Inhibition of Nitric Oxide Synthase Decreases Anesthetic Requirements of Intravenous Anesthetics in Xenopus laevis

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Background: Acute inhibition of nitric oxide synthase (NOS) has been demonstrated to reduce the anesthetic requirements of volatile anesthetics. Recent data suggest that not only volatile but also intravenous anesthetic agents interact with nitric oxide (NO) metabolism. The aim of this study was to examine the effect of NOS inhibition by nitro-L-arginine-methyl-ester (L-NAME) on the anesthetic action of the intravenous anesthetics thiopental, propofol, and ketamine.

Methods: The anesthetic potencies of thiopental, propofol, and ketamine were determined in Xenopus laevis tadpoles in the absence and presence of L-NAME. Anesthesia was defined as loss of righting reflex for 5 s. A nonlinear logistic regression curve was fitted to the data and half-maximal effective concentrations (EC50) were calculated. A second set of experiments was performed with different concentrations of L-NAME in the presence of the previously determined EC50 of the intravenous anesthetics.

Results: The EC50 of the anesthetics thiopental, propofol, and ketamine were determined to be 25.5 ± 2.0 μM, 1.9 ± 0.1 μM, and 59.7 ± 0.7 μM, respectively. The addition of L-NAME shifted the concentration-response curves to the left in a concentration-dependent manner. In the presence of 1 mM L-NAME, the EC50 of thiopental was reduced by 43%, the EC50 of propofol by 26%, and the EC50 of ketamine by 63%. The addition of D-NAME did not change the EC50 values of the three anesthetics. In the presence of L-arginine, the effect of L-NAME on the EC50 of thiopental was reversed. When administered by itself in a concentration range from 0.1 μM to 10 mM, L-NAME did not alter the behavior of the tadpoles.

Conclusions: The results of the present study show that acute inhibition of NOS by L-NAME results in reduced anesthetic requirements of the intravenous anesthetics thiopental, propofol, and ketamine. This interaction of acutely administered L-NAME and intravenous anesthetics indicates that the NO–cyclic guanosine 3′,5′-monophosphate system is involved in mediating the anesthetic effect of these compounds. (Key words: Intravenous anesthetics: thiopental; propofol; ketamine. Nitric oxide synthase inhibition: L-NAME. Anesthetic requirements.)

NITRIC oxide (NO), first described as endothelium-derived relaxing factor, was subsequently shown to play an important role as a messenger system involved in neuronal signal transduction. Instead of acting at a specific receptor located at the surface of a cell membrane, NO acts intracellularly at the enzyme guanylyl cyclase, thus increasing tissue concentrations of cyclic guanosine 3′,5′-monophosphate (cGMP). Several receptor systems, including N-methyl-D-aspartate (NMDA), muscarinic, and gamma-aminobutyric acid receptors and α2-adrenoceptors, have been shown to mediate their actions via the NO-cGMP pathway.

Nitric oxide is synthesized intracellularly by oxidation of a guanidine nitrogen atom of the amino acid L-arginine, yielding citrulline and NO. The reaction is catalyzed by the enzyme nitric oxide synthase (NOS) of which three isoforms have been described and cloned, a neuronal form, an endothelial form, and an inducible or immunologic form primarily found in macrophages. Analogs of L-arginine, such as nitro-L-arginine-methyl-ester (L-NAME), are competitive inhibitors of NOS, with no apparent specificity for one of the subtypes.

The role of NO in the regulation of consciousness is still a matter of discussion. Volatile anesthetics have been shown to affect the activity of NOS and to decrease cGMP levels in the brain. Therefore, it has been suggested that the anesthetic action of halothane is at least partly mediated by an effect on NO-cGMP metabolism. However, findings on the effect of NOS inhibition remain controversial. After administration of NOS inhibitors, the minimal alveolar concentration (MAC) of halo-
thane is either decreased or remained unchanged.\textsuperscript{5,6} In rats chronically treated with L-NAME and in mice deficient of the neuronal NOS gene, the MAC of volatile anesthetics was not modified.\textsuperscript{7,8}

Intravenous anesthetics such as thiopental reduce NOS activity and cerebral cGMP levels.\textsuperscript{9,10} Although an effect of the anesthetic activity of intravenous anesthetics has been postulated, no data are available on the interaction of the NO pathway and the anesthetic action of intravenous anesthetics. If the anesthetic effect of intravenous compounds involves the NO pathway similar to inhalational anesthetics, then an inhibition of this signal transduction pathway should decrease anesthetic requirements. To test this hypothesis, the effect of L-NAME on thiopental, propofol, and ketamine requirements was determined in \textit{Xenopus laevis} tadpoles. This model is well suited to determine anesthetic requirements because steady-state conditions are attained. Further, the response of tadpoles to anesthetics is similar to that of mammals, including humans.\textsuperscript{11}

**Methods**

With approval of the local animal care committee, experiments were performed comparing requirements of the intravenous anesthetics thiopental, propofol, and ketamine in \textit{X. laevis} tadpoles pretreated with the NOS inhibitor L-NAME with the requirements of control groups. The anesthetic potencies of the intravenous anesthetics were determined at 23 ± 1°C in early pre-limb-bud tadpoles about 1 cm long. Measurement of concentration–response curves was achieved by placing groups of ten tadpoles in 500-ml beakers containing aqueous oxygenated solutions of the anesthetics studied (pH, 7.0; titrated using hydrogen chloride or sodium oxide). Anesthesia was defined as loss of righting reflex, as described previously.\textsuperscript{12,13} Briefly, after exposure to the anesthetic solution for 10 min, tadpoles were tipped with a flame-polished glass pipette. Inability of a tadpole to right itself within 5 s was scored as anesthesia. Testing was repeated every 10 min for up to 120 min. To ensure that steady-state conditions were attained, the time needed to reach a plateau in the response after exposure to several concentrations of the intravenous anesthetics was determined. A constant level of response was generally reached after 40–60 min. The half-maximal effective concentration (EC\textsubscript{50}) was measured at 90 min assuming that equilibrium conditions were achieved after this period. Concentration–response curves were determined with at least eight different concentrations of thiopental, propofol, or ketamine. The experiments were repeated three times, and the data were pooled. Thus for each anesthetic 240–280 animals were studied. Each animal was only included once per concentration and anesthetic. Determinations of anesthetic potencies in presence of L-NAME or its enantiomer D-NAME were performed in a similar manner. L-NAME or D-NAME were added in a fixed concentration of 1 mM, whereas the concentrations of thiopental, propofol, and ketamine were varied. Two similar sets of experiments were performed exposing tadpoles to L-NAME ranging from 0.1 μM to 10 mM in concentration in the absence and presence of previously determined EC\textsubscript{50} values of thiopental, propofol, and ketamine. In a further experiment, the competitive nature of the NOS inhibition was evaluated by exposing tadpoles to varying concentrations of thiopental in presence of 1 mM L-NAME and 10 mM L-arginine.

After each experiment, tadpoles were placed in beakers containing tap water until full recovery of righting reflex was confirmed. No tadpole was used for more than one experiment. Concentration–response curves were analyzed according to the method of Waard\textsuperscript{14} for quantal biologic responses. Half-maximal effective concentrations and slopes of the curves were calculated and reported as mean ± SEM. For comparison of experiments, variances in EC\textsubscript{50} were calculated from the SEM, the sum yielding the estimated variance of the difference in the EC\textsubscript{50}. The ratio of the difference to the SEM was then referred to as a standard normal distribution. The problem of multiplicity was addressed using the Bonferroni correction to maintain a significance level of 0.05.

**Results**

Exposure of \textit{X. laevis} tadpoles to different concentrations of thiopental, propofol, or ketamine resulted, similar for each compound, in an increased fraction of anesthetized animals with increasing concentrations of the anesthetic. Calculated logistic concentration–response plots showed a sigmoidal curve (fig. 1). Thiopental exhibited an EC\textsubscript{50} of 25.5 ± 2.0 μM, with a slope of the concentration response curve of 2.1 ± 0.3. Addition of 1 mM L-NAME resulted in an EC\textsubscript{50} of 14.6 ± 0.5 μM (slope 2.3 ± 0.3), thus increasing the anesthetic effect of thiopental by 43%. Propofol administered alone showed an EC\textsubscript{50} of 1.9 ± 0.1 μM (slope 3.7 ± 0.2),
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![Graphs showing concentration-response curves for anesthetic agents with and without L-NAME.]

whereas in the presence of 1 mM L-NAME, the EC₅₀ was reduced by 26% to 1.4 ± 0.1 μM (slope 3.7 ± 0.1). The EC₅₀ of ketamine was 59.7 ± 0.7 μM (slope 2.9 ± 0.1), and it was reduced by 63% to 22.0 ± 0.3 μM (slope 3.1 ± 0.1) with the addition of L-NAME.

The increase in anesthetic activity of the three intravenous anesthetic agents depended on the concentration of L-NAME added. In presence of a concentration of one of the anesthetic compounds sufficient to anesthetize 50% of the animals, L-NAME increased the fraction of anesthetized animals with increasing concentrations (fig. 2), resulting in an EC₅₀ of 89.2 ± 2.4 μM, 101.2 ± 6.1 μM, and 84.6 ± 3.1 μM, for thiopental, propofol, and ketamine, respectively. Slopes of the concentration-response curves ranged from 1.0 to 1.3. L-NAME administered alone did not alter the behavior of the tadpoles in a concentration range from 0.1 μM to 10 mM. Adding 1 mM D-NAME did not change the EC₅₀ values of all three anesthetics (thiopental, 26.8 ± 3.7 μM; propofol, 2.0 ± 0.4 μM; ketamine, 57.2 ± 6.3 μM). In presence of 1 mM L-NAME and 10 mM L-arginine, the EC₅₀ of thiopental was 21.7 ± 4.3 μM.

Discussion

The results of the present study show that treatment with the NOS inhibitor L-NAME reduces the anesthetic requirements of the intravenous anesthetics thiopental, propofol, and ketamine in a concentration-dependent manner. The enantiomer D-NAME did not change the effect of the anesthetic compounds, and adding L-argi-
nine reversed the effect of L-NAME on the anesthetic potency of thiopental. The magnitude of the effect on the anesthetic action of the intravenous agents was similar to that determined for the volatile anesthetics halothane and isoflurane. The decrease in the EC50 of ketamine was greater than the EC50s of thiopental and propofol. When administered by itself, L-NAME did not influence the tadpole righting reflex, suggesting that inhibition of the NO pathway alone is not sufficient to induce an anesthetic state.

Anesthetic potencies were determined in tadpoles because it has been shown previously that steady-state conditions of aquatic animals with surrounding solutions are readily achieved. The effects of anesthetics on tadpoles have been studied in great detail, exhibiting similarities to other vertebrate species including humans. Because of the steady-state conditions, pharmacokinetic influences on drug actions can be excluded, although differences in binding to plasma proteins may occur. However, the EC50 values determined with this experimental design cannot be compared directly with plasma concentrations in humans because no anesthetic adjuncts were administered and because concentration–response curves were determined under steady-state conditions. Although this animal model has been used since the beginning of the century to measure the anesthetic effect of a compound, its limitations are the sensitivity at different points in the central nervous system that may not be mediating the anesthetic effect and the fact that measured concentrations do not necessarily bear a constant relation to the concentrations that provide clinical anesthesia.

The mechanisms by which anesthetics act are largely unknown, although advances have been made to define their actions at a molecular level. Anesthesia has been attributed to the effects of anesthetics on various cellular subsystems, including neuronal cell membranes and membrane proteins, such as receptors and ion channels, as well as second messenger systems. One of the cellular signal transduction pathways involves endothelial-derived relaxing factor, which has been identified as NO or a close derivative that releases NO. After diffusion into neighboring cells, NO binds with high affinity to the heme group of the enzyme guanylyl cyclase, leading to conformational changes that increase the activity of the enzyme, resulting in increased cGMP concentrations. Nitric oxide is synthesized by a variety of tissues, including neuronal tissue of the central nervous system. High densities of NOS in the central nervous system have been demonstrated in the cerebellum, hypothalamus, midbrain, striatum, and hippocampus. Increases in cGMP concentrations in neuronal tissues after stimulation of excitatory NMDA, glutamate, and kainate receptors are mediated by NO release. Second messenger systems involving the NO pathway have been proposed to be of early evolutionary origin. Nitric oxide synthesizing enzymes have, for example, been found in horseshoe crabs, arthropods that have remained unchanged for the past 500 million years. Activity of NOS has also been shown in metamorphosing and adult X. laevis.

The neurotransmitter NO has been suggested to be involved in nociception and maintenance of wakefulness, and lack of NO is supposed to result in impairments of consciousness such as heat stroke, cerebral malaria, ethanol intoxication, and opioid narcosis. Inhibition of NOS reduces MAC values of halothane, indicating a role for NO in the mediation of consciousness. Determination of MAC involves a painful stimulus, and thus reduction of MAC by NOS inhibitors may be caused by analgesia rather than by decreased levels of consciousness. The current study defined anesthesia as loss of righting reflex in tadpoles induced by a nonpainful stimulus, and thus a distinction can be made between anesthetic and analgesic effects.

Intravenous anesthetics have been shown to exert actions at several neuronal receptor pathways. The ob-
served decrease in anesthetic requirements by NOS inhibition may be caused by an interaction of L-NAME with a neuronal signal transduction pathway involving the NO-cGMP system. Several neuronal signal transduction pathways relevant to anesthesia have been shown to include the NO-cGMP system such as the NMDA receptor-, the muscarinic receptor-, and the α₂-adrenoceptor-mediated pathways.\textsuperscript{21,26,31} Although initially intravenous anesthetics have been reported to exert only minor effects on the NO-cGMP system,\textsuperscript{32} recent data suggest a more complex view. Findings that pentobarbital, ketamine, fentanyl, or midazolam did not reduce rat brain NOS activity may be due to a lack of superoxide and hydrogen peroxide—removing enzymes in the assay mixture.\textsuperscript{2} Galley and Webster\textsuperscript{8} reported a decrease in the neuronal form of NO activity after administration of intravenous anesthetics, including thiopental, ketamine, etomidate, and midazolam, when superoxide dismutase and catalase were added.

The barbiturate thiopental as well as propofol are thought to exert their anesthetic action at the gamma-aminobutyric acid receptor, the prevalent inhibitory receptor of the central nervous system.\textsuperscript{33} Gamma-aminobutyric acid receptors have been shown to be colocalized with NOS in many areas of the central nervous system.\textsuperscript{34} Release of gamma-aminobutyric acid and its receptor function are regulated by NO and cGMP.\textsuperscript{35,36} Similar to inhalational anesthetics, barbiturates have been shown to suppress neuronal cGMP increases stimulated by NMDA and kainate receptors.\textsuperscript{37} However, a study by Terasako et al.\textsuperscript{10} showed that thiopental, in contrast to inhalational anesthetics, decreases cerebellar cGMP concentrations stimulated by administration of sodium nitroprusside, an NO donor independent of NOS, thus providing evidence for a direct action of thiopental at the guanylyl cyclase.

Cholinergic transmission is also involved in mediating the anesthetic state.\textsuperscript{30,38} In rat brain, muscarinic receptors are localized in the same areas as NOS, and the NO-cGMP system was found to be involved in mediating the effects of muscarinic agonists.\textsuperscript{39,40} In spinal cord tissue, acetylcholine releases NO by acting on muscarinic receptors, thus increasing peripheral cGMP levels and causing analgesic effects.\textsuperscript{41} Antinociception mediated by muscarinic receptors has been shown to be antagonized by NOS inhibitors.\textsuperscript{42} Thus NO-mediated analgesia cannot account for the decrease in EC\textsubscript{50} of intravenous anesthetics observed in the current study because administration of the NOS inhibitor L-NAME leads to decreasing cGMP levels. In addition, no painful stimuli is applied when the righting reflex is used as an anesthetic end point, suggesting that higher integrative neuronal processes rather than analgesic effects are involved.\textsuperscript{43}

Ketamine is a noncompetitive inhibitor of NMDA receptors, suggesting that the NMDA receptor is involved in ketamine-mediated anesthesia.\textsuperscript{44,45} Further, ketamine inhibits NO synthesis stimulated by NMDA and non-NMDA-receptor-specific analogs. Based on these findings, investigators have speculated that inhibition of neuronal cGMP levels induced by NMDA receptor blockade is responsible for generating an anesthetic state.\textsuperscript{46} However, in NOS-deficient mice, ketamine reduced the minimum alveolar concentration of isoflurane, thus indicating that ketamine acts on receptors different from the NMDA receptors or that NMDA receptors mediate their intracellular effects via pathways independent of NO release.\textsuperscript{47}

The MAC of isoflurane in knockout mice without neuronal NOS activity was shown to be unaffected by the administration of the NOS inhibitor L-NAME, whereas in healthy mice anesthetic requirements were reduced. In addition, MAC values in NOS knockout mice were similar to those determined in healthy mice, and baseline consciousness was not affected. It has been suggested that the unaffected response to anesthetics in NOS-deficient mice can be explained by the presence of multiple pathways to maintain vital functions such as wakefulness.\textsuperscript{48} Effective mechanisms must be postulated that compensate for a lack of NOS activity. This is further supported by findings of the effect of long-term administration of L-NAME on MAC values of halothane in rats. Although cerebellar NOS activities and cerebellar concentrations of cGMP were decreased in rats treated with L-NAME for 5 days, halothane MAC did not differ from untreated rats.\textsuperscript{49} These findings indicate that NO is not the sole messenger for maintaining consciousness or pain perception but that multiple systems ensure a high level of redundancy.\textsuperscript{48} This hypothesis is supported by our findings that acute administration of L-NAME in the absence of intravenous anesthetics did not cause a change in the animals' behavior. General anesthetics exert their effects simultaneously at various receptor systems in the central nervous system, which mediate their effects through several different signal transduction pathways, thus reducing redundancy. Suppression of only one of these pathways such as the NO-cGMP system in the absence of a general anesthetic is not sufficient to induce an anesthetic state.

Despite conflicting data regarding the effect of the
intravenous anesthetics thiopental, propofol, and ketamine on the NO-cGMP pathway, the results of the present study clearly show that acute inhibition of NOS by L-NAME results in reduced anesthetic requirements of these compounds. Although experiments on knockou mice and long-term NOS-inhibited animals found no alterations in anesthetic requirements for volatile anesthetics, the interaction of acutely administered L-NAME and intravenous anesthetics indicates that the NO-cGMP system is involved in mediating the anesthetic effect of these compounds.

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