Isoflurane-induced Cerebral Hyperemia in Neuronal Nitric Oxide Synthase Gene Deficient Mice

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Background: Nitric oxide (NO) has been reported to play an important role in isoflurane-induced cerebral hyperemia *in vivo.* In the brain, there are two constitutive isoforms of NO synthase (NOS), endothelial NOS (eNOS), and neuronal NOS (nNOS). Recently, the mutant mouse deficient in nNOS gene expression (nNOS knockout) has been developed. The present study was designed to examine the role of the two constitutive NOS isoforms in cerebral blood flow (CBF) response to isoflurane using this nNOS knockout mouse.

Methods: Regional CBF (rCBF) in the cerebral cortex was measured with laser-Doppler flowmetry in wild-type mice (129/SV or C57BL/6) and nNOS knockout mice during stepwise increases in the inspired concentration of isoflurane from 0.6 vol% to 1.2, 1.8, and 2.4 vol%. Subsequently, a NOS inhibitor, N^ω-nitro-L-arginine (L-NNA), was administered intravenously (20 mg/kg), and 45 min later, the rCBF response to isoflurane was tested again. In separate groups of wild-type mice and the knockout mice, the inactive enantiomer, N^ω-nitro-D-arginine (D-NNA) was administered intravenously in place of L-NNA. Brain NOS activity was measured with radio-labeled L-arginine to L-citrulline conversion after treatment with L-NNA and D-NNA.

Results: Isoflurane produced dose-dependent increases in rCBF by 25 ± 3%, 74 ± 10%, and 108 ± 14% (SEM) in 129/SV mice and by 32 ± 2%, 71 ± 3%, and 96 ± 7% in C57BL/6 mice at 1.2, 1.8, and 2.4 vol%, respectively. These increases were attenuated at every anesthetic concentration by L-NNA but not by D-NNA. Brain NOS activity was decreased by 92 ± 2% with L-NNA compared with D-NNA. In nNOS knockout mice, isoflurane increased rCBF by 67 ± 8%, 88 ± 12%, and 112 ± 18% at 1.2, 1.8, and 2.4 vol%, respectively. The increase in rCBF at 1.2 vol% was significantly greater in the nNOS knockout mice than that in the wild-type mice. Administration of L-NNA in the knockout mice attenuated the rCBF response to isoflurane at 1.2 and 1.8 vol% but had no effect on the response at 2.4 vol%.

Conclusions: In nNOS knockout mice, the cerebral hyperemic response to isoflurane is preserved by compensatory mechanism(s) that is NO-independent at 2.4 vol%, although it may involve eNOS at 1.2 and 1.8 vol%. It is suggested that in wild-type mice, eNOS and nNOS contribute to isoflurane-induced increase in rCBF. At lower concentrations (1.2 and 1.8 vol%), eNOS may be involved, whereas at 2.4 vol%, nNOS may be involved. (Key words: Anesthetics, volatile: isoflurane. Brain, cerebral blood flow: laser-Doppler flowmetry. Gases: nitric oxide. Genetics: nitric oxide synthase. Pharmacology: nitric oxide synthase inhibitor.)

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THE volatile anesthetic, isoflurane, has been shown to cause cerebral hyperemia in several species.[1-4] Nitric oxide (NO), the widely recognized vasodilator and neuromodulator molecule,[5] has been reported to participate in isoflurane-induced cerebral vasodilation *in vivo.*[4,6,7] In the brain, under normal physiologic conditions, NO is produced by the two constitutive isoforms of nitric oxide synthase (NOS), endothelial NOS (eNOS), and neuronal NOS (nNOS). In principle, NO from either eNOS or nNOS may participate in the cerebral hyperemic response to isoflurane. Koenig *et al.*[7]

have demonstrated that light-dye–induced endothelial injury abolished the cerebrovascular dilation of cerebral surface vessels to isoflurane, suggesting that an intact endothelium, and possibly eNOS, was an essential component of vasodilation in these vessels. However, intraparenchymal microvessels, which are not affected by the light-dye treatment, also may contribute to the hyperemic response to isoflurane and may be under the influence of NO from nNOS[8] in addition to eNOS. Cerebral vasodilation also is modulated by anesthetic-induced changes in neuronal activity and cerebral metabolism.[2,9] Recent studies suggest that cerebral blood flow (CBF) is directly coupled to neuronal activity in part via NO produced by nNOS in neuronal cells and processes.[10] Thus, anesthetic modulation of neuronal activity and NO production by nNOS also may play an important role in cerebral vasodilation. If one could determine which NOS isoform is involved in the anesthetic-induced cerebral hyperemia, it would help to elucidate the cellular source and mechanism of action of NO in the cerebrovascular effect of these anesthetics. Moreover, it has been reported that nNOS-derived NO exacerbates acute ischemic injury, whereas eNOS-derived NO has a protective property.[11] Therefore, volatile anesthetics also may influence ischemic brain injury, depending on their effects on particular NOS isoforms. In two previous studies,[6,7] the NOS inhibitor, N^{ω}-nitro-L-arginine methyl ester (L-NAME), was used to demonstrate the importance of basal NO production in the cerebral hyperemic response to isoflurane. Because L-NAME nonselectively inhibits nNOS and eNOS, the relative contribution of these two NOS isoforms to isoflurane-induced cerebral hyperemia remains unclear.

Recently, the nNOS deficient mouse (nNOS knockout or nNOS$^{-/-}$) was developed by targeted disruption of the nNOS gene.[12] The residual catalytic activity of NOS in the brain of nNOS$^{-/-}$ mouse is very low ($<5\%$) and probably derives from eNOS.[12] The nNOS$^{-/-}$ mouse displays a normal cerebrovascular responsiveness to hypercapnia,[13] which is independent of residual eNOS activity because it is not attenuated by the NOS inhibitor, N^{ω}-nitro-L-arginine (L-NNA). These data suggest the relative importance of nNOS in the wild-type mice and reflect the physiologic redundancy of NO-independent mechanisms in nNOS knockout mice in the hypercapnic response.

These findings prompted us to investigate the cerebral hyperemic response to isoflurane according to a similar paradigm. In the current study, we sought to determine whether the isoflurane-induced cerebral hyperemic response is present in the nNOS knockout mouse and, if present, to determine whether this response is affected by the inhibition of the residual NOS activity with L-NNA. For this purpose, regional CBF (rCBF) in the cerebral cortex was measured with laser Doppler flowmetry in nNOS knockout mice and in wild-type mice during stepwise increases in the concentration of isoflurane.

Methods

The experimental procedures and protocols used in this investigation were reviewed and approved by the Institutional Animal Care and Use Committee.

Surgical Procedures and Cerebral Blood Flow Monitoring

Experiments were performed using nNOS knockout mice (nNOS$^{-/-}$ mice) and two strains of wild-type mice. We used 129/SV and C57BL/6 wild-type mice (Taconic Farms, Germantown, NY) because nNOS knockout mice were derived from these two strains as previously described.[12] Anesthesia was induced by sodium pentobarbital (50 mg/kg, intraperitoneal) and was maintained by inhalation of isoflurane, 0.6%, and administration of pentobarbital (10 mg/kg, hourly). It has been reported that the minimum alveolar concentration (MAC) of isoflurane is not different between wild-type and nNOS knockout mice (1.0 MAC = 1.2 vol%).[14] The animals were tracheally intubated, paralyzed by gallamine (5 mg/kg intraperitoneal) to avoid movements by cough at high concentration of isoflurane that may interfere the measurements, and the lungs were mechanically ventilated with a small animal ventilator (SAR-830, CWE, Ardmore, PA) with 30% O_2 in N_2. End-tidal CO_2 tension (ET_{CO2}) was measured by microcapnometer (Capstar-100, IITC Inc., Woodland Hills, CA, or Columbus instruments, Columbus, OH) that reliably detected carbon dioxide at sampling flow rates of 10–15 ml/min. This sampling rate has been reported not to interfere with the respiration of the animals.[15] Body temperature was maintained at $37 \pm 1°C$ using a thermostat-controlled water circulated heating mat. The right femoral artery was cannulated with PE-10 tubing to facilitate the measurement of arterial pressure and arterial blood gases. Arterial P_{O2}, P_{CO2}, and pH were measured with gas analyzer (ABL-300, Radiometer, Denmark, or 178B, Ciba-Corning, Medfield, MA). A femoral vein was cannulated for the infusion of drugs and physiologic crystalloid solution. Inspired oxygen and isoflurane concentrations were continuously monitored (POET II™, Criticare Systems, Inc., Milwaukee, WI). Regional cerebro-

cortical blood flow was determined by laser-Doppler flowmetry (PF3, Perimed, Sweden). The techniques used for monitoring CBF in mice with laser-Doppler flowmetry have been described previously.[3] Our laser-Doppler flowmetry provides measured values in perfusion units that do not convert to absolute (physical) units of CBF. Nevertheless, a good correlation between the relative changes in laser-Doppler flowmetry and in CBF as measured by the H_2 clearance technique has been found.[16] Further, the laser-Doppler flowmetry measurements in perfusion units have shown remarkably good animal-to-animal reproducibility that gave us the confidence to analyze and report flow data in perfusion units for certain comparative purposes. The head of the mouse was placed in a stereotaxic apparatus (Model 900, David Kopf, Tujunga, CA). After incising the skin and exposing the skull, the laser-Doppler flow probe was positioned using a micromanipulator so that there were no visible pial vessels in the field of measurement. The probe did not touch the cranium, and a drop of mineral oil was used to improve optical coupling between the probe and the tissue. ET_{CO2}, mean arterial blood pressure (MAP), rCBF, and isoflurane concentrations were recorded on an 8 channel polygraph (Astro-Med, Inc., West Warwick, RI) or computer data acquisition system (Mac lab/8, AD Instruments, Medford, MA). After surgery, a 30-min equilibration period was allowed for stabilization of the preparation under 0.6 vol% isoflurane and pentobarbital anesthesia.

Experimental Protocols

The mice were assigned to six groups, and experiments were performed according to the general protocol to be described. The experimental groups were: group 1, 129/SV wild-type mice with L-NNA treatment (n = 7); group 2, C57BL/6 wild-type mice with L-NNA treatment (n = 6); group 3, neuronal NOS knockout mice (nNOS$^{-/-}$) with L-NNA treatment (n = 6); group 4, 129/SV wild-type mice with N$^\omega$-nitro-D-arginine (D-NNA) treatment (n = 5); group 5, C57BL/6 wild-type mice with D-NNA treatment (n = 5); and group 6, neuronal NOS knockout mice (nNOS$^{-/-}$) with D-NNA treatment (n = 6). The general experimental protocol was as follows. The concentration of isoflurane was increased in a stepwise manner from 0.6 vol% (baseline) to 1.2, 1.8, and 2.4 vol%. Each inspired concentration of isoflurane was maintained for 15 min. Although ideal site for the measurement of isoflurane concentration is alveoli, it has been reported that anesthetic uptake is more rapid in smaller animals[17] and has been suggested that the difference of the concentration between alveolar and inspired gas may become negligible.[14] After the

cumulative exposure to isoflurane, the inspired concentration of isoflurane was reduced to 0.6 vol% and held constant for 30 min. The NOS inhibitor, L-NNA (20 mg/kg) or its less active enantiomer D-NNA (20 mg/kg), was then administered intravenously. L-NNA was used in groups 1, 2, and 3, whereas D-NNA was used in groups 4, 5, and 6. Forty-five min later, the inspired concentration of isoflurane was increased again from 0.6 vol% to 1.2, 1.8, and 2.4 vol% and maintained for 15 min at each level. L-NNA has been chosen as a nonselective, competitive inhibitor of NOS because it has been reported to be more potent than L-NMMA and to have no muscarinic effect as L-NAME does.[18,19] The dosage was selected based on the previous reports[18] to provide an enough inhibition of nNOS activity to block physiologic CBF response by L-NNA.[20] To account for the possible involvement of a time factor and confirm the stereospecific NOS inhibitory action of L-NNA, we performed experiments with D-NNA infusion in each experimental group in wild-type and knockout mice. Regarding the order of administration of isoflurane at various concentrations, we found that once rCBF reached a steady state at high isoflurane concentration, it did not return to baseline within 30 min, which would have made it difficult to complete an experimental protocol with a reversed or random design. Therefore, we chose the cumulative administration. To maintain the arterial blood pressure during experimental period, methoxamine, an α_1-adrenergic receptor agonist, was infused intravenously. The rate of infusion was varied in the range of 0.5 – 2 μg/min, which was adjusted to attain the same level of MAP as the baseline. It has been previously reported that α_1-adrenergic agonists infused intravenously have a negligible effect on CBF.[21,22] We have used phenylephrine and methoxamine to support the mean arterial blood pressure during the challenges to carbon dioxide[23] and volatile anesthetics[3] and found that these adrenergic agents did not influence the cerebral hyperemic response. Methoxamine was used in the present study because this drug has been proven to be a more specific α_1-receptor agonist than phenylephrine[24] and to maintain carbon dioxide reactivity well (Okamoto and Hudetz, unpublished observations).

Assessment of NOS Catalytic Activity

Immediately after the experiment, the brains were removed, and the cerebral cortex was separated and rapidly frozen in liquid nitrogen and stored at −70°C until NOS activity was determined. NOS activity was measured by the conversion of [^{3}H]L-arginine to [^{3}H]L-citrulline ac-

cording to high-pressure liquid chromatography (HPLC) method originally described by Carlberg.[25] The cerebral cortical tissue was homogenized in 20 mM HEPES buffer (pH, 7.4). The homogenate was then centrifuged at 9000 *g* for 10 min at 4°C. The supernatant (each sample including 150 µg protein determined by protein assay) was incubated with [³H]L-arginine (0.2 µCi) in 100 µl of 20 mM HEPES buffer containing 2 mM $CaCl_2$, 1 mM NADPH, 1.25 µg/ml calmodulin, 2.5 µM FAD, 1 µM FMN, and tetrahydrobiopterin for 5 min at 37°C, and reaction was stopped by adding 50 µl of 20 mM EDTA solution (pH, 5.5) and frozen in liquid nitrogen. Products were separated by HPLC on a LC-18 DB column (Supelco, Bellefonte, PA) at a flowrate of 1.5 ml/min. Products were monitored using an on-line radioactive flow detector (A-100, Radiomatic Instruments, Median, CT). Results were expressed as pmole citrulline produced $\cdot$ mg protein$^{-1} \cdot$ min^{-1}.

Drugs

Isoflurane was purchased from Abbot laboratories (North Chicago, IL). Pentobarbital, gallamine, $CaCl_2$, calmodulin, HEPES, NADPH, FAD, FMN, EDTA, and tetrahydrobiopterin were purchased from Sigma Chemical Co. (St. Louis, MO.). [³H]L-arginine was purchased from Amersham Laboratories (Buckinghamshire, England). N$^\omega$-nitro-L-arginine and N$^\omega$-nitro-D-arginine were purchased from Calbiochem-Novabiochem (San Diego, CA).

Data Analysis and Statistics

Baseline rCBF was determined from 3-min long record under steady state conditions at 0.6 vol% isoflurane. The rCBF response to increasing isoflurane concentration was obtained from the last 3 min of rCBF record of the 15-min period at each anesthetic concentration. The rCBF response was expressed as percent change from baseline value. Regional cerebrovascular resistance (rCVR) was estimated by dividing MAP by rCBF. Data were expressed as mean $\pm$ SEM. Statistical significance of the effect of isoflurane at three concentrations before and after L-NNA or D-NNA was tested separately in each group of mice using repeated measures analysis of variance (ANOVA) with two-within-factor nested design. To compare the response between the different strains of mice, we used one factor ANOVA for each concentration of isoflurane before and after L-NNA or D-NNA separately. A *P* value of less than 0.05 was considered to be significant.

Results

Approximately 30 min after completion of the surgical procedure and final positioning of the probe for laser Doppler flowmetry, all animals were hemodynamically stable. Baseline values of MAP, rCBF, and rCVR in experimental groups of mice are presented in Table 1. As reported before,[13] MAP was slightly lower in the nNOS knockout mice than in the wild-type mice. However, there were no significant differences in baseline rCBF and rCVR among four groups of mice. Arterial blood pH, P_{CO2}, and P_{O2} values in control conditions also are presented in Table 1. There were no significant differences in arterial blood gas/pH among the experimental groups.

The effects of isoflurane before (control) and after the administration of L-NNA in 129/SV wild-type mice are presented in figure 1A. During the first exposure to isoflurane, rCBF increased dose-dependently by 25 $\pm$ 3%, 74 $\pm$ 10%, and 108 $\pm$ 13% at 1.2, 1.8, and 2.4 vol%, respectively. Subsequent administration of L-NNA significantly attenuated the rCBF response to isoflurane at each concentration; the rCBF response was 12 $\pm$ 2%, 33 $\pm$ 4%, and 48 $\pm$ 5% at 1.2, 1.8, and 2.4 vol%, respectively ($P < 0.01$ at each level *vs.* before L-NNA). The effects of isoflurane in C57BL/6 wild-type mice are presented in figure 1B. The rCBF responses to isoflurane before and after L-NNA were almost the same in C57BL/6 as in 129/SV mice except that L-NNA had a smaller effect in C56BL/6 (69 $\pm$ 7%) than in 129/SV mice at 2.4% ($P < 0.05$ *vs.* 129/SV mice). The effects of isoflurane and subsequent administration of L-NNA in nNOS knockout mice are presented in figure 1C. In this group, isoflurane increased rCBF dose-dependently by 67 $\pm$ 8%, 88 $\pm$ 12%, and 112 $\pm$ 18% at 1.2, 1.8, and 2.4 vol%, respectively. At 1.2 vol%, the increase in rCBF was significantly greater in the nNOS knockout mice than in either 129/SV or C57BL/6 wild-type mice ($P < 0.05$). No such difference was observed at 1.8 or 2.4 vol%. Administration of L-NNA significantly attenuated the cerebral hyperemic response to isoflurane at the lower concentrations (17 $\pm$ 7% increase in rCBF at 1.2 vol% and 69 $\pm$ 13% increase at 1.8 vol%; $P < 0.01$ *vs.* before L-NNA). L-NNA did not alter the rCBF response to isoflurane at 2.4 vol% (114 $\pm$ 19%). The response to isoflurane after L-NNA was significantly greater in nNOS knockout mice than in 129/SV mice at 1.8 and 2.4 vol%, and it was significantly greater at 2.4 vol% when compared with C57BL/6 mice.

The effects of isoflurane before and after the adminis-

Table 1. Baseline MAP, rCBF, rCVR, and Arterial Blood Gas/pH in the Experimental Groups

	Group 1 129/SV (L-NNA, n = 7)	Group 2 C57BL/6 (L-NNA, n = 6)	Group 3 nNOS$^{-/-}$ (L-NNA, n = 6)	Group 4 129/SV (D-NNA, n = 5)	Group 5 C57BL/6 (D-NNA, n = 5)	Group 6 nNOS$^{-/-}$ (D-NNA, n = 6)
MAP (mmHg)	81 ± 4	83 ± 3	72 ± 2*	80 ± 3	81 ± 2	73 ± 2*
rCBF (PU)	113 ± 9	118 ± 4	105 ± 14	108 ± 8	111 ± 12	106 ± 7
rCVR (mmHg/PU)	0.73 ± 0.05	0.73 ± 0.03	0.73 ± 0.06	0.75 ± 0.06	0.71 ± 0.07	0.71 ± 0.03
pH	7.29 ± 0.01	7.28 ± 0.02	7.29 ± 0.03	7.29 ± 0.06	7.30 ± 0.01	7.30 ± 0.01
Pa_{O_2} (mmHg)	121 ± 9	118 ± 2	133 ± 8	123 ± 6	134 ± 13	125 ± 13
Pa_{CO_2} (mmHg)	37.1 ± 1.9	37.2 ± 3.0	39.0 ± 2.9	35.9 ± 2.3	36.2 ± 2.1	37.8 ± 2.0

Values are mean ± SEM. Baseline values are measured under 0.6 vol% isoflurane + pentobarbital (10 mg/kg per hour).

MAP = mean arterial pressure; rCBF = regional cerebral blood flow; rCVR = regional cerebrovascular resistance; PU = perfusion units; nNOS$^{-/-}$ = neuronal nitric oxide synthase-deficient mice.

* $P < 0.05$ versus 129/SV and C57BL/6 wild-type mice.

tration of D-NNA in 129/SV and C57BL/6 wild-type mice and nNOS knockout mice are presented in figure 2. The inactive enantiomer, D-NNA, had no effect on the isoflurane-induced rCBF increase. Incidentally, the results indicated no difference in the CBF response between the two repeated exposures of isoflurane in any of the groups. As seen in figure 1, the rCBF response to 1.2 vol% isoflurane was significantly greater in nNOS knockout mice than in both wild-type mice. Figure 3 illustrates the effects of L-NNA and D-NNA on baseline rCBF and rCVR in both strains of wild-type mice and nNOS knockout mice. Intravenous administration of L-NNA decreased rCBF and increased rCVR in three strains of mice. The changes in rCBF and rCVR with L-NNA were not significantly different among the 129/SV mice, C57BL/6 mice, and nNOS knockout mice. In contrast to L-NNA, D-NNA had no effect on the rCBF and rCVR in any strains of mice.

In absolute unites, the brain NOS catalytic activity in L-NNA-treated mice (n = 6, from group 1) and D-NNA-treated mice (n = 5, from group 4) was 1.3 ± 0.9 and 16.5 ± 2 pmole citrulline produced · mg protein^{-1} · min^{-1}, respectively. L-NNA decreased the brain NOS catalytic activity by 92 ± 2% compared with D-NNA.

Discussion

Since the discovery of a biosynthetic pathway for the formation of NO in the brain,[26] NO has been suggested to be an important physiologic messenger and modulator of cerebrovascular tone.[5,18,27] In the cerebral vasodilator response to volatile anesthetics such as halothane and isoflurane, NO has been implicated to play a role, at least *in vivo*, in several species.[4,6,28,29] In the brain,

there are two isoforms of NOS, *i.e.*, eNOS and nNOS, that may participate in this response. These NOS isoforms are distributed in many cell types, including the endothelium,[30] neurons,[27] astrocytes,[31] and perivascular nerves.[32,33] Because it has been reported that nNOS rather than eNOS plays an important role in the cerebral hyperemic response to hypercapnia,[34] it is possible that nNOS also may play a role in the cerebrovascular response to isoflurane. However, the relative importance of nNOS *versus* eNOS in the CBF response to isoflurane remains unclear because former studies were performed using NOS inhibitors such as L-NNA or L-NAME, which inhibit NOS in a nonselective manner.

A mutant mice strain deficient in nNOS gene expression was recently developed and was chosen in the current study to investigate how the two constitutive isoforms of NOS may contribute to the isoflurane-induced cerebral hyperemia. We found that the response to isoflurane in the nNOS knockout mice was maintained at every concentration of this anesthetic between 1.2 vol% and 2.4 vol% at levels identical to those seen in the wild-type mice. Clearly, disruption of the nNOS gene left the CBF regulation basically intact during the administration of isoflurane. A significant degree of vasodilation to isoflurane was present in the knockout mice after the administration of the nonselective NOS inhibitor L-NNA. Because the brain NOS activity is extremely low after L-NNA administration in the knockout mouse, an involvement of NOS in this situation can be safely excluded. However, it is not possible to determine conclusively from these experiments the degree to which nNOS contributed to cerebral vasodilation in the wild-type mice. Nevertheless, this result suggests that, after targeted disruption of the neuronal NOS
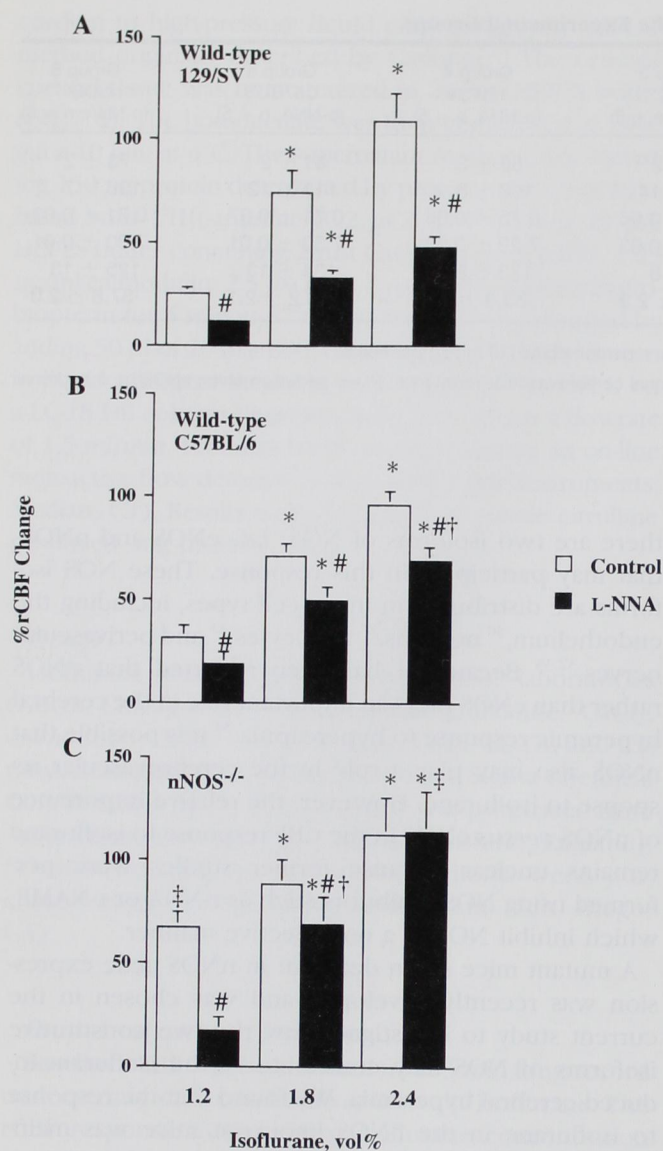
Fig. 1. Isoflurane increases regional cerebral blood flow (rCBF) in a dose-dependent manner in wild-type mice; 129/SV (*A*) and C57BL/6 (*B*), and nNOS knockout mice (nNOS$^{-/-}$, *C*) before (control) and after the administration of L-NNA (20 mg/kg, intravenous). L-NNA significantly attenuates the rCBF response to isoflurane at every concentration in the wild-type mice. In nNOS knockout mice, L-NNA attenuates rCBF at 1.2 and 1.8 vol% but has no effect on the response at 2.4 vol%. Data are expressed as mean ± SEM, n = 6–7 animals. *$P <$ 0.01 *vs.* lower concentration of column with same fill. #$P <$ 0.05 *vs.* control; †$P <$ 0.05 *vs.* 129/SV mice; ‡$P <$ 0.05 *vs.* either 129/SV or C57BL/6 wild-type mice at the same anesthetic concentration and same column fill.

gene, cellular mechanisms, other than the nNOS based-pathway, play a role in the cerebrovasodilator response to isoflurane. For example, arachidonic acid metabolites may be involved in the response.[4] Further, because

L-NNA attenuated the hyperemic response to lower concentrations of isoflurane (1.2 and 1.8 vol%), some non-neuronal isoforms of NOS were probably involved in vasodilation in the nNOS$^{-/-}$ mice. It has been reported that in nNOS knockout mice, residual brain NOS activity is less than 5% of that in wild-type mice and is derived
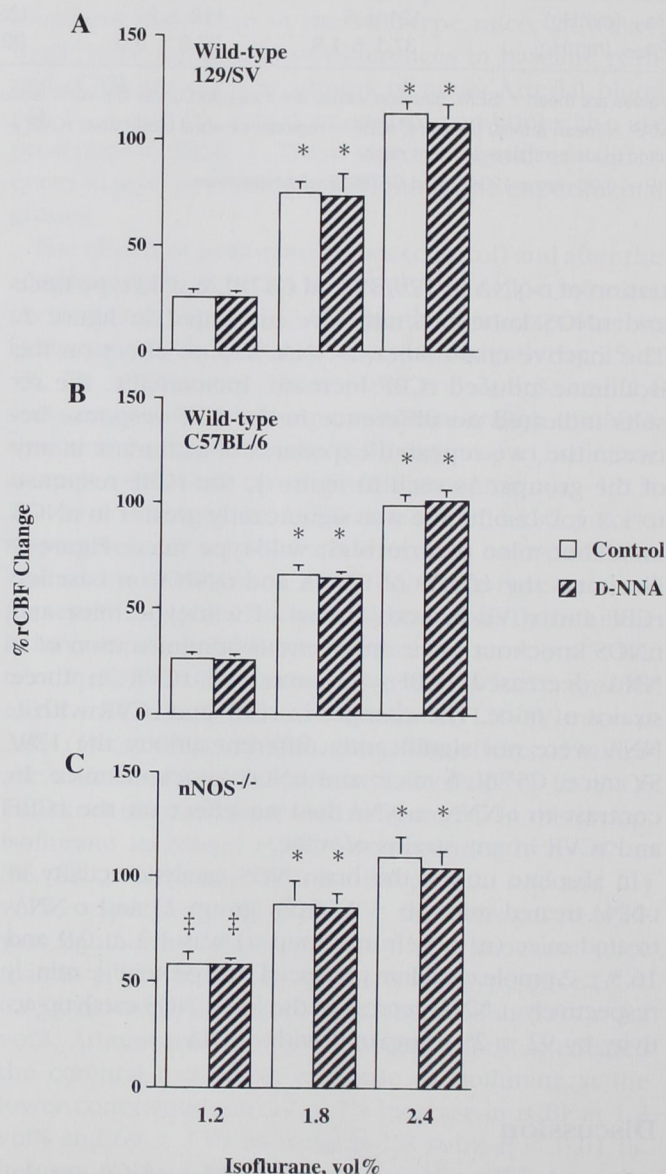

Fig. 2. Intravenous administration of D-NNA (20 mg/kg intravenous) does not affect the rCBF response to isoflurane in wild-type mice; 129/SV (*A*) and C57BL/6 (*B*), and nNOS knockout mice (nNOS$^{-/-}$, *C*) before (control) and after D-NNA. Data are expressed as mean ± SEM, n = 5–6 animals. *$P <$ 0.01 *vs.* lower concentration of column with same fill, ‡$P <$ 0.05 *vs.* either 129/SV or C57BL/6 wild-type mice at the same anesthetic concentration and same column fill.
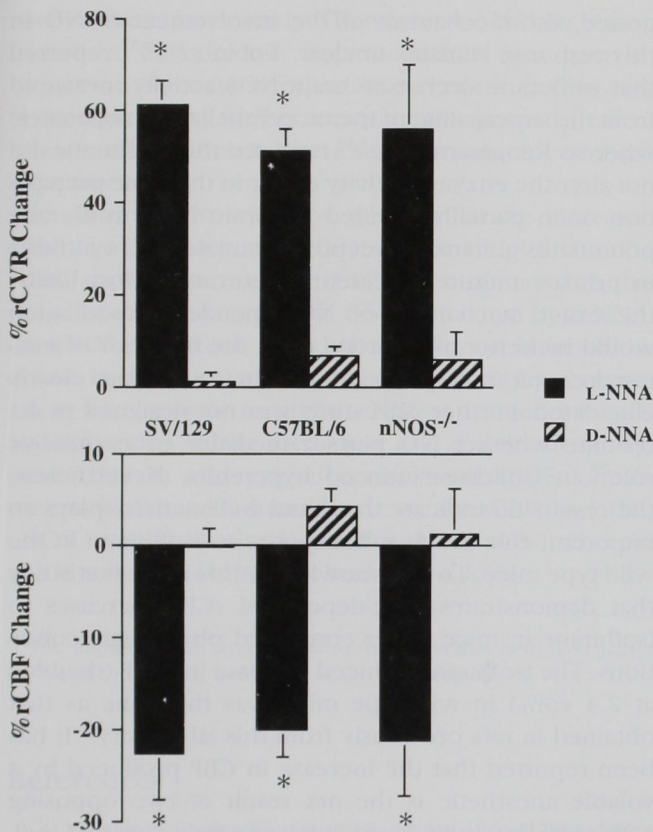
Fig. 3. Intravenous administration of L-NNA (20 mg/kg intravenous) decreases the baseline rCBF and increases the regional cerebrovascular resistance (rCVR) measured at 45 min after L-NNA in wild-type mice; 129/SV and C57BL/6, and nNOS knockout mice (nNOS$^{-/-}$). The effects of L-NNA on rCBF or rCVR are not different among the three strains of mice. D-NNA has no significant effect on baseline rCBF and rCVR in any of the three strains. Data are expressed as mean ± SEM, n = 5–7 animals. *$P < 0.01$ L-NNA *vs.* D-NNA.

from eNOS but not from inducible (Ca^{2+}-calmodulin independent) NOS.[12] Immunohistochemical staining of NOS is absent in neurons and perivascular nerve fibers but is preserved in arteriolar endothelium in the nNOS$^{-/-}$ strain.[13] Therefore, our present findings that the rCBF response to isoflurane was attenuated by L-NNA at 1.2 and 1.8 vol% in the nNOS knockout mice suggest that eNOS may be involved in the isoflurane-induced hyperemic response at these concentrations. Because the rCBF response to isoflurane at 1.2 vol% was greater in the nNOS knockout than in the wild-type mice, this increases the possibility that eNOS expression was upregulated in compensation for the loss of nNOS in the nNOS$^{-/-}$ mice. NO derived from eNOS and nNOS has been reported to contribute to the maintenance of rest-

ing CBF by us and other investigators.[23,24] In the present study, nNOS knockout mice and wild-type mice had equal baseline rCBF levels, and the effects of L-NNA on the baseline rCBF and rCVR were similar in the nNOS knockout and the wild-type mice. Therefore, it is suggested that the L-NNA inhibitable total NOS activity was similar in these strains of mice and that NO derived from another NOS isoform may have compensated for the loss of NO from nNOS. These findings are consistent with the view that in nNOS knockout mice, eNOS may play a compensatory role in the maintenance of resting rCBF and its response to certain vasodilatory stimuli. This hypothesis is challenged by the finding that cerebrovascular response to acetylcholine and eNOS immunostaining are unchanged in the nNOS knockout mice.[13] Further, it has recently been reported[35] that nNOS knockout mice express an abnormal nNOS isoform resulting from an alternative splicing that skips the targeted exon of the nNOS gene. This isoform retains 80% of the synthetic activity of normal nNOS and may contribute along with eNOS to a small (5–10%) residual NOS activity of the brain of the knockout mice. However, this isoform may have abnormal subcellular distribution, and its functional role in cerebral vasodilation remains to be clarified. The presence of an abnormal nNOS in the brain does not impact our conclusions with respect to the stated NO-independence of isoflurane-induced hyperemia in nNOS knockout mice at the high anesthetic concentration. It cannot be excluded that the abnormal nNOS isoform may contribute to the isoflurane-induced hyperemia in the knockout mice at low anesthetic concentration. Because the expression of the abnormal nNOS isoform is low in the wild-type mice, it probably has little, if any, role in the hyperemic response to isoflurane in these animals.

Our results obtained with L-NNA further suggested that the NO system is involved in the response to isoflurane at every concentration investigated in wild-type mice. This is in contrast to the nNOS knockout mice wherein, despite its presence, eNOS failed to contribute to isoflurane-induced cerebrovasodilation at 2.4 vol%. These results do not directly indicate whether in the wild-type mice, eNOS or nNOS played the principal role in isoflurane-induced vasodilation. Extrapolation of our results from nNOS knockout mice to normal physiology is not straightforward because compensatory changes occur in knockout animals, and we do not yet know how these compensatory changes influence the various pathways involved in isoflurane-induced vasodilation. We have no reason to believe that a contribution

from eNOS to vasodilation in wild-type mice would have been ablated in the knockout mice. Therefore, the most logical conclusion is that in the wild-type mice, cerebral vasodilation to isoflurane at high concentration depended principally on the nNOS rather than eNOS. In support of this conclusion, we now have preliminary data suggesting that normal nNOS activity is required for the vasodilator response to volatile anesthetics in cerebral microvessels using nNOS specific inhibitor, 7-nitroindazole (Harkin, *et al.,* unpublished data). Taken together, these data suggest that the NO-dependent cerebrovasodilator response at 2.4% isoflurane may be partially nNOS-dependent, whereas either eNOS or nNOS may be involved at 1.2% and 1.8%. This would increase the intriguing possibility that different isoforms of NOS may be involved at different concentration of isoflurane in the hyperemic response. Although we do not know the exact mechanism of this isoform-selective involvement of NOS in the hyperemic response, it is conceivable that isoflurane may affect eNOS and nNOS activity differently *in vivo.* For example, eNOS activity may be increased at 1.2% and suppressed at 2.4%, whereas nNOS may not be suppressed even at 2.4% because of the greater capacity derived from neuromodulatory circuits involving NMDA and GABA receptors.[10,36] Further studies, possibly using eNOS knockout mice or eNOS and nNOS double knockout mice, will be necessary to elucidate which NOS isoform is involved in the hyperemic response to isoflurane at different concentrations.

As control subjects, we used 129/SV wild-type mice and C57BL/6 wild-type mice. We used both strains because the nNOS knockout mice are chimeric because they were obtained by disrupting the nNOS gene in J1 embryonic stem cell line derived from 129/SV mouse strain and injecting it into the blastocytes of C57BL/6 mice.[12] The rCBF response to isoflurane in these two strains of wild-type mice was similar, although the inhibitory effect of L-NNA at 2.4 vol% was somewhat less in C57BL/6 mice than in 129/SV mice. Despite this difference, the rCBF response after L-NNA in the nNOS knockout mice at 2.4 vol% was significantly greater than in either stain of wild-type mice. The ideal control for nNOS knockout mouse would be the heterozygous mouse with positive nNOS gene, and we would expect that the results from the heterozygous mice were between those obtained in C57BL/6 mice and in 129/SV mice.

Although an important role of NO in isoflurane-induced cerebral vasodilation *in vivo* is strongly sug-

gested, the mechanism of the involvement of NO in this response remains unclear. Tobin *et al.*[37] reported that isoflurane decreased brain NOS activity measured from the supernatant of the rat cerebellar homogenates, whereas Rengasamy *et al.*[38] reported that isoflurane did not alter the enzyme activity either in the same preparation or in partially purified rat brain NOS. Isoflurane potentiates glutamate receptor-stimulated NO synthesis in primary culture of cerebral neurons.[10] Most likely, the exact mechanism of NO-dependent vasodilation would be better understood once the behavior of neuronal circuit in response to anesthetics is more clearly elucidated. Further, this study was not designed to determine whether NO plays a mediator or permissive role[29] in isoflurane-induced hyperemia. Nevertheless, the results do indicate that basal NOS activity plays an important role in the full response to isoflurane in the wild-type mice. To our knowledge, this is the first study that demonstrates dose-dependent rCBF increases to isoflurane in mice under controlled physiologic conditions. The isoflurane-induced increase in rCBF (doubled at 2.4 vol%) in wild-type mice was the same as that obtained in rats previously from this laboratory.[3] It has been reported that the increase in CBF produced by a volatile anesthetic is the net result of two opposing actions of the drug: direct cerebrovasodilation and indirect vasoconstriction coupled to an induced decrease in cerebral metabolic rate.[9] In this regard, it has been shown that isoflurane caused a greater increase in CBF when the metabolic depressant effect was eliminated by previous administration of barbiturate or propofol in animals and humans.[2,39] In the present study, we used isoflurane superimposed on basal pentobarbital anesthesia and could obtain a substantial increase in rCBF, minimizing the metabolism-related attenuation of the rCBF response during the exposure to isoflurane. Because GABA and NOS have been shown to colocalize in the cortical neurons[40] and because NO was shown to modulate GABA release in various regions of the brain,[41] it is possible that the knockout mice react differently to pentobarbital from wild-type mice, resulting in a different interpretation of the present data. However, NOS-positive neurons comprise only a small fraction of GABA-containing cells,[40] and it has been reported that in the cerebral cortex, the NOS inhibitor L-NNA fails to modulate GABA receptor function.[42] Thus, it is unlikely that the disruption of nNOS gene would influence all or the majority of GABA-ergic effects. Further, there was no difference in baseline CBF between wild-type mice and knockout mice at the dosage of pentobarbital

used in the present study. Therefore, we had no reason to believe that the use of pentobarbital may have an impact on the data interpretation. Future studies may be conducted to further examine the potential effect of various baseline anesthetics on the outcome of these experiments.

In summary, in wild-type mice isoflurane increased rCBF in a dose-dependent manner, at 1.2, 1.8, and 2.4 vol%, and the increase in rCBF was attenuated in this range of concentrations by L-NNA but not by D-NNA. In nNOS knockout mice, the isoflurane-induced increase in rCBF was maintained, most likely by compensatory mechanisms involving eNOS at 1.2 and 1.8 vol% and NO-independent mediators at 2.4 vol%. It is suggested that, in wild-type mice, eNOS and nNOS contribute to isoflurane-induced increase in rCBF and that eNOS may be involved at 1.2 and 1.8 vol%, whereas nNOS may at least be involved at 2.4 vol%.

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