Role of adenosine A$_{2B}$ receptors in vasodilation of rat pial artery and cerebral blood flow autoregulation

HWA KYOUNG SHIN,1 YUNG WOO SHIN,2 AND KI WHAN HONG1,3

Departments of 1Pharmacology and 2Internal Medicine, College of Medicine, Pusan National University, Pusan 602-739; and 3Center for Biofunctional Molecules, Pohang University of Science and Technology, Pohang 790-600, Korea

Shin, Hwa Kyoung, Yung Woo Shin, and Ki Whan Hong. Role of adenosine A$_{2B}$ receptors in vasodilation of rat pial artery and cerebral blood flow autoregulation. Am. J. Physiol. Heart Circ. Physiol. 278: H339–H344, 2000.—This study was aimed to investigate the underlying mechanism of vasodilation induced by the activation of A$_{2B}$ adenosine receptors in relation to cerebral blood flow (CBF) autoregulation. Changes in pial arterial diameters were observed directly through a closed cranial window. N’-nitro-L-arginine methyl ester (L-NAME, nitric oxide synthase inhibitor) significantly suppressed the concentration-dependent vasodilations induced by adenosine and 5’-N-ethylcarboxamido-adenosine (NECA) but not the vasodilation by CGS-21680 (A$_{2A}$-receptor agonist). Moreover, NECA-induced vasodilation was suppressed by alloxazine (1 µmol/l) but not by ZM-241385 (1 µmol/l, A$_{2A}$ antagonist), which suggests mediation by A$_{2B}$ receptor activation. Otherwise, the level of nitrite/nitrate was concentration dependently increased in the artificial cerebrospinal fluid (CSF) when adenosine and NECA were suffused over the cortical surface, L-NAME, and alloxazine, but not ZM-241385, largely inhibited their releases. The lower limit of CBF autoregulation was little affected following pretreatment with L-NAME or alloxazine. Thus it is suggested that adenosine-induced vasodilation via activation of A$_{2B}$-adenosine receptors of the rat pial artery is coupled to the production of nitric oxide, which contributes little to CBF autoregulation.

5’-N-ethylcarboxamido-adenosine; N’-nitro-L-arginine methyl ester; alloxazine; nitrite/nitrate

Adenosine A$_2$ receptors are regarded as coupling to stimulation of adenylate cyclase activity and the production of cAMP (8) and to mediate the dilator response of cerebral arterioles to adenosine (6, 24). Adenosine level increases in the brain with moderate hypotension, and its increment is related to the regulation of cerebral blood flow (CBF) (33). The adenosine A$_2$ receptors are further classified as A$_{2A}$ and A$_{2B}$ subtypes based on the receptor affinity (2). Cloning techniques have confirmed the existence of distinct A$_{2A}$ and A$_{2B}$ subtypes of A$_2$ receptor (29). Although the precise physiological functions of the A$_{2B}$ receptors remain undefined, roles for the A$_{2B}$ receptors have been suggested in the regulation of neuroglia functions (7), myocardial contractility (18), vascular smooth muscle tone (20), and intestinal chloride secretion (30).

Because of the lack of specific agonists or antagonists for the A$_{2B}$-adenosine receptor, little is known about the physiological role for the A$_{2B}$ receptors in cerebral autoregulation. Brackett and Daly (1) measured adenosine-evoked accumulations of cAMP in cultured NIH 3T3 fibroblasts showing the A$_{2B}$-subtype receptor. In this cell line, N’-N-ethylcarboxamido-adenosine (NECA) was highly selective for the A$_{2B}$ receptors, whereas 2-p-(2-carboxyethyl)-phenethylamino-5’-N-ethylcarboxamido adenosine (CGS-21680) (19), which is highly selective for A$_{2A}$-subtype receptors in the pheochromocytoma PC12 membranes, has a much lower potency in the fibroblasts. CGS-21680 was demonstrated to be selective for the A$_{2A}$ compared with the A$_{2B}$-subtype receptors in the rat hippocampus and striatum (19). Of the adenosine receptor antagonists, alloxazine has been demonstrated to be selective for the A$_{2B}$-subtype receptor with a selectivity of about ninefold for the NIH 3T3 fibroblasts over the PC12 A$_{2A}$ receptor (1), whereas ZM-241385 is highly selective for the A$_{2A}$ subtype (24).

According to the functional characterization of adenosine receptors in the vascular beds, NECA was also demonstrated to be a relatively selective agonist for the adenosine A$_{2B}$ receptors in the guinea pig aorta (10) and the rat mesenteric artery (28).

Recently, Hong et al. (13) demonstrated that when the cortical surface is suffused with cAMP, the release of adenosine is increased in the artificial cerebral spinal fluid (CSF), and they suggested that the cAMP-adenosine pathway as a viable metabolic mechanism is implicated in the production of adenosine in the rat pial artery and contributes to the regulation of vasodilation in response to hypotension.

On the other hand, Martin and Potts (21) documented that the rat renal artery contains A$_{2B}$-adenosine receptors that are located on the endothelium, and the A$_{2B}$ receptors cause release of nitric oxide. Moreover, nitric oxide and nitric oxide synthase expression have been evidenced in the cAMP-induced pial artery vasodilation (26). However, to our knowledge, the role of the adenosine A$_{2B}$-receptor subtype has not been clearly characterized in the pial arteries in relation to a physiological role such as the vasodilator response to acute hypotension and CBF autoregulation.

We therefore aimed to examine the effects of L-NAME on the vasodilation and nitrite/nitrate formation evoked...
by adenosine and NECA (adenosine A_{2B}-receptor predominant agonist), respectively, in comparison with those elicited by CGS-21680 (adenosine A_{2A}-receptor predominant agonist). Thereafter, we determined whether the lower limit of CBF autoregulation was shifted or not under pretreatment with ZM-241385 (A_{2A}-receptor antagonist), alloxazine (A_{2B}-receptor antagonist), and L-NAME (nitric oxide synthase inhibitor).

### MATERIALS AND METHODS

#### Preparation of animals
Male Sprague-Dawley rats (250–320 g) were anesthetized with urethane (1 g/kg ip) and placed on a heating pad (Homeothermic Blanket System, Harvard Apparatus, South Natick, MA) to maintain a constant rectal temperature (37 ± 0.5°C). After a tracheostomy, the rat was mechanically ventilated with room air by a respirator (model 683, Harvard Apparatus) after immobilization with 5 mg/kg gallamine triethiodide. The mean PCO2 was monitored with a capnometric analyzer (CapStar-100, IITC Life Science, Woodland Hills, CA). Catheters were placed in a carotid artery for measurement of systemic arterial blood pressure (Statham P23D pressure transducer, Gould, Cleveland, OH) and in a femoral artery for withdrawing and sampling arterial blood. The blood was collected before and after installation of a cranial window for blood gas and pH determination (STAT Profile 3, Nova Biomedicals, Boston, MA). The mean arterial blood gases and pH were within normal limits as shown in Table 1.

#### Measurement of vessel diameter
Pial microvessels were visualized through an implanted closed cranial window. The head was fixed in a prone position with a stereotaxic apparatus (Stoelting, Wood Dale, IL), and a square-shape (5 × 5 mm) burr hole was made over the right parietal cortex. The dura was resected with caution. Pial precapillary microvessels were visualized through the cranial window, where prewarmed artificial CSF saturated with a gas mixture of 95% O2-5% CO2 was constantly suﬀused over the cortical surface at 0.3 ml/min. Cerebral microvessels were allowed to equilibrate for 60–90 min after the installation of the cranial window. The image of the pial microvessels was captured with a charge-coupled device video camera (VDC 3900, Sanyo, Japan) through a stereomicroscope (model SMZ-2T, Nikon, Japan) at a speed of 30 frames per second. The vessel diameter was measured by a width analyzer (C3161, Hamamatsu Photonics, Hamamatsu, Japan) through a stereomicroscope (model SMZ-2T, Nikon, Japan). It was fed to a television monitor for direct observation.

#### Measurement of lower limit of CBF
The animal’s head was fixed in a stereotaxic instrument, and the animals spontaneously breathed room air. After a craniotomy was performed, the CBF to the pial artery over the parietal cortical surface was measured by using laser-Doppler flowmetry (Laserflo BPM2, Vasamedics, St. Paul, MN) equipped with a 1-mm-diameter needle probe (model P-433–5 Needle probe, Vasa medics, St. Paul, MN). After careful section of the dura mater, the probe was placed lateral to, but near the pial artery, in the open window and advanced into the CSF for 0.2-mm above the surface of the cortex. After measurement of two baselines at intervals of 10 min, the prewarmed artificial CSF saturated with a gas mixture of 95% O2-5% CO2 (37°C) containing antagonists was applied locally to the open window with a bolus volume of 100 µl for three times every 10 min. The baseline CBF levels were not altered during application of the antagonists by the concentrations used in this experiment. The antagonist concentration was expressed as micromoles per liter. The laser-Doppler flowmetry outputs were regarded as arbitrary units, and the changes in CBF were expressed as a percentage of the baseline CBF.

#### Nitrite/nitrate determination
Nitric oxide production, as assessed by nitrite/nitrate concentrations in the artificial CSF, was determined by using the Griess reagent (Promega, Madison, WI). Briefly, aliquots (3 ml) of artificial CSF drained from the cortical surface were collected for 5 min during the suffusion containing each concentration of adenosine or NECA in the absence and the presence of inhibitors. They were concentrated and dried using a centrifugal vaporizer (Eyela, CVE 200D, Japan). The samples were dissolved in the 300 µl of artificial CSF and incubated at room temperature for 15 min. Thereafter, 50 µl of each sample were added to the wells containing 50 µl of the sulfanilamide solution and allowed to react for 5–10 min at room temperature while protected from light. After adding 50 µl of 0.1% N-1-naphthylethylenediamine dihydrochloride solution to all wells, we analyzed total amounts of NO2/NO3 by measuring absorbance at 540 nm by a microplate reader (Power Wave 340, Bio-Tek Instruments). All samples were run in duplicates or triplicates.

#### Drugs
Adenosine and L-NAME were purchased from Sigma Chemical (St. Louis, MO). 2-(Carboxyethyl)-5-phenethylamin-5’-N-ethylcarboxamido adenosine (CGS-21680), NECA, and alloxazine (benzoylpteridine-2,4(1H,3H)-dione) were from Research Biochemicals International (Natick, MS). ZM-241385 (4’-2-[7-amino-2-(2-furyl)[1,2,4]triazol-2,3-a][1,3,5]-triazin-5-yl amino]ethyl)phenol) was obtained from Tocris Cookson (Bristol, UK) and dissolved in dimethyl sulfoxide to make a stock solution of 10 mmol/l. Rp-adenosine-3’,5’-cyclic monophosphorothioate (Rp-cAMPS) and Rp-8-bromoguanosine 3’,5’-cyclic monophosphorothioate sodium salt (Rp-8-BrcGMPS) were obtained from Biomol Research.

#### Statistical analysis
All data are expressed as means ± SE. A Student’s t-test was used for analyzing values between the data of vehicle and inhibitor-treated groups (EC_{25} and lower limit of CBF autoregulation). Two-way repeated-measures ANOVA was used for the comparison of time-dependent arterial diameter changes in response to agonists between

### Table 1. Physiological variables: MABP and blood gas analysis

<table>
<thead>
<tr>
<th>Variables</th>
<th>Before</th>
<th>After Experiments</th>
</tr>
</thead>
<tbody>
<tr>
<td>n</td>
<td>101</td>
<td>101</td>
</tr>
<tr>
<td>MABP, mmHg</td>
<td>106 ± 5</td>
<td>107 ± 4</td>
</tr>
<tr>
<td>PCO2, mmHg</td>
<td>31.4 ± 2.6</td>
<td>32.6 ± 1.4</td>
</tr>
<tr>
<td>PO2, mmHg</td>
<td>104.5 ± 5.7</td>
<td>98.3 ± 4.6</td>
</tr>
<tr>
<td>pH</td>
<td>7.41 ± 0.02</td>
<td>7.42 ± 0.01</td>
</tr>
</tbody>
</table>

Values are means ± SE; n, number of rats. MABP, mean arterial blood pressure.
inhibitor-treated and untreated groups. ANOVA for repeated measurement followed by Dunnett's method was used for statistical analysis of CBF between baseline value and the value at each blood pressure level. P < 0.05 was accepted as statistically significant.

RESULTS

Under control conditions, mean arterial blood pressure was within the normal ranges (Table 1), and the baseline pial arterial diameters were 37.3 ± 4.6 μm (n = 101 rats).

Effects of L-NAME, ZM-241385, and alloxazine. Adenosine (0.01–10 μmol/l), CGS-21680 (0.01–10 μmol/l), and NECA (0.01–1 μmol/l) exerted concentration-dependent vasodilations of the pial arterioles. The EC25 values for adenosine and NECA were significantly increased by pretreatment with L-NAME from 0.10 ± 0.02 to 1.87 ± 0.07 μmol/l (n = 7; mean dose ratio, 18.7; P < 0.001) and from 0.02 ± 0.01 to 0.33 ± 0.07 μmol/l (n = 6; mean dose ratio, 16.5; P < 0.05), respectively, with significantly decreased maximum dilation (P < 0.05). Suffusion with artificial CSF containing 10 μmol/l NECA caused a slight fall in arterial blood pressure. Thus the concentration of NECA used to elicit vasodilation was between 0.01 and 1 μmol/l for vasodilation. However, CGS-21680-induced vasodilation was little influenced by L-NAME (Fig. 1). The baseline pial arterial diameters were not changed on suffusion of 10 μmol/l L-NAME from 30 min before experiment.

The concentration-dependent vasodilation evoked by adenosine (0.01–10 μmol/l) was significantly inhibited not only by 1 μmol/l of ZM-241385 but also by 1 μmol/l of alloxazine with significantly increased EC25 values and decreased dilatation magnitude at 10 μmol/l of adenosine. Otherwise, the NECA-induced vasodilation was significantly suppressed by alloxazine (1 μmol/l) with significantly increased EC25 value (from 0.02 ± 0.01 to 0.60 ± 0.04 μmol/l; n = 4; mean dose ratio, 30; P < 0.001). However, pretreatment with ZM-241385 (1 μmol/l) caused a decrease in dilatation magnitude at 1 μmol/l of NECA with little change in EC25 value (Fig. 2).

Release of nitrite and nitrate. The adenosine (0.01–10 μmol/l)- and NECA (0.01–1 μmol/l)-stimulated nitrite/nitrate levels released from suffused artificial CSF were increased in a concentration-dependent manner, and they were markedly inhibited by pretreatment with L-NAME (10 μmol/l) (Fig. 3). In contrast, CGS-21680 caused increase in nitrite/nitrate releases, which were not significantly influenced by L-NAME (Fig. 3).

NECA (0.01 and 1 μmol/l)-stimulated nitrite/nitrate releases were significantly suppressed by alloxazine (1 μmol/l, P < 0.05) but not by ZM-241385 (1 μmol/l), suggesting that adenosine-stimulated nitrite/nitrate release was mediated via activation of A2B subtype receptors (P < 0.05) (Fig. 4).

Effect of Rp-cAMPS and Rp-8-BrcGMPS. To assess the signal transduction mechanisms underlying CGS-21680- and NECA-induced vasodilation, we examined the effects of Rp-cAMPS and Rp-8-BrcGMPS on vasodilation. As shown in Fig. 5, CGS-21680 (10 μmol/l)-induced vasodilation was significantly inhibited by Rp-cAMPS (10 μmol/l, P < 0.01) but not by Rp-8-BrcGMPS (10 μmol/l). In contrast, NECA (1 μmol/l)-induced vasodilation was not inhibited by Rp-cAMPS (10 μmol/l) but by Rp-8-BrcGMPS (10 μmol/l, P < 0.001).

Effects of inhibitors on CBF autoregulation. Mean arterial blood pressure was 105.5 ± 4.6 mmHg (n = 101) under resting conditions. CBF to the pial arteries was well preserved despite a decrease in mean arterial blood pressure to ~55 mmHg. When the blood pressure level further decreased, CBF fell steeply depending on the magnitude of the fall in blood pressure thereafter. The lower limit of autoregulation was defined as the blood pressure at which CBF decreased by 10% of its value at resting mean arterial blood pressure. After pretreatment with either 10 μmol/l of alloxazine, an
A2B-receptor antagonist, or 100 µmol/l of L-NAME, a nitric oxide synthase inhibitor, with a bolus volume of 100 µl for three times every 10 min, the lower limit of CBF autoregulation was not altered. By contrast, the lower limit of CBF autoregulation, when assessed under pretreatment with ZM-241385 (1 µmol/l, a selective A2A-receptor antagonist), significantly shifted to a higher blood pressure level (control, 54.3 ± 4.7 to 67.0 ± 2.1 mmHg, P < 0.05) (Fig. 6).

**DISCUSSION**

The purpose of this investigation was to clarify the involvement of nitric oxide in adenosine-induced vasodilation mediated by the A2B-subtype receptors in rat pial arterioles and its physiological importance in CBF autoregulation. The major findings are as follows: 1) adenosine- and NECA-induced vasodilations were significantly suppressed by L-NAME, whereas CGS-21680-induced vasodilation was not affected by it; 2) NECA-induced vasodilation was significantly inhibited by alloxazine, an A2B-subtype receptor antagonist, but not by ZM-241385, a selective adenosine A2A receptor antagonist; 3) the nitrite/nitrate levels released into the suffusate were significantly increased by adenosine and NECA, and their release was largely inhibited by L-NAME and alloxazine, but not by ZM-241385; and 4) the lower limit of CBF autoregulation was little affected under pretreatment with either L-NAME or alloxazine.

Adenosine produces receptor-mediated activation of adenyl cyclase and dilation of intracerebral (15) and pial (23) arteries. Headrick and Berne (11) reported that adenosine mediates relaxation in the guinea pig aorta by endothelium-dependent and -independent mechanisms, and the receptors involved in these relaxations are characteristic of the A2-adenosine subtype. Vials and Burnstock (31) further documented that in the guinea pig coronary artery, a major part of vasodilator action of adenosine is directly mediated via A2 receptors on the smooth muscle, and activation by adenosine of A2 purinoceptors on endothelial cells induces relaxation via production of nitric oxide.
Recently, we observed that adenosine-induced vasodilation in the rat pial artery was mediated via activation of adenosine $A_{2A}$- and $A_{2B}$-subtype receptors, but not $A_1$ subtype. Whereas a part of the vasodilator action of adenosine is mediated directly via adenosine $A_{2A}$-subtype receptors (13), in our study increasing concentrations of NECA as well as adenosine elicited an enhanced nitric oxide production in association with vasodilations in a concentration-dependent manner. Moreover, the agonist effects were significantly inhibited by L-NAME, an inhibitor of nitric oxide synthase (27). Li et al. (17) reported an enhancement of nitric oxide synthase inhibition and by alloxazine have led us to postulate that NECA elicits vasodilation by stimulating the release of nitric oxide mediated via adenosine $A_{2B}$ receptors.

In our in vivo study, it was not easy to measure the intracellular nucleotides/protein kinase concentrations in the pial arteries. Instead, we demonstrated that NECA-induced vasodilation was significantly inhibited by Rp-8-BrcGMPS, an inhibitor of cGMP kinase, but not by Rp-cAMPS, a preferential inhibitor of cAMP-dependent protein kinase in agreement with the result of Dubey et al. (5). By contrast, CGS-21680-induced vasodilation was not suppressed by Rp-8-BrcGMPS but by Rp-cAMPS. Thus it is likely that NECA evokes vasodilation via a pathway that does not involve adenyl cyclase-cyclic protein kinase A but involves a guanylyl cyclase-protein kinase G pathway.

On the other hand, Hong et al. (12, 14) demonstrated that calcitonin gene-related peptide is implicated in the autoregulatory vasodilation of the pial artery in response to hypotension, and its effect is closely related with accumulation of intracellular cAMP. Recently, an involvement of the metabolic cAMP-adenosine pathway was demonstrated as a likely mechanism in the production of adenosine that acted as a regulator of vasodilation in response to hypotension (13). In this study, we predicted that, after pretreatment with L-NAME, the lower limit of autoregulation would shift to higher blood pressure levels. However, L-NAME failed to alter the lower limit of CBF autoregulation in comparison to the pre-L-NAME levels, suggesting that nitric oxide is not involved in the autoregulatory vasodilation following a decrease in mean arterial blood pressure. These results are consistent with the reports of Wang et al. (32) and Buchanan and Phillis (3). Pretreatment with alloxazine did not influence the...
lower limit of CBF autoregulation. Thus it is unlikely that the vasodilation coupled to nitrite/nitrate formation that is mediated by activation of adenosine A2B-subtype receptors is involved in the modulation of CBF autoregulation in the pial artery. By contrast, pretreatment with ZM-241385 (a selective A2A-receptor antagonist) (24) caused a significant shift of the lower limit of CBF autoregulation to higher blood pressure levels, suggesting that activation of adenosine A2A-, but not A2B-, subtype receptors is predominantly implicated in the mediation of autoregulatory vasodilation in response to hypotension and CBF autoregulation.

The findings of the present study indicate that adenosine-induced vasodilation via activation of A2B receptors of rat pial arteries is coupled to the production of nitric oxide; however, these effects contribute little physiologically to the CBF autoregulation.

This study was supported in part by a Research Fund for Basic Medical Development of Korea Ministry of Education (to K. W. Hong) and by the Center for Biofunctional Molecules (supported by Korea Science & Engineering Foundation), Pohang University of Science and Technology, Pohang, Korea (to K. W. Hong). Address correspondence and reprint requests to: K. W. Hong, Dept. of Pharmacology, College of Medicine, Pusan National University, Ami-Dong 1-Ga, Seo-Gu, Pusan 602-739, Korea (E-mail: kwhong@yowon.pusan.ac.kr).

Received 2 July 1999; accepted in final form 14 September 1999.

REFERENCES


