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Effects of adenosine receptor antagonists on pial arteriolar dilation during carbon dioxide inhalation

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Abstract

The role of adenosine in the cerebrovascular response to carbon dioxide inhalation was evaluated in two sets of experiments. The pial circulation was recorded by a Laser-Doppler flow probe placed over a closed cranial window in methoxyflurane anesthetized rats. Topical application of the nonselective adenosine receptor antagonist caffeine (1 mM), the selective A_1 receptor antagonist 8-cyclopentyl-1,3-dipropylxanthine (DPCPX,1 μM), or the selective A_{2A} receptor antagonist 4-(2-[7-amino-2-(2-furyl)[1,2,4]triazolo[2,3-*a*]triazin-5-yl amino]ethyl) phenol (ZM 241385, 1 μM) all failed to affect mean arterial blood pressure, basal cerebral blood flow, or the carbon dioxide-evoked hyperemia. Systemically administered caffeine (20 mg/kg) also had no significant effects. However, following the systemic administration of the nonselective nitric oxide synthase inhibitor, N^G-nitro-L-arginine methyl ester (L-NAME, 20 mg/kg), the topical application of both caffeine and ZM 241385 (but not DPCPX) significantly reduced the carbon dioxide-evoked hyperemia. L-NAME (20 mg/kg) administered intravenously, evoked a significant increase in mean arterial blood pressure, a slow progressive decline in cerebral blood flow and, during brief (60–90 s) periods of 7.5% carbon dioxide inhalation, a significant decrease in arterial blood pressure. L-NAME failed to reduce the carbon dioxide-evoked increase in cerebral blood flow as measured by the area under the curve (AUC), although it did reduce the peak flow response. Topically applied L-NAME (1 mM) failed to alter mean arterial blood pressure, basal cerebral blood flow, or the carbon dioxide-evoked increases in cerebral blood flow.

In a second series of experiments, we evaluated the ability of 10% carbon dioxide inhalation for 8 min to elicit a release of adenosine from the cerebral cortex. Adenosine levels in the cortical superfusates rose significantly during periods of carbon dioxide inhalation. The data suggest that following the removal of the confounding effects of nitric oxide, which are unlikely to be mediated locally, a significant contribution by adenosine A_{2A} receptor activation to the carbon dioxide-evoked cortical hyperemia was evident.

Keywords: Adenosine; Cerebral blood flow; Hypercapnia; Nitric oxide (NO); ZM 241385

1. Introduction

Variations in arterial carbon dioxide levels have marked effects on cerebrovascular flow. Hypercapnia results in arterial dilation and an elevated cerebral blood flow, whereas hypocapnia leads to arterial constriction and a reduced cerebral blood flow. The cerebrovascular changes are thought to be due solely to changes in arterial carbon dioxide levels, as alterations in arterial pH, independent of changes in arterial carbon dioxide levels, have minimal effects on cerebral blood flow (Harper and Bell, 1963). Proposed mechanisms to account for the effects of elevated arterial carbon dioxide levels on cerebral blood flow include changes in the pH of brain extracellular fluid (Kontos et al., 1977), activation of neural pathways (Ingvar, 1958; Irikura et al., 1995; Shalit et al., 1967) and the involvement of vasodilatory agents, including adenosine and nitric oxide.

A role for adenosine was initially proposed based on the observations that systemically administered caffeine, an adenosine receptor antagonist, significantly inhibited carbon dioxide (10%)-induced increases in cerebral flow recorded in a rat venous outflow model (Phillis and DeLong, 1987). Conversely, two agents which potentiate adenosine's action, dipyridamole and deoxycoformycin, enhanced peak flow rates during brief (30–90 s) periods of carbon dioxide inhalation, suggesting that adenosine, a potent vasodilator, could play a role in coupling arterial carbon dioxide levels to cerebral blood flow. These initial

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results were extended by demonstrations that topically applied adenosine deaminase, the enzyme which converts adenosine to the inactive metabolite inosine, attenuated pial arteriolar dilation during hypercapnic episodes in rat and cat cranial window studies (Simpson and Phillis, 1991; Wei and Kontos, 1993).

Negative findings in respect to adenosine's involvement in hypercapnia-evoked increases in cerebral blood flow have been reported by other investigators, who found that theophylline, another adenosine antagonist, had no effect on the carbon dioxide-evoked cerebral hyperemia (Emerson and Raymond, 1981; Hoffman et al., 1984; Morii et al., 1987; Wei and Kontos, 1993). Interestingly, following a topical application of theophylline, topically applied adenosine deaminase failed to attenuate the vasodilatory effects of carbon dioxide inhalation (Wei and Kontos, 1993), suggesting that the presence of an intact adenosine receptor is necessary for expression of the effect of adenosine deaminase. Subsequent studies with selective adenosine receptor agonists and antagonists established that the A_{2A} receptor subtype mediates adenosine-induced dilation of intracerebral arterioles in the rat brain, with a possible contribution of A_{2B} receptors (Ngai et al., 2001). The A_{2A} receptor antagonist used in one study, 4-(2-[7-amino-2-(2-furyl)[1,2,4]triazolo[2,3-*a*]triazin-5-yl amino]ethyl) phenol (ZM 241385), applied topically failed to attenuate hypercarbic vasodilation (Meno et al., 2001).

Hypercapnia is frequently associated with electroencephalographic changes of cortical arousal (high frequency, low voltage EEG activity), which accompanies the increase in cerebral blood flow (Dell, 1958; Gerrits et al., 2001). Ingvar (1958) suggested that in addition to the direct action of carbon dioxide (pH) on cortical arterioles, the vasodilation results from an indirect action mediated by the cortical arousal response. Changes in the excitability of individual cortical neurons during carbon dioxide inhalation have been demonstrated, with excitation at moderate carbon dioxide levels (Krnjevic et al., 1965). Neuronal activity is one of the major factors regulating cerebral blood flow, with cerebrovasodilation being elicited by stimulation of a variety of afferent inputs including sciatic nerve stimulation, vibrissal stimulation and pontine reticular formation stimulation (Iadecola et al., 1994a). In several studies, these vascular responses were attenuated by nitric oxide synthase inhibitors or adenosine receptor antagonists (Dirnagl et al., 1994; Gotoh et al., 2001; Iadecola et al., 1994a,b, Ko et al., 1990; Meno et al., 2001). There is also evidence that nitric oxide is involved in setting the level of cortical arousal and may thus affect cerebral blood flow. Systemically administered N^G-nitro-L-arginine methyl ester (L-NAME) inhibits electrocortical arousal in rats (Bagetta et al., 1993) and 7-nitroindazole, an inhibitor of neuronal nitric oxide synthase induces a state of central depression with motor deficits (Dzoljic et al., 1996). Inhibition of brain nitric oxide synthase could therefore result in reductions in cerebral blood flow occurring in response to reduced neuronal activity.

Studies on the role of nitric oxide have frequently used agents that inhibit the activity of nitric oxide synthase, the enzymes which synthesize nitric oxide from its precursor, L-arginine. The majority of such studies have demonstrated that nitric oxide synthase inhibitors, administered either systemically or topically, attenuate the hypercapnia-evoked increase in cerebral blood flow in a variety of animal models (see Iadecola et al., 1994a for a summary of results). The effect of nitric oxide synthase inhibitors has been stereospecific, dose-dependent, and partially reversible following the administration of L (but not D-)-arginine. Degrees of attenuation of the carbon dioxide responses have been variable, ranging from 30% to 90%, depending on route of administration, concentration of inhaled carbon dioxide, and dose of inhibitor. It has been suggested that nitric oxide may have a "permissive" action, setting the stage for other vasodilatory influences, by elevating cGMP levels in arterial smooth muscle cells (Iadecola et al., 1994b; Okamoto et al., 1997). Pelligrino and Wang (1998) have reviewed the potential crosstalk between cGMP generated by nitric oxide and cAMP triggered by adenosine A_{2A} receptor activated adenylate cyclase in vascular regulation.

In an attempt to reconcile the conflicting observations on adenosine's role in hypercapnia-evoked increases in cerebral blood flow, Estevez and Phillis (1997) evaluated the contributions of nitric oxide and adenosine, as well as cortical electroencephalographic arousal, to hypercapnic hyperemia. Systemically administered caffeine (10 mg/kg) frequently enhanced the ability of carbon dioxide inhalation to initiate electroencephalographic arousal, and when this occurred, its ability to depress the increases in cerebral blood flow was reduced or even absent. Caffeine did not affect normocapnic basal cerebral blood flow rates. The selective A_{2A} receptor antagonist 9-chloro-2-(2-furyl)[1,2,4]-triazolo[1,5-*c*]quinazolin-5-amine (CGS 15943;1 mg/kg) also significantly depressed carbon dioxide-induced arteriolar vasodilation. Administration of L-NAME (20 mg/kg) elicited a slowly developing, progressive, decline in cerebral blood flow, even though mean arterial blood pressure had increased, and attenuated the vasodilatory response to carbon dioxide inhalation. In preparations in which caffeine reduced, but did not abolish, the hypercapnia-evoked response to carbon dioxide, a subsequent administration of L-NAME resulted in a complete block. Conversely, caffeine administration further depressed or abolished that portion of carbon dioxide responses which was resistant to L-NAME. These data suggested that both nitric oxide and adenosine may contribute to pial arteriolar vasodilation during hypercapnia and that carbon dioxide-induced cortical arousal, with enhanced neuronal activity, contributes to the vascular response.

A limitation of the above study was that caffeine, CGS 15943 and L-NAME were administered systemically, with potential effects at various levels of the central nervous

system, autonomic nervous system, and cardiovascular system. To further evaluate the role of adenosine and nitric oxide in hypercapnia-evoked arteriolar vasodilation, we have re-examined the effects of caffeine, L-NAME and the more selective A_{2A} and A_1 receptor antagonists, ZM 241385 and 8-cyclopentyl-1,3-dipropylxanthine (DPCPX, respectively), administered topically and/or systemically using a closed cranial window over the rat cerebral cortex.

2. Materials and methods

2.1. Cerebral blood flow studies

This study was conducted on 59 male Sprague–Dawley rats (300–350 g wt; Charles River). Anesthesia was induced with halothane and, after insertion of a tracheal cannula, maintained with methoxyflurane (Penthrane) in 25% oxygen in nitrogen. Body temperature was controlled at 37 °C with a rectal probe and an electronically regulated abdominal heating pad. One femoral artery was cannulated for measurement of arterial blood pressure and to obtain samples for pH and blood gas measurements. A femoral vein was cannulated for drug administration. Animals (52) to be used for cerebral blood flow experiments were mounted in a Narashige SN2 stereotaxic frame and the dorsal surface of the skull was exposed by a longitudinal incision along the midline. The periosteum of the skull was scraped off and the underlying bone kept clean and dry. A craniectomy was then performed over the left side parietal cortex, 1.5 mm lateral to the midline between bregma and lambda using an electrical drill and No. 6 and No. 1 fine steel burs. Bone bleeding was controlled with a fine cautery probe or bone wax. The thinned bone was lifted off with Dumont microforceps. The dura mater was then covered with a small piece of wet tissue paper. A thin circular line of cyanoacrylate adhesive (3 M Vetbond, No. 1469C, 3 M, St. Paul, MN) was applied to the exposed bone around the craniectomy followed by a layer of dental acrylic (Kerr, Romulus, MI). Four polyethylene tubes (2 (PE50) for afferent flow, 1 (PE60) for efferent drainage flow and 1 (PE50) for the temperature probe) were pressed down into the soft acrylic and another circle of acrylic was added to the doughnut. A glass cover slip was then pressed onto the doughnut before it hardened to create a smooth flat surface onto which a cover slip would eventually be mounted. The dura/arachnoid complex was incised with a 26G hypodermic needle tip and reflected with microforceps. Dural bleeding was controlled by temporary application of minute paper pledgets. The diameter of the cranial window was approximately 2.5 mm. The surface of the acrylic doughnut was next covered with a layer of Nexaband cyanoacrylate (Veterinary Product Laboratories, Phoenix, AZ) and the lumen of the doughnut filled with artificial cerebrospinal fluid (artificial cerebrospinal fluid) so that when the cover slip was pressed down, no air bubbles were present in the closed cranial window. The two inlet ports had previously been flushed and filled with artificial cerebrospinal fluid to remove any air.

Each input channel was connected to a heat exchanger, the temperature of which was controlled by water circulating through a heated water bath. The temperature of the closed cranial window was maintained at 36.5–37 °C by adjusting the temperature in the heat exchangers. Superfusate flow rate was maintained at 0.25 ml/min, using a Harvard Apparatus infusion pump. Brain herniation was prevented by maintaining intracranial pressure at 3–5 mm Hg through adjustment of the height of the outflow tube. Superfused artificial cerebrospinal fluid had the following composition: Na^+, 155.8 mEq/l; K^+, 2.95 mEq/l; Ca^{2+}, 2.5 mEq/l; Mg^{2+}, 1.85 mEq/l; Cl^-, 141.13 mEq/l; HCO_3^- 22 mEq/l; dextrose 66.5 mg/dl; urea, 40.2 mg/dl. In order to avoid bubble formation during passage through the heat exchanger, warmed artificial cerebrospinal fluid was degassed with a vacuum pump prior to use and its pH adjusted to 7.3 using hydrochloric acid.

Artificial cerebrospinal fluid was superfused through one afferent tube with topically applied pharmacological agents in artificial cerebrospinal fluid applied through the other port.

Changes in inspired gases were achieved by connecting the Penthrane vaporizer to gas cylinders containing either 25% oxygen in nitrogen or 7.5% carbon dioxide, 25% oxygen in nitrogen. The gases flowed through individual, matched, Kontes Glass flow probes to ensure comparable rates of flow. Hypercapnic challenges were achieved by unclamping the carbon dioxide line and clamping the 25% oxygen in nitrogen line for 60 s (or for 90 s in a few animals) at 10-min intervals, with a return to the carbon dioxide-free solutions after each 60-s exposure.

After three consistent carbon dioxide responses had been recorded, the appropriate pharmacological agent was administered either intravenously or topically and a succession of three to four carbon dioxide challenges was recorded. Depending on the particular experiment, a topically applied drug could be washed out at this point to evaluate recovery, or another pharmacological agent, or mixture of agents passed through the cranial window.

A screw was inserted in the skull for continuous electroencephalographic recording on a Grass Polygraph. Arterial blood pressure recordings were also obtained, together with cortical blood flow records obtained using a Laser-Doppler probe. The probe was positioned in close apposition to the cover-slip surface, and adjusted both to avoid large pial vessels and locate a point at which stable resting flow rates of 50–60 tissue perfusion units (TPU) were recorded by the Transonic Systems BLF 21D flowmeter, with flow increases during carbon dioxide inhalations. Blood gas analyses during normocapnia and at the end of hypercapnic episodes were carried out at two to three time points during each experiment. As each rat could be subjected to some 15–20 carbon dioxide challenges, it would not have been beneficial to the animal to

remove the large quantities of blood required for repeated evaluations.

The effects of several pharmacological agents on carbon dioxide-induced increases in cerebral blood flow were evaluated in this study. These include the nitric oxide synthase inhibitor N^G-nitro-L-arginine methyl ester (L-NAME), administered either systemically (20 mg/kg) or topically (10^{-3} M) in artificial cerebrospinal fluid; a nonselective adenosine antagonist, caffeine (20 mg/kg or 1 μM) in artificial cerebrospinal fluid; the adenosine A_{2A} selective antagonist, 4-(2-[7-amino-2-(2-furyl)[1,2,4]triazolo[2,3-*a*]triazin-5-yl amino]ethyl) phenol (ZM 241385, Zeneca Pharmaceuticals, Cheshire, U.K.) (1 μM), in 0.1% dimethylsulfoxide in artificial cerebrospinal fluid; and an adenosine A_1 receptor antagonist, 8-cyclopentyl-1,3-dipropylxanthine (DPCPX, Sigma, St. Louis, MO) (1 μM) in 0.1% dimethylsulfoxide in artificial cerebrospinal fluid. 0.1% dimethysulfoxide did not affect carbon dioxide-induced increases in blood flow (see also Ngai et al., 2001).

Because the Laser-Doppler flow monitor displays blood flow readings in arbitrary tissue perfusion units that do not allow measurement of cerebral blood flow in terms of absolute values, the alterations in carbon dioxide during hypercapnia were measured in terms of the area under the curve (AUC) of the carbon dioxide response, which was digitized utilizing Sigma Scan (Jandel Scientific, Corte Madera, CA, USA). This was measured by tracing the blood flow response from the time it increased over basal flow until it returned back to basal flow levels. Transient, single point alterations in perfusion units were ignored. The AUC thus represented both the magnitude and duration of the blood flow response to hypercapnea. The average of three control carbon dioxide responses was obtained (Fig. 1). Data from three carbon dioxide exposures following drug application was normalized as a percentage of the preceding mean control response. Statistical differences were determined by one-way analysis of variance (ANOVA) with contrasts to the appropriate control group. A $P < 0.05\%$ was accepted as denoting a statistically significant difference. Statistically significant effects of L-NAME on basal cerebral blood flow and the peak carbon dioxide response (the maximum blood flow increase over basal flow, ignoring the duration of the blood flow response to hypercapnea) were determined by a Student's *t*-test.

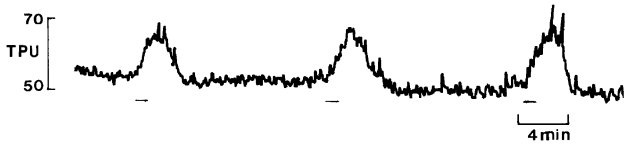

Fig. 1. Three control responses of cerebral blood flow (in tissue perfusion units (TPUs)) to 60-s periods of 7.5% carbon dioxide inhalation as recorded by a doppler flow probe. The black bars under the traces represent the periods of hypercapnia. Measurement of the area under the curve (AUC) is described in Materials and methods.

2.2. Carbon dioxide-evoked adenosine efflux

Experiments were conducted on seven male Sprague–Dawley rats. Following anesthesia-induction, tracheal cannulation, anesthesia maintenance with Penthrane, and the insertion of femoral arterial and venous cannulae, the dorsal surfaces of both cerebral cortices were exposed. After reflection of the dura/arachnoid layer, oval cortical cups, suspended in flexible mounting brackets were placed on both cortices with sufficient light pressure to ensure that fluid could not leak into or from the cups. The dorsal surface of the head around the cups was covered with a stabilizing gel of 3% agar in artificial cerebrospinal fluid. A monopolar electroencephalograph electrode was placed in each cup. Electroencephalographic recordings and arterial blood pressure were recorded on a Grass polygraph. Two hundred microliters of artificial cerebrospinal fluid was pipetted into both cups. Cup fluid was maintained at 37 °C with a heat lamp, and the rat's temperature was maintained at the same temperature with an electronically controlled abdominal heating pad. During the course of the experiment, cup contents were collected at 8-min intervals and replaced with 200 μl of artificial cerebrospinal fluid.

A standard sequence of events was followed with all seven rats. Following the collection of 2×8-min basal artificial cerebrospinal fluid samples, the animals inhaled a mixture of 10% carbon dioxide and 25% oxygen in nitrogen for 8 min and were then reconnected to the 25% oxygen in nitrogen cylinder for three collection periods, followed by one or more periods of carbon dioxide inhalation with three collections in between. The collected superfusate samples were collected in individual chilled microvials, centrifuged in a refrigerated centrifuge at $1200 \times g$ and then briefly stored at −20 °C pending assay. Adenosine concentrations in the artificial cerebrospinal fluid samples were measured by high performance liquid chromatography using previously published methods (Walter et al., 1988). Significant effects of carbon dioxide inhalation on adenosine efflux were determined using ANOVA with contrasts to the preceding basal collection period. $P < 0.05$ was accepted as denoting a significant drug effect.

All animal procedures described in this report were in accordance with the NIH Guide to the Care and Use of Laboratory Animals and were approved by the Wayne State University Animal Investigation Committee.

3. Results

3.1. Physiological variables

Arterial blood gas and pH values (mean ± S.E.M.) in the 52 rats of the first experimental series prior to 60 s, 7.5% carbon dioxide challenges were: pH, 7.40 ± 0.004; arterial carbon dioxide levels, 34.8 ± 1.1 mm Hg; and arterial oxygen levels, 96.9 ± 2.8 mm Hg. Mean arterial blood

pressure was 103.9 ± 9.2 mm Hg. Inhalation of 7.5% carbon dioxide in 25% oxygen resulted in significant reductions in arterial pH 7.31 ± 0.01 ($P < 0.001$); with significant elevations in arterial carbon dioxide levels 48.4 ± 1.2 ($P < 0.001$); and in arterial oxygen levels 108.6 ± 3.7 ($P < 0.05$). Mean arterial blood pressure was unaffected by carbon dioxide inhalation. Electroencephalographic arousal was infrequently observed during the brief carbon dioxide inhalations.

Arterial blood gas and pH values for the seven adenosine release rats prior to the 8 min, 10%, carbon dioxide challenges were: pH, 7.40 ± 0.006; arterial carbon dioxide levels, 33.7 ± 2.3 mm Hg, and arterial oxygen levels, 106.6 ± 3.1 mm Hg, with a mean arterial blood pressure of 102.8 ± 3.6 mm Hg. Following 8 min of 10% carbon dioxide in 25% oxygen inhalation, the blood gas values were: arterial carbon dioxide levels, 59.8 ± 2.5 ($P < 0.001$); arterial oxygen levels, 132.0 ± 2.2 ($P < 0.001$); with a pH of 7.22 ± 0.01 ($P < 0.001$). Mean arterial blood pressure was not affected by carbon dioxide inhalation. Electroencephalographic arousal was observed in five of the seven animals in this group during carbon dioxide inhalation.

3.2. Cerebral blood flow

Inhalation of 7.5% carbon dioxide resulted in increases in cerebral blood flow above basal normocapnic levels. The fact that carbon dioxide-enriched gas flowed through the Penthrane vaporizer en route to the rats resulted in a relatively slow rise in the rate of flow, which peaked shortly after the restoration of carbon dioxide-free gas flow, and then returned to basal levels during the subsequent 2–3 min. As a percentage of normocapnic values, the increases in cerebral blood flow were generally in the range of 40–100%. The incidence of carbon dioxide-evoked electroencephalographic arousal, that is associated with more dramatic increases in cerebral blood flow (Estevez and Phillis, 1997), was minimized by using brief (60 s) periods of carbon dioxide administration. As stated above, arousal occurred more frequently in the second group of animals, administered 10% carbon dioxide for 8-min periods.

3.3. Caffeine and DPCPX

The effects of systemically administered caffeine (20 mg/kg), a nonselective adenosine receptor antagonist, were evaluated in eight rats. Systemic caffeine had no significant effects on blood pressure or basal flow rates. It also failed to block the carbon dioxide-evoked hyperemia, as measured by response area (AUC). However, it did cause a 20% reduction ($P < 0.05$) in the peak height of the response. Topically administered caffeine (1 mM) elicited a small, nonsignificant reduction in the carbon dioxide-evoked increases in cerebral blood flow (Fig. 2).

The A_1 selective adenosine receptor antagonist DPCPX (1 μM) did not affect MABP, basal cortical blood flow rates or the carbon dioxide-induced increase in cerebral blood flow.

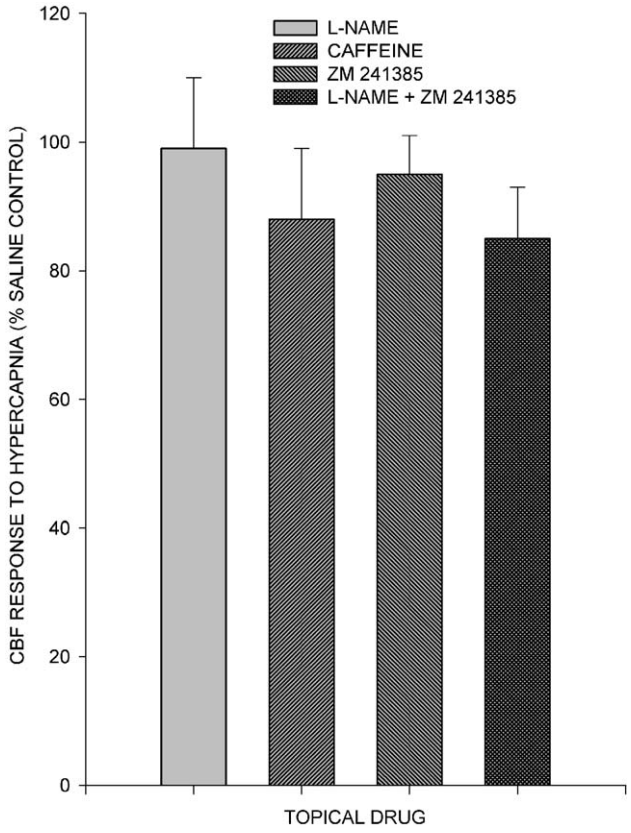

Fig. 2. Bargraphs illustrating the increases in cerebral blood flow during brief (60–90 s) periods of inhalation of 7.5% carbon dioxide in 25% oxygen in nitrogen, measured by a Laser-Doppler probe. The carbon dioxide-evoked increases in flow following topical applications of L-NAME (1 mM), caffeine (1 mM), ZM 41385 (1 μM) and L-NAME plus ZM 41385 in artificial cerebrospinal fluid are presented as percentages of the response during control applications of artificial cerebrospinal fluid, measured as the area under the curve (AUC) of three successive carbon dioxide exposures in each rat (mean ± S.E.M.).

3.4. ZM 241385

This A_{2A} selective receptor antagonist was applied topically on the cerebral cortex of nine rats in a 0.1% dimethylsulfoxide artificial cerebrospinal fluid solution. At this concentration, dimethylsulfoxide did not affect either basal cerebral blood flow or the carbon dioxide-evoked hyperemia. When applied by itself, ZM 214385 (0.1 or 1.0 μM) did not alter either basal cerebral blood flow or the response to carbon dioxide inhalation (Fig. 2).

3.5. L-NAME

The nonselective nitric oxide synthase inhibitor, L-NAME, was administered both systemically and topically. Systemic L-NAME (20 mg/kg) evoked a rapid and significant increase in blood pressure of approximately 20–35

mm Hg magnitude. The increase in pressure then gradually declined over the following 20–50 min. L-NAME was also observed to cause a transient fall of several mm Hg in mean arterial blood pressure during carbon dioxide inhalation, even though this effect had not been noticeable in pre-L-NAME controls. Systemic L-NAME elicited a slow, progressive decline in cerebral blood flow from a control level of 58.1 ± 2.3 to 45.8 ± 2.3 TPUs ($P < 0.05$) in 19 rats. The decrease in cerebral blood flow occurred even though L-NAME administration had elicited a rapid increase in mean arterial blood pressure, which then declined slowly to its original level. As assessed by the area (AUC) of the carbon dioxide-evoked increase in cerebral blood flow, systemically administered L-NAME failed to depress the carbon dioxide hyperemic response. However, when measured by the percentage peak height increase above basal flow levels, it did cause a 20% reduction in peak height ($P < 0.05$), which appeared to have been a consequence of the carbon dioxide-induced fall in mean arterial blood pressure. This is consistent with the result of a previous study in which L-NAME depressed the peak height percentage increase in carbon dioxide-evoked flow above preceding basal flow rates (Estevez and Phillis, 1997). In essence, L-NAME appears to have reduced the peak height of the carbon dioxide response while lengthening its duration. Exposure to L-NAME was also associated with an increase in the "spontaneous" waves of increases and decreases in blood flow (vasomotion) that were recorded by the flow probe. These changes are associated with cyclical contractions and relaxations of pial arterioles, which have been recorded by a video camera (Ngai et al., 1995), following topical application of N^G-nitro-L-arginine, another nitric oxide synthase inhibitor.

The effects of topically applied L-NAME (1 mM; for 51.2 ± 5.2 min) were evaluated in five rats. It failed to significantly reduce either basal flow rates or the carbon dioxide-evoked increases (AUC) in cerebral blood flow (Fig. 2) when applied for periods of up to 40 min.

3.6. Drug interactions

Evidence presented in a previous report demonstrated that both adenosine and nitric oxide contribute to hypercapnia-induced cerebrovasodilation in rats (Estevez and Phillis, 1997) and the effects of a simultaneous blockade of both systems were more effective than either alone. However, in that study, all agents were applied systemically. The possibility of local interactions was approached by evaluating the effects of topical applications of caffeine and ZM 241385 following systemic exposure of the cerebral cortex to L-NAME, or alternatively of topical application of both L-NAME and ZM 241385.

The results with caffeine and ZM 241385 application following a 30-min prior exposure to systemic L-NAME (20 mg/kg i.v.) are illustrated in Fig. 3. Following an L-NAME (20 mg/kg) application, caffeine (1 mM), applied topically, caused a significant 41% reduction in the carbon dioxide-elicited increases in cerebral blood flow in four rats (Fig. 3) with some recovery after 20–30 min of perfusion with caffeine-free artificial cerebrospinal fluid. Similar effects (Fig. 3) were observed following the topical application of ZM 241385 (1 μM) to L-NAME (20 mg/kg) pretreated rats ($n = 5$). Slow recovery was evident following washout of the ZM 241385. The depressor effect of carbon dioxide inhalation following the systemic administration of L-NAME (20 mg/kg) in this group of rats averaged 10.2 ± 0.8 mm Hg. The depressor effect was not significantly affected by topical application of either caffeine (8.8 ± 0.6 mm Hg) or ZM 41385 (8.9 ± 1.2 mm Hg). Topical applications of DPCPX (1 μM), a selective A_1 adenosine receptor antagonist failed to affect the carbon dioxide-evoked hyperemias in another four rats pretreated with L-NAME (20 mg/kg) (Fig. 3).

Topical administration of ZM 241385 (1 μM) was followed 30 min later by the application of artificial cerebrospinal fluid containing ZM 241385 (1 μM) together with topical L-NAME (0.1 mM, four rats). The combined topical application of ZM 241385 and L-NAME failed to significantly affect the responses to carbon dioxide in four rats

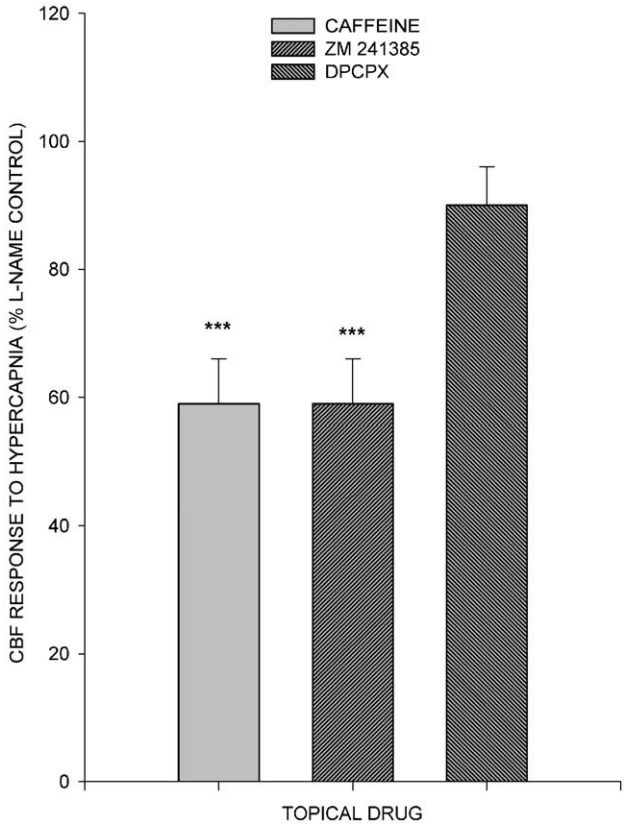

Fig. 3. Bargraphs illustrating the effects of prior systemic administration of L-NAME (20 mg/kg) on the increases in cerebral blood flow elicited by 7.5% carbon dioxide inhalation during topical applications of caffeine (1 mM), ZM 41385 (1 μM), and DPCPX (1 μM). The bars are calculated as percentages of the control response during systemic L-NAME administration (mean ± S.E.M.). ***$P < 0.001$.

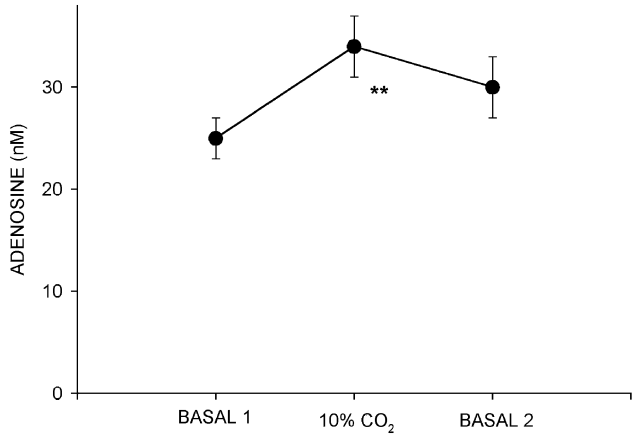

Fig. 4. Levels of adenosine in 200 μl cortical superfusate samples collected at 8-min intervals prior to; after 8 min of 10% carbon dioxide inhalation; and 8 min after termination of carbon dioxide inhalation. Results are from seven rats, each with bilateral cortical cups, exposed to two to three periods of carbon dioxide inhalation. $^{**}P < 0.01$.

(Fig. 2), but did reduce the basal cortical blood flows from 47.3 ± 5.0 to 38.0 ± 3.7 TPUs.

3.7. Hypercapnia-evoked adenosine efflux

Adenosine efflux into bilateral cortical superfusates during 8-min periods of carbon dioxide inhalation was measured in seven rats, with each being exposed to two to three carbon dioxide inhalations at 24-min intervals. The results are illustrated in Fig. 4 in which the basal level of adenosine in cerebral cortical superfusates is 25 nM. At the end of the 8-min periods of carbon dioxide inhalation, adenosine levels had risen to 34 nM ($P < 0.01$), with some recovery towards basal levels in the initial post-carbon dioxide collection period.

4. Discussion

In the present study, we have analyzed the role of adenosine in the cerebrovascular microcirculation's response to hypercapnia, with particular emphasis on the potential interactions between adenosine and nitric oxide. Previous studies in this laboratory using systemically administered adenosine receptor antagonists and potentiators of adenosine's actions had led to the suggestion that adenosine was involved in hypercapnia-evoked cerebral vasodilatation (Estevez and Phillis, 1997; Phillis and DeLong, 1987). This proposal received support from experiments showing that topically applied adenosine deaminase reduced carbon dioxide-evoked cortical arteriolar dilation during carbon dioxide inhalation (Simpson and Phillis, 1991; Wei and Kontos, 1993). Other investigators have failed to observe decreases in carbon dioxide-evoked hyperemias following administration of the adenosine receptor antagonists theophylline and 8-*p*-sulfophenyltheophylline (Hoffman et al., 1984; Morii et al., 1987; Pelligrino et al., 1995; White et al., 1998). More recently, Ngai et al. (2001) have demonstrated that the adenosine receptor on rat intracerebral arterioles that primarily mediates vasodilation by adenosine is of the A_{2A} type and can be selectively antagonized by topically applied ZM 241385. Neither DPCPX, an A_1 receptor-selective antagonist, nor 3-ethyl-5-benzyl-2-methyl-4-phenylethyl-6-phenyl-1,4(±)dihydropyridine-3,5-dicarboxylate (MRS-1191), an A_3 receptor-selective antagonist, attenuated adenosine vasodilation. Meno et al. (2001) further demonstrated that intravenously administered ZM 241385 did not block hypercarbic vasodilation, reiterating earlier claims that adenosine is not involved in the cerebrovascular response to hypercapnea.

An initial primary objective of the present study was therefore to examine the reason why systemically or topically applied ZM 241385 had no significant antagonistic action on carbon dioxide-evoked dilation of cerebral arterioles, as reported by Meno et al. (2001). The data obtained appeared to support these observations as we failed to observe any reductions in the carbon dioxide response following ZM 241385 application by either route. The nonspecific adenosine receptor antagonist caffeine also failed to reduce carbon dioxide-evoked cerebral arteriolar vasodilations when used in conjunction with the cortical window technique.

Based on the data obtained in the previous experiments on the role of nitric oxide in carbon dioxide-evoked arteriolar dilation, which demonstrated dual contributions by nitric oxide and adenosine (Estevez and Phillis, 1997), it was decided to explore this avenue further by blocking nitric oxide formation with L-NAME. Removal of the nitric oxide contribution to the carbon dioxide-evoked arteriolar vasodilation clearly revealed a significant reduction in the hyperemia following topical application of caffeine, or of the A_{2A}-selective adenosine receptor blocker ZM 241385. Earlier and subsequent publications have suggested that nitric oxide may play a "permissive" role in the cerebral blood flow response to hypercapnia (Iadecola et al., 1994b; Okamoto et al., 1997; Wang et al., 1999). A permissive role of nitric oxide implies that while a certain background level of nitric oxide is required for a vasodilatory response to occur, increases in nitric oxide levels are not required for the dilation to take place. In this sense, the presence of nitric oxide allows other mediators to relax vascular smooth muscle.

In several respects the results obtained with L-NAME in this study appear to be inconsistent with the previous reports from both this and some other laboratories. However, the literature on the effects of nitric oxide synthase inhibitors on carbon dioxide-evoked increases in cerebral cortical blood flow is replete with such differences. Variability in outcome may depend on a number of factors, including species of animal or human subjects (McPherson et al., 1995; Schmetterer et al., 1997; Thompson et al., 1996; White et al., 1998), anesthesia (Gellhorn, 1953; Goadsby, 1994; Iadecola et al., 1994a; Levasseur and Kontos, 1989; Morii et al., 1987;

Phillis and DeLong, 1987; Schmetterer et al., 1997; Wolk et al., 1995a), duration of carbon dioxide exposure (Bremer and Thomas, 1936; Gerrits et al., 2001; Ingvar, 1958; Krnjevic et al., 1965; Somjen et al., 1987), techniques used for assessment of blood flow (Irikura et al., 1995), the route of administration of drugs used to modulate flow and the appropriate choice of pharmacological agents (Iadecola et al., 1994a,b; Irikura et al., 1994; Reiner and Zagvazdin, 1998).

Systemically administered nonselective nitric oxide synthase inhibitors raise mean arterial blood pressure while at the same time reducing cerebral cortical blood flow, which is indicative of an increase in basal arteriolar tone, whereas topical application causes neither (Fabricius et al., 1996; present results). The selective neuronal nitric oxide synthase inhibitor, 7-nitroindazole (7-NI) when administered systemically, causes a small, nonsignificant decrease in mean arterial blood pressure, an 18% reduction in basal cortical blood flow and a 77% decrease in the response to carbon dioxide inhalation. However, when applied topically, it only slightly reduces the carbon dioxide-induced hyperemia (Fabricius et al., 1996). The authors suggested that neither cortical endothelial nitric oxide synthase nor neuronal nitric oxide synthase activity was important for the carbon dioxide effect. Indeed, following deletion of either neuronal (Irikura et al., 1994) or endothelial (Ma et al., 1996) nitric oxide synthase in mice, hypercapnia continues to be associated with an increased cerebral blood flow.

Another anomalous effect of systemic L-NAME was to cause a fall in mean arterial blood pressure during carbon dioxide administration, which resulted in a significant decline in peak increases in cortical blood flow (Estevez and Phillis, 1997), but not in the area under curve (AUC) measurements of response in the present experiments. This did not occur when L-NAME was administered topically. A similar effect of systemically administered L-NAME has been described by Wolk et al. (1995a) in paralyzed, urethane anesthetized rats, where in 11 rats, L-NAME reversed a pressor effect of brief hypercapnia induced by 10% carbon dioxide into a depressor response with a reduced carbon dioxide-induced cerebral blood flow response. However, in five rats, L-NAME did not abolish the central pressor effect of hypercapnia and significantly augmented the carbon dioxide-induced vasodilatory response by reducing cortical vascular resistance. In conclusion, it was suggested that while nitric oxide does not mediate the vasodilatory effects of a brief hypercapnia in the cerebral cortex, it is critical for the neurogenic pressor effect of carbon dioxide inhalation (Wolk et al., 1995a,b).

It is likely important that the effect of local adenosine receptor blockade on the carbon dioxide response was evident following systemic but not topical L-NAME application. This implies that the effects of L-NAME are mediated either centrally via neurogenic mechanisms, in the medullary blood pressure regulatory centers where nitric oxide is a neuromodulator (Wolk et al., 1995a,b), or on larger upstream vessels where nitric oxide is known to mediate the shear stress response (Zanzinger, 1999). It is also important to note that even following the combined blockade of adenosine receptors and nitric oxide formation, there remained a robust hyperemic response to carbon dioxide inhalation.

In conclusion, the data presented in this report provide evidence that a brief exposure to carbon dioxide elicits a release of adenosine from the cerebral cortex and that this adenosine binds to local A_{2A} receptors causing vasodilation and increased blood flow. The findings are also consistent with evidence that adenosine is involved in the carbon dioxide and acidosis-induced dilations of coronary arterioles in the isolated, perfused, rat heart (Phillis et al., 1998). The lack of effect on the cerebral blood flow response to hypercapnea when adenosine receptor antagonists were applied in the presence of intact nitric oxide signaling pathways indicates that there is likely to be considerable redundancy in the regulation of cerebral blood flow. However, combined blockage of nitric oxide synthase activity with L-NAME and of adenosine A_{2A} receptors with ZM 241385, revealed an underlying adenosinergic mechanism.

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