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Regional differences in mechanisms of cerebral circulatory response to neuronal activation

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Gotoh, Jun, Tang-Yong Kuang, Yasuaki Nakao, David M. Cohen, Peter Melzer, Yoshiaki Itoh, Hazel Pak, Karen Pettigrew, and Louis Sokoloff. Regional differences in mechanisms of cerebral circulatory response to neuronal activation. *Am J Physiol Heart Circ Physiol* 280: H821–H829, 2001.—Vibrissal stimulation raises cerebral blood flow (CBF) in the ipsilateral spinal and principal sensory trigeminal nuclei and contralateral ventroposteromedial (VPM) thalamic nucleus and barrel cortex. To investigate possible roles of adenosine and nitric oxide (NO) in these increases, local CBF was determined during unilateral vibrissal stimulation in unanesthetized rats after adenosine receptor blockade with caffeine or NO synthase inhibition with N^G-nitro-L-arginine methyl ester (L-NAME) or 7-nitroindazole (7-NI). Caffeine lowered baseline CBF in all structures but reduced the percent increase during stimulation only in the two trigeminal nuclei. L-NAME and 7-NI lowered baseline CBF but reduced the percent increase during stimulation only in the higher stations of this sensory pathway, i.e., L-NAME in the VPM nucleus and 7-NI in both the VPM nucleus and barrel cortex. Combinations of caffeine with 7-NI or L-NAME did not have additive effects, and none alone or in combination completely eliminated functional activation of CBF. These results suggest that caffeine-sensitive and NO-dependent mechanisms are involved but with different regional distributions, and neither fully accounts for the functional activation of CBF.

adenosine; nitric oxide; caffeine; 7-nitroindazole; N^G-nitro-L-arginine

NUMEROUS STUDIES HAVE ESTABLISHED that neuronal functional activation is associated with increases in both cerebral energy metabolism and blood flow (CBF) in components of the activated neural pathway (8, 18, 30–32). The increases in energy metabolism evoked by functional activation have been shown to be proportional to the increases in spike frequency in the afferent inputs to the activated areas (13, 32) and to be due mainly to activation of Na^+-K^+-ATPase activity (21, 32). The mechanisms mediating the increases in CBF during functional activation remain, however, largely undefined. A popular hypothesis proposed by Roy and Sherrington (27) was that CBF is intrinsically regulated by products of energy metabolism to meet the altered metabolic demands associated with functional activity. This hypothesis received support from subsequent findings that CBF is raised by increased CO_2 tension, lowered pH, and decreased oxygen tension, all expected consequences of increased tissue metabolism, and reduced by changes in these chemical factors in the opposite direction, to be expected with decreased metabolism (14). Since then, many other endogenous agents that affect cerebral blood vessels, e.g., nitric oxide (NO), adenosine, adenine nucleotides, K^+, prostaglandins, vasoactive intestinal peptide, etc., have been identified and considered as possible candidates, but not one of them, alone or in combination with others, has yet been proven to account fully or even to be essential for the enhancement of CBF by neuronal activation.

Inhibition of cerebral glucose utilization by hypoglycemia or pharmacological doses of 2-deoxyglucose (2-DG) has been found to result in marked increases in CBF (3, 10). Inasmuch as these conditions depress cerebral energy metabolism, the increases in CBF cannot be ascribed to vasodilator products of glucose and/or oxygen metabolism. Recently, Horinaka et al. (11) found that caffeine dose dependently attenuates to the point of complete elimination the elevation of CBF in hypoglycemia. Caffeine is a nonselective antagonist of adenosine receptors, and adenosine has vasodilator properties that have led to its consideration as a potential mediator of the adjustment of CBF to functional and metabolic activities (25, 28, 37). The present studies were undertaken to determine whether caffeine might also inhibit the enhancement of CBF by functional neuronal activation. A number of studies, all carried out in anesthetized animals and most often with laser-Doppler flowmetry applied only to the cerebral cortex, have implicated NO in the functional activation of CBF (2, 4, 7, 12, 19). In contrast, studies with the nonspecific NO synthase inhibitor, N^G-nitro-L-arginine methyl ester (L-NAME), in which CBF was quan-

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titatively determined in unanesthetized animals, have failed to support such a role for NO (1, 33, 36). To our knowledge, the effects of the specific inhibitor of neuronal NO synthase, 7-nitroindazole (7-NI), have never been examined in unanesthetized animals. We therefore also compared in this study the effects of caffeine, L-NAME, and 7-NI, each administered alone, and combinations of caffeine with each of the NO synthase inhibitors. The studies were carried out in unanesthetized rats, functional activation was elicited by vibrissal stimulation, and absolute rates of CBF were quantitatively determined in four stations of the whisker-to-barrel cortex pathway.

MATERIALS AND METHODS

Chemicals

2-[1-^{14}C]DG (2-[^{14}C]DG; sp. act. 51 mCi/mmol) and 4-[*N-methyl*-^{14}C]iodoantipyrine ([^{14}C]IAP; sp. act. 54 mCi/mmol) were obtained from NEN (Boston, MA). Caffeine, L-NAME, 7-NI, and DMSO were obtained from Sigma (St. Louis, MO).

Animals

All procedures performed on animals were in strict accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals and were approved by the local Animal Care and Use Committee.

Normal, adult, male Sprague-Dawley rats (290–410 g) were obtained from Charles River Laboratories (Wilmington, MA). They were maintained on a 12:12-h light-dark cycle with humidity and temperature controlled at normal levels and were allowed food and water ad libitum until deprived of food but not water ~16 h before the experiment. On the day of the experiment, the rats were anesthetized with halothane (5% for induction, 1.0–1.5% for maintenance) in 70% N_2O-30% O_2, and polyethylene catheters (PE-50; Clay-Adams, Parsippany, NJ) were inserted in both femoral arteries and one femoral vein. Also, the vibrissae on the right side of the face were cut close to the skin to minimize spurious stimulation on the unstimulated control side. Lidocaine ointment (5%) was applied to the surgical wounds after closure. Loose-fitting plaster casts were then applied to the lower torso to prevent locomotion but to allow free movement of the head, forelimbs, and upper torso. Body temperature was maintained at 37°C throughout the experiment by a servo-controlled infrared heating lamp connected to a rectal thermistor.

After at least 3 h for recovery from surgery and anesthesia, vibrissal stimulation and measurement of either local CBF or local cerebral glucose utilization ($lCMR_{Glc}$) were initiated. The vibrissae on the left side of the face were stroked forward and backward continuously with a small paint brush at a rate of 2–4 strokes/s throughout the 1-min period of measurement of local CBF or the 45-min period of measurement of $lCMR_{Glc}$.

Local CBF was determined during unilateral vibrissal stimulation in eight groups of rats. *Groups 1*, *2*, and *3* were injected intravenously with saline, 20 mg/kg of caffeine, or 40 mg/kg of caffeine, respectively, 15 min before initiation of whisker stroking and measurement of CBF. *Group 4* was injected intravenously with 30 mg/kg of L-NAME 30 min before vibrissal stimulation and CBF measurement. *Groups 5* and *6* were injected intraperitoneally with either DMSO alone or 30 mg/kg of 7-NI dissolved in DMSO, respectively, 30 min before onset of whisker stroking and CBF measurement. *Groups 7* and *8* were injected with a combination of 20 mg/kg of caffeine and either L-NAME or 7-NI, respectively, with each drug injected as above. In two additional groups of rats, $lCMR_{Glc}$ was measured in the four stations of the whisker-to-barrel cortex pathway during similar vibrissal stimulation; one was a saline-treated control group, and the other was injected intravenously with 40 mg/kg of caffeine 15 min before onset of vibrissal stimulation and measurement of $lCMR_{Glc}$. Because energy metabolism is probably more directly linked to functional activity, $lCMR_{Glc}$ was measured to assess whether caffeine effects on the CBF responses to functional activation were secondary to alterations in the intensity of neuronal functional activation or directly on mechanisms of regulation of CBF during activation.

Physiological Variables

Mean arterial blood pressure (MABP) was measured with a Digi-Med Blood Pressure Analyzer (Micro-Med, Louisville, KY). Hematocrit was determined in arterial blood samples centrifuged in a Microfuge B (Beckman Instruments, Fullerton, CA). Arterial blood P_{O_2}, P_{CO_2}, and pH were measured with a Corning pH/blood gas analyzer (model 288; Corning Medical, Medfield, MA). Arterial plasma glucose content was measured in a Beckman Glucose Analyzer 2 (Beckman Instruments).

Because caffeine stimulated respiration and lowered arterial P_{CO_2}, caffeine-treated rats were placed in transparent plastic bags that were intermittently ventilated with 5% CO_2 in humidified room air at a rate of 1–5 l/min as needed to maintain arterial P_{CO_2} within normal limits. The saline-treated control rats were treated similarly, except that the bags were ventilated with humidified room air. Arterial P_{CO_2}, P_{O_2}, pH, and MABP were measured before drug or saline administration and again just before measurements of CBF or $lCMR_{Glc}$.

Measurement of Local CBF

Local CBF was determined by the autoradiographic [^{14}C]IAP method as previously described (29), except that the [^{14}C]IAP (40 μCi in 0.8 ml of physiological saline) was continuously infused via the femoral venous catheter by a computer-driven infusion pump (model 2400–003; Harvard Apparatus, South Natick, MA) programmed to produce a constantly rising arterial [^{14}C]IAP concentration. Timed arterial samples were collected on weighed filter paper discs throughout the infusion period and later were assayed for [^{14}C]IAP concentration by liquid scintillation counting as previously described (29). At a precisely recorded time ~1 min after onset of the infusion, the rat was decapitated, and the brain was removed, frozen in isopentane maintained at −40 to −50°C with dry ice, and cut into 20-μm sections in a cryostat at −22°C. The frozen brain sections were mounted on glass coverslips, immediately dried on a hot plate at ~60°C, and autoradiographed together with calibrated [^{14}C]methylmethacrylate standards on Kodak EMC-1 X-ray film (Kodak, Rochester, NY). Local tissue concentrations of [^{14}C]IAP were determined by densitometric analysis of the autoradiograms, and rates of local CBF were calculated from the local tissue concentrations and the time course of the arterial [^{14}C]IAP concentration by means of the operational equation of the method (29) and the computer program developed by G. Mies (Max Planck Institut für Neurologische Forschung, Köln, Germany) for use with the NIH Image program (W. Rasband, National Institute of Mental Health, Bethesda, MD) and Macintosh computer.

Measurement of $lCMR_{Glc}$

$lCMR_{Glc}$ was measured bilaterally in the spinal and principal trigeminal nuclei, ventroposteromedial (VPM) thalamic nucleus, and barrel region of the sensory cortex by the quantitative autoradiographic 2-[^{14}C]DG method (34) during unilateral vibrissal stimulation. Vibrissal stimulation was begun simultaneously with the intravenous injection of a pulse of 125 μCi/kg of 2-[^{14}C]DG that initiated the period of measurement of $lCMR_{Glc}$. Timed arterial blood samples were then drawn and centrifuged, and the time courses of the plasma glucose and 2-[^{14}C]DG concentrations over the ~45-min experimental period were determined as previously described (34). At the end of the experimental period, the rats were decapitated, and their brains were frozen, sectioned, and autoradiographed as described above. Local tissue concentrations of ^{14}C in the four stations of the whisker-to-barrel cortex pathway were determined by the same computerized image-processing system used for the determination of local CBF, and $lCMR_{Glc}$ was calculated from the local tissue ^{14}C concentrations and the time courses of the arterial plasma glucose and 2-[^{14}C]DG concentrations according to the operational equation of the method (34).

Statistics

In all animals, local CBF or $lCMR_{Glc}$ was determined bilaterally in the four stations of the whisker-to-barrel cortex pathway. Local CBF was also determined bilaterally in 18 structures outside the pathway. Data are expressed as means ± SE for the number of animals indicated in Figs. 1–5 and Table 1. For each structure, a side-to-side comparison was made with a paired *t*-test on the absolute values of CBF or $lCMR_{Glc}$. Statistical analyses of the percent difference between the two sides were made on logarithmically transformed percentages. Homogeneity of groups with respect to side-to-side percentage differences for each structure was first analyzed by a one-way ANOVA followed by pair-wise *t*-tests between groups. Statistical significance of differences between groups in the absolute values of local CBF, $lCMR_{Glc}$, or physiological variables was determined by Student's *t*-test or one-way ANOVA followed by Dunnett's *t*-test when multiple groups were compared against a single group.

RESULTS

Effects of Caffeine Administration

Effects on behavior and physiological variables. Caffeine administration evoked hyperventilation and behavioral changes, e.g., sniffing, intense grooming, head bobbing, chewing, and paw pushing, that persisted throughout the experimental period. These effects were more striking with the larger dose of caffeine. Effects of hyperventilation on arterial P_{CO_2} were controlled by intermittent addition of 5% CO_2 to the inspired air as needed, but arterial P_{O_2} rose significantly and pH tended to fall after caffeine administration (Table 1).

Effects of caffeine on baseline CBF and functional activation of CBF. In both control and caffeine-treated groups, none of the 18 structures outside the whisker-to-barrel cortex pathway that were examined exhibited statistically significant side-to-side differences in CBF (Fig. 1). Both doses of caffeine (20 and 40 mg/kg), however, produced dose-dependent, statistically significant ($P < 0.05$ or <0.01) reductions in CBF in most of these structures (Fig. 1). Caffeine also tended to reduce CBF in the unstimulated side of the four structures of the whisker-to-barrel cortex pathway, but these reductions reached statistical significance ($P < 0.05$) only with the 40 mg/kg dose in the spinal trigeminal nucleus, VPM nucleus, and barrel cortex (Fig. 2).

In the saline-treated controls, unilateral vibrissal stimulation markedly raised CBF in the ipsilateral spinal trigeminal (+68%) and principal sensory trigeminal (+60%) nuclei and the contralateral VPM nucleus (+29%) and barrel cortex (+51%) above the levels in the homologous structures of the unstimulated side ($P < 0.005$; Fig. 2). Both doses of caffeine reduced the percent increase in CBF due to stimulation to about half in the spinal ($P < 0.01$) and principal trigeminal ($P < 0.04$) nuclei (Fig. 2). Caffeine also tended to dose dependently lower the percent increase in CBF due to stimulation in the VPM thalamic nucleus and to enhance the percent increase in the barrel cortex, but these effects failed to achieve statistical significance (Fig. 2).

Effects of caffeine on the functional activation of local glucose utilization. Energy metabolism is probably more directly coupled to neuronal functional activity than blood flow. Therefore, to assess whether the effects of caffeine on the percentage of the changes in CBF evoked by vibrissal stimulation were secondary to alterations in metabolism or intensity of neuronal activation or were due to an action on the mechanisms of

Table 1. *Physiological variables immediately before measurement of cerebral blood flow*

Treatment	*n*	Mean Arterial Blood Pressure, mmHg	Arterial P_{CO_2}, mmHg	Arterial P_{O_2}, mmHg	Arterial pH
Saline	7	112 ± 1	35 ± 0.5	85 ± 1	7.45 ± 0.01
Caffeine (20 mg/kg)	10	114 ± 3	37 ± 2	114 ± 4†	7.40 ± 0.01*
Caffeine (40 mg/kg)	8	115 ± 4	36 ± 1	109 ± 4†	7.42 ± 0.01
Saline + L-NAME (30 mg/kg)	5	131 ± 2†	36 ± 1	92 ± 1	7.41 ± 0.004
Caffeine (20 mg/kg) + L-NAME (30 mg/kg)	7	135 ± 2†	38 ± 1	107 ± 3*	7.38 ± 0.01†
Saline + DMSO	6	122 ± 2	36 ± 1	92 ± 3	7.44 ± 0.01
Saline + 7-NI in DMSO (30 mg/kg)	6	119 ± 2	40 ± 1	97 ± 6	7.40 ± 0.01
Caffeine (20 mg/kg) + 7-NI in DMSO (30 mg/kg)	8	122 ± 3	39 ± 1	107 ± 8†	7.38 ± 0.02†

Values are means ± SE obtained immediately before measurement of cerebral blood flow (CBF) in no. of animals (*n*) indicated. L-NAME, N^G-nitro-L-arginine methyl ester; 7-NI, 7-nitroindazole. All treatments were administered intravenously except for DMSO, which was administered intraperitoneally. *$P < 0.05$ and †$P < 0.01$ compared with saline-treated controls.

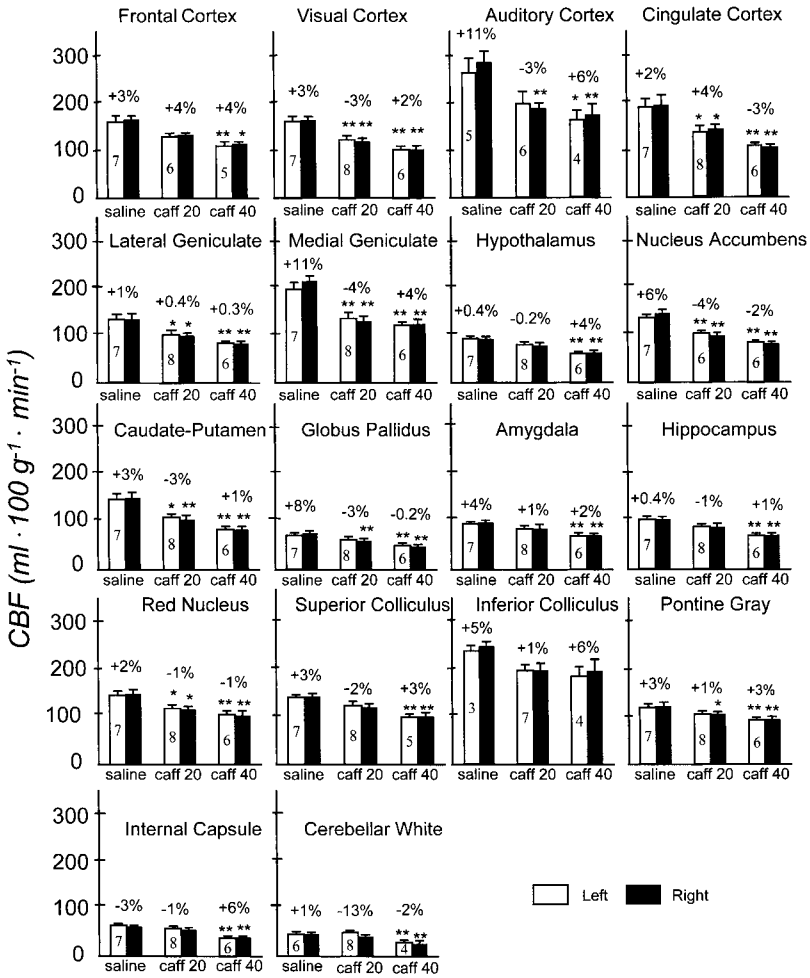

Fig. 1. Effects of caffeine on cerebral blood flow (CBF) during stimulation of vibrissae on the left side of the face in structures outside the whisker-to-barrel cortex pathway. Bar heights and error bars represent mean CBF ± SE for no. of animals indicated in bars. The %values represent the means of the individual percentage of differences between left and right sides for that structure in all of the animals. None of these structures exhibited a statistically significant difference between stimulated and unstimulated sides. Caff 20 and caff 40, doses of 20 and 40 mg/kg caffeine, respectively. *$P <$ 0.05 and **$P < 0.01$, statistically significant different CBF in caffeine-treated animals compared with saline-treated control group (determined by Dunnett's t-test for multiple comparisons with a single control group).

coupling of CBF to functional activity, we examined the effects of caffeine on the changes in $lCMR_{Glc}$ evoked by vibrissal stimulation. Unilateral vibrissal stimulation markedly raised $lCMR_{Glc}$ in the stimulated sides of all four stations of the pathway, and these increases were not reduced by the caffeine treatment (Fig. 3). In fact, in the barrel cortex, caffeine statistically significantly ($P < 0.05$) enhanced the percentage of increase in $lCMR_{Glc}$ due to stimulation (Fig. 3).

Effects of Inhibitors of NO Synthase on CBF Response to Functional Activation

L-NAME is a nonspecific inhibitor of endothelial, neuronal, and inducible NO synthase activities, whereas 7-NI specifically inhibits only the neuronal isoform. Because of uncertainty about which of these isoforms, if any, was involved in the CBF response to functional activation, we examined the effects of both inhibitors.

Effects of 7-NI. Because 7-NI had to be dissolved in DMSO and administered intraperitoneally, it was also necessary to examine the effects of intraperitoneal DMSO alone. Compared with values obtained in the saline-treated controls, DMSO had no statistically significant effects on any of the physiological variables (Table 1), baseline CBF, or percentage of increases in CBF evoked by vibrissal stimulation (Fig. 4). 7-NI lowered baseline CBF statistically significantly ($P <$ 0.05 by Dunnett's t-test for multiple comparisons) in the spinal and principal trigeminal nuclei, but comparable reductions in the VPM nucleus and barrel cortex were statistically significant only by Student's t-test ($P < 0.04$) and not the Dunnett's test (Fig. 4). Almost exactly opposite to the effects of caffeine (i.e., statistically significantly reduced percentage of increases in CBF due to vibrissal stimulation in the two trigeminal nuclei and not in the VPM nucleus and barrel cortex), 7-NI had no effects in the two brain stem nuclei but significantly diminished the responses in the VPM nucleus ($P \approx 0.05$) and barrel cortex ($P < 0.04$; Fig. 4).

Effects of L-NAME. Intravenous administration of L-NAME (30 mg/kg) had no effects on arterial P_{CO_2} and P_{O_2} but raised MABP from 113 ± 6 (mean ± SD) before to 131 ± 5 mmHg 30 min after its injection ($P < 0.01$). Despite the higher MABP during the measurement of CBF (Table 1), L-NAME treatment lowered baseline CBF on the unstimulated side in all four stations of the whisker-to-cortex pathway by ~25–41% below the levels in the saline-treated controls (Fig. 4). The reductions in baseline CBF cannot be attributed to lowered energy metabolism because systemic administration of L-NAME has been found to have no effects on $lCMR_{Glc}$ in a variety of cerebral structures in unanesthetized rats (35). The increases in MABP and cerebral vascular resistance and reductions in baseline CBF produced by

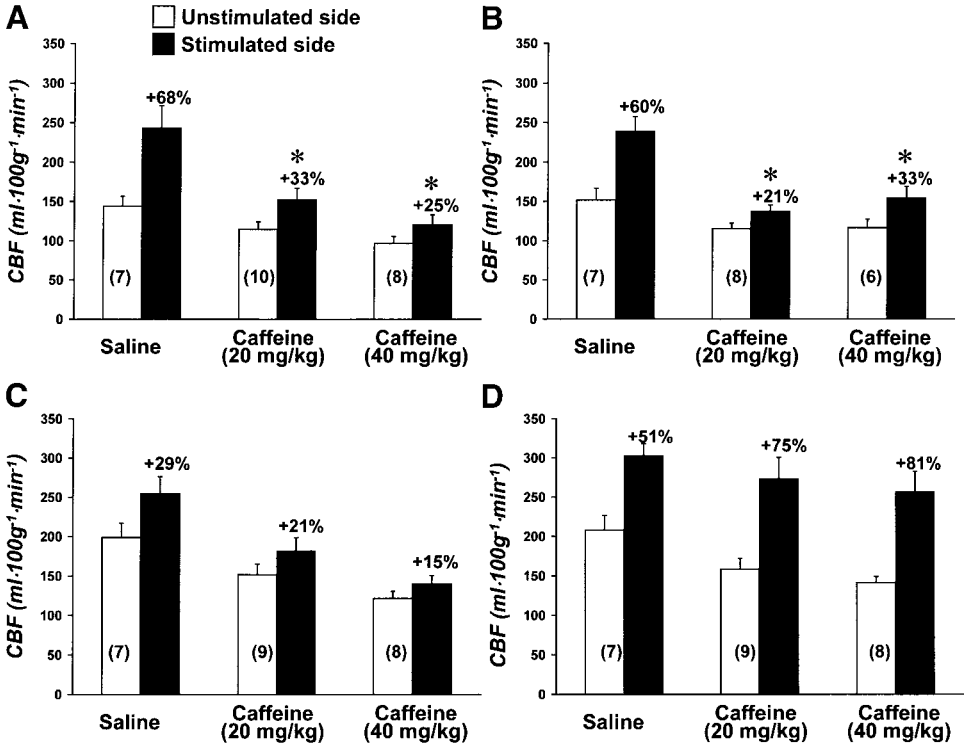

Fig. 2. Effects of 20 and 40 mg/kg intravenous doses of caffeine on baseline blood flow and increases in blood flow evoked by functional activation in four stations of the whisker-to-barrel cortex pathway. *A*: spinal trigeminal nucleus; *B*: principal sensory trigeminal nucleus; *C*: ventral posteromedial (VPM) thalamic nucleus; *D*: barrel field of sensory cortex. Bar heights and error bars represent means ± SE of no. of animals indicated. See *Statistics* for statistical tests. All side-to-side differences were statistically significant ($P < 0.02$); the %values shown are the percentage of differences between the two sides. Also, the 40 mg/kg dose of caffeine statistically significantly ($P < 0.05$) reduced the CBF values in the spinal trigeminal nucleus, VPM nucleus, and barrel cortex on the unstimulated side below the values in the saline-treated control rats. *$P < 0.05$, statistically significant reduction in percent enhancement of blood flow due to vibrissal stimulation compared with those seen in saline-treated control rats.

L-NAME therefore indicate that it effectively inhibited a tonic vasodilator influence of NO in both the systemic and cerebral circulatory beds. Despite its effects on baseline CBF, however, L-NAME did not statistically significantly alter the percent enhancement of CBF evoked by vibrissal stimulation in any of the stations of the pathway, except in the VPM thalamic nucleus ($P < 0.05$; Fig. 4).

Effects of Combinations of Caffeine with Either L-NAME or 7-NI

Although caffeine, L-NAME, and 7-NI reduced the percent enhancement of CBF due to vibrissal stimulation in selected structures, they did not fully eliminate the functional activation of CBF in any of them. Because of the possibility of multiple mechanisms of reg-

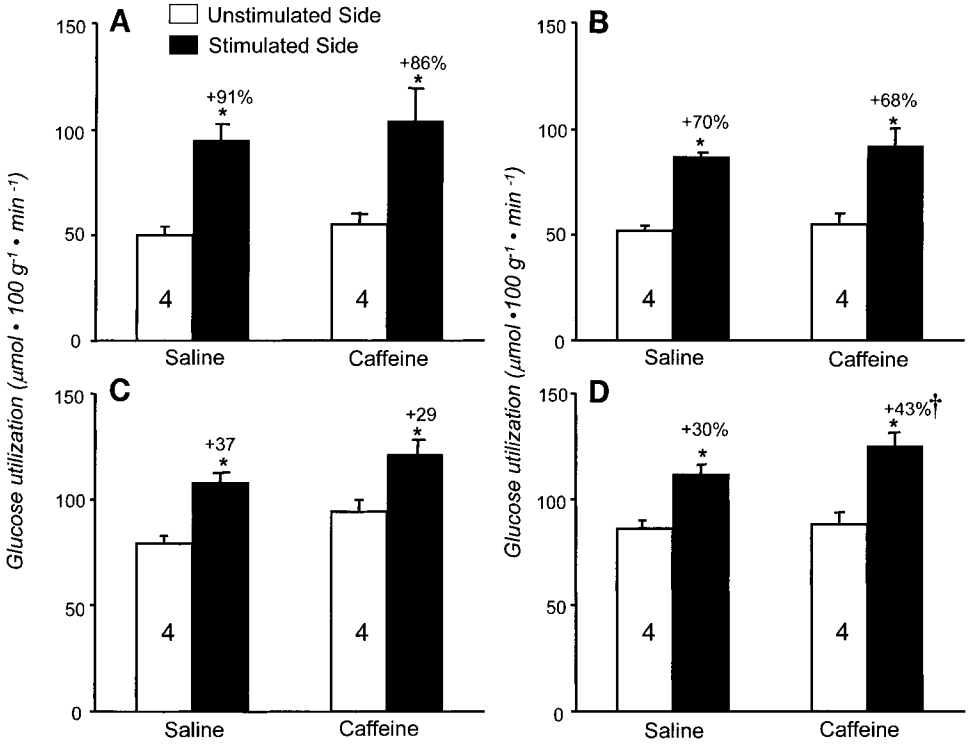

Fig. 3. Effects of caffeine (40 mg/kg) on activation of local cerebral glucose utilization by unilateral vibrissal stimulation in four stations of whisker-to-barrel cortex sensory pathway. *A*: spinal trigeminal nucleus; *B*: principal sensory trigeminal nucleus; *C*: ventral posteromedial thalamic nucleus; *D*: barrel field of sensory cortex. Bar heights and error bars represent mean CBF ± SE for no. of animals indicated. *$P < 0.01$, statistically significant difference between stimulated and unstimulated sides (by paired *t*-test). †$P < 0.05$, statistically significantly greater percentage of stimulation of CBF in caffeine-treated rats than in saline-treated control group (by grouped *t*-test applied to logarithmic transforms of the individual %differences between stimulated and unstimulated sides).

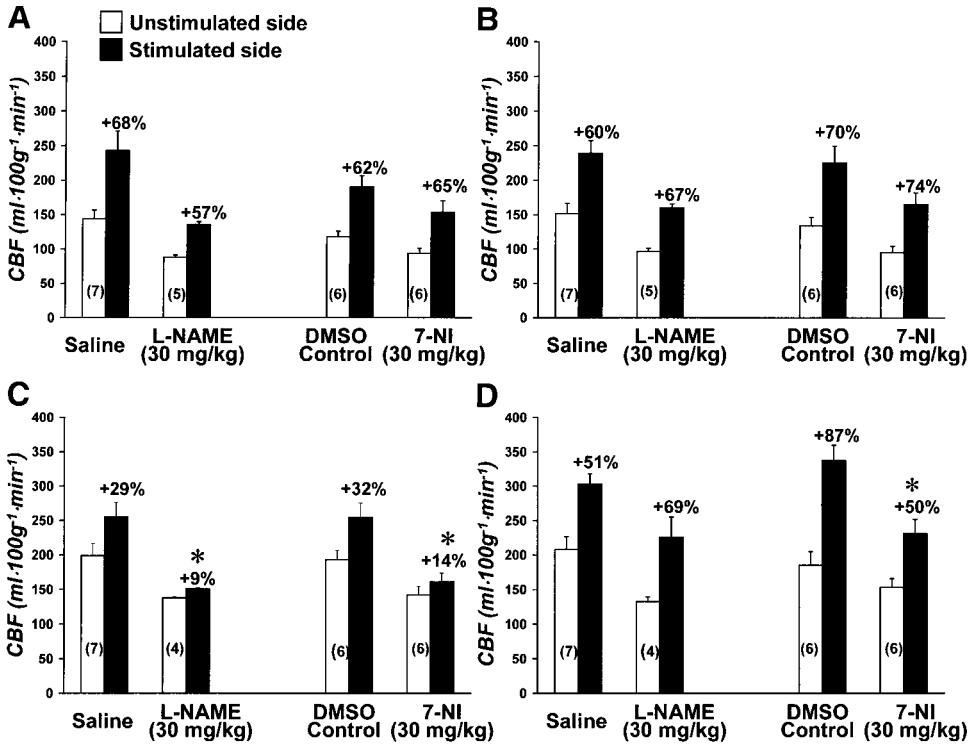

Fig. 4. Effects of 30 mg/kg N^G-nitro-L-arginine methyl ester (L-NAME) administered intravenously and of 30 mg/kg of 7-nitroindazole (7-NI) administered intraperitoneally on baseline blood flow and on functional activation of blood flow in four stations of the whisker-to-barrel cortex pathway. *A*: spinal trigeminal nucleus; *B*: principal sensory trigeminal nucleus; *C*: ventral posteromedial thalamic nucleus; *D*: barrel field of sensory cortex. Bar heights and error bars represent means ± SE of no. of animals indicated. See *Statistics* for statistical tests. All side-to-side differences were statistically significant ($P < 0.03$); the %values shown are the percentage of differences between the two sides. Also, baseline CBF (i.e., CBF on the unstimulated side) was statistically significantly lower ($P < 0.05$) in all four structures of the pathway in the rats treated with L-NAME or 7-NI than in the saline-treated control rats. *Statistically significantly lower percentage of increase in blood flow due to vibrissal stimulation compared with that seen in saline-treated ($P < 0.05$) or DMSO-treated ($P \leq 0.05$) rats.

ulation of CBF, both caffeine-sensitive and NO-sensitive, in which blockade of one might result in compensation by the other, we examined the effects of caffeine in combination with either 7-NI or L-NAME. There were no additive effects of caffeine and either of the NO synthase inhibitors. The results (Fig. 5) were essentially the same as those seen with caffeine (Fig. 2) or either of the NO synthase inhibitors (Fig. 4) alone.

DISCUSSION

Caffeine was recently found to attenuate in a dose-dependent manner to the point of complete extinction

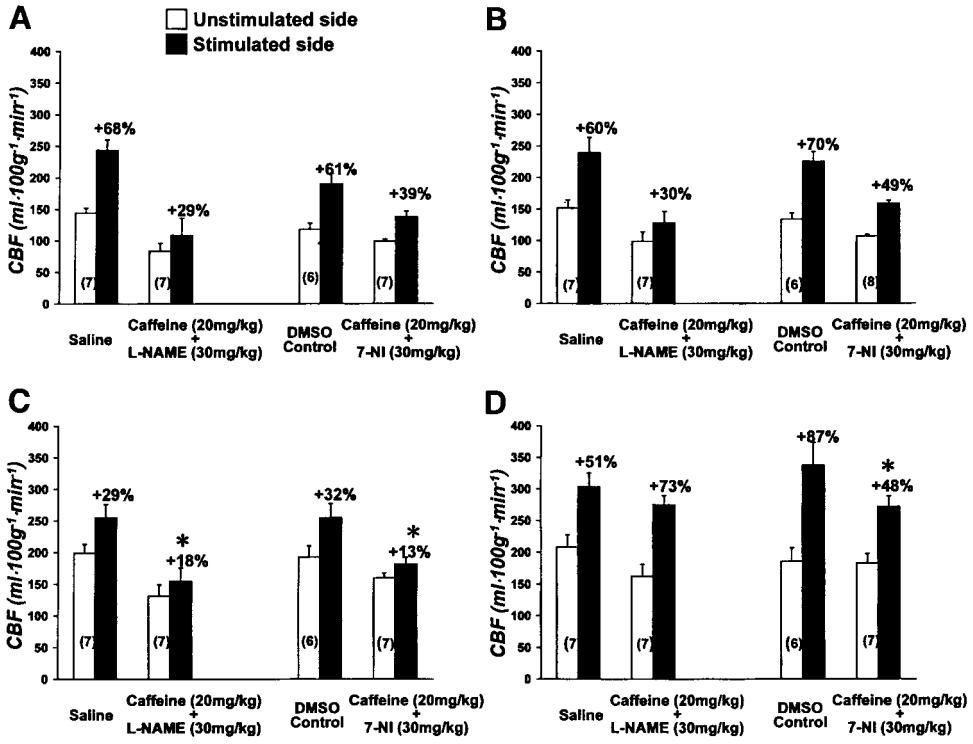

Fig. 5. Effects of combined administration of 20 mg/kg of caffeine administered intravenously with either 30 mg/kg L-NAME administered intravenously or 30 mg/kg 7-NI administered intraperitoneally on baseline blood flow and on functional activation of blood flow in four stations of the whisker-to-barrel cortex pathway. *A*: spinal trigeminal nucleus; *B*: principal sensory trigeminal nucleus; *C*: ventral posteromedial thalamic nucleus; *D*: barrel field of sensory cortex. Bar heights and error bars represent means ± SE of no. of animals indicated. See *Statistics* for statistical tests. All side-to-side differences were statistically significant ($P < 0.03$); the percentage of values shown are the means of the individual percentage of differences between the two sides. *$P < 0.05$, statistically significant reduction in the percentage of increases in blood flow due to vibrissal stimulation compared with those seen in normal saline-treated or DMSO-treated control rats.

the marked increases in CBF that occur in insulin-induced hypoglycemia, a condition in which levels of adenosine and its metabolic degradation products are greatly elevated in brain (11). Because adenosine is known to have vasodilator actions, and caffeine is a nonselective adenosine receptor antagonist, these findings suggested that adenosine was the mediator of the enhancement of CBF in hypoglycemia. In the present study, we have attempted to determine if it might also mediate the increases in CBF associated with neuronal functional activity. Previous studies suggesting a role for adenosine in the functional activation of CBF were based on changes in pial arterial diameters (15) or laser-Doppler flow patterns (7) induced in the sensory cortex by topical applications of test agents to the cortex of anesthetized animals. Because of reported differences in the effects of NO synthase inhibitors on functional activation of CBF in anesthetized and unanesthetized animals and in different stations of an activated pathway (1, 2, 4, 7, 12, 19, 33, 36), in the present study we measured absolute perfusion rates in four stations of the whisker-to-barrel cortex pathways under more physiological conditions, e.g., parenteral administration of the test compounds to unanesthetized animals.

The results indicate that caffeine-sensitive mechanisms do contribute to the functional activation of CBF in some but not all stations of the activated pathway. Vibrissal stimulation markedly raised CBF in the four stations examined, but caffeine statistically significantly reduced the percent enhancement only in the spinal and principal sensory trigeminal nuclei and not at all in the VPM thalamic nucleus and barrel cortex. Even in the two structures in which it was effective, however, it failed to abolish the increases in CBF. The limited effects of caffeine cannot be explained by inadequate dosage. The 20 mg/kg dose had been sufficient to block completely the increases in CBF in insulin-induced hypoglycemia (11) and appeared to exert close to maximal effects in the present study inasmuch as the 20 and 40 mg/kg doses had comparable effects on both baseline CBF and the increases evoked by vibrissal stimulation. In fact, the higher dose of caffeine, if anything, magnified the stimulation-induced increase in CBF in the barrel cortex where it also enhanced the metabolic response to stimulation. Possibly increased production of metabolic products with vasodilator actions might have overshadowed the inhibitory effects of caffeine. This may explain why we observed no inhibition by caffeine in the barrel cortex in contrast to previous studies in which topical application of theophylline or adenosine deaminase to the somatosensory cortex reduced functional activation of CBF (7, 15).

Although caffeine failed to abolish the functional activation of CBF in the brain stem nuclei, the reductions there were quite substantial. Many drugs suppress neuronal functions, and it was conceivable that caffeine, acting on adenosine receptors on neurons or on other cellular processes, might have reduced the CBF responses by lowering the intensity of neuronal functional activation rather than by inhibiting the adjustment of CBF to functional activity. This was apparently not the case. Stimulation-dependent activation of local glucose utilization is directly related to neuronal spike activity (32), and vibrissal stimulation markedly stimulated $lCMR_{Glc}$ in all four stations of the pathway, particularly in the brain stem nuclei. Caffeine, however, altered neither baseline nor functionally activated levels of glucose utilization in the brain stem nuclei, indicating that the attenuation of the CBF responses there was not due to diminished neuronal activation.

The methylxanthines, caffeine and theophylline, are potent nonspecific inhibitors of adenosine receptors, but they also have other actions, e.g., phosphodiesterase inhibition, calcium mobilization, benzodiazopine receptor binding, etc. We chose caffeine because of its lesser effects on baseline CBF and also because it is less potent than theophylline with respect to some of those other actions of the methyxanthines. The lower dose of caffeine that we used, i.e., 20 mg/kg, has been considered the upper limit before risking actions unrelated to adenosine receptor antagonism (9, 26), and our higher dose, 40 mg/kg, did not reduce the functional activation of CBF in the brain stem nuclei to any greater degree. Therefore, it is likely that the effects of caffeine we observed were maximal and predominantly due to adenosine receptor antagonism.

The fact that caffeine maximally reduced CBF responses to functional activation by about one-half in only two of the four stations of the pathway indicated that other vasodilator mechanisms independent of adenosine were involved. There are many endogenous compounds in brain that can influence cerebral vascular tone, and one or more of them could be involved in redundant mechanisms of CBF regulation during neuronal functional activation. NO is one such candidate. Several studies have reported that NO synthase inhibition by drugs markedly reduce or eliminate the CBF responses to vibrissal stimulation (6, 7, 12). Also, studies in which residual NO synthase activity was inhibited with nitroarginine in mice lacking either the neuronal or the endothelial NO synthase gene have implicated NO derived from neuronal NO synthase as the mediator (2, 19). Cholet et al. (4, 5) observed regional differences in these effects; functional activation of CBF in the trigeminal nuclei was unaffected by neuronal NO synthase inhibition with 7-NI but was totally eliminated in the VPM thalamic nucleus and sensory cortex. Dirnagl and colleagues (7) examined the effects of both adenosine receptor antagonists and NO synthase inhibitors and concluded that both adenosine and NO were involved with interacting modes of action. All of these studies were carried out in anesthetized animals, sometimes with the NO synthase inhibitors applied topically to the cortex. There have been contradictory reports on whether superfusion of the cortex with the L-nitroarginine suppresses somatosensory evoked potentials (17, 22, 23). If so, then attenuation of functional activation of CBF by nitroarginine so administered might reflect more suppression of neuronal functional activation rather than the CBF re-

sponse to functional activation. It should be noted, however, that restoration of NO levels in the tissue by NO donor agents after topical application of nitroarginine restores the CBF response to vibrissal stimulation (16). In all reported studies in which nitroarginine was administered systemically or intrathecally to unanesthetized animals, no effects on the percent enhancement of CBF elicited by vibrissal stimulation were observed (1, 33, 36).

Therefore, to reexamine the role of NO in the CBF responses to vibrissal stimulation in unanesthetized rats, we included experiments on the effects of L-NAME, a nonspecific inhibitor of all isoforms of NO synthase, and 7-NI, a specific inhibitor of only the neuronal isoform. These inhibitors were administered alone or in combination with caffeine to determine if the residual CBF responses in the trigeminal nuclei after caffeine treatment were mediated by NO. The intravenous administration of L-NAME raised MABP and lowered baseline CBF, indicating effective inhibition of, at least, the endothelial NO synthase. 7-NI was injected intraperitoneally 30 min before onset of vibrissal stimulation and measurement of CBF; this dose, mode of administration, and timing had previously been shown to inhibit brain NO synthase activity by 75–89% in various brain regions, including components of the whisker-to-barrel cortex pathway (5, 20). In our study, 7-NI did not affect blood pressure, probably because it inhibited only neuronal and not endothelial NO synthase, but it did lower baseline CBF, indicating tonic influence of neuronal NO synthase activity on the cerebral circulation. Nevertheless, despite the evidence that they had inhibited NO synthase activity, neither inhibitor had any effect on the percentage of enhancements of CBF by vibrissal stimulation in the two trigeminal nuclei. On the other hand, both reduced the CBF response in the VPM thalamic nucleus, and 7-NI had the same effect in the barrel cortex as well. Therefore, the effects of 7-NI were almost opposite to those of caffeine. Administration of combinations of NO synthase inhibitors and caffeine yielded results essentially like those obtained with each of the drugs alone; there were no additive effects, indicating that residual responses remaining after blockade of adenosine receptors or NO formation were not mediated by NO or adenosine, respectively.

Our results confirm the findings of Cholet et al. (4, 5) that inhibition of neuronal NO synthase activity with 7-NI suppresses CBF responses to vibrissal stimulation in the VPM thalamic nucleus and barrel cortex but not in the trigeminal nuclei. However, they did not examine the effects of L-NAME. In our earlier studies (1, 33), L-NAME did not statistically significantly affect the percent enhancement of CBF elicited by vibrissal stimulation in any of the four structures of the whisker-to-barrel cortex pathway, despite inhibitions of total brain NO synthase activity by >50% after systemic and more than 80% after intracisternal administration. The effects of L-NAME in the present study were, in general, similar. Despite evidence that it had inhibited at least endothelial NO synthase activity (e.g., elevated arterial blood pressure and cerebral vascular resistance), it had no significant effects on the CBF responses to stimulation in any of the four structures except for the VPM nucleus. The reason for this difference is unclear, possibly a spurious statistical result in one or the other study because of limited sample sizes. The results obtained with 7-NI, however, suggest a role for NO, at least in some regions of the brain. The difference in the effects of L-NAME and 7-NI supports a role for neuronal NO synthase and suggests that the dose of L-NAME was sufficient to inhibit only endothelial and not neuronal NO synthase activities.

On the basis of our previous findings with L-NAME (1, 33), we had concluded that, although NO exerted a tonic vasodilator influence on the cerebral circulation, it did not mediate the enhancement of CBF by sensory stimulation. The results of the present study indicate, however, that mechanisms sensitive either to caffeine or to neuronal NO synthase inhibition may contribute, but with opposite regional patterns of distribution within the whisker-to-barrel cortex pathway. It may be of significance that the caffeine effects are confined to the trigeminal nuclei, the direct projection zones from the peripheral sense organs that contain the primary synapses of the pathway, and are absent in the thalamus and cortex where the secondary and higher-level synapses reside, whereas NO produced by neuronal NO synthase appears to operate in the regions containing the secondary and not primary synapses. In any case, neither the caffeine-sensitive nor the NO-dependent mechanisms, alone or in combination, account fully for the functional activation of CBF in any region. They may participate in but do not mediate the adjustment of the CBF to functional activity, and other mechanisms must also be involved. Involvement of cyclooxgenase-2 in the enhancement of CBF in the sensory cortex by vibrissal stimulation has been reported recently (24). The nature of the participation of these various mechanisms is unclear, but in the case of NO recent evidence suggests that its role is permissive (16). The complexity of multiple regulatory mechanisms with different regional distributions presents a formidable problem for future studies.

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