the brachial artery increased forearm blood flow, as measured with venous occlusion plethysmography, to 2.9±0.4, 4.3±0.5, 5.6±1.1, and 9.6±2.1 in the patients and to 2.8±0.6, 4.4±1.0, 9.0±1.7, and 17.0±3.1 mL/min/dL in controls (P<0.05). However, adenosine-induced vasodilation in the presence of dipyridamole (100 μg/min/dL) was similar in both groups (P=0.9). Additionally, in isolated erythrocytes, adenosine uptake was accelerated by incubation with homocysteine (half-time 6.4±0.3 versus 8.1±0.5 minutes, P<0.001) associated with increased intracellular formation of S-adenosylhomocysteine (P<0.0001).

Conclusions—in hyperhomocysteinemia, adenosine-induced vasodilation is impaired but is restored by dipyridamole. Accelerated cellular adenosine uptake probably accounts for these observations. These impaired actions of adenosine could well contribute to the cardiovascular complications of hyperhomocysteinemia. (Arterioscler Thromb Vasc Biol. 2005;25:109-114.)

Key Words: adenosine ■ hyperhomocysteinemia ■ dipyridamole ■ forearm ■ nucleoside transport

Hyperhomocysteinemia is an independent risk factor for atherosclerosis and thrombembolism. It is poorly understood which mechanism is responsible for these cardiovascular complications.

Recently, we and others have drawn attention to a new hypothesis, focusing on the influence of homocysteine on the metabolism of the endogenous nucleoside adenosine.1,2 According to this hypothesis, a homocysteine-induced fall in extracellular adenosine contributes to the cardiovascular sequelae of hyperhomocysteinemia. Fundamental to this is the reversibility of the reaction in which S-adenosylhomocysteine (AdoHcy) is hydrolyzed to form homocysteine and adenosine.3 Although the equilibrium constant of this reaction favors AdoHcy synthesis, under physiological conditions AdoHcy is hydrolyzed to homocysteine and adenosine, because both reaction products are rapidly metabolized. In hyperhomocysteinemia, the reaction shifts toward AdoHcy synthesis at the expense of free intracellular adenosine. Subsequently, facilitated diffusion of extracellular adenosine into the cells through the dipyridamole-sensitive equilibrative nucleoside transporter is enhanced, limiting stimulation of membrane-bound adenosine receptors (Figure 1).

By stimulation of these receptors, extracellular adenosine induces several effects, which could protect against the development of atherosclerosis and thrombosis and against ischemia-reperfusion injury.1,4 Particularly in situations of hypoxia or ischemia, when the concentration of adenosine increases rapidly, these effects work in concert to protect the affected tissue.5 Previous animal studies suggest that the effect of intracellular AdoHcy formation on the transmembranous adenosine gradient and thus diffusion of extracellular adenosine into the cells is most pronounced in these very situations of high concentrations of adenosine, thus limiting adenosine receptor stimulation when most needed.6,7 Therefore, we speculate that in hyperhomocysteinemia, decreased extracellular adenosine concentrations contribute to the development of the associated cardiovascular problems.
Figure 1. Simplified representation of the biochemical reactions relevant to our hypothesis. Under normal conditions, adenosine is continuously produced by the hydrolysis of AdoHcy (left). In hyperhomocysteinemia, adenosine production from AdoHcy hydrolysis is hampered or the reaction is even reversed, and AdoHcy will accumulate at the expense of free adenosine (right). Dipyridamole inhibits cellular adenosine uptake by blockade of the ENT. AMP indicates adenosine mono-phosphate; 5′-NT, 5′-nucleotidase; ADA, adenosine deaminase; AK, adenosine kinase; Ino, inosine; Met, methionine; ENT, equilibrative nucleoside transporter.

In the present study, we addressed this issue in patients with severe hyperhomocysteinemia. We estimated basal intravascular and muscle interstitial adenosine concentration by microdialysis. Secondly, we measured adenosine-induced forearm vasodilation. According to our hypothesis, accelerated cellular adenosine uptake would decrease the amount of free extracellular adenosine able to stimulate adenosine receptors and, consequently, would attenuate adenosine-induced vasodilation in this patient group. Inhibition of cellular adenosine uptake by dipyridamole should restore this diminished response. Additionally, we aimed to more directly demonstrate homocysteine-induced increased intracellular formation of AdoHcy and subsequent accelerated adenosine uptake in isolated human erythrocytes.

Methods

Subjects
After approval of the local ethics committee, adult patients with hyperhomocysteinemia due to homozygous cystathionine β-synthase deficiency from our outpatient clinic were asked to participate if their fasting total plasma homocysteine was more than 20 μmol/L despite treatment. Exclusion criteria were mental retardation, previous vascular events, asthma, and oral anticoagulation. Twelve patients were considered eligible, and nine agreed to participate and stopped one week before the experiment) and alendronic acid (70 mg weekly in 2 patients). A control group of eight healthy volunteers was composed with similar age and body mass index as the patient group.

Experimental Protocol
Immediately after insertion of the microdialysis probes, dialysate sampling was started. Two hours after insertion, both microdialysis probes were removed and suspended in isotonic saline for in vitro calibration, as previously described.

Subsequently, baseline FBF was measured during infusion of saline followed by infusion of increasing dosages of adenosine (0.5, 1.5, and 15 μg/min/dL forearm). After 30 minutes of equilibration, baseline FBF measurement was repeated, followed by infusion of dipyridamole (100 μg/min/dL) and increasing dosages of adenosine (0.15, 0.5, and 1.5 μg/min/dL) on top of dipyridamole infusion. In combination with dipyridamole, we used lower concentrations of adenosine because of the well-known potentizing effect of dipyridamole on adenosine-induced vasodilation. Finally, maximal vasodilation was measured during postocclusive reactive hyperemia to test for possible structural vascular changes in the patient group, as described previously.

Adenosine Uptake in Isolated Erythrocytes
In 6 additional healthy volunteers, erythrocytes were isolated for in vitro experiments. In hyperhomocysteinemia, erythrocytes are relevant in the regulation of circulating endogenous adenosine because adenosine is efficiently taken up by erythrocytes through the dipyridamole-sensitive transporter and because homocysteine and AdoHcy are increased in erythrocytes of patients with hyperhomocysteinemia.

Freshly isolated erythrocytes were resuspended in MOPS buffer to obtain a 2% solution. Fifty-μL portions were incubated at 37°C with 1-homocysteine (100 μmol/L) in DTT and with DTT alone for 10 minutes (paired experiments). Subsequently, adenosine was added in a final concentration of 1 μmol/L. After 0, 3, 6, 10, and 15 minutes, adenosine uptake and deamination were completely blocked with high-dose dipyridamole (10 μmol/L) and ethyro-9-(2-hydroxynon-3-yl)-adenine (8 μmol/L), respectively. Subsequently, after centrifugation through a dibutylphltalate layer, the adenosine concentration in the supernatant and the AdoHcy concentration in the erythrocytes were determined. The effect of homocysteine on adenosine uptake was maximal after 6 minutes of uptake. To investigate the adenosine concentration dependency of this homocysteine effect, an additional series of experiments was conducted with 6 minutes of adenosine uptake but with a variable adenosine concentration ranging from 0.125 μmol/L to 2 μmol/L (n = 4). Finally, we determined the inhibiting effect of dipyridamole (0.2 μmol/L, 5-minute incubation) on the accelerating effect of homocysteine with 6 minutes of adenosine uptake (n = 2).

Drugs and Solutions
Solutions of adenosine (Adencor, Sanofi-Synthelabo) and dipyridamole (Persantin, Boehringer Ingelheim) were freshly prepared with NaCl 0.9% as solvent. 1-Homocysteine was freshly prepared in DTT and with DTT alone for 10 minutes (paired experiments). Subsequently, adenosine was added in a final concentration of 1 μmol/L. After 0, 3, 6, 10, and 15 minutes, adenosine uptake and deamination were completely blocked with high-dose dipyridamole (10 μmol/L) and ethyro-9-(2-hydroxynon-3-yl)-adenine (8 μmol/L), respectively. Subsequently, after centrifugation through a dibutylphltalate layer, the adenosine concentration in the supernatant and the AdoHcy concentration in the erythrocytes were determined. The effect of homocysteine on adenosine uptake was maximal after 6 minutes of uptake. To investigate the adenosine concentration dependency of this homocysteine effect, an additional series of experiments was conducted with 6 minutes of adenosine uptake but with a variable adenosine concentration ranging from 0.125 μmol/L to 2 μmol/L (n = 4). Finally, we determined the inhibiting effect of dipyridamole (0.2 μmol/L, 5-minute incubation) on the accelerating effect of homocysteine with 6 minutes of adenosine uptake (n = 2).

Analytical Procedures
Dialysate adenosine concentration was determined by high-performance liquid chromatography (HPLC) with reversed-phase ion-pairing separation and UV detection. Plasma homocysteine was determined by reverse phase (RP)-HPLC as described previously. Plasma AdoHcy and AdoMet were determined by tandem mass spectrometry, based on the work of Struyts et al.
Statistics

Values are expressed as mean±SE unless otherwise stated. P<0.05 is considered statistically significant. Because plasma concentrations of homocysteine, AdoHcy, AdoMet, and vitamins did not show a Gaussian distribution (P>0.1; Shapiro–Wilks test for normality), the Mann–Whitney test was used to compare groups. Other baseline parameters were normally distributed, and consequently a Student t test was used.

To compare adenosine-induced vasodilation between the two groups, a repeated measures ANOVA was used. Finally, for each subject, the area under the curves (AUC) of change in FBF was calculated for the adenosine-induced vasodilation with and without dipyridamole. The ratio of the AUC (with dipyridamole) and the AUC (without dipyridamole) was used to quantify the effect of dipyridamole. Because these ratios were not normally distributed, a Mann–Whitney test was used to compare groups.

In the in vitro experiments, the decrease of extracellular adenosine in time was fitted according to one phase exponential decay (GraphPad Prism version 4.00 for Windows, GraphPad Software), and half times were compared with paired Student t test.

Results

Baseline Characteristics

Demographic data are shown in Table 1. Plasma concentrations of total homocysteine, AdoHcy, and AdoMet were higher in the patient group (P<0.005). Treatment with high-dose folic acid and pyridoxine resulted in higher plasma concentrations of folate and vitamin B6 in the patient group (P<0.005). In one patient, we were not able to insert the intravenous probe and intrarterial cannula, and consequently only data on interstitial adenosine were available.

Microdialysis Experiments

Immediately after intramuscular insertion, dialysate adenosine concentration is known to be high because of myocyte damage and decreases to baseline level within one hour.9 To estimate basal interstitial adenosine concentration, we averaged the dialysate concentration of the two consecutive dialysate samples taken 1.5 hours after insertion. In the patient group, basal adenosine concentration was 96±21 nmol/L (n=7; 2 patients were excluded because of extremely low concentration of creatine and phosphocreatine in the first microdialysis sample, indicating misplacement of the probe), whereas in the control group basal adenosine concentration was 73±9 nmol/L (n=8, P=0.3).

The dialysate adenosine concentration from the intravascular probe was at steady-state immediately after insertion. Therefore, we averaged the values of all dialysate samples to one value. In the patient group this baseline concentration yielded 142±33 nmol/L (n=8), whereas in the control group it yielded 135±25 nmol/L (n=8, P=0.7). Adenosine recovery of the intramuscular and intravascular probes was 48±4% and 47±5% for patients and 47±4% and 39±2% for controls, respectively (P=0.9 and 0.2, respectively).

Plethysmography Experiments

Baseline FBF in the infused arm was 2.0±0.2 and 2.5±0.5 mL/min/dL for patients and controls, respectively (n=8; P=0.4). During infusion of increasing adenosine dosages, FBF in the patient group was 2.9±0.4, 4.3±0.5, 5.6±1.1, and 9.6±2.1 mL/min/dL, respectively (Figure 2). In the control group, FBF was 2.8±0.6, 4.4±1.0, 9.0±1.7, and 17.0±3.1 mL/min/dL, respectively. This adenosine-induced vasodilation was attenuated in the patient group (P<0.05).

After 30-minute equilibration, baseline FBF was 2.7±0.3 and 3.1±0.7 mL/min/dL in the patient group and control group, respectively (n=8; P=0.6). Infusion of dipyridamole increased FBF to 4.3±0.5 mL/min/dL in patients and to 4.7±1.0 in controls (P=0.7 between groups). Subsequent infusion of adenosine on top dipyridamole increased FBF to 10.9±1.6, 16.3±2.6, and 27.2±2.9 mL/min/dL in patients and to 8.8±1.4, 14.8±2.4, and 24.4±4.0 in controls, respectively (P=0.9). The ratio of the AUC for adenosine-induced vasodilation with dipyridamole and without dipyridamole was 0.40±0.08 in the patient group and 0.16±0.03 in the control group (P<0.05). Moreover, with both experimental groups taken together, there was a negative correlation between total plasma homocysteine concentration and the
TABLE 2. Systemic Effects of Adenosine Infusion

<table>
<thead>
<tr>
<th></th>
<th>Heart Rate</th>
<th>Mean Arterial Pressure</th>
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<tr>
<td></td>
<td>Patients</td>
<td>Controls</td>
</tr>
<tr>
<td>Baseline 1</td>
<td>62±4</td>
<td>57±2</td>
</tr>
<tr>
<td>Ado 0.5</td>
<td>61±4</td>
<td>55±1</td>
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<tr>
<td>Ado 1.5</td>
<td>63±4</td>
<td>56±1</td>
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<tr>
<td>Ado 5.0</td>
<td>62±4</td>
<td>56±1</td>
</tr>
<tr>
<td>Ado 15.0</td>
<td>65±3*</td>
<td>57±2</td>
</tr>
<tr>
<td>Baseline 2</td>
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<td>57±2</td>
</tr>
<tr>
<td>Dipy</td>
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<td>57±2</td>
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<tr>
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<td>69±4</td>
<td>60±2</td>
</tr>
<tr>
<td>Dipy + ado 0.5</td>
<td>70±4</td>
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</tr>
<tr>
<td>Dipy + ado 1.5</td>
<td>76±5†</td>
<td>70±2†</td>
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</tbody>
</table>

*Significant increase in heart rate as compared to baseline 1 (P<0.05) and †baseline 2 (P<0.001). No differences between groups.

AUC for adenosine-induced vasodilation (Spearman $r=-0.53$, $P=0.035$) and a positive correlation between plasma homocysteine and the effect of dipyridamole expressed as the ratio of the AUC's for adenosine-induced vasodilation with and without concomitant infusion of dipyridamole (Spearman $r=0.59$, $P=0.015$).

During the experiment, neither mean arterial pressure (MAP) nor the FBF in the control arm differed between the patient group and the control group ($P>0.1$; ANOVA for repeated measures). The effects of adenosine on heart rate and blood pressure are shown in Table 2. Concomitant infusion of adenosine with dipyridamole was associated with an increase in heart rate in both groups (ANOVA for repeated measures, $P<0.001$). Also, infusion of adenosine without dipyridamole was associated with an increase in heart rate in the patient group ($P<0.05$). Blood pressure was not influenced by adenosine infusion. There were no differences between both groups.

Finally, minimal forearm vascular resistance (MAP/FBF) during postocclusive reactive hyperemia was 2.1±0.2 and 2.4±0.2 AU for patients and controls, respectively ($P=0.3$).

Adenosine Uptake in Isolated Erythrocytes

Plasma homocysteine of the 6 subjects in this study averaged 9.1±0.9 $\mu$mol/L. Adenosine uptake into the erythrocytes and subsequent metabolism results in a decrease of extracellular adenosine in time (Figure 3a). Adenosine uptake was accelerated by incubation with homocysteine (half time 6.4±0.3 versus 8.1±0.5 minutes, $P<0.001$). Intracellularly, AdoHcy increased in time in cells incubated with homocysteine, but not in control samples (Figure 3b; $P<0.0001$, ANOVA for repeated measures). At 6 minutes, adenosine in the supernatant was lower in the homocysteine-incubated cells. This absolute homocysteine-induced difference in adenosine concentration significantly increased when the initial adenosine concentration was varied from 0.125 $\mu$mol/L to 2.0 $\mu$mol/L (Figure 3c and 3d; $P<0.05$, ANOVA for repeated measures).

Finally, dipyridamole significantly inhibited the accelerating effect of homocysteine on cellular adenosine uptake. Homocysteine decreased the free extracellular adenosine concentration after 6 minutes of adenosine uptake with 14.5±0.5% in the absence and with 2.9±0.5% in the presence of dipyridamole (Figure 4; $P<0.05$, n=2).

Discussion

In the present study, we show for the first time that adenosine-induced forearm vasodilation is attenuated in patients with hyperhomocysteinemia due to classical homocystinuria, and that this attenuation is completely restored by coinfusion of the adenosine uptake inhibitor dipyridamole.

The association between hyperhomocysteinemia and atherosclerosis and thrombosis was first established in patients with classical homocystinuria.17 Without treatment, 50% of suchlike patients experience a vascular event before the age of 30 years.18 Treatment is aimed at minimizing the biochemical abnormalities and consists of pyridoxine (vitamin B6) and folic acid and, if necessary, betaine anhydricum and vitamin B12.19 This treatment regimen significantly improves vascular outcome.20 Our patients continued to have elevated plasma homocysteine levels despite treatment. Because of the defective degradation of homocysteine to cystathionine, homocysteine can only be remethylated to methionine or converted to AdoHcy. This altered metabolic profile is illustrated by the increased plasma concentrations of AdoHcy and...
AdoMet in our patient group. Previous in vivo experiments on vascular function in this patient group are scarce and demonstrated impaired flow-mediated dilation and carotid artery wall hypertrophy.\textsuperscript{21–23}

In the present study, adenosine-induced forearm vasodilation was reduced in patients with hyperhomocysteinemia. Based on the observations that dipyridamole restores this impaired vasodilation and that in erythrocytes homocysteine accelerates cellular adenosine uptake, we conclude that this impaired adenosine-induced vasodilation is caused by accelerated cellular adenosine uptake. Adenosine-induced vasodilation is partly endothelium-dependent.\textsuperscript{24} We did not use alternative endothelium-dependent vasodilators to test for endothelial function in our patient group. However, as shown by previous studies in patients with classical homocystinuria, endothelial dysfunction could well also be present in our patients. Could endothelial dysfunction or structural vascular damage, both previously described in a comparable patient group, challenge our conclusion? In our opinion this is not the case. The observation that dipyridamole restored adenosine-induced vasodilation suggests that accelerated adenosine uptake through the equilibrative nucleoside transporter, rather than endothelial dysfunction, accounts for the attenuated vasodilation. It needs to be realized that dipyridamole is proposed to have alternative mechanisms of actions besides inhibition of nucleoside transport.\textsuperscript{25} Phosphodiesterase inhibition could theoretically potentiate endothelium-dependent cGMP-mediated dilation, thus improving this portion of adenosine-induced vasodilation. However, in an identical experimental model as in the present study, our group has previously shown that dipyridamole-induced local vascular effects are indeed solely caused by adenosine uptake inhibition. Dipyridamole potentiated the vasodilator response to adenosine,\textsuperscript{10} and dipyridamole-induced vasodilation (100 \( \mu \)g/min/dL) was inhibited by the adenosine receptor antagonist theophylline.\textsuperscript{26} Moreover, we showed in isolated erythrocytes that homocysteine indeed accelerates cellular adenosine uptake, which is counteracted by dipyridamole. Although the effects of homocysteine in this model are rather modest, these in vitro observations provide additional evidence that accelerated cellular adenosine uptake might account for the observed impaired adenosine-induced vasodilation.

Minimal forearm vascular resistance was similar in both groups. This parameter accurately reflects structural arteriolar status,\textsuperscript{27} indicating that in our patient group, at least in the forearm vascular bed, no structural vascular changes were present. It should be mentioned that reactive hyperemia is shown to be partially adenosine-dependent.\textsuperscript{28} This portion of the hyperemia in our study would theoretically be diminished in the patients with hyperhomocysteinemia. However, because of the maximal stimulus for vasodilation after 13 minutes of ischemia, other mediators probably compensate for this decrease in adenosine-induced vasodilation.\textsuperscript{27}

In previous experiments, it was shown in guinea pig hearts\textsuperscript{29–30} and rat brain\textsuperscript{31} that perfusion with homocysteine (thiolactone) decreased organ release of adenosine. The results from our in vitro experiments showed that also in human erythrocytes, homocysteine accelerates cellular uptake of adenosine. Moreover, we demonstrated that this is indeed associated with increased intracellular formation of AdoHcy. Finally, this effect of homocysteine is more pronounced at higher concentration of adenosine (Figures 2 and 3). This observation could well explain why baseline endogenous adenosine, as estimated by microdialysis, was not reduced in our patient group. This is in strong contrast to the study by Chen et al, which demonstrated an \( \approx \)50% reduction in endogenous baseline adenosine concentration induced by mild elevation of plasma homocysteine from 6.7±0.4 to 14.7±0.5 \( \mu \)mol/L.\textsuperscript{7} This discrepancy could be caused by the differences between man and rat concerning protein-binding of homocysteine,\textsuperscript{6} AdoHcy hydrolase activity,\textsuperscript{3} and characteristics of the equilibrative nucleoside transporter.\textsuperscript{32} Also, the experimental methionine-induced hyperhomocysteinemia in the rat model differs from the hyperhomocysteinemia in our patient group, possibly affecting tissue and cellular distribution of homocysteine. But most importantly, the results from our in vitro studies suggest that the effect of AdoHcy synthesis on the rate of cellular adenosine uptake is limited to situations of high extracellular adenosine concentrations, as for example in ischemia, when myocardial interstitial adenosine concentration can increase up to 40-fold in pigs.\textsuperscript{7} A recent microdialysis study on the pig heart showed that local application of homocysteine reduced dialysate adenosine concentration during hypoxia, but not during normoxia.\textsuperscript{7} Likewise, Kloor et al concluded from a rat study that homocysteine is the rate-limiting factor for AdoHcy synthesis in hypoxic conditions when tissue levels of adenosine are elevated, whereas in normoxia the availability of free adenosine is rate-limiting.\textsuperscript{6} This suggests that also in vivo, the effect of homocysteine on adenosine concentration is more pronounced and therefore more easily detected in situations of high concentrations of adenosine than under normoxic baseline conditions. Unfortunately, for practical reasons we were not able to collect microdialysis samples during infusion of adenosine or during ischemia.

We have shown that the vasodilating effect of adenosine, which was reduced in hyperhomocysteinemia, was com-

![Figure 4. Extraglucal concentration of adenosine after 0 and 6 minutes of adenosine uptake by isolated human erythrocytes in the absence (open bars) and presence (filled bars) of homocysteine. Dipyridamole significantly reduces the accelerating effect of homocysteine on adenosine uptake (\( P<0.05 \), \( n=2 \)).](image-url)
pletely restored by the nucleoside uptake inhibitor dipyridamole. Extrapolating this finding, dipyridamole would be beneficial in patients with hyperhomocysteinemia by preserving the protective cardiovascular effects of adenosine in hypoxia or ischemia. To our best knowledge, dipyridamole has never been tested systematically in such patients in clinical trials.

Several limitations of the present study need to be discussed. First, we used a group of patients with classical homocystinuria, using high doses of vitamins, including vitamin B6 and folic acid. It remains to be established whether changes in adenosine metabolism are also important in patients with mild hyperhomocysteinemia. Considering the vitamin therapy in our patient group, we are not aware of any actions of these vitamins on nucleoside transport. Secondly, considering the large interindividual variation, microdialysis may lack sufficient sensitivity to exclude subtle differences in baseline adenosine concentration between the two study groups. Moreover, the values obtained with microdialysis might not reflect the adenosine concentrations in specific microenvironments, such as near the endothelial lining.

In conclusion, in patients with severe hyperhomocysteinemia, adenosine-induced effects are impaired, which could contribute to the cardiovascular complications of this disease.

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