The Involvement of Nitric Oxide and Guanylate Cyclase on the Adenosine A2B Receptor-induced Cerebral Blood Flow Responses in the Rats

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Abstract - This study was performed to investigate the regulatory mechanism of cerebral blood flow of adenosine A2B receptor agonist in the rats, and to define whether its mechanism is mediated by nitric oxide (NO) and guanylate cyclase. In pentobarbital-anesthetized, pancuronium-paralyzed and artificially ventilated male Sprague-Dawley rats, all drugs were applied topically to the cerebral cortex. Blood flow from cerebral cortex was measured using laser-Doppler flowmetry. Topical application of an adenosine A2B receptor agonist, 5'-N-ethylcarboxamidoadenosine (NECA; 4 μmol/l) increased cerebral blood flow. This effect of NECA (4 μmol/l) was blocked by pretreatment with NO synthase inhibitor, Nω-nitro-L-arginine methyl ester (L-NAME; 40 μmol/l) and guanylate cyclase inhibitor, LY-83,583 (10 μmol/l). These results suggest that adenosine A2B receptor increases cerebral blood flow. It seems that this action of adenosine A2B receptor is mediated via the NO and the activation of guanylate cyclase in the cerebral cortex of the rats.

Keywords □ Adenosine A2B receptor, 5'-N-ethylcarboxamidoadenosine, cerebral blood flow, nitric oxide, guanylate cyclase

INTRODUCTION

The action of adenosine as a neurotransmitter or neuromodulator responsible for cardiovascular regulation has been suggested (Barraco et al., 1987; Brecht et al., 1991). It is generally accepted that systemic hypoxia exerts strong dilator influences upon the vasculature of skeletal muscle and the brain due to the action of locally released vasodilator substances. Adenosine that mediates cerebral vasodilation during systemic hypoxia is released from the endothelium (Coney and Marshall, 1998). Adenosine is coupled to adenylate cyclase via 2 types of receptor: A1 receptor that mediates an inhibition of adenylate cyclase and A2 receptor that mediates a stimulation of adenylate cyclase (Choca et al., 1987; Gerber and Gähwiler, 1994; Jiang et al., 1992; Van Calker et al., 1979). The A1 and A2 receptors have distinct distribution in the central nervous system (Bruns et al., 1987; Stone et al., 1988). The adenosine A2 receptors are further classified as adenosine A2A receptor and A2B receptor subtypes based on the potency of agonist and the receptor affinity (Fredholm et al., 1994). Cloning techniques have confirmed the existence of distinct A2A receptor and A2B receptor subtypes of A2 receptor (Stehle et al., 1992). And adenosine may cause vasodilatation by increasing intracellular cAMP, so adenosine A1 receptor may produce vasodilatation in cerebral cortex. The role for adenosine in the regulation of cerebral blood flow has been proposed by a number of investigators (Dirmagl et al., 1994; Wysham et al., 1986). Adenosine A2 receptor agonist produces a substantial increase in cerebral blood flow but adenosine A1 receptor agonist has minimal effects (Coney and Marshall, 1998).

Nitric oxide (NO) is a potent vasodilator that regulates the vascular tone in several vascular beds, including the brain, therefore NO might be of importance for the increase of cerebral blood flow (Akgoren et al., 1994). And NO is a key coupling compound that links changes in cerebral blood flow and metabolism (Goadsby et al., 1992). Adenosine causes NO release from cultured cortical astrocytes and mobilization of calcium from intracellular stores rather than influx is involved in the adenosine-induced activation of NO synthase (Janigro et al., 1996). NO production leads to activation of guanylate cyclase and may help couple cerebral blood flow and metabolism (Dirmagl et al., 1993). In the cerebral and other vascular...
beds, activation of guanylate cyclase cause relaxation of vascular smooth muscle (Hyman et al., 1989). However, little is known about the regulatory mechanism of cerebral blood flow of adenosine A$_{2n}$ receptor agonist in the rats. This study was performed to examine the regulatory mechanism of cerebral blood flow of adenosine A$_{2n}$ receptor agonist in the rats, and to define whether its mechanism is mediated by NO and guanylate cyclase.

**MATERIALS AND METHODS**

The experimental animals, male Sprague-Dawley rats (250-300 gm), were categorized into four groups. The first group of these groups was treated only with 5'-N-ethylcarboxamidoadenosine (NECA; 4 µmol/l), an adenosine A$_{2n}$ receptor agonist, topically to the cerebral cortex. The second group was treated with N$\text{'}$-nitro-L-arginine methyl ester (L-NAME; 40 µmol/l), a NO synthase inhibitor, topically to the cerebral cortex 10 min before the injection of 4 µmol/l of NECA. The third group was treated with LY-83,583 (10 µmol/l), an guanylate cyclase inhibitor, topically to the cerebral cortex 10 min before the injection of 4 µmol/l of NECA. Another group was sham-operated animal group. All drugs were purchased from RBI chemical company (USA) and SIGMA chemical company (USA). All drugs except LY-83,583 were dissolved in artificial cerebrospinal fluid (composition: 120.00 mM NaCl, 2.8 mM KCl, 22.00 mM NaHCO$_3$, 1.45 mM CaCl$_2$, 1.00 mM Na$_2$HPO$_4$, 0.876 mM MgCl$_2$) prior to administration and applied topically to the cerebral cortex. LY-83,583 was dissolved in 1% ethanol prior to administration and applied topically to the cerebral cortex.

The drug administrations were performed in pentobarbital-anesthetized (50 mg/kg, i.p.), pancuronium-paralyzed (0.1 mg/kg/min, i.v.) and artificially ventilated (Harvard, USA), male Sprague-Dawley rats (250-300 gm). Rectal temperature was maintained at 37±0.5°C with a heating pad, and the rats were placed in a stereotaxic instrument (Kopf, USA) in the prone position and the parietal bone was removed by gradually thinning the bone bilaterally between the temporal and transverse suture lines using a dental burr: a square-shape (5 x 5 mm) burr hole was made over the right parietal cortex and bone wax was used to achieve hemostasis. The dura mater was carefully removed from the left parietal cortex and the surface of the cortex was superfused with a artificial cerebrospinal fluid. A barrier was fixed caudal to the craniotomy so that when a drug was applied topically to the cortex

Cerebral blood flow levels were measured with a laser-Doppler flowmetry (Laserflo BPM 403A) equipped with a 1-mm-diameter needle probe through the cranial window, where prewarmed artificial CSF saturated with a gas mixture of 95% O$_2$-5% CO$_2$ was constantly suffused over the cortical surface. 'Zero' blood flows were determined in each preparation after sacrifice at the conclusion of the experiment. Blood pressure and heart rate were continuously monitored via a femoral arterial catheter (PE-50) connected to a pressure transducer (Statham P23, USA) and a polygraph (Grass, USA). PO$_2$, PCO$_2$ and pH of arterial blood were measured with blood gas analyzer before and after stereotaxic experiment. All results are expressed as mean±SEM with P<0.05 and P<0.01 considered as the level of significance. The statistical analysis of mean values was performed by analysis of variance (ANOVA). Student's t-test for paired data was also used for statistical evaluation of the results.

**RESULTS**

A representative experiment showing the effects of topical application of NECA on cerebral blood flow is illustrated in Table I and Fig. 1. Topical application of NECA caused an increase in cerebral blood flow that reached a maximum in 30 min after application. Pretreatment with alloxazine (4 µmol/l), an adenosine A$_{2n}$ receptor antagonist, blocked the NECA-induced cerebral blood flow responses. The increase in cerebral blood flow evoked by NECA was dose-dependent (2, 4 and 6 µmol/l of NECA increased the cerebral blood flow by 23±3, 71±6 and 97±13%, respectively; n=10; Fig. 1). Topical application of NECA (4 µmol/l) caused an increase in cerebral blood flow that reached a maximum in 30 min after application (n=10, Table I, Fig. 2). Baseline cerebral blood flow for these rats were 100±11.2%. Topical application of an equivalent volume of artificial cerebrospinal fluid did not affect the basal cerebral blood flow. Cerebral blood flow was not affected in sham-operated animal group.

Pretreatment with L-NAME (40 µmol/l) significantly attenuated the NECA-induced cerebral blood flow responses (n=10, Table I, Fig. 3 and Fig. 5). Baseline cerebral blood flow for these rats were 100±14.7%. Another different groups were treated with L-NAME (20 µmol/l, 60 µmol/l) affected NECA-induced cerebral blood flow responses concentration-dependently. Therefore in this study authors chose the middle concentration.

Pretreatment with LY-83,583 (10 µmol/l) significantly attenuated the NECA-induced cerebral blood flow responses (n=10,
Table 1. Percent changes in cerebral blood flow of 5'-N-ethylcarboxamidoadenosine (NECA; 4 umol/l) only, NECA (4 umol/l) after pretreatment with N^3^-nitro-L-arginine methyl ester (L-NAME; 40 umol/l) and NECA (4 umol/l) after pretreatment with LY-83,583 (LY; 10 umol/l). Pretreatment with L-NAME or LY-83,583 have no effects on cerebral blood flow. Cerebral blood flow is not affected in sham-operated animal group. Number of rats in each group is 10. Data are the mean±S.E. *p<0.05, compared to 0 time cerebral blood flow.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Cerebral Blood Flow (%)</th>
<th>Minute after Treatment</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>0</td>
</tr>
<tr>
<td>NECA</td>
<td>100±11.2</td>
<td>135±13.6*</td>
</tr>
<tr>
<td>L-NAME+NECA</td>
<td>100±13.8</td>
<td>103±12.7</td>
</tr>
<tr>
<td>LY+NECA</td>
<td>100±14.2</td>
<td>105±14.2</td>
</tr>
<tr>
<td>sham-operated</td>
<td>100±11.6</td>
<td>103±12.9</td>
</tr>
</tbody>
</table>

Fig. 1. Dose-dependent increase of cerebral blood flow (CBF) by topical application of 5'-N-ethylcarboxamidoadenosine (2, 4 and 6 umol/l). Data represent mean±S.E.. Significant differences were determined by ANOVA with a multiple comparisons test; *p<0.01 (2 umol/l vs. 4 umol/l), **p<0.01 (2 umol/l vs. 6 umol/l and 4 umol/l vs. 6 umol/l). All doses significantly differ from p<0.01 as compared to basal CBF.

Fig. 2. Effects of topical application of 5'-N-ethylcarboxamidoadenosine (NECA; 4 umol/l) on cerebral blood flow responses in rats. Values are mean±S.E. in 10 rats.

Fig. 3. Effects of topical application of 5'-N-ethylcarboxamidoadenosine (NECA; 4 umol/l) after pretreatment with N^3^-nitro-L-arginine methyl ester (L-NAME; 40 umol/l) on cerebral blood flow responses in rats. Values are mean±S.E. in 10 rats.

Fig. 4. Effects of topical application of 5'-N-ethylcarboxamidoadenosine (NECA; 4 umol/l) after pretreatment with LY-83,583 (10 umol/l) on cerebral blood flow responses in rats. Values are mean±S.E. in 10 rats.

Table I, Fig. 4 and Fig. 6). Baseline cerebral blood flow for these rats were 100±14.2%. Another different groups were treated with LY-83,583 (5 μmol/l, 15 μmol/l) affected NECA-induced cerebral blood flow responses concentration-dependently. Therefore in this study authors chose the middle concentration.
Topical application of L-NAME (40 μmol/l) or LY-83,583 (10 μmol/l) had no effects on cerebral blood flow. No significant changes in PO$_2$, PCO$_2$ and pH of arterial blood were seen following stereotaxic experiment (Table II).

**DISCUSSION**

In this present study, topical application of NECA in anesthetized and artificially ventilated rats elicited an increase in cerebral blood flow. Adenosine plays an important role in many physiological processes. Its actions are mediated by specific cell surface receptors coupled to G proteins (Fredholm et al., 1994; Olah and Stiles, 1996). Four subtypes of adenosine receptors have been cloned: A$_1$, A$_2A$, A$_2B$ and A$_3$. Significant advancement has been made in the understanding of the molecular pharmacology and physiological relevance of adenosine receptors (Feoktistov and Biaggioni, 1997). It is also recognized that adenosine A$_2B$ receptors are coupled to intracellular pathways different from those of adenosine A$_2A$ receptors, a finding that may provide the basis for their distinct physiological role. Adenosine A$_2B$ receptors have been implicated in mast cell activation and asthma, vasodilation, regulation of cell growth, intestinal function and modulation of neurosecretion (Feoktistov and Biaggioni, 1997). Pharmacological identification of A$_2B$ receptors, based on their affinity and characteristic order of potency for receptors. Adenosine A$_2B$ receptors participate in the regulation of vascular tone (Webb et al., 1992). Therefore adenosine-induced vasodilation is mediated via adenosine A$_2B$ receptors. In this present study, topical application of NECA, an adenosine A$_2B$ receptor agonist, in anesthetized and artificially ventilated rats elicited an increase in cerebral blood flow. Adenosine is a potent dilator of cerebral vessels (Ibayashi et al., 1991; Ngai and Winn, 1993) and has been implicated in the regulation of cerebral blood flow (Phillis, 1989; Winn et al., 1981). Ngai (Ngai and Winn, 1993) investigated the receptors involved in adenosine-induced dilation in cerebral resistance arterioles and adenosine acts primarily via A$_2$ receptors to elicit cerebral vasodilation. Some experimental evidences suggest that adenosine receptor plays a critical role in the mediation of cerebral blood flow responses (Van Calker et al., 1979; Hong et al., 1994; Hong et al., 1996; Hong et al., 1999), and some experimental evidences suggest that adenosine A$_2$ receptor agonist produces a substantial increase in cerebral blood flow (Coney and Marshall, 1998; Van Wylen et al., 1989). Systemic hypoxia exerts strong dilator influences upon the vasculature of skeletal muscle and the brain due to the action of locally released vasodilator substances. Adenosine plays a major part in this vasodilatation (Mian and Marshall, 1991; Skinner and Marshall, 1996; Thomas and Marshall, 1994). Adenosine makes a major contribution to the vasodilatation induced in the cerebral cortex of the rat by systemic hypoxia. Adenosine is involved in coupling of cerebral blood flow to neuronal activation. Adenosine A$_2$ receptor agonist pro-

**Table II. Summary of blood gas analysis**

<table>
<thead>
<tr>
<th>Variables</th>
<th>Before Experiment</th>
<th>After Experiment</th>
</tr>
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<tbody>
<tr>
<td>PaCO$_2$, mmHg</td>
<td>83.8±2.7%</td>
<td>89.1±4.1%</td>
</tr>
<tr>
<td>PaO$_2$, mmHg</td>
<td>39.9±2.4%</td>
<td>40.1±1.8%</td>
</tr>
<tr>
<td>pH</td>
<td>7.20±0.36</td>
<td>7.29±0.41</td>
</tr>
</tbody>
</table>

![Fig. 5. Changes in cerebral blood flow (CBF) of 5'-N-ethylcarboxamido-adenosine (NECA; 4 umol/l) only and NECA (4 umol/l) after pretreatment with 5'-nitro-L-arginine methylester (L-NAME; 40 umol/l) topically. Values are recorded at the end of 30 minutes after topical application of NECA. Values are the mean±S.E. in 10 rats. *p<0.01, compared to NECA only group.](image)

![Fig. 6. Changes in cerebral blood flow (CBF) of 5'-N-ethylcarboxamido-adenosine (NECA; 4 umol/l) only and NECA (4 umol/l) after pretreatment with LY-83,583 (LY; 10 umol/l) topically. Values are recorded at the end of 30 minutes after topical application of NECA. Values are the mean±S.E. in 10 rats. *p<0.01, compared to NECA only group.](image)
duces a substantial increase in cerebral blood flow but adenosine A_1 receptor agonist has minimal effects (Coney and Marshall, 1998). But little is known about the regulatory mechanism of cerebral blood flow of adenosine A_2B receptor agonist.

This effect of NECA (4 μmol/l) was blocked by pretreatment with NO synthase inhibitor, N^{G}-nitro-L-arginine methyl ester (L-NAME; 40 μmol/l). NO is a potent vasodilator and a key coupling compound that links changes in cerebral blood flow and metabolism (Goadsby et al., 1992). Adenosine is involved in coupling of cerebral blood flow to neuronal activation and NO is involved in this response as well, so there is an interaction between the vasodilator pathways of adenosine and NO (Dirmag et al., 1994). Vials and Burnstock (Vials and Burnstock, 1993) documented that in the guinea pig coronary artery, a major part of vasodilator action of adenosine is directly mediated via adenosine A_2 receptors on the smooth muscle and, by activation of adenosine A_2 purinoreceptors on endothelial cells, induces relaxation via production of NO. Therefore our results suggest that the effects of NECA on the cerebral blood flow is mediated by NO synthase.

This effect of NECA (4 μmol/l) was blocked by pretreatment with guanylate cyclase inhibitor, LY-83,583 (10 μmol/l). The level of cyclic GMP in vascular smooth muscle is an important regulator of blood flow. Many vasodilators work through increases in cyclic GMP (Hyman et al., 1989; Zhou and Torphy, 1991). Generally NO production leads to increase in the level of cyclic GMP and may help couple cerebral blood flow and metabolism (Dirmag et al., 1993). It is also believed that NO is involved in the coupling of neurotransmitter receptor stimulation with cellular cyclic GMP responses (Garthwaite, 1988). In this present study adenosine A_2B receptor is mediated by stimulation of guanylate cyclase. Therefore adenosine A_2B receptor may increase cerebral blood flow by increasing intracellular cGMP. So adenosine A_2B receptor may produce vasodilation in cerebral cortex by stimulation of guanylate cyclase. We demonstrated that NECA-induced vasodilation in cerebral cortex was significantly inhibited by guanylate cyclase inhibitor, LY-83,583. Therefore the regulation of cerebral blood flow in adenosine A_2B receptor is mediated by guanylate cyclase. In further study we may determine the level of cGMP level to confirm this evidence.

In conclusion, our results show that adenosine A_2B receptor increases cerebral blood flow and this action of adenosine A_2B receptor is mediated via the NO and the activation of guanylate cyclase in the cerebral cortex of the rats.

REFERENCES


