Endothelial Release of Nitric Oxide Contributes to the Vasodilator Effect of Adenosine in Humans

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Background The endogenous nucleoside adenosine plays an important role in the regulation of vascular tone, especially during ischemia. Experimental data derived from animal models suggest that nitric oxide (NO) contributes to the vasodilator effect of adenosine. The primary purpose of this investigation was to determine whether the endothelial release of NO contributes to adenosine-induced vasodilation in humans.

Methods and Results Venous occlusion plethysmography was used to assess the forearm blood flow (FBF) responses to graded intra-arterial infusions of adenosine (1.5 to 500 μg/min). Dose-response curves were constructed before and during intra-arterial infusion of the NO synthase inhibitor Nω-monomethyl-L-arginine (L-NMMA) (2 mg/min, n=6) or vehicle (n=6). Before infusion of L-NMMA, adenosine caused a dose-dependent increase in FBF from 2.3 to 15.9 mL·min⁻¹·dL⁻¹. During concurrent infusion of L-NMMA, adenosine increased FBF from 1.7 to 10.0 mL·min⁻¹·dL⁻¹, and this change from baseline was significantly reduced compared with that before L-NMMA (P<.05). L-NMMA also attenuated the FBF response to adenosine when the basal constrictor effect of L-NMMA was prevented by coinfusion of the NO donor sodium nitroprusside (n=6, P<.01). In contrast, L-NMMA did not affect the FBF response to intra-arterial infusion of the endothelium-independent vasodilator verapamil (from 2.0 to 13.9 mL·min⁻¹·dL⁻¹ before L-NMMA and from 1.3 to 13.6 mL·min⁻¹·dL⁻¹ during L-NMMA; n=6, P=NS). The second objective of this study was to determine whether the adenosine-induced release of NO is mediated by activation of endothelial potassium channels, putatively coupled to adenosine receptors. Thus, the FBF response to adenosine was measured before and during infusion of the ATP-dependent potassium channel blocker tolbutamide (1 mg/min, n=6), or the potassium channel blocker quinidine (0.5 mg/min, n=6). The adenosine-mediated increments in FBF were not attenuated by either potassium channel blocker.

Conclusions Adenosine-induced vasodilation in humans is mediated, at least in part, by endothelial release of NO. The transducing mechanism of this phenomenon is not known, but it does not appear to involve the activation of either ATP-dependent or quinidine-sensitive potassium channels. (Circulation. 1995; 92:2135-2141.)

Key Words • nitric oxide • vasodilation • endothelium • adenosine
Selected Abbreviations and Acronyms

FBF = forearm blood flow
L-NMMA = N\textsuperscript{\text{\(\text{\textminus}\)}}-monomethyl-L-arginine
NO = nitric oxide
SNP = sodium nitroprusside
cells.\textsuperscript{13,14} In addition, potassium channel activation and NO release can be triggered nonspecifically by increments in shear stress related to the raised flow.\textsuperscript{15}

In the present study we evaluated whether the endothelial release of NO plays an important role in the vasodilating effects of adenosine in humans and, if it does, whether the aforementioned potential mechanisms are involved in that NO release. Human data on this subject are especially important given the large interspecies differences in endothelial pharmacology.\textsuperscript{16} To address these questions, the effects of adenosine were assessed in vivo by use of the perfused forearm technique, both before and during blockade of the production of NO by the NO synthase inhibitor L-NMMA and before and during blockade of potassium channels by the sulfonyleurea derivative tolbutamide and the antiarrhythmic drug quinidine. These studies, as well as appropriate control experiments, allow us to conclude that the vascular effects of adenosine in humans are based, at least in part, on the endothelial release of NO. This adenosine-induced release of NO is not mediated by activation of ATP-dependent potassium channels or by quinidine-sensitive potassium channels.

**Methods**

Thirty-nine healthy volunteers participated in the study, and all gave written informed consent before participation. The health status of each volunteer was determined by medical history and a physical examination as well as by laboratory investigations to assess lipid levels. Demographic data of the participants are shown in Table 1. Subjects who had high blood pressure (>145/90 mm Hg) or high concentrations of serum cholesterol (LDL greater than the 75th percentile for age and sex) or who smoked cigarettes were excluded from participation because these features can interfere with the NO pathway.\textsuperscript{17,20}

Each subject participated in one experiment of approximately 4 hours’ duration. The experiments were performed in a quiet, temperature-controlled room (22°C). The participants were all asked to abstain from food, alcohol, and caffeine for at least 12 hours before the test. Because caffeine acts as a potent adenosine receptor antagonist in the human cardiovascular system,\textsuperscript{21} we measured plasma caffeine levels at the time of the test to ensure compliance with respect to the abstinence recommendations. The subjects remained in the supine position throughout the test. After each subject’s arrival in the laboratory, the left brachial artery was cannulated under local anesthesia with a 20-gauge catheter, which was used for intra-arterial drug infusion as well as for blood pressure monitoring (Gould Inc). Drugs were delivered with an automatic syringe infusion pump (Harvard Apparatus).

FBF recordings were started after an equilibration period of at least 40 minutes. FBF was measured in each arm by venous occlusion mercury-in-Silastic strain-gauge plethysmography (Hokanson EC4, D.E. Hokanson) as previously described.\textsuperscript{19} Both arms rested in slings at heart level, with the forearms slightly elevated to ensure a sufficient venous return. To be sure that FBF recordings referred predominantly to the forearm skeletal muscle circulation, the hand circulation was occluded during all FBF recordings by a wrist cuff inflated 100 mm Hg above the systolic blood pressure.\textsuperscript{22}

**Assessment of the Role of NO in the Vasodilator Response to Adenosine and Verapamil**

In the first subgroup of six subjects, the vasodilator response to increasing dosages of adenosine was investigated. Baseline measurements were taken during infusion of placebo (glucose 5%) into the brachial artery. Adenosine was then infused intravenously in six increasing dosages for 4 minutes per dose (adenosine dosages were 1.5, 5, 15, 50, 150, and 500 \(\mu\)g/min). Measurements were performed during the last 2 minutes of each infusion. After an equilibration interval of 60 minutes in which the FBF was allowed to return towards baseline levels, new baseline recordings were obtained during placebo infusion. Subsequently, infusion of L-NMMA into the brachial artery was started at a dose of 2 mg/min. After 15 minutes of the L-NMMA-infusion alone, hemodynamic measurements were taken to evaluate the vasoactive effects of L-NMMA. Thereafter, the six increasing adenosine dosages were again administered and coinfused with L-NMMA. The total duration of L-NMMA infusion was approximately 40 minutes. In an additional series of three subjects, we verified that infusion of L-NMMA at 2 mg/min for 40 minutes induced a steady-state vasoconstrictor response that did not progress over time.

In the next group of six subjects, similar experiments were performed with a similar time schedule and drug dosages of adenosine and L-NMMA, but with these experiments the L-NMMA infusion was accompanied by a low intra-arterial dose of the NO donor SNP. Previous studies have shown that L-NMMA infusion induces a vasoconstrictor response in the forearm vascular bed by inhibiting the basal release of NO from the endothelium.\textsuperscript{23} From a methodological viewpoint, this change in baseline could complicate the interpretation of the results, given that the baseline vascular resistance is an important determinant of the response to a vasodilator stimulus\textsuperscript{24} and that the achieved concentrations of adenosine are proportionally higher in a vasoconstricted state. From pilot studies, we determined that an intra-arterial dose of SNP of 0.2 \(\mu\)g/min was appropriate to counteract the vasoconstrictor response to L-NMMA. Therefore, this dose of SNP was coinfused with L-NMMA. The “clamping” of the NO-mediated vascular tone by the concomitant infusion of L-NMMA and SNP has recently been shown to be a useful approach in animal experiments on this subject.\textsuperscript{25} An additional six subjects were studied for reasons of time control. The same time schedule was used as in the other adenosine experiments; however, the second dose-response measurement was performed in the presence of placebo instead of active drug. This study was done to ensure that the forearm vasodilator response to adenosine did not change over the 4-hour experimental session.

In theory, NO release from the endothelium may have been triggered nonspecifically by the adenosine-induced increase of shear stress resulting from the high FBFs. To evaluate this nonspecific stimulus of NO release, we measured FBF responses to the endothelium-independent calcium entry blocker verapamil before and after L-NMMA (2 mg/min) in another six subjects. Verapamil was used in four dose-steps of 5 minutes each (10, 30, 100, 300 \(\mu\)g/min). Because verapamil has a longer half-life than adenosine, we waited for 90 minutes to wash out the drug after the first series of verapamil infusion. Apart from these details, the time schedule was similar to that used in the previous experiments.

**Assessment of the Role of Potassium Channel Activation in the Vasodilator Response to Adenosine**

It recently was shown that adenosine receptors are coupled to ATP-dependent potassium channels.\textsuperscript{11} These channels can be blocked specifically by sulfonyleurea derivatives, not only in
the pancreatic β cells but also at the level of the vessel wall. Therefore, adenosine dose-response studies were performed in a group of six volunteers before and during the intraarterial infusion of tolbutamide (1 mg/min). The infusion of tolbutamide started 15 minutes before the first adenosine dose. Because systemic dosages of tolbutamide may induce hypoglycemia by stimulating insulin release and because insulin increases skeletal muscle blood flow and alters vascular responsiveness to drugs in the forearm model, arterial blood samples for glucose and insulin were taken just before and after tolbutamide infusion to demonstrate that no systemic effects occurred at this low dose.

In an additional six healthy volunteers, the vascular response to adenosine was assessed before and during the intra-arterial infusion of 0.5 mg/min quinidine. Studies have shown by use of the patch-clamp technique that quinidine blocks several types of potassium channels, including those activated by adenosine. To limit the cumulative dose of quinidine in healthy volunteers, we used only the four highest adenosine dosages (instead of all six) in these subjects. Measurements of FBF and subsequent administration of the four adenosine dosages were started after 10 minutes of quinidine infusion, and the total infusion time of quinidine was restricted to 30 minutes (cumulative dose, 15 mg).

Drugs
Adenosine was prepared for each experiment by dilution of Adenocard vials (6 mg per 2 mL, Fujiwasa Pharmaceutical Co) in saline. N\textsuperscript{\textbeta}monomethyl-L-arginine acetate, purchased from Calbiochem, was diluted in glucose 5% just before the experiments. SNP was purchased from Elkins-Sinn, Inc. Protected from light, it was dissolved in glucose 5% just before administration. Verapamil HCl (American Reagent Laboratories Inc) was diluted in glucose 5%. For the tolbutamide experiments, sterile tolbutamide sodium (Orinase Diagnostic, The Upjohn Co) was dissolved in saline (NaCl 0.9%). For the quinidine studies, we used quinidine gluconate vials (Eli Lilly) of 800 mg/10 mL diluted in glucose 5%. Depending on the experiment, glucose 5% or saline was used as placebo infusion. All drugs and placebo infusions were administered at the same infusion rate of 0.4 mL/min.

Statistics and Calculations
The effects of adenosine were analyzed by comparison of the hemodynamic variables at baseline and at the six dosage levels by one-way ANOVA with repeated measures. Post hoc comparisons between the different dosages were made by Scheffe’s F tests. The paired t test was used for the assessment of the effects of L-NMMA, tolbutamide, or quinidine on baseline parameters. To evaluate the effect of the intervention (NO synthase blockade, potassium channel blockade) on the adenosine or verapamil responses, two-way repeated-measures ANOVA was performed on the changes from baseline. Because the mean arterial blood pressure was not affected by either drug infusion (see “Results”), FBF changes were assumed to represent changes in forearm vascular tone. Differences were considered to be statistically significant at P<.05 (two-tailed). All results are presented as mean±SEM unless otherwise indicated.

Results
Vasodilator Response to Adenosine
For assessment of the vascular response to graded adenosine infusion, the data of the first dose-response curve for all experiments with the six adenosine dosages were pooled (n=24). Table 2 summarizes the results of this analysis. Adenosine induced a dose-dependent increase in FBF, which was significantly different from baseline for the third through sixth dosage levels. The dose dependency was supported by significant differences between dosages (Scheffe’s F tests, P<.05). One hour after cessation of adenosine infusion, the FBF was identical to the baseline level. As shown in Table 2, there were no changes in flow in the noninfused forearm or in blood pressure or heart rate during adenosine infusion, arguing against any systemic effect of the dosages used.

In the subset of experiments in which the adenosine infusions were repeated after 1 hour without any intervention (time controls), adenosine increased FBF the first time from 2.3±0.4 to 15.2±4.7 mL/min\textsuperscript{-1}•dL\textsuperscript{-1} and the second time from 1.9±0.7 to 16.0±7.2 mL/min\textsuperscript{-1}•dL\textsuperscript{-1}. There was no significant difference between the FBF changes from baseline between the first and second series of measurements.

Plasma caffeine levels could not be detected in 20 of 23 subjects in whom it was measured (lower limit of assay, 0.5 mg/L). In the remaining three subjects, caffeine concentration ranged from 1.8 to 4.0 mg/L. Despite detectable caffeine levels in these three subjects, each showed a vasodilator response to adenosine that was comparable to that observed in the others, justifying inclusion of their data in the results. Moreover, statistical analysis after exclusion of these subjects did not affect the outcome of the findings.

Vasoconstrictor Response to L-NMMA
To assess the effect of L-NMMA on basal FBF, the data from six L-NMMA–adenosine experiments, three L-NMMA time-control experiments, and six L-NMMA–verapamil experiments were pooled. After 15 minutes of intra-arterial L-NMMA infusion, FBF decreased significantly from 2.1±0.2 to 1.5±0.1 mL/min\textsuperscript{-1}•dL\textsuperscript{-1} (n=15, paired t test; P<.01). In contrast, the FBF of the contralateral noninfused arm remained constant during this infusion (1.8±0.1 before and 1.8±0.1 mL/min\textsuperscript{-1}•dL\textsuperscript{-1} during L-NMMA infusion). Moreover, L-NMMA infusion did not change the mean arterial blood pressure (81.3±2.3 versus 79.1±2.7 mm Hg, P=NS) or heart rate (56.1±2.2 versus 54.9±2.1 beats per minute, P=NS), indicating that local L-NMMA infusion did not affect systemic hemodynamics. In the three subjects in whom L-NMMA was the only drug given, the FBF fell from 1.8±0.3 mL/min\textsuperscript{-1}•dL\textsuperscript{-1} at baseline to 1.3±0.2 and 1.6±0.1 mL/min\textsuperscript{-1}•dL\textsuperscript{-1} after 15 and 40 minutes, respectively, arguing against a progressive vasoconstrictor effect after 15 minutes of infusion.

Effects of NO Synthase Blockade on Vasodilator Responses
Fig 1 illustrates the FBF response to the six increasing adenosine dosages during the placebo and L-NMMA infusions. Adenosine in the presence of placebo induced a dose-dependent increase in FBF from 2.3±0.2 to 15.9±3.1 mL/min\textsuperscript{-1}•dL\textsuperscript{-1}. During coinfusion with L-NMMA, adenosine increased FBF from 1.7 to 10.0 mL/min\textsuperscript{-1}•dL\textsuperscript{-1}, this response being significantly reduced compared with the first dose-response curve. The concomitant infusion of L-NMMA and adenosine elicited no significant changes in contralateral FBF (from 1.8±0.2 mL/min\textsuperscript{-1}•dL\textsuperscript{-1} at baseline to 2.3±0.3 mL/min\textsuperscript{-1}•dL\textsuperscript{-1} at the highest adenosine dose), mean arterial pressure (from 80±4 to 83±4 mm Hg), or heart rate (from 55±2 to 54±2 beats per minute).
In the second series of experiments, the vasoconstrictor effect of L-NMMA was counteracted by concomitant infusion of SNP. Again, in the presence of placebo, adenosine caused a dose-dependent forearm vasodilator effect with an increase of FBF from 1.9±0.4 to 14.9±2.0 mL·min⁻¹·dL⁻¹ (Fig 2). After conditions returned to baseline, the combined infusion of L-NMMA and SNP did not significantly change FBF (2.0±0.3 versus 2.2±0.3 mL·min⁻¹·dL⁻¹; n=6, P=NS). The subsequent administration of the six adenosine dosages elicited dose-dependent increments in FBF from 2.2±0.3 to 9.8±2.4 mL·min⁻¹·dL⁻¹; the changes from baseline were significantly reduced compared with those before administration of L-NMMA and SNP (P<.01) (Fig 2). No significant changes occurred in contralateral FBF (from 79±2 to 81±3 mm Hg), or heart rate (from 53±2 to 53±3 beats per minute).

Effects of Potassium Channel Blockade on Adenosine Responses

Fig 3 demonstrates the effects of tolbutamide on the vasodilator response to adenosine. Tolbutamide infusion into the brachial artery did not change the baseline FBF (2.3±0.3 versus 2.2±0.3 mL·min⁻¹·dL⁻¹). Moreover, the adenosine-induced increase in FBF was not reduced by tolbutamide.

Fig 4 illustrates the effects of quinidine on the forearm vasodilator response to adenosine infusion. In this series, adenosine increased FBF from 1.6±0.3 to 9.0±0.8 mL·min⁻¹·dL⁻¹. After equilibration, the FBF returned to a baseline value of 1.5 mL·min⁻¹·dL⁻¹. Ten minutes of quinidine infusion increased the baseline FBF significantly to 2.9±0.7 mL·min⁻¹·dL⁻¹ (P<.05). However, regional quinidine infusion did not significantly affect the adenosine-induced increase in FBF from baseline.

Throughout these two series of experiments, there were no changes in contralateral FBF, blood pressure, or heart rate. The plasma insulin concentrations averaged 4.4±1.2 µU/mL before and 4.5±1.2 µU/mL after tolbutamide administration (P=NS), and the glucose levels were 84±3 and 81±2 mg/dL, respectively (P=NS).
The vasodilator response to adenosine in humans is mediated, at least in part, by the endothelial release of NO. The following evidence supports this conclusion: (1) blockade of NO synthase by L-NMMA significantly reduced the FBF response to adenosine, (2) the vehicle-control experiments ensured that this reduced response could not be attributed to time; and (3) the observation that L-NMMA attenuated adenosine responses could not be attributed to a change in basal vascular resistance, because restoration of baseline conditions by the addition of a low dose of SNP did not change the results. We reasoned that the coinfusion of SNP and L-NMMA was the most ideal approach to correct for the L-NMMA-associated change in baseline vascular resistance. Addition of exogenous NO by infusion of SNP was thought to restore the baseline biology of the vascular wall and recondition its responsiveness to vasoreactive stimuli. Also, the vasoconstrictor effect of L-NMMA (when infused alone) did not progress over time and thus could not account for the L-NMMA-mediated reduction in adenosine responses. Apart from the pathophysiological consequences of our conclusion, it is obvious that the use of adenosine for the assessment of endothelium-independent vasodilation in peripheral resistance vessels is not a valid approach in human research.

Several recent studies in animals support our observations, but others do not. Species differences and different experimental conditions may contribute to this discrepancy. In the perfused-forearm technique used in this study, the drug is administered into the vascular lumen, so the exposure to adenosine is much higher for the vascular endothelium than for the underlying smooth muscle cell layer, especially because of the efficient uptake of adenosine by the endothelium. In several in vitro vascular preparations, the exposure is more balanced, and therefore direct relaxant effects on smooth muscle cells mediated by stimulation of A_{1}-adenosine receptors may have been more pronounced. Shear Stress as a Mechanism of Action

A number of transducing mechanisms should be considered as mediators for the ability of adenosine to release endothelium-derived NO. For example, the adenosine-induced release of NO might have been triggered nonspecifically by the increase in flow (shear stress) rather than by the specific stimulation of endothelial adenosine receptors. However, in previous studies in which the same technique was used, the intra-arterial infusion of L-NMMA reduced the relaxant effects of acetylcholine but not those of SNP, arguing against a contribution of flow-related NO release during drug-induced elevations of FBF. Furthermore, in the present study, L-NMMA did not attenuate the vasodilator response to the calcium entry blocker verapamil, an endothelium-independent vasodilator, despite production of blood flow responses comparable to those observed in the middle of the dosage range of adenosine. Our data therefore point toward a more specific interaction between L-NMMA and adenosine. We and others previously showed that low intrabrachial dosages of the adenosine receptor antagonists caffeine and theophylline are able to block the forearm vasodilator response to adenosine, enabling us to postulate that the adenosine-mediated release of NO results from stimulation of endothelial adenosine receptors. Unfortunately, selective agonists and antagonists for the different subtypes of adenosine receptors are not available for human use, so it is not possible at this time to determine whether the adenosine-induced NO release in humans is mediated by stimulation of endothelial A_{1}- or A_{2a}-adenosine receptors.

Role of Potassium Channels

We postulated that activation of potassium channels may serve as an intermediate step, transducing stimulation of adenosine receptors to the synthesis and/or release of NO from the endothelium. Because equipotent dosages of the related sulfonylurea derivative glibenclamide have been shown to attenuate the vasodilator response to pharmacological opening of K\textsubscript{ATP} channels in the forearm vascular bed, we think that our negative observation of tolbutamide cannot be attributed to ineffective dosing. Consequently, our data support the conclusion that opening of the K\textsubscript{ATP} channel does not contribute to the vasodilator response to intra-arterial adenosine infusion in humans. In theory, K\textsubscript{ATP} channel opening might have contributed to the vasoactive effects of adenosine at two different levels. At the level of the endothelium, K\textsubscript{ATP} channel opening may hyperpolarize endothelial cells, and may thereby activate endothelial NO release by increasing the influx of calcium. In contrast to increases of calcium influx in endothelial cells after hyperpolarization, calcium influx decreases after membrane hyperpolarization in vascular smooth muscle cells. Therefore, opening of potassium channels also results in vasodilation at the level of vascular smooth muscle cells. It must be emphasized that because of our intraluminal administration of adenosine, its effects may have been predominantly endothelium dependent, and therefore we think that our tolbutamide data do not exclude a role of K\textsubscript{ATP} channel activation in the smooth muscle-relaxing effects of adenosine.
Although we realize that quinidine has several pharmacological properties, including sodium channel blockade and antiadrenergic effects, its recently established potassium channel-blocking properties have made this drug an additional tool for our study, especially because quinidine was able to attenuate the vasodilator response to adenosine in animal studies.13 Quinidine increased basal FBF but did not attenuate the vasodilator response to adenosine. The effect of quinidine on the baseline flow may well be explained by its α-adrenergic-blocking properties. In line with the reasoning on tobutamide, our data argue against a role of quinidine-sensitive potassium channels in the adenosine-mediated release of NO but do not exclude an interaction between quinidine and adenosine at the level of vascular smooth muscle cells.

Pathophysiological Implications

Apart from effects on vascular tone, adenosine has other important properties, including inhibition of platelet aggregation,44 inhibition of leukocyte activation,45 and presynaptic inhibition of noradrenaline release.46 Furthermore, adenosine appears to mediate ischemic preconditioning in the myocardium.47 The release of endogenous adenosine may also contribute to the reactive hyperemic response after ischemia,14,48,50-53 as well as to exercise-induced vasodilation.49 In recent years, NO has been demonstrated to affect several of these pathophysiological phenomena in a similar way.14,48,50-53 Because adenosine is released from tissue, including the endothelium, during anoxia or ischemia,44 the currently observed relation between adenosine and endothelial NO release makes endogenous adenosine a likely candidate for triggering NO release during ischemia. We believe that our results create the foundation for future studies on the interrelation between the release of endogenous adenosine and that of NO in several pathophysiological conditions in humans.

Acknowledgments

This research was supported by an NIH program project grant in vascular biology and medicine (HL-48743). M.A.C. is a recipient of an NHLBI academic award in systemic and pulmonary vascular medicine (HL-02663). The authors gratefully acknowledge Joanne Normandin for manuscript preparation.

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