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Cerebellar Nitric Oxide Is Increased during Isoflurane Anesthesia Compared to Halothane Anesthesia

A Microdialysis Study in Rats

Alex L. Loeb, Ph.D.,*† Nichelle R. Raj, B.S.,* David E. Longnecker, M.D.*

Background: **This study examined the influences of isoflurane *versus* halothane anesthesia on basal and agonist-stimulated nitric oxide in the cerebellum of intact rats. Nitric oxide was measured using the hemoglobin-trapping method in an *in vivo* microdialysis technique. This method uses the stoichiometric reaction of nitric oxide with oxyhemoglobin to produce methemoglobin and nitrate; the change in methemoglobin concentration is measured spectrophotometrically to estimate nitric oxide concentration.**

Methods: **Male Wistar rats were anesthetized with isoflurane (1.4%) or halothane (1.2%), mechanically ventilated and paralyzed (intravenous pancuronium, 1 mg/kg). Microdialysis probes were implanted into the cerebellum. Bovine oxyhemoglobin dissolved in artificial cerebrospinal fluid was pumped through the probe (2 μl/ min) and assayed at 15-min intervals. The glutamatergic agonist, kainic acid (KA, 5 mg/kg, intraarterially), was used to stimulate nitric oxide production. N^G-nitro L-arginine methyl ester (L-NAME, 40 mg/kg, intravenously) was used to inhibit nitric oxide synthase.**

Results: **Unstimulated cerebellar nitric oxide concentrations were stable and greater during anesthesia with isoflurane (532 ± 31 nM; mean ± SEM) than with halothane (303 ± 23 nM). L-NAME pretreatment reduced nitric oxide concentrations during isoflurane, but not halothane, anesthesia. Infusion of KA increased nitric oxide in both groups; however, the increase in nitric oxide was significantly greater during isoflurane anesthesia. Pretreatment with L-NAME inhibited the response to KA in both groups.**

Conclusions: **Nitric oxide production in the cerebellum, monitored by microdialysis, was greater during isoflurane anesthesia than during halothane anesthesia. Increased nitric oxide production during isoflurane anesthesia would be expected to impact central neuronal function and cerebral blood flow and vascular resistance. (Key words: Cerebellum; halothane; isoflurane; kainic acid; microdialysis; nitric oxide; rat.)**

NITRIC OXIDE (NO) has important functions in many physiologic systems, including the brain[1] and cardiovascular system.[2] In neurons, NO synthesis can be stimulated by several compounds, including the excitatory amino acid glutamate.[3–5] In the brain, NO has been shown to influence the release of neurotransmitters, neuronal function and plasticity, neurotoxicity, and cerebral blood flow,[1,6] primarily through the stimulation of cyclic GMP production in target cells.[1–5,7]

Several studies indicate that anesthesia interferes with NO/cyclic GMP system in the brain. Nitric oxide synthesis inhibition has been reported by some,[8,9] but not others,[10] to increase volatile anesthetic potency in rats and to prevent glutamatergic agonist-stimulated cyclic GMP accumulation in cerebellar slices.[11] Antagonists of the glutamatergic receptor, such as ketamine and MK-801, prevented glutamate-stimulated NO production in cultured neurons[5] and also increased the potency of general anesthetics.[12] Using primary cultures of rat cortical neurons, we[13] have shown that exposure to low, clinically relevant concentrations of isoflurane potentiated NO production in response to glutamatergic agonists; however, halothane and enflurane did not. Rengasamy *et al.*[14] recently reported that NO production was enhanced by isoflurane, but not halothane, in rat cerebellar slices.

Although the role of NO in anesthesia or consciousness is controversial, centrally released NO might influence at least some forms of neuronal transmission as well as local brain blood flow and vascular resistance. Our previous data[13] demonstrating an enhanced response to

* Assistant Professor, Departments of Anesthesia and Pharmacology.

† Research Specialist, Department of Anesthesia; Current address: Cytometrics, Inc., Philadelphia, Pennsylvania.

‡ R. D. Dripps Professor and Chair.

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Address reprint requests to Dr. Loeb: Cytometrics, Inc., 615 Chestnut Street, Philadelphia, Pennsylvania 19106. Address electronic mail to: loeb@cytometrics.com

glutamatergic agonists during exposure to isoflurane *in vitro* were consistent with observations that NO-dependent circulatory control was enhanced during isoflurane compared with halothane[15,16] or pentobarbital[17] anesthesia. The goal in the present studies was to use a microdialysis technique to determine whether the anesthetics halothane or isoflurane altered NO concentrations in the cerebella of anesthetized rats, a location where NOS is highly expressed.[3,18]

Materials and Methods

The chemicals used in the present study were obtained from the following sources: kainic acid, bovine hemoglobin, N^G-nitro L-arginine methyl ester (L-NAME), and all buffer constituents from Sigma (St. Louis, MO); halothane from Halocarbon Laboratories (Hackensack, NJ); isoflurane from Ohmeda (Liberty Corner, NJ); pancuronium bromide from Elkins-Sinn (Cherry Hill, NJ).

Male Wistar rats (250–400 g) were anesthetized with either halothane or isoflurane. A tracheostomy was performed, and anesthesia was maintained with halothane (1.2%) or isoflurane (1.4%) (concentrations corresponding to 1 MAC for each in rats[19,20]). Rats were mechanically ventilated ($F_{I_{O_2}}$ = 0.3) and then paralyzed with intravenous pancuronium bromide (1 mg/kg) to facilitate ventilation.[15] Catheters were placed in the aortic arch *via* the right carotid artery, the femoral artery, and the femoral vein for direct blood pressure monitoring and drug administration. All rats received a saline infusion of 1 ml/h. Body temperature was maintained at 37°C to 38°C with a heating pad. Arterial blood (200 μl) was sampled periodically throughout the experiments and analyzed immediately for pH, Pa_{O_2}, and Pa_{CO_2} using a Corning 168 blood gas analyzer. Pa_{CO_2} was maintained at normal values (approximately 35–40 mmHg) by ventilation rate.

Cranial Surgery

Rats were placed into a stereotaxic device (David Kopf Instr.). The skull was exposed by making a cranial incision running approximately 2 cm sagittally. The skin was retracted, and the remaining tissue carefully scraped from the skull until the coronal and sagittal sutures were evident. The bregma was used as a zero reference (horizontal) for positioning the microdialysis probe and for locating the underlying brain structures, using stereotaxic coordinates.[21] A small hole was made in the skull using a hand-held drill, and the underlying meninges was incised using a 27-gauge needle. The probe, attached to a micromanipulator on the stereotaxic device, was then lowered through the hole to a position just above the brain surface. Using this position as a zero reference (vertical), the microdialysis probe was lowered into the left cerebellum using the micromanipulator (1 mm lateral, −12.3 mm posterior, −6.5 mm ventral from bregma). The location of the probe was subsequently verified by histology.

Microdialysis techniques can be used to measure local production and release of certain compounds in discrete brain regions *in vivo.*[22] Using a hemoglobin trapping method,[23,24] microdialysis can be used to monitor brain NO concentrations. This relatively new technique takes advantage of the stoichiometric and irreversible binding of NO to hemoglobin and avoids the use of a bioassay for a second messenger, such as cyclic GMP, to monitor NO production. Binding of NO to oxyhemoglobin produces methemoglobin and nitrate.[25,26] The change in methemoglobin absorbance can then be correlated to a change in NO production.

Bovine hemoglobin was used to detect NO. Oxyhemoglobin (2 μM, final concentration) was prepared as previously described[25] in artificial cerebral spinal fluid (ACSF, consisting of NaCl, 121 mM; KCl, 3.5 mM; $NaHCO_3$, 25 mM; NaH_2PO_4, 1 mM; $MgCl_2$, 1.2 mM; $CaCl_2$, 1 mM; pH, 7.35). Oxyhemoglobin, diluted in ACSF (ACSF-Hb), was prepared daily, filtered (0.2 μm), and kept protected from light and on ice until use. Conversion of oxyhemoglobin to methemoglobin was quantitated by observing a difference in absorbance (401–411 nm) of the hemoglobin solution after exposure to NO donor compounds, exactly as described by Feelish and Noack.[27]

Microdialysis Probe

Brain microdialysis probes (MD-2204, Bioanalytical Systems) with 320 μm outer diameter, 4 mm membrane length, and low molecular weight cut off membrane (<5,000 d) were used. Probes were implanted *in vivo,* as described previously. In some cases, probes were placed *in vitro* as well, in a small foil-wrapped beaker containing ACSF (37°C). The *in vitro* probe served as a "blank" to control for absorbance not caused by NO. ACSF and ACSF-Hb were loaded into 1.25 ml gas tight syringes (Hamilton-Fisher Scientific, Pittsburgh, PA) and pumped through the probe using a syringe pump (Harvard 22, Harvard Apparatus, Holliston, MA) at 2 μl/min. ACSF-Hb syringes were wrapped with foil and cooled using refrigerant packs to limit autoxidation[24] and to prevent oxyhemoglobin deg-

radation from the heat generated by the syringe pump. The connecting tubing from the syringe to the probe (approximately 27 cm with 3–4 μl volume) was exposed to room temperature, allowing the hemoglobin solution to warm gradually before entering the probe. After probe implantation, ACSF alone was pumped through the probe for 60 min, before changing to an ACSF-Hb–containing syringe. ACSF-Hb was pumped through the probes for an additional 30 min before samples were collected for analysis. Preliminary data indicated that an additional 1.5 h were required before NO concentrations became stable. Others have shown a similar equilibration time was necessary for neurotransmitter levels to stabilize after probe implantation.[28,29] Outflow from the probe was collected into microcentrifuge tubes that were kept in a foil-wrapped container on ice. Thirty microliter samples were collected (over 15 min) in each tube. Tubes were kept on ice until analysis. Sample optical density at 401 and 411 nm was measured using a spectrophotometer (DU65, Beckman Instruments, Fullerton, CA) equipped with a 5-carat microcell.

Determination of Nitric Oxide Production

The hemoglobin-trapping technique is based on the stoicheometric reaction of NO with oxyhemoglobin to produce methemoglobin and nitrate.[25,26] The oxidation of oxyhemoglobin by NO to methemoglobin produces a characteristic increase in the hemoglobin absorbance spectrum at 401 nm with no change in the absorbance at the isosbestic point at 411 nm.[25–27] Thus the difference between the absorbance at 401 and 411 nm can be used to calculate the concentration of methemoglobin formed. To calculate the increase in methemoglobin, and therefore NO trapped within a dialysate sample, the difference in absorbance (*e.g.*, 401–411) was divided by the molar extinction coefficient for the reaction ($\Delta\epsilon_{401-411}$ = 38 $mM^{-1} \cdot cm^{-1}$), exactly as described[25] using the formula: [NO] = $OD_{401-411}/(\Delta\epsilon_{401-411}) \cdot$ (path length). The path length in our cuvette was 1 cm. The use of the isosbestic point measurement is necessary to correct for any slight changes in the concentration of hemoglobin that may occur in the system.[25,26] The theoretical limit of detection in our system was 26 nM.

To determine the change in absorbance caused by the production of NO *in vivo,* it was necessary to determine the hemoglobin autoxidation rate over the time course of the experiment (several hours) and to correct for the non–NO-related absorbance of the hemoglobin solution itself. For these experiments, the same hemoglobin solution was divided into two syringes and was pumped simultaneously through two microdialysis probes using the same syringe pump; one probe was placed *in vivo* and the other *in vitro,* as described previously. Because methemoglobin is produced spontaneously within oxygenated hemoglobin solutions, by reactions unrelated to the formation of NO,[26] it was necessary to know the rate of spontaneous oxidation of hemoglobin in parallel control solutions (the *in vitro* probe) to determine the portion of the change in the 401–411 value caused by NO. Subtracting the 401–411 value of the solution passing through the *in vitro* probe from 401–411 value of the solution passing through the rat brain effectively corrected for the changes resulting from autoxidation and was analogous to using an external reference. The autoxidation rate was found to be constant over the time course of a single experiment and did not vary from day to day (equivalent to 30 ± 2 nM per 15-min period). In experiments where the change in NO production after drug administration was determined, autoxidation was estimated by subtracting a constant based on the predetermined autoxidation rate from the observed 401–411 value to obtain a corrected value. The corrected value was used to calculate NO production.

Protocols

Unstimulated Nitric Oxide Production. Nitric oxide concentrations were determined quantitatively using the two-microdialysis probe technique in control animals and in those pretreated with the NOS inhibitor N^G-nitro L-arginine methyl ester (L-NAME, 40 mg/kg intravenously). L-NAME was dissolved in 0.5 ml saline and administered over 2.5 min at least 60 min before collection of baseline dialysate samples to obtain maximal inhibition of brain NO synthase.[30] After the equilibration period in either control or L-NAME-treated rats, dialysate samples were collected every 15 min. The average NO concentrations during the final 90 min of dialysis were used to determine the basal NO value (an equivalent time period was used for stimulated NO production experiments).

Stimulated Nitric Oxide Production. The glutamate receptor agonist kainic acid (KA, 5 mg/kg) was infused over 5 min into the aortic arch catheter to stimulate brain NO production. Administration of the drug by this route ensured that it would be delivered to the brain *via* the left carotid artery and circle of Willis as well as the vertebral arteries. The preferred method of drug delivery using microdialysis would have been directly through the probe. However, preliminary studies indicated that

mixing KA with the oxyhemoglobin dialysate interfered with the spectrophotometric assay. In separate groups of rats, L-NAME (40 mg/kg, intravenous) was administered (as described previously) at least 60 min before administration of KA. A single microdialysis probe was used in these experiments. Samples were collected every 15 min over the same time period used for the unstimulated NO production experiments. The change in NO produced by KA was calculated by averaging the 401–411 value of the dialysates in the three samples preceding the infusion of KA to obtain an initial value at t = 0. The amount of NO produced after KA administration was calculated based on the change in 401–411 value from the initial value after correction for hemoglobin degradation.

Statistical Analysis. Data are presented as mean ± SEM. The differences within and between multiple groups over time were determined using two-way analysis of variance (ANOVA) with repeated measures followed by Newman Kuel's multiple comparisons test. Differences between independent groups were analyzed by one way ANOVA.

Results

Unstimulated Cerebellar Nitric Oxide Production

After the equilibration period in either control or L-NAME treated rats, dialysate samples were collected every 15 min for 90 min using the two microdialysis probe technique to calculate NO concentrations in rat cerebellum. Linear regression analysis indicated there was no significant trend of increase or decrease in NO concentration over this time period in any group and so the average NO concentration over the observation period was calculated for each group. NO concentrations were significantly greater during isoflurane (532 ± 31 nM) than halothane (303 ± 23 n*M*) anesthesia (n = 6 in each group). Pretreatment with L-NAME significantly reduced NO concentrations in rats anesthetized with isoflurane (365 ± 65 n [n = 7]), but not halothane (281 ±61 nM [n = 10]) when compared to NO concentrations observed in the absence of the NOS inhibitor in each group.

Stimulated Nitric Oxide Production

In cultured rat cortical neurons, we demonstrated previously that NO production in response to glutamatergic agonists, including KA, was enhanced in the presence of isoflurane, but unaffected by halothane.[13] To determine

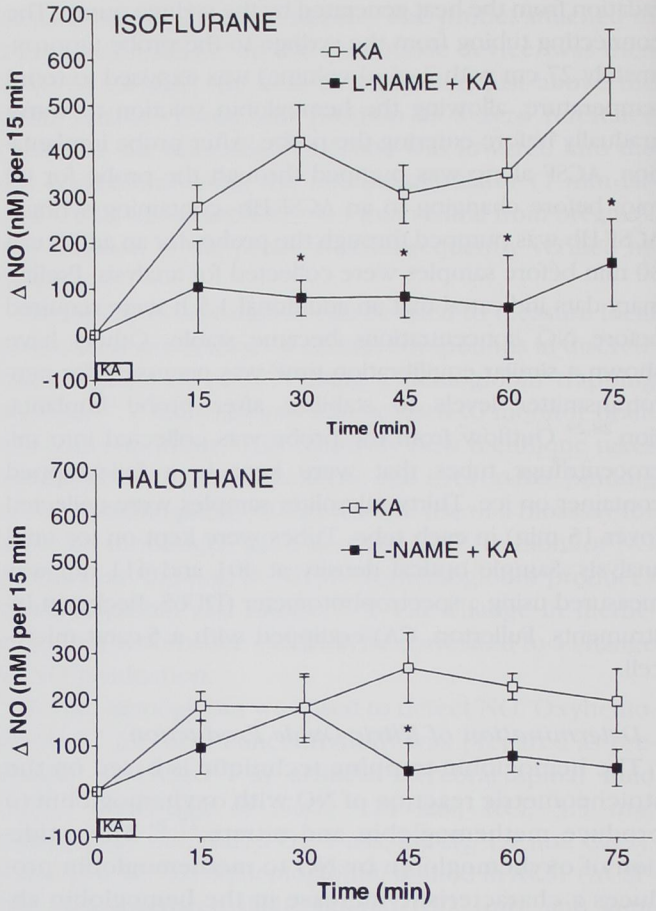

Fig. 1. KA administration increased cerebellar NO during isoflurane or halothane anesthesia. Pretreatment with L-NAME (40 mg/kg) inhibited KA-stimulated NO production (n = 5 in each group). *$P < 0.05$, KA *versus* KA + L-NAME.

whether the same phenomenon occurred in the cerebellum *in vivo,* KA was administered to rats during halothane or isoflurane anesthesia in the presence or absence of L-NAME. In these studies, NO production was determined using a single microdialysis probe. The change in NO concentration after KA was determined over the same time course described previously for determination of unstimulated NO concentrations. All data were analyzed together using two-way repeated measures ANOVA to compare responses in both anesthetic groups, but the response of each anesthetic group was graphed separately for improved clarity of display. KA significantly increased NO (two-way repeated measures ANOVA; F = 2.522; $P = 0.004$; fig. 1). NO increased more during isoflurane than during halothane anesthesia at t = 30 min (isoflurane 414 ± 82 nM *vs.* halothane 183 ± 65 nM) and t = 75 min (isoflurane 560 ± 93 nM *vs.* halothane 196 ± 69 nM). During isoflurane anesthesia,

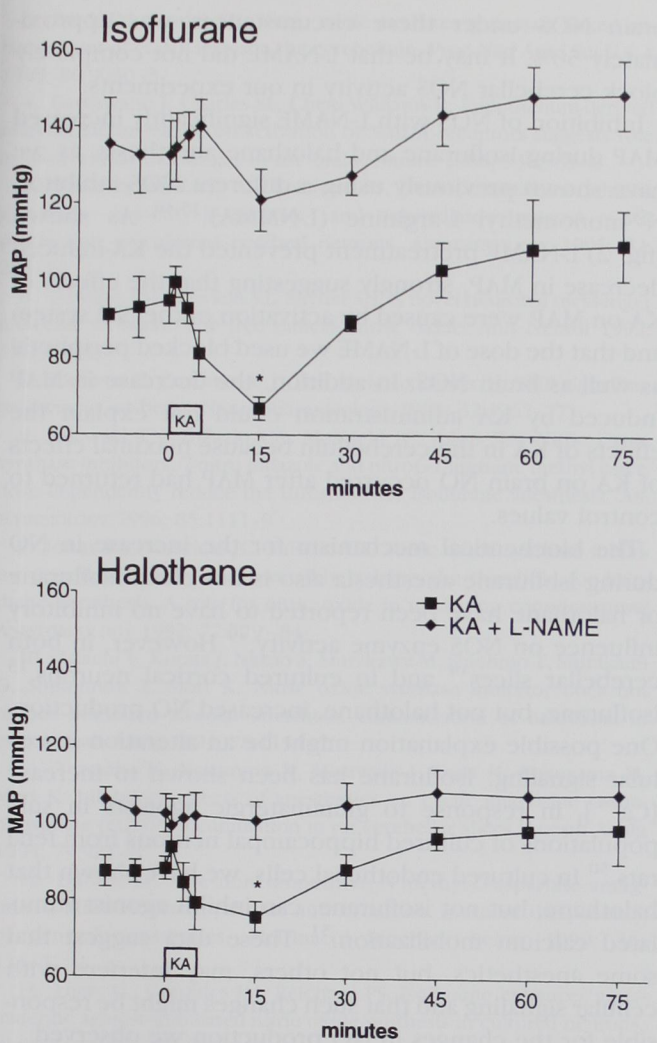

Fig. 2. Effect of KA administration on mean arterial blood pressure (MAP) during anesthesia with isoflurane (*top*) or halothane (*bottom*) in the presence or absence of L-NAME (40 mg/kg, intravenous, 1-h pretreatment). Data are from the same rats as shown in figure 1. There were five rats in each group. *$P < 0.05$ compared to $t = 0$. Time of KA administration is indicated by the box.

KA increased NO at all time points compared to t = 0, and L-NAME inhibited the effect of KA throughout the observation period. In contrast, during halothane anesthesia, KA-stimulated an increase in NO in control rats at t = 45 min only. L-NAME pretreatment prevented the effect of KA. There were no differences in NO between the two groups pretreated with L-NAME.

Intra-arterial KA administration produced a triphasic effect on MAP in the rats (fig. 2): a transient increase in MAP lasting approximately 1–3 min, followed by a decrease in MAP, with the minimum MAP observed at approximately 15 min, and finally, an increase back to control or higher values. The only significant effect of KA on MAP was the transient decrease in MAP at t = 15 min in both groups that recovered to control values by t = 30 min. L-NAME significantly increased MAP in both groups as expected. After L-NAME treatment, KA administration did not significantly alter MAP during halothane or isoflurane anesthesia.

Discussion

The data presented here demonstrate that NO in the cerebella of intact rats can be measured by microdialysis and the hemoglobin-trapping method. NO concentrations were greater during isoflurane anesthesia than during halothane anesthesia. KA administration stimulated NO production in the presence of either anesthetic, with the increase greater during isoflurane anesthesia than halothane anesthesia. Increased NO during isoflurane would be expected to influence both central neuronal function and cerebral vasculature.

These findings support our previous reports indicating that the contribution of NO to cardiovascular control *in vivo* was greater during isoflurane anesthesia than halothane anesthesia,[15,16] and that isoflurane enhanced NO production in cultured neurons stimulated by KA.[13] Consistent with our observation that isoflurane anesthesia was associated with enhanced NO-dependent regional blood flow as compared with halothane anesthesia,[15] Lee *et al.*[31] found that isoflurane induced a larger increase in erythrocyte flow in the rat brain microcirculation than did halothane. In addition, Sturaitis *et al.*[17] reported that isoflurane-, but not pentobarbital-, anesthetized dogs responded to the cholinergic agonist oxotremorine with an increase in cerebral blood flow that was prevented by L-NAME in most brain regions. When superfused over a cranial window preparation in fentanyl/N_2O anesthetized rats, 1% isoflurane produced approximately 17% dilation of pial arterioles,[32] whereas 1% halothane produced approximately 10% dilation,[33] both of which were inhibited by NOS inhibitors.

The effect of the volatile anesthetics on neuronal NO production is not well understood; however, an effect of anesthetics on neuronal NO production could alter cardiovascular responses as well as anesthetic state. We speculate that an increase in response to agonist stimulation could be responsible for enhanced global brain blood flow or increased flow to specific brain areas during isoflurane anesthesia. Isoflurane has been shown to improve brain function during conditions of altered oxygen delivery to tissue,

such as systemic hemorrhage[34] or regional ischemia of the human[35] or animal[36] brain. The mechanism(s) by which isoflurane specifically produces these effects is not clear but may be due, at least in part, to enhanced NO concentrations. For example, increased brain NO (produced by L-arginine infusion) has been reported to promote NO-dependent vasodilation, to increase regional cerebral blood flow, and to reduce infarct volume after middle cerebral artery occlusion.[37] Conversely, blockade of NO production has been reported to alter anesthetic potency,[8,9] to increase the duration of bicuculline-induced seizures[38] and the severity of kainic acid-induced seizures[39] in rats, and to inhibit cortical spreading depression in rabbits[40] and cats.[41] These data suggest that NO may be a key compound that links changes in cerebral blood flow and metabolism, is an endogenous anticonvulsant, or a neuroprotectant during ischemia.

The present experiments did not allow us to determine the source of the NO that we measured; however, the most likely sources were from neurons or glia.[18,42,43] Faraci *et al.*[44] asked whether the cerebral hyperemia in response to KA stimulation in rabbits was a result of NO release from vascular as well as nonvascular tissue. They found that KA produced NO-mediated dilation of cerebral arterioles *in vivo* but did not alter diameters in isolated cerebral vessels. Their data indicated that KA did not directly stimulate NO-dependent vasodilation of cerebral blood vessels. However, their data did not exclude the possibility that KA receptor-containing neurons within a neuronal network could have stimulated the release of acetylcholine or another neurotransmitter from nearby neurons that in turn could stimulate the release of NO from cerebrovascular endothelial cells.

Pretreatment with L-NAME significantly reduced basal levels of NO in the cerebellum during anesthesia with isoflurane, but not halothane, even though L-NAME prevented agonist stimulated increases in NO during anesthesia with either drug. Although some investigators have reported NOS inhibitors to reduce basal NO production in brain,[45–48] others have not.[23,24,29] The reasons for these different findings are not readily apparent, but may be the result of the specific techniques, brain locations studied, or anesthetics used. However, in all the studies cited previously, pretreatment with a NOS inhibitor prevented agonist-stimulated increases in NO production. In studies of the effects of NOS inhibition on cerebral blood flow and NOS activity, Iadecola *et al.*[30] reported that systemic administration of maximally effective doses of NOS inhibitors induced rapid and sustained increases in MAP, but that maximal inhibition of brain NOS under these circumstances was approximately 50%. It may be that L-NAME did not completely block cerebellar NOS activity in our experiments.

Inhibition of NOS with L-NAME significantly increased MAP during isoflurane and halothane anesthesia, as we have shown previously using a different NOS inhibitor, N^G-monomethyl L-arginine (L-NMMA).[15,16] As shown (fig. 2) L-NAME pretreatment prevented the KA-induced decrease in MAP, strongly suggesting that the effects of KA on MAP were caused by activation of the NO system and that the dose of L-NAME we used blocked peripheral as well as brain NOS. In addition, the decrease in MAP induced by KA administration could not explain the effects of KA in the cerebellum because maximal effects of KA on brain NO occurred after MAP had returned to control values.

The biochemical mechanism for the increase in NO during isoflurane anesthesia also is not clear. Isoflurane or halothane have been reported to have no inhibitory influence on NOS enzyme activity.[49] However, in both cerebellar slices[14] and in cultured cortical neurons,[13] isoflurane, but not halothane, increased NO production. One possible explanation might be an alteration in cellular signaling. Isoflurane has been shown to increase $[Ca^{2+}]_i$ in response to glutamatergic agonists in subpopulations of cultured hippocampal neurons from fetal rats.[50] In cultured endothelial cells, we have shown that halothane, but not isoflurane, can inhibit agonist stimulated calcium mobilization.[51] These data suggest that some anesthetics, but not others, may interfere with cellular signaling and that such changes might be responsible for the changes in NO production we observed.

In conclusion, NO production in the cerebellum, monitored by intracranial microdialysis, was greater during isoflurane than halothane anesthesia. The glutamatergic agonist KA stimulated NO production significantly in the presence of halothane or isoflurane, although the magnitude and time course of the response was different, depending on the anesthetic. Increased NO production during isoflurane anesthesia would be expected to impact neuronal function and cerebral blood flow.

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