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The Involvement of Nitric Oxide and Guanylate Cyclase on the Adenosine A_{2B} 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 A_{2B} 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 A_{2B} 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, N^G-nitro-L-argine methylester (L-NAME; 40 μmol/l) and guanylate cyclase inhibitor, LY-83,583 (10 μmol/l). These results suggest that adenosine A_{2B} receptor increases cerebral blood flow. It seems that 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.

Keywords □ Adenosine A_{2B} 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; Bredt *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 vasodilatation during systemic hypoxia is released from the endothelium (Coney and Marshall, 1998). Adenosine is coupled to adenylate cyclase via 2 types of receptor : A_1 receptor that mediates an inhibition of adenylate cyclase and A_2 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 A_1 and A_1 receptors have distinct distribution in the central nervous system (Bruns *et al.*, 1987; Stone *et al.*, 1988). The adenosine A_2 receptors are further classified as adenosine A_{2A} receptor and A_{2B} receptor subtypes based on the potency of agonist and the receptor affinity (Fredholm *et al.*, 1994). Cloning techniques have confirmed the existence of distinct A_{2A} receptor and A_{2B} receptor subtypes of A_2 receptor (Stehle *et al.*, 1992). And adenosine may cause vasodilatation by increasing intracelluar 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 (Dirnagl *et al.*, 1994; Wysham *et al.*, 1986). Adenosine A_2 receptor agonist produces a substantial increase in cerebral blood flow but adenosine A_1 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 (Dirnagl *et al.*, 1993). In the cerebral and other vascular

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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_{2B} receptor agonist in the rats. This study was performed to examine the regulatory mechanism of cerebral blood flow of adenosine A_{2B} 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_{2B} receptor agonist, topically to the cerebral cortex. The second group was treated with N^G-nitro-L-argine methylester (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 umol/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 Na2HPO4, 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_{2B} 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 I. Percent changes in cerebral blood flow of 5'-N-ethylcarboxamidoadenosine (NECA; 4 umol/l) only, NECA (4 umol/l) after pretreatment with N^G-nitro-L-argine methylester (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 o time cerebral blood flow.

Treatment	Cerebral Blood Flow (%)								
	Minute after Treatment								
	0	5	10	15	30	45	60	75	90
NECA	100±11.2	135±13.6*	137±12.5*	149±15.1*	164±18.1*	163±17.7*	164±17.9*	163±16.6*	163±18.8*
L-NAME+NECA	100±13.8	103±12.7	105±14.3	103±13.6	104±11.4	106±12.8	105±14.6	106±16.1	105±12.8
LY+NECA	100±14.2	105±14.2	107±12.8	108±13.7	107±12.7	109±15.1	107±14.1	110±15.8	108±16.3
sham-operated	100±11.6	103±12.9	106±13.6	107±16.5	108±13.2	107±12.7	109±13.6	109±14.2	110±14.9

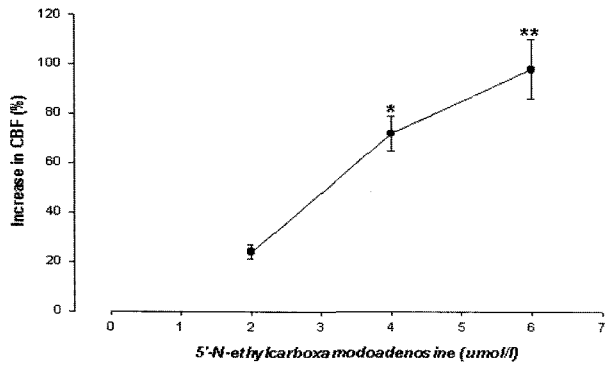

Fig. 1. Dose-dependent increase of cerebral blood flow (CBF) by topical application of 5'-*N*-ethylcarboxamidoadenosine (2, 4 and 6 umol/l). Data represent mean±S.E.. Significant differences were determined by ANOVA with a multiple comparisons test; *p<0.01 (2 umol/l vs. 4 umol/l), **p<0.01 (2 umol/l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

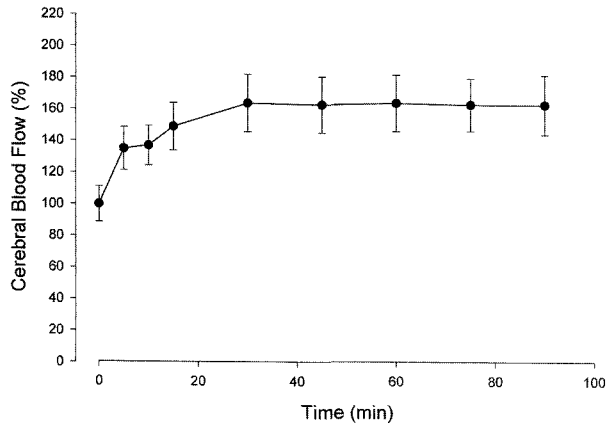

Fig. 2. Effects of topical application of 5'-*N*-ethylcarboxamidoadenosine (NECA; 4 umol/l) on cerebral blood flow responses in rats. Values are mean±S.E. in 10 rats.

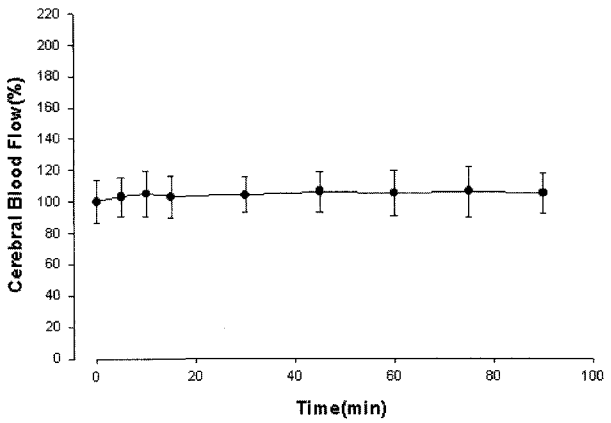

Fig. 3. Effects of topical application of 5'-*N*-ethylcarboxamidoadenosine (NECA; 4 umol/l) after pretreatment with N^G-nitro-L-argine methylester (L-NAME; 40 umol/l) on cerebral blood flow responses in rats. Values are mean±S.E. in 10 rats.

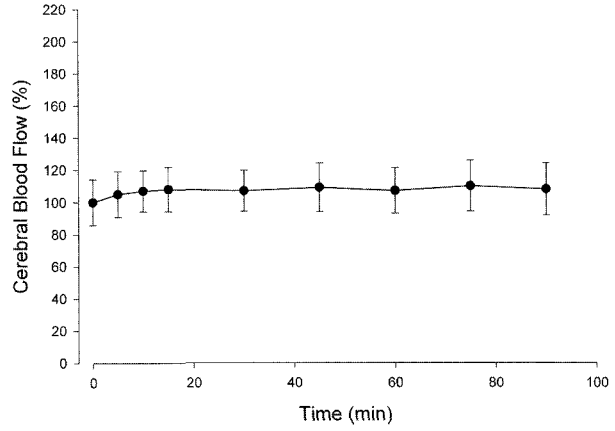

Fig. 4. Effects of topical application of 5'-*N*-ethylcarboxamidoadenosine (NECA; 4 umol/l) after pretreatment with LY-83,583 (10 umol/l) on cerebral blood flow responses in rats. Values are mean±S.E. in 10 rats.

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.

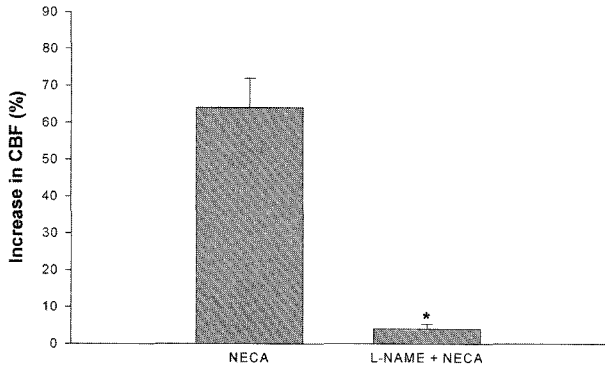

Fig. 5. Changes in cerebral blood flow (CBF) of 5'-*N*-ethylcarboxamidoadenosine (NECA; 4 umol/l) only and NECA (4 umol/l) after pretreatment with N^G-nitro-L-argine methylester (L-NAME; 40 umol/l) topically. Values are recorded at the end of 30 minutes after topical application of NECA. Values are the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

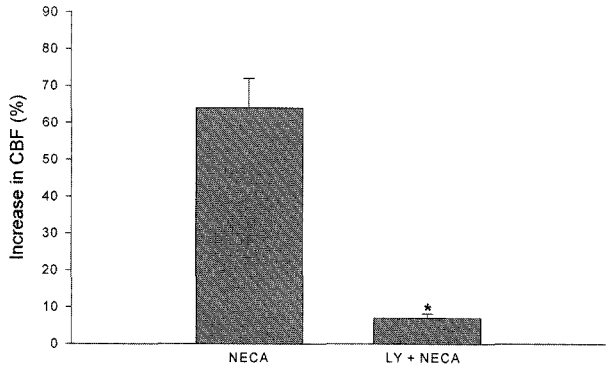

Fig. 6. Changes in cerebral blood flow (CBF) of 5'-*N*-ethylcarboxamidoadenosine (NECA; 4 umol/l) only and NECA (4 umol/l) after pretreatment with LY-83,583 (LY; 10 umol/l) topically. Values are recorded at the end of 30 minutes after topical application of NECA. Values are the mean±S.E. in 10 rats. *p<0.01, compared to NECA only group.

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).

Table II. Summary of blood gas analysis

Variables	Before Experiment	After Experiment
$PaCO_2$, mmHg	83.8±2.7%	89.1±4.1%
PaO_2, mmHg	39.9±2.4%	40.1±1.8%
pH	7.20±0.36	7.29±0.41

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-

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-argine methylester (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 (Dirnagl *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 activation by adenosine of A_2 purinoceptors 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 (Dirnagl *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 intracelluar cGMP. So adenosine A_{2B} receptor may produce vasodilatation in cerebral cortex by stimulation of guanylate cyclase. We demonstrated that NECA-induced vasodilatation 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.

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