Selective Anesthetic Inhibition of Brain Nitric Oxide Synthase

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Background: It has been postulated that nitric oxide (NO) is a neurotransmitter involved in consciousness, analgesia, and anesthesia. Halothane has been shown to attenuate NO-mediated cyclic guanosine monophosphate accumulation in neurons, and a variety of anesthetic agents attenuate endothelium-mediated vasodilation, suggesting an interaction of anesthetic agents and the NO-cyclic guanosine monophosphate pathway. However, the exact site of anesthetic inhibitory action in this multistep pathway is unclear. The current study examines effects of volatile and intravenous anesthetic agents on the enzyme nitric oxide synthase (NOS) in brain.

Methods: NOS activity was determined by in vitro conversion of [14C]arginine to [14C]citrulline. Wistar rats were decapitated and cerebellum quickly harvested and homogenized. Brain extracts were then examined for NOS activity in the absence and presence of the volatile anesthetics halothane and isoflurane, and the intravenous agents fentanyl, midazolam, ketamine, and pentobarbital. Dose–response curves of NOS activity versus anesthetic concentration were constructed. Effects of anesthetics on NOS activity were evaluated by analysis of variance.

Results: Control activities were 57.5 ± 4.5 pmol· mg protein⁻¹·min⁻¹ in the volatile anesthetic experiments and 51.5 ± 6.5 pmol· mg protein⁻¹·min⁻¹ in the intravenous anesthetic experiments. NOS activity was not affected by ketamine (≤1 × 10⁻⁴ M), pentobarbital (≤ 5 × 10⁻⁴ M), fentanyl (≤1 × 10⁻⁴ M), and midazolam (≤ 1 × 10⁻³ M). Halothane decreased NOS activity to 36.7 ± 2.5 (64% of control), P < 0.01 from control), 23.8 ± 4.3 (41%, P < 0.01 from control and <0.05 from 0.5% halothane), 25.2 ± 3.8 (44%, P < 0.01 from control and <0.05 from 0.5% halothane), and 19.7 ± 2.8 (34%, P < 0.01 from control and <0.05 from 0.5% halothane) pmol· mg protein⁻¹·min⁻¹ at 0.5, 1.0, 2.0, and 3.0% vapor. Isoflurane decreased NOS activity to 48.9 ± 6.1 (85% of control), 46.0 ± 3.2 (80%, P < 0.05 from control), 40.3 ± 5.1 (70%, P < 0.05 from control), and 34.2 ± 4.0 (60%, P < 0.05 from control and 0.5% and 1.0% isoflurane) pmol· mg protein⁻¹·min⁻¹ at 0.5, 1.0, 1.5, 2.0% vapor, respectively.

Conclusions: Volatile anesthetics inhibit brain NOS activity in an in vitro system, but the intravenous agents examined have no effect at clinically relevant concentrations. This inhibition suggests a protein–anesthetic interaction between halothane, isoflurane, and NOS. In contrast, intravenous agents appear to have no direct effect on NOS activity. Whether intravenous agents alter signal transduction or regulatory pathways that activate NOS is unknown. (Key words: Anesthetics, gases: nitric oxide. Anesthetics, intravenous: fentanyl, ketamine, midazolam. Anesthetics, volatile: halothane, isoflurane. Hypnotics, barbiturates: pentobarbital. Protein/enzyme interaction: nitric oxide synthase.)

NITRIC oxide (NO) is a labile, gaseous neurotransmitter within the central nervous system and spinal cord.¹⁻¼ One recent study found that inhibition of NO production by intravenous administration of arginine analogues (agents that block nitric oxide synthase [NOS]), reduces minimum alveolar concentration (MAC) of volatile anesthetics.⑤ Further support for possible involvement of NO in the action of halothane comes from the known inhibitory effect of halothane on cyclic guanosine monophosphate (cGMP) production in brain.③ Pentobarbital also decreases cGMP formation in many brain regions.③ The observation that N-methyl-D-aspartate–induced increases in cytosolic cGMP levels⑥,7 are mediated by NO, suggested that NO functions as an intracellular agent involved in signal transduction in brain.

In addition, volatile anesthetics cause reversible inhibition of endothelium-derived relaxing factor (EDRF) production in rat thoracic aorta⑧,9; EDRF is now rec-
ognized to be NO generated by endothelial NOS (type III). The site at which anesthetics affect this pathway is not known. Uggeri et al. suggested that volatile anesthetics inhibit both receptor- and non-receptor-mediated EDRF formation proximal to guanylate cyclase activation, one site of which may be NOS.

Accordingly, we hypothesized that volatile anesthetics inhibit brain NOS activity. The current study evaluates effects of volatile and intravenous anesthetic agents on activity of NOS (type I) isolated from rat brain.

Materials and Methods

Tissue Preparation

The experimental protocol was approved by the Johns Hopkins institutional animal care committee, which conforms to the Guiding Principles in the Care and Use of Animals as approved by the Council of the American Physiological Society. Male Wistar rats (200–300 g) were decapitated and brain was quickly harvested, rinsed briefly in 4°C buffer containing Tris 50 mm, and ethylendiamine tetracetic acid 2 mm, pH 7.4. A cerebellar hemisphere was weighed, placed in 20 volumes (weight/volume) of buffer and homogenized with a Cole Parmer ultrasonic Homogenizer 4710 (Chicago, IL) for 10 s. Tissue homogenate was then centrifuged at 10,000 × g at 4°C for 15 min. Supernatant was collected and kept on ice until ready for assay. All assays were performed in duplicate or triplicate in six to ten animals.

Assay Reaction

NOS activity was measured by quantifying conversion of arginine to citrulline using a modification of the techniques described by Bredt and Snyder. Reactions with intravenous agents were conducted in 10 × 75 mm borosilicate tubes; reactions with volatile agents were performed in 1.2 ml foil-sealed gas tight tubes. All reactions were performed at 22°C in mixture containing [14C]arginine 500 nm, CaCl₂ 1 mm, and β-nicotinamide adenine dinucleotide phosphate 1 mm in a total volume of 200 μl. Reactions were initiated by addition of 25 μl of cerebellar tissue supernatant. Specificity of the NOS reaction was assessed by addition of the specific NOS inhibitor, L-nitroarginine methylester, 100 μM to a serial set of tubes.

Experiments requiring the addition of soluble intravenous anesthetic agents were performed by adding each of the anesthetic agents in increasing concentrations from 1 × 10⁻⁹ M to 5 × 10⁻⁸ M before the addition of tissue extract. Each of the agents tested was soluble in water (75 μl) which was added to the reaction mixture to yield a total volume of 200 μl. Concurrent controls without addition of agent were similarly prepared. Reactions incubated for 30 min at 22°C. Reaction mixture pH was confirmed (pH 7.4) after addition of each agent at all concentrations.

To evaluate the effect of volatile anesthetics on NOS activity, reaction mixtures were bubbled for 3 min with dry filtered air containing halothane (0, 0.5, 1.0, 2.0, or 3.0%) or isoflurane (0, 0.5, 1.0, 1.5, or 2%) using calibrated vaporizers (Fluotec MK III, Cyprane, Keighley, Yorkshire, England and Ohmeda, BOC Healthcare). Preliminary experiments established that 3 min was adequate to ensure equilibrium of the reaction mixture with vapor presented; no alteration of pH occurred as a result of gas administration. Concurrent controls were the tubes presented with air only while the vaporizers were in the off position. The 25 μl tissue extract was then added and the vapor (or air alone) presented for an additional 2 min. The tubes were then sealed airtight and the reactions allowed to proceed at 22°C for 30 min.

All reactions were terminated with addition of 2 ml of buffer containing 30 mM hydroxyethylpipеразинэтиленсульфонная кислота and 3 mM ethylendiamine tetracetic acid, pH 5.5. The mixtures were then applied to chromatography columns preapplied with 0.5 ml DOWEX AG50WX-8 ion exchange resin (Na⁺ form, pH 7.0). This resin allows citrulline (neutral amino acid) to elute while arginine (cationic [+] charge) is bound. Columns were then rinsed with 2 × 1 ml water to ensure complete elution of citrulline. Chromatography column extraction of [14C]arginine was 99–99.6% efficient. When 200,000 disintegrations per minute of [14C]arginine were applied by our technique in absence of NOS, 800–1,600 disintegrations per minute eluted through the resin. Efficiency of [14C]citrulline recovery after passage through the column is 94–96%. L-Nitroarginine methylester (100 μM) produced >97.5% inhibition of enzymatic activity. The eluant containing [14C]citrulline was quantified by liquid scintillation spectroscopy (Beckman LS 1800) with an efficiency of 90–94%. Protein added to each tube was measured according to the method of Bradford. Volatile anesthetic concentrations in reaction mixtures were measured by gas chromatography (Hewlett-Packard 5880, Avondale, PA) after extraction in heptane. Concentrations ranged from 0–2 mM. Preliminary data demonstrate constant velocity over time without substrate limitation. Reac-
tion velocities of arginine conversion to an equimolar ratio of NO and [14C]citrulline were calculated and data expressed as enzyme activity per milligram protein (picomoles citrulline per milligram protein per minute). Inhibition of enzyme activity is expressed as percent inhibition of control (100%).

Isoflurane was obtained from Anaquest (BOC Health Care, Madison, WI), and halothane from Ayerst (New York, NY); gases were delivered using calibrated vaporizers and a filtered, compressed air gas source at 1 l/min. [14C]Arginine (339 mCi/mmol; 1 Ci = 37 GBq) was obtained from New England Nuclear–Du Pont, NEC-267E, Boston, Mass. Intravenous anesthetic agents were obtained as follows: ketamine (Parke-Davis, Morris Plains, NJ), pentobarbital (Elkins-Sinn, Cherry Hill, NJ), fentanyl (Janssen Pharmaceutica, Piscataway, NJ), and midazolam (Roche Laboratories, Nutley, NJ). All other chemicals were obtained from Sigma Chemical (St. Louis, MO).

Statistical Analysis
Data are presented as mean ± standard error of the mean. Inhibition of NOS activity is reported (calculated velocity) and illustrated as percent of control activity. Statistical significance of each point in inhibition curves was determined from raw data by one-way analysis of variance and post hoc Dunnett’s test.

Results

NOS activity in cerebellar tissue in the absence of anesthetics was 57.5 ± 4.5 pmol · mg protein⁻¹ · min⁻¹ (mean ± SE) for the volatile anesthetic experiments and 51.5 ± 6.5 pmol · mg protein⁻¹ · min⁻¹ for the intravenous anesthetic experiments (P = 0.46 by t test). Exposure of the solutions to bubbling air had no apparent effect on NOS activity and any evaporation of solution which may have occurred was without loss of NOS activity.

Effects of isoflurane and halothane on NOS activity are displayed in figure 1A. Halothane (n = 6) inhibited NOS activity; residual activities were 57.5 ± 4.5 (100 ± 7.8%), 36.7 ± 2.5 (63.9 ± 4.3%, P < 0.01 from control), 23.8 ± 4.3 (41.4 ± 7.3%, P < 0.01 from control and <0.05 from 0.5% halothane), 25.2 ± 3.8 (43.9 ± 6.6%, P < 0.01 from control and <0.05 from 0.5% halothane), and 19.7 ± 2.8 pmol · mg protein⁻¹ · min⁻¹ (34.3 ± 4.8% of control, P < 0.01 from control and <0.05 from 0.5% halothane) at 0, 0.5, 1.0, 2.0 and 3.0% vapor concentration. Isoflurane (n = 6) dose-dependently inhibited NOS activity with residual activity of 57.5 ± 5.4 (100 ± 8.7%), 48.9 ± 6.1 (85 ± 10.6%), 46.0 ± 3.2 (80.0 ± 5.6%, P < 0.05 from control), 40.3 ± 5.1 (70.0 ± 8.9%, P < 0.05 from control), and 34.2 ± 4.0 (59.5 ± 7.0% of control, P < 0.05 from control and 0.5% and 1.0% isoflurane) at 0, 0.5, 1.0, 1.5, and 2.0% vapor. Potency of inhibition as a function of MAC revealed similar curves, with halothane demonstrating greater inhibition at any given MAC multiple or vapor concentration (fig. 1B).

Pentobarbital and ketamine demonstrated no apparent effect on NOS activity at concentrations as high as 1 × 10⁻⁴ M (table 1). At 5 × 10⁻⁴ M, less than 10% inhibition was observed with pentobarbital and ketamine.
Table 1. NOS Activity in the Presence of Increasing Concentrations of Intravenous Anesthetic Agents

<table>
<thead>
<tr>
<th>Concentration (m)</th>
<th>Pentobarbital</th>
<th>Ketamine</th>
<th>Midazolam</th>
<th>Fentanyl</th>
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</thead>
<tbody>
<tr>
<td>1 x 10^{-8}</td>
<td></td>
<td></td>
<td>51.4 ± 1.6</td>
<td>58.7 ± 4.3</td>
</tr>
<tr>
<td>5 x 10^{-8}</td>
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<td></td>
<td>53.8 ± 1.9</td>
<td>61.3 ± 4.4</td>
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<td>1 x 10^{-7}</td>
<td>52.6 ± 1.4</td>
<td>51.3 ± 0.9</td>
<td>47.4 ± 3.3</td>
<td>60.3 ± 4.9</td>
</tr>
<tr>
<td>5 x 10^{-7}</td>
<td>52.0 ± 1.4</td>
<td>51.3 ± 1.2</td>
<td>52.6 ± 3.5</td>
<td>54.2 ± 3.4</td>
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<tr>
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<td>52.7 ± 1.4</td>
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</tr>
<tr>
<td>1 x 10^{-5}</td>
<td>51.5 ± 1.7</td>
<td>51.3 ± 1.2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>5 x 10^{-5}</td>
<td>51.8 ± 1.8</td>
<td>52.4 ± 1.7</td>
<td></td>
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</tr>
<tr>
<td>1 x 10^{-4}</td>
<td>47.6 ± 2.3</td>
<td>49.1 ± 1.3</td>
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</table>

Control activity = 51.5 ± 6.5 pmol/mg protein per min. Data are expressed as mean ± SE (n = 8–10).

*pH altered from 7.35–7.45 range.

(92.4 ± 4.4 and 95.3 ± 2.6% of control respectively). Midazolam demonstrated less than 10% inhibition at 1 x 10^{-5} M (90.8 ± 6.3% of control); this was not different than control. Fentanyl had no effect on NOS activity in concentrations up to 1 x 10^{-5} M (100.7 ± 8.99% of control). At 1 x 10^{-4} M (midazolam and fentanyl) and 1 x 10^{-5} M (pentobarbital and ketamine), the agents exceeded the capacity of the buffering system resulting in large alterations in pH; thus reduced activity at these concentrations were likely due to altered incubation conditions (data not shown).

Discussion

Results of the current study demonstrate inhibition of NOS by volatile anesthetics at the molecular level. The inhibition appears to be dose-dependent (0–1% halothane, and 0–2% isoflurane) and present for both halothane and isoflurane. When inhibition of NOS activity is compared as a fraction of MAC, the effective dose for 25% inhibition is 0.34 MAC for halothane and 1.02 MAC for isoflurane. The dose required for 50% inhibition is 0.87 MAC for halothane and >1.73 MAC isoflurane (highest concentration tested). The intravenous anesthetic agents, which are known to operate via specific receptor systems at the level of the plasma membrane, appear to have no direct influence on NOS, an intracellular enzyme.

Johns et al. have reported that inhibition of NOS decreases the threshold of halothane anesthesia in the rat. This inhibition may result in decreased levels of consciousness, and augment analgesia, sedation or anesthetic effects. Further, this same group has demonstrated that volatile anesthetics attenuate vascular EDRF production. In their study, inhibition of EDRF release was not exclusively receptor mediated, and was proximal to the activation of guanylate cyclase. Our data suggest that volatile anesthetics can inhibit NOS in vitro at the molecular level.

NO has been identified as an intermediary neurotransmitter in brain, spinal cord, and peripheral tissues, and may modulate nociception in these tissues. Administration of l-nitroarginine methylster in the intrathecal space reduces thermal hyperalgesia in a model of neuropathic pain in the rat, suggesting that sustained NO production mediates hyperalgesia. However, analgesia is induced in peripheral tissues by local administration of morphine, acetycholine and nitroprusside (an NO donor). Acetylcholine-induced analgesia is blocked by prior local administration of NOS inhibitors such as L-N^6-monomethyl arginine. Although NO is involved in processing of nociception, its actions appear to be site dependent, and further elucidation of the role of NO is necessary. It is unknown if volatile anesthetics mediate changes in NOS in these tissues to effect changes in processing nociception.

No universal mechanism of general anesthetic action is broadly accepted, although theories of lipid-anesthetic and protein-anesthetic interactions have been proposed. Reported data clearly demonstrate direct interaction between inhalational anesthetic agents and
select enzymes and proteins.\textsuperscript{17–21} These interactions are felt to occur at hydrophobic binding sites on the proteins, with halothane being consistently more potent than isoflurane. Results of the current study demonstrate that volatile anesthetics can inhibit NOS activity in a system which does not contain intact cellular membranes. The nature of the interaction between halogenated anesthetics and NOS remains to be elucidated. Direct binding to hydrophobic sites on the enzyme could prevent binding of substrate (arginine) or necessary cofactors (5,6,7,8-tetrahydrobiopterin, flavin mononucleotide [FMN], flavin adenine dinucleotide [FAD], \(\beta\)-nicotinamide adenine dinucleotide phosphate, \(\text{Ca}^{2+}\), and calmodulin), or induce conformational changes in enzyme. Another possibility is direct interaction of volatile agent with cofactor or substrate.

Results of the current study are consistent with published reports by Johns et al.,\textsuperscript{5} Uggeri et al.,\textsuperscript{8} and Kant et al.\textsuperscript{3} In these studies, halothane (and isoflurane\textsuperscript{5}) inhibited NO-mediated phenomena (anesthesia/consciousness, vasodilation, and cGMP formation, respectively). However, our study documents \textit{in vitro} enzyme effects rather than \textit{in situ} inhibition of NO production or action: effects which could be due to modulation of regulatory proteins, signal transduction or effector mechanisms. A decrease in NO production would account for the findings of Kant et al.,\textsuperscript{3} where halothane decreased cGMP production. cGMP production in multiple areas of brain has been directly correlated with NO production.\textsuperscript{6,7} Hart et al. recently reported attenuation of NO stimulated-guanylate cyclase activation by halothane in vascular smooth muscle,\textsuperscript{21} and similar attenuation might also occur in brain. Volatile anesthetics appear to have activity at multiple steps in the EDRF, or NO, pathway.

Volatile anesthetics induce cerebral vasodilation which is attenuated by inhibitors of NOS, suggesting that NO participates in this vasodilation response. This vascular action of volatile anesthetics appears to be at odds with our observations that NOS is inhibited by these agents. Perhaps volatile anesthetics elicit actions \textit{in vivo} which lead to NO production despite partial enzyme inhibition. Further work in this area is needed.

In the current study, we could not demonstrate direct inhibition of NOS activity by intravenous anesthetic agents. The intravenous agents tested act \textit{via} specific receptor systems (table 2) which normally elicit discrete intracellular responses upon activation by their endogenous ligands. These processes are incompletely defined and some may involve NOS activation. Thus, the current study does not rule out indirect effects of these agents on NO production. For example, N-methyl\(\text{-}\text{D-}\text{-}\text{aspartate}–\text{glutamate} \) receptors\textsuperscript{4,7} and morphine\textsuperscript{13} (presumably \textit{via} opioid receptors) stimulate NOS activity in select neurons. Barbiturates inhibit release of aspartate and glutamate (excitatory amino acids) and enhance release of \(\gamma\)-aminobutyric acid (inhibitory neurotransmitter) in many central nervous system regions.\textsuperscript{16} Barbiturates also reduce the sensitivity of neurons in many areas of the central nervous system to glutamate, whereas halothane has no such action.\textsuperscript{22} It is postulated that ketamine’s anesthetic action is mediated \textit{via} blockade of the \(\text{Ca}^{2+}\) channel associated with the N-methyl\(\text{-}\text{D-}\text{-}\text{aspartate} \) receptor, and might reduce availability of intracellular \(\text{Ca}^{2+}\) as a cofactor for NOS. Fentanyl and midazolam may also alter signal transduction or regulation of NOS \textit{via} action at opioid or benzodiazepine receptors respectively. However, the current study clearly demonstrates that there is no direct interaction between these agents and NOS. The concentrations tested span the clinically relevant concentrations achieved in anesthesia.

In conclusion, the current study demonstrates inhibition of brain NO production \textit{in vitro} by volatile anesthetics (but not intravenous agents) similar to the inhibition of vascular EDRF production reported by Uggeri et al.\textsuperscript{8} Because NOS is highly membrane-associated \textit{in vivo}, it is possible that membrane effects of volatile anesthetics may further alter NOS activity. Although our study suggests a direct volatile anesthetic–NOS interaction, it does not establish that inhibition of NO is the mechanism of anesthesia of volatile agents. The parallel between inhibition of NO production and anesthetic action of volatile anesthetics is consistent with inhibition of NO production (or action) as a possible or contributory mechanism of general anesthetic action, however causation has not been demonstrated.

<table>
<thead>
<tr>
<th>Table 2. Intravenous Anesthetic Agents, Clinical Plasma Concentrations, and Proposed Sites of Action</th>
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<tr>
<td><strong>Agent</strong></td>
</tr>
<tr>
<td>Pentobarbital</td>
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<td>Ketamine</td>
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<tr>
<td>Midazolam</td>
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<td>Fentanyl</td>
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General theories of action include enhancement of inhibitory, or inhibition of excitatory neurotransmission via these receptors.
ANESTHETIC INHIBITION OF NITRIC OXIDE SYNTHASE

 References


