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Halothane and Isoflurane Inhibit Vasodilation Due to Constitutive but Not Inducible Nitric Oxide Synthase

Implications for the Site of Anesthetic Inhibition of the Nitric Oxide/Guanylyl Cyclase Signaling Pathway

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Background: **Inhalational anesthetics inhibit the nitric oxide-guanylyl cyclase signaling pathway, but the site of this inhibition is not yet clear. This study was designed to test the hypothesis that receptor activation or downstream signaling events leading to nitric oxide synthase activation are important sites for this inhibition by comparing the effect of anesthetics on vasodilation caused by the calcium-dependent constitutive endothelial nitric oxide synthase *versus* the calcium-independent inducible nitric oxide synthase.**

Methods: **Endothelium-intact or -denuded rat thoracic aorta rings preincubated with or without lipopolysaccharide were mounted for isometric tension measurement, constricted with phenylephrine, then relaxed with methacholine in the presence or absence of halothane (1–3%) or isoflurane (1–3%). The cyclic guanosine 3,5-monophosphate content in the endothelium-denuded rings preincubated with or without lipopolysaccharide in the presence or absence of 3% halothane or 3% isoflurane was quantified by radioimmunoassay. The activity of partially purified inducible nitric oxide synthase from activated mouse macrophage was assayed in the presence or absence of halothane (1–4%) or isoflurane (1–5%) by the conversion of ^{3}H-L-arginine to ^{3}H-L-citrulline.**

Results: **Halothane and isoflurane inhibited methacholine-stimulated, nitric oxide-mediated vasorelaxation in endothelium-intact aortic rings. Neither halothane nor isoflurane affected the vasorelaxation caused by basal endothelial nitric oxide synthase or inducible nitric oxide synthase activity. Neither anesthetic altered the cyclic guanosine 3,5-monophosphate increase caused by inducible nitric oxide synthase in the lipopolysaccharide-treated rings.**

Conclusions: **The results demonstrate that halothane and isoflurane inhibit only receptor/calcium-activated nitric oxide synthase action and that direct inhibition of nitric oxide synthase, soluble guanylyl cyclase, or an interaction with nitric oxide are not responsible for anesthetic inhibition of endothelium-dependent vasorelaxation. (Key words: Anesthetics, volatile: halothane; isoflurane. Artery, endothelium: endothelium-derived relaxing factor; nitric oxide. Enzymes, nitric oxide synthase: endothelial; inducible. Nucleotides: cyclic guanosine 3,5-monophosphate. Vascular smooth muscle: vasodilation.)**

ENDOTHELIUM-DERIVED relaxing factor, first discovered as a potent vasodilator produced by endothelium[1] is now known as nitric oxide or a chemically related compound.[2] Extensive studies have demonstrated that nitric oxide is an agonist for soluble guanylyl cyclase and that this nitric oxide-guanylyl cyclase signaling pathway is present in a variety of tissues.[3,4] The enzymes responsible for the synthesis of nitric oxide from L-arginine in mammalian tissue are known as nitric oxide synthase.[4] There are three major isoforms of nitric oxide synthase.[5] Two are constitutive enzymes, one normally expressed in the endothelium and one in neurons. A third inducible isoform can be produced in a variety of cells including smooth muscle cells[6] and macrophages[7] only after induction by endotoxin or cytokines such as tumor necrosis factor-α and interferon-γ. Both constitutive and inducible isoforms contain a heme moiety and require β-nicotinamide adenine dinucleotide phosphate (reduced form, NADPH), flavin adenine dinucleotide, flavin mononucleotide, and tetrahydrobiopterin as cofactors.[5,8] The constitutive isoforms also are calcium and calmodulin dependent, whereas the inducible isoform has a tightly bound cal-

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modulin subunit and does not require calcium for activation (fig. 1).[5,9]

Nitric oxide is an important mediator for the excitatory synaptic transmission of N-methyl-D-aspartate, glutamate, and kainate in the brain.[10–12] It is proposed that some anesthetics may suppress excitatory transmission to achieve anesthesia through inhibiting the formation or action of nitric oxide. Johns *et al.*[13] have demonstrated that nitro^{G}-L-arginine methyl ester (L-NAME), a specific nitric oxide synthase inhibitor, dose dependently and reversibly reduces the minimum alveolar concentration of halothane anesthesia in rats, suggesting an important relationship between the nitric oxide-guanylyl cyclase signaling pathway and anesthesia or level of consciousness. In addition, inhalational anesthetics such as halothane, enflurane, isoflurane, and sevoflurane have been demonstrated to inhibit endothelium-dependent vasodilation in arterial rings.[14–16] However, the mechanisms underlying these effects are controversial.[17] Early studies suggest that the site of inhibition is proximal to soluble guanylyl cyclase activation.[14,15,18,19] Some more recent reports, however, indicate that inhalational anesthetics may also inhibit the formation or release of nitric oxide or may work as a scavenger to inactivate nitric oxide after its formation or may even interfere with the activation of soluble guanylyl cyclase by nitric oxide.[20–23] Our recent studies, using partially purified enzymes, however, clearly demonstrate that inhalational anesthetics neither affect the basal or agonist-stimulated soluble or particulate guanylyl cyclase activity nor directly inhibit the endothelial or brain nitric oxide synthase activity *in vitro*.[24,25] Our study, using an endothelium-smooth muscle coculture model, further excluded the possibility of the activation of guanylyl cyclase by nitric oxide as the inhibitory site for inhalational anesthetics.[26]

In light of these observations, we hypothesized that the receptor activation or downstream signaling events leading to nitric oxide synthase activation are sites of inhibition for inhalational anesthetics on the nitric oxide-guanylyl cyclase signaling pathway. Because the signaling pathway after the activation of constitutive or inducible nitric oxide synthase is identical, the lack of inhibition of inducible nitric oxide synthase-induced vasorelaxation by anesthetics would imply that anesthetics do not affect activated nitric oxide synthase enzymatic function, nitric oxide itself, guanylyl cyclase activation, or effects of cyclic guanosine 3,5-monophosphate (cGMP) in causing vasorelaxation (fig. 1). We therefore tested our hypothesis by comparing the effects of inhalational anesthetics on calcium-/calmodulin-dependent and calcium-/calmodulin-independent nitric oxide synthase activation in rat aortic rings preincubated with or without lipopolysaccharide (LPS), measuring the agonist-stimulated constriction and relaxation as well as cGMP changes. The effect of inhalational anesthetics on partially purified inducible nitric oxide synthase activity also was investigated to confirm the results of our aortic ring study.

Materials and Methods

Preparation of Vascular Rings

Male Sprague-Dawley rats (weighing 300–350 g) were killed in accordance with our institutional Research and Animal Welfare Committee standards. The descending thoracic aorta was gently removed and placed in ice-cold modified Krebs' buffer (all in mM: NaCl 111, KCl 5, NaH_2PO_4 1, $MgCl_2$ 0.5, $NaHCO_3$ 25, $CaCl_2$ 2.5, dextrose 11.1). The aorta was then dissected clean of fat and surrounding connective tissue and cut into 2.5–3.0-mm ring segments. The rings were then incubated in Dulbecco's Modified Eagle's Medium (Gibco, Grand Island, NY) containing 4,500 mg/l D-glucose and L-glutamine either with or without 500 ng/ml LPS for 5 h at 37°C and continuously gassed with air and 5% CO_2.[27]

Isometric Tension Measurements

The rings were either left with their endothelia intact or denuded of endothelium by gentle rotation on a forceps. The rings were then mounted on Grass Ft-03 force transducers (Grass, Quincy, MA) at 2.0 g resting tension in 37°C water-jacketed 25-ml tissue baths containing 10 ml modified Krebs' buffer continuously gassed with air and 5% CO_2. Indomethacin (28 μM), an inhibitor of cyclooxygenase metabolism of arachidonic acid,[14] was added to the buffer throughout all experiments to prevent formation of vasoactive prostanoid metabolites. The buffer was changed every 15 min during a 60-min equilibration period. Endothelial-intact status was confirmed by constricting rings with 10^{-7} M phenylephrine followed by relaxing them with 10^{-6} M methacholine. If they relaxed more than 40% to methacholine they were considered to be endothelium-intact rings. Endothelium-denuded rings showed no relaxation. Rings were then washed and reequilibrated to basal tension.

Eight rings of each experiment were divided into four duplicate groups (one used for the anesthetic study,

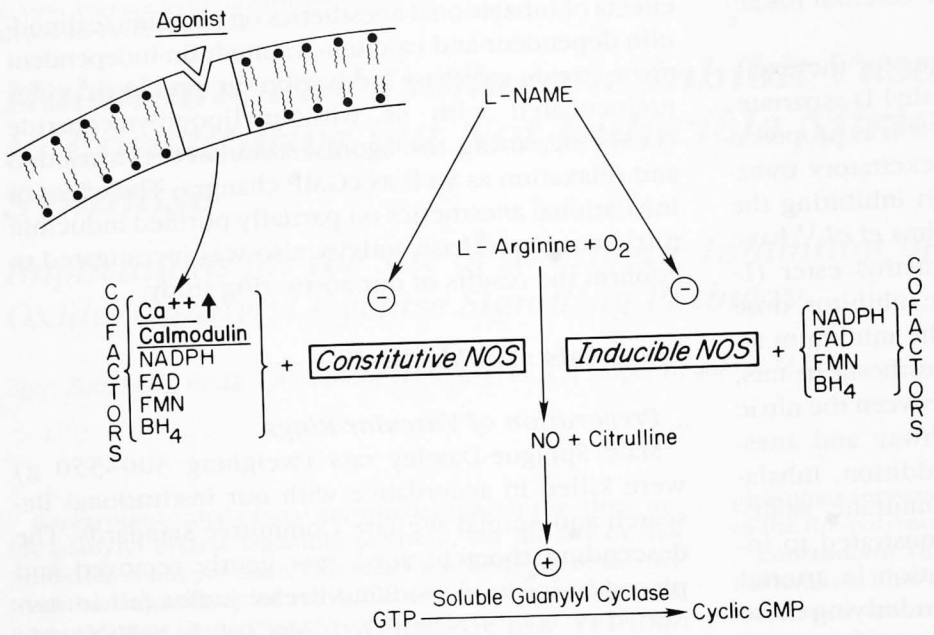

Fig. 1. Diagram of nitric oxide-guanylyl cyclase signaling pathway showing the difference between the constitutive and inducible nitric oxide synthase pathways.

the other one used as a time-control): 1) endothelium-intact, 2) endothelium-denuded, 3) LPS-preincubated and endothelium-intact, and 4) LPS-preincubated and endothelium-denuded rings. The experimental protocols were as follows: Dose-response curves for phenylephrine (10^{-8} to 10^{-5} M) were first obtained to individualize the EC60 dose for each ring. This EC60 dose (60% maximal contractile dose) was used to achieve active tension and the rings were then subjected to methacholine (10^{-7}–10^{-5} M). The values obtained were considered as preanesthetic control and the same experimental procedure was repeated in the presence or absence (time-control experiments) of 1%, 2%, or 3% halothane or isoflurane. Halothane or isoflurane was added to the rings 5 min before the addition of phenylephrine by a calibrated vaporizer in line with the air and 5% CO_2 gas at a flow rate of 4 l/min. Preliminary gas chromatographic studies suggested that the concentration of halothane or isoflurane in the buffer reached plateau after 5 min of gassing under these experimental conditions.[14,24] Postanesthetic controls were then obtained in the absence of anesthetics. The ability of L-NAME, a competitive inhibitor of nitric oxide synthase, to reverse the relaxation caused by LPS induction or by methacholine was investigated by adding 300 μM L-NAME 10 min before the addition of the same EC60 dose of phenylephrine to each of the rings. These reversal experiments were done to measure the portion of relaxation due to the nitric oxide-guanylyl cyclase signaling pathway in the total relaxation caused by LPS or methacholine.

Cyclic Guanosine 3,5-Monophosphate Analysis of Rings

Denuded rat descending thoracic aortic rings were prepared and incubated with 3×10^{-7} M phenylephrine for 6 min at 37°C in the presence or absence of 3% halothane or 3% isoflurane preincubated as described earlier. The rings were then flash-frozen in dry ice-cooled acetone. Cyclic GMP was extracted by homogenizing each ring in 1 ml of 0.1 N ice-cold hydrochloride. After centrifugation at 1000*g* for 10 min, the supernatant was analyzed for cGMP content by radioimmunoassay (^{125}I kit, Amersham, Buckinghamshire, UK).[28] Protein content was determined by dissolving the homogenate in 0.66 N NaOH and analyzing the total dissolved protein with the Bio-Rad protein assay method (Richmond, CA).[29]

Partially Purified Inducible Nitric Oxide Synthase Assay

Mouse RAW 264.7 macrophages were cultured in RPMI 1640 (Gibco) containing 10% fetal bovine serum. The confluent macrophages were then activated to express inducible nitric oxide synthase by incubating with LPS (300 ng/ml) in the same medium for 24 h at 37°C. Partially purified inducible nitric oxide synthase was prepared in a manner similar to that previously described.[7]

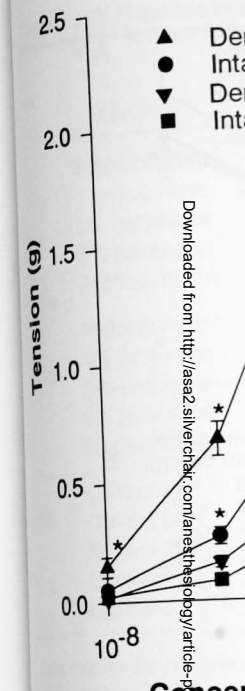

Fig. 2. Phenylephrin
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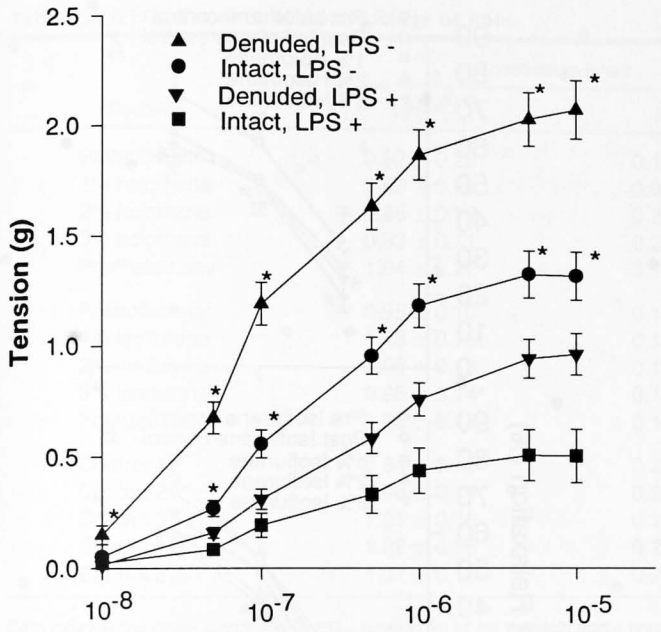


Fig. 2. Phenylephrine dose-response curve for rat thoracic aortic artery rings preincubated in the presence or absence of lipopolysaccharide (LPS+ and LPS−, respectively) and with or without endothelium (intact and denuded, respectively). Each data point represents mean ± SEM with n = 24–28 animals. *$P < 0.05$ compared to the lipopolysaccharide-treated counterpart.

Briefly, LPS stimulated macrophages were collected and washed twice with Dulbecco's phosphate-buffered saline (*p*H 7.4, Gibco). The cells were then homogenized by a tissue grinder fitted with a polytetrafluorethylene pestle in 50 mM Tris-HCl (*p*H 7.4) containing 0.1 mM ethylenediaminetetraacetic acid, 0.1 mM EGTA, 0.5 mM dithiothreitol, 1 μM pepstatin, and 2 μM leupeptin at 4°C. Homogenates were centrifuged at 100,000*g* for 60 min at 4°C. The supernatant was collected and used as the source of inducible nitric oxide synthase. The protein content in the supernatant also was measured with the Bio-Rad protein assay method.[29]

Nitric oxide synthase activity was determined by measuring the formation of ^{3}H-L-citrulline from ^{3}H-L-arginine as described.[7] Enzymatic reactions were performed in the reaction mixture (final volume 250 μl) containing 50 mM Tris-HCl (*p*H 7.4), 0.1 mM L-citrulline, 0.1 mM NADPH, 10 μM tetrahydrobiopterin, and 50 μM ^{3}H-L-arginine in the presence or absence of halothane (1–4%) or isoflurane (1–5%) for 10 min at 37°C. Preliminary time-course data demonstrated a linear increasing activity of the inducible nitric oxide synthase over the initial 10-min period of incubation under the current experimental conditions. Enzymatic reactions were terminated by adding 2 ml ice-cold stop buffer containing 20 mM sodium acetate (*p*H 5.5), 1 mM L-citrulline, 2 mM ethylenediaminetetraacetic acid, and 0.2 mM EGTA. The ^{3}H-L-citrulline produced was then separated from ^{3}H-L-arginine by Dowex AG 50W-X8 (Na+ form, Bio-Rad Laboratories, Hercules, CA) column.[7,25]

Data Analysis

Data are presented as mean ± SEM. The percent relaxation in the isometric tension study was calculated by dividing methacholine-induced relaxation (in grams) from the stable phenylephrine plateau constriction by the phenylephrine plateau constriction (in grams) and multiplying by 100. Statistical comparisons were made using paired Student's *t* test when comparing isometric tension of the same aortic rings treated with or without inhalational anesthetics or using one-way analysis of variance followed by Neuman-Keuls means comparison testing between different groups of aortic rings in the isometric tension study, cGMP study or partially purified inducible nitric oxide synthase activity study. $P < 0.05$ was considered significant. Each data point represents the mean of the data from at least six animals.

Drugs and Chemicals

Phenylephrine, methacholine, indomethacin, L-NAME, and L-citrulline were obtained from Sigma (St. Louis, MO). Halothane was obtained from Halocarbon Laboratories (Hackensack, NJ), isoflurane from Ohmeda Caribe Inc (Liberty Corner, NJ). Dowex AG 50W-X8 (Na+ form) and Bio-Rad protein assay reagent were obtained from Bio-Rad. Gasses (95% air and 5% CO_2) were obtained from Roberts Oxygen Company (Waynesboro, VA).

Results

Isometric Tension Study

Lipopolysaccharide significantly decreased the peak tension and shifted the dose-response curve of phenylephrine to the right in both endothelium-intact and -denuded aortic rings (fig. 2). The phenylephrine EC60 was 2.20×10^{-7} M and 1.54×10^{-7} M, respectively, for endothelium-intact and -denuded rings without LPS treatment, which were significantly different from those of

their counterparts with LPS treatment (3.88×10^{-7} M and 4.14×10^{-7} M, respectively, n = 24–28, $P < 0.05$).

Halothane and isoflurane significantly inhibited (at 2% or 3% of halothane or isoflurane) endothelium-dependent relaxation caused by methacholine in the rings without LPS treatment (figs. 3A and 3B). This inhibition was reversible because methacholine caused the same extent of relaxation in the postanesthetic control as that in the preanesthetic control (figs. 3A and 3B). This inhibition is not owing to the different experimental cycles because the parallel time-control experiments showed virtually identical magnitude of relaxation caused by methacholine over the five experimental cycles (fig. 3C).

Neither halothane nor isoflurane at concentrations of 1–3% affected the basal tension of rings in any groups studied. The LPS-exposed aortic rings developed less than 40% of the phenylephrine EC60 tension of the nonexposed counterparts (table 1). Halothane reversibly inhibited the phenylephrine EC60 tension in endothelium-denuded rings without LPS treatment. Thus, the phenylephrine EC60 tension of endothelium-denuded rings in the presence of 3% halothane was significantly lower than that of posthalothane control ($P < 0.05$). Similarly, 3% isoflurane also significantly inhibited the phenylephrine EC60 tension compared to that of the postisoflurane control in both endothelium-intact and -denuded rings without LPS treatment ($P < 0.05$). Isoflurane (3%) also significantly inhibited the phenylephrine EC60 tension compared to that of postisoflurane control in endothelium-denuded rings with LPS treatment ($P < 0.05$). However, the phenylephrine EC60 tension in the endothelium-intact rings incubated with LPS was neither affected by halothane nor isoflurane. Halothane also failed to affect the phenylephrine EC60 tension in the endothelium-intact, LPS-treated rings (table 1). The parallel time control experiments excluded the possibility that the phenylephrine EC60 tension changes described earlier were caused by different experimental cycles (Table 1).

L-NAME (300 μM) significantly increased the phenylephrine EC60 tension of both endothelium-intact and -denuded rings treated with LPS ($P < 0.05$; fig. 4). However, the phenylephrine EC60 tension of these rings in the presence of 300 μM L-NAME was still significantly lower than that of the rings without LPS treatment in the presence of 300 μM L-NAME (fig. 4), suggesting that 300 μM L-NAME only partially reversed the effects of LPS on the phenylephrine EC60 tension of these rings, which is consistent with previous work

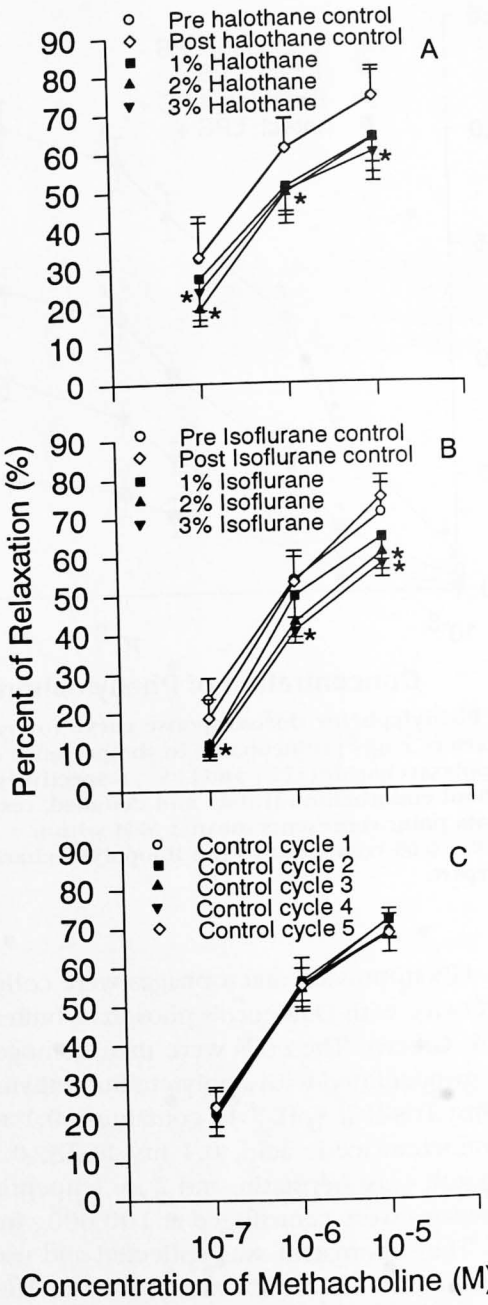


Fig. 3. Effect of halothane (*A*) or isoflurane (*B*) on methacholine-induced, endothelium-dependent vasodilation of rat thoracic aortic rings precontracted with phenylephrine (EC60 dose). The time-control experiment (*C*). Each data point represents mean ± SEM with n = 6–8 animals. *$P < 0.05$ compared to the preanesthetic and postanesthetic controls.

from our laboratory.[27] However, 300 μM L-NAME abolished the response to methacholine of endothelium-intact rings without LPS treatment (1 μM methacholine relaxed these rings preconstricted with phenylephrine EC60 only by 1.25 ± 0.66% in the presence of 300 μM

Table 1. PE EC_{60} Tension of Aortic Rings of Rats

Condition	Endothelium Intact		Endothelium Denuded	
	LPS (−)	LPS (+)	LPS (−)	LPS (+)
Prehalothane	0.92 ± 0.25	0.12 ± 0.03	1.56 ± 0.32	0.54 ± 0.09
1% halothane	0.89 ± 0.12	0.09 ± 0.02	1.56 ± 0.21	0.59 ± 0.16
2% halothane	0.86 ± 0.12	0.28 ± 0.16	1.54 ± 0.26	0.87 ± 0.20
3% halothane	0.83 ± 0.11	0.28 ± 0.16	1.42 ± 0.30*	0.86 ± 0.15
Posthalothane	1.04 ± 0.22	0.15 ± 0.04	2.04 ± 0.25	0.93 ± 0.12
Preisoflurane	0.95 ± 0.10	0.15 ± 0.06	1.61 ± 0.09	0.44 ± 0.13
1% isoflurane	1.13 ± 0.15	0.14 ± 0.07	1.74 ± 0.21	0.47 ± 0.12
2% isoflurane	1.06 ± 0.13	0.14 ± 0.07	1.74 ± 0.22	0.46 ± 0.13
3% isoflurane	0.95 ± 0.14*	0.16 ± 0.10	1.56 ± 0.20*	0.36 ± 0.09*
Postisoflurane	1.20 ± 0.18	0.13 ± 0.06	2.00 ± 0.26	0.48 ± 0.12
Control 1	0.84 ± 0.13	0.24 ± 0.08	1.57 ± 0.15	0.58 ± 0.10
Control 2	0.96 ± 0.14	0.21 ± 0.07	1.74 ± 0.17	0.59 ± 0.13
Control 3	1.01 ± 0.16	0.19 ± 0.06	1.70 ± 0.16	0.73 ± 0.18
Control 4	1.02 ± 0.16	0.22 ± 0.07	1.89 ± 0.15	0.90 ± 0.20
Control 5	1.07 ± 0.15	0.25 ± 0.10	1.90 ± 0.18	0.91 ± 0.20

Data indicate the phenylephrine (PE) EC_{60} tension (g) of rat thoracic aortic rings preincubated in the presence or absence of lipopolysaccharide (LPS+ and LPS−, respectively) and with or without endothelium. Values are mean ± SEM in grams with n = 6–8 animals.

EC_{60} = 60% maximal contractile dose of phenylephrine.

* $P < 0.05$ *versus* the postanesthetic control.

L-NAME, n = 12, $P > 0.05$ comparing 1.25 ± 0.66% to 0).

Cyclic Guanosine 3,5-Monophosphate

Cyclic guanosine 3,5-monophosphate content in the endothelium-denuded rings was significantly increased by exposure to LPS ($P < 0.05$). However, neither 3% halothane nor 3% isoflurane significantly decreased the cGMP content in the LPS-treated rings (fig. 5).

Inducible Nitric Oxide Synthase Activity

Neither halothane (1–4%) nor isoflurane (1–5%) significantly altered the inducible nitric oxide synthase activity (fig. 6).

Discussion

Several studies indicate that inhalational anesthetics inhibit the nitric oxide-guanylyl cyclase signaling pathway.[13–16,30,31] However, the site(s) at which this inhibition takes place are not clear. The proposed sites include the synthesis, release, or transport of nitric oxide as well as the activation of guanylyl cyclase.[17] We investigated the possible inhibitory sites using rat aortic rings treated with or without LPS.

Lipopolysaccharide has been demonstrated to induce expression of the inducible nitric oxide synthase isoform in endothelium and vascular smooth muscle cells as well as macrophages.[7,32,33] In the current study, L-NAME, a specific nitric oxide synthase inhibitor, significantly increased the phenylephrine EC60 of the LPS-treated aortic rings, suggesting the induction of inducible nitric oxide synthase. Because inducible nitric oxide synthase has calmodulin tightly bound in its resting state, it is continuously activated without additional calcium.[5,9] The observation that neither halothane nor isoflurane significantly increased the phenylephrine EC60 tension in the LPS-treated rings suggests that neither inhalational anesthetic inhibits the nitric oxide production of these vascular rings. The cGMP data further suggest that halothane and isoflurane do not inhibit the inducible nitric oxide synthase activity because the cGMP increase caused by inducible nitric oxide synthase was not affected by either anesthetic. Consistent with this, neither halothane nor isoflurane significantly inhibited the partially purified inducible nitric oxide synthase activity. Therefore, direct inhibition of nitric oxide synthase enzymatic function or any distal point in the nitric oxide-guanylyl cyclase-cGMP pathway is not the major site at which these two anesthetics inhibit the nitric oxide-guanylyl cyclase signaling pathway.

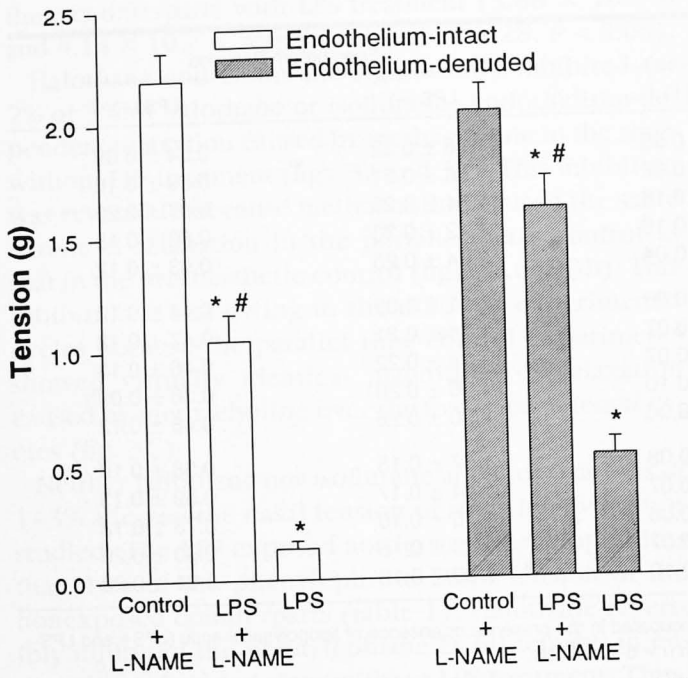


Fig. 4. Effect of 300 μM L-NAME on the phenylephrine EC60 tension of LPS-treated rat thoracic aortic rings. Each data point represents mean ± SEM with n = 12–14 animals. *$P < 0.05$ compared to the control plus L-NAME group. #$P < 0.05$ compared to the group of LPS alone.

This is consistent with the results of a study conducted in our laboratory that demonstrated that inhalational anesthetics at concentrations ranging from 1% to 4% produced no significant effect on either endothelial or brain nitric oxide synthase activity *in vitro* under a variety of experimental conditions.[26] However, a study by Tobin *et al.*[20] showed that halothane and isoflurane at clinically relevant concentrations (0.5–2%) inhibited isolated rat brain nitric oxide synthase activity. The reason for these controversial results is not known. However, consistent with our results, Tagliente[34] recently reported that halothane at different concentrations caused no significant change in the Michaelis constant (Km) for L-arginine or maximum velocity (V_{max}) of nitric oxide synthase, suggesting that the mechanism of anesthetic action of halothane is not mediated by direct alteration of nitric oxide synthase activity.

Alternatively, guanylyl cyclase has been proposed as the site for inhalational anesthetic inhibition of the nitric oxide-guanylyl cyclase signaling pathway. This has been suggested by arterial ring studies using sodium nitroprusside, nitroglycerin, or nitric oxide as the vessel relaxants[35] and by evaluating the effect of anesthetics on a partially purified guanylyl cyclase enzyme system.[22,23,36] However, a variety of studies using similar models have not confirmed these observations.[14,24,36] We prepared partially purified soluble and particulate guanylyl cyclases from rat brain and demonstrated that halothane, enflurane, or isoflurane at a very wide range of concentrations did not affect the basal or agonist-stimulated activity of partially purified guanylyl cyclase *in vitro*.[24] Consistent with these results, another study employing endothelium smooth muscle cell coculture methods, using intact cells, also strongly suggested that halothane and isoflurane did not affect the activation of guanylyl cyclase by sodium nitroprusside, nitroglycerin, or nitric oxide.[26] The current study provides further evidence that halothane and isoflurane do not inhibit guanylyl cyclase or the subsequent actions of cGMP in eliciting vascular relaxation. If the activation of guanylyl cyclase or the action of cGMP is the site of inhibition, the increase of cGMP in the LPS-treated rings should be significantly inhibited by halothane or isoflurane and the decrease in constriction to phenylephrine of the LPS-treated rings should be reversed by these two anesthetics. These two effects have not been observed in this study; therefore, current evidence strongly suggests that the inhibitory sites for inhala-

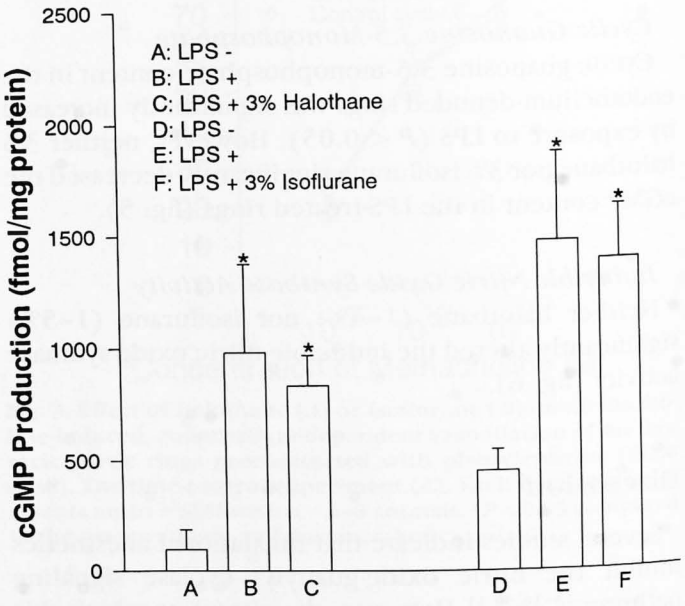
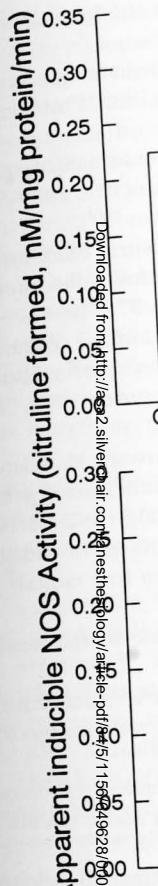


Fig. 5. Effect of halothane and isoflurane on the cyclic guanosine monophosphate content of endothelium-denuded rat thoracic rings. Rings were preincubated in the presence or absence of lipopolysaccharide (LPS+ and LPS−, respectively). Each data point represents mean ± SEM with n = 6 animals. *$P < 0.05$ compared to the rings without lipopolysaccharide treatment.



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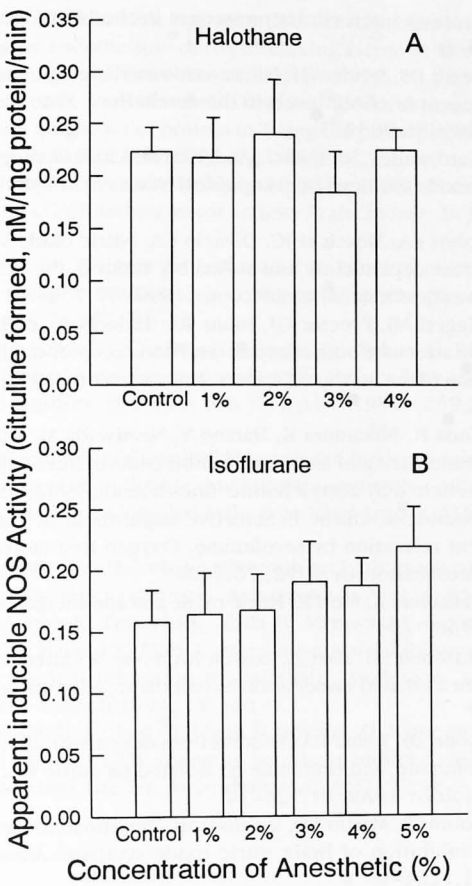

Fig. 6. Effect of halothane (*A*) and isoflurane (*B*) on the activity of partially purified inducible nitric oxide synthase of activated mouse macrophages. Each data point represents mean ± SEM with n = 9.

tional anesthetics on the nitric oxide-guanylyl cyclase signaling pathway are proximal to guanylyl cyclase.

Endothelial nitric oxide synthase is a constitutive form of nitric oxide synthase, which requires calcium for activation[5] (fig. 1). Methacholine acts on the muscarinic receptor on the endothelial cell surface, resulting in a receptor-mediated increase in cytosolic calcium from both extracellular and intracellular sources and a subsequent increase in production of nitric oxide.[14] Methacholine may also cause the release of endothelium-derived hyperpolarizing factor to induce vasorelaxation, mainly in small blood vessels.[37] The contribution of hyperpolarizing factor to the vasorelaxation caused by methacholine in our current experiments is minimal because 300 μM L-NAME abolished the vasorelaxation by methacholine. Our results demonstrate that both halothane and isoflurane reversibly inhibited the vascular ring relaxation caused by methacholine. This inhibition occurred in the presence of indomethacin, which inhibits the production of vasoactive prostanoid metabolites; the production of which may be stimulated by methacholine as well as by inhalational anesthetics,[19] confirming the previous vascular ring studies in the absence of indomethacin.[14,15,18,19] The results also showed that neither halothane nor isoflurane affected the basal endothelial nitric oxide synthase activity because neither of them affected the basal tension in those endothelium-intact rings. Therefore, agonist-stimulated receptor activation and/or subsequent events leading to an increase in cytosolic calcium and nitric oxide synthase activation may be important sites for the inhalational anesthetic inhibition of the nitric oxide-guanylyl cyclase signaling pathway (fig. 1).

Inhalational anesthetics have been demonstrated to have significant effects on cytosolic calcium concentration in multiple cell types, including endothelial cells, through an effect on calcium movement into the cells, either by changing calcium influx through receptor- or voltage-activating membrane calcium channels or by an alteration in calcium release from or uptake into the sarcoplasmic reticulum.[38,39] Using fluorescent dye, Uhl *et al.*§ and Loeb *et al.*[40] reported that halothane significantly inhibited the endothelial cell calcium transient stimulated by the agonists bradykinin and adenosine triphosphate. Inhalational anesthetics also have been shown to impair receptor activation. Halothane has been shown to shorten acetylcholine receptor kinetics,[41] and isoflurane has been shown to cause flickering of the acetylcholine receptor.[42] Many inhalational anesthetics (such as halothane, enflurane, and isoflurane) have been shown to interfere with the coupling between muscarinic receptors and their G proteins.[43–45] Therefore, it is clear from the literature that inhalational anesthetics can impair receptor activation and the cytosolic calcium responses caused by agonists. Consistent with this idea, a study from our laboratory demonstrated that inhalational anesthetics inhibited the receptor-mediated and nonreceptor-mediated but calcium-dependent nitric oxide synthase activation in rat aortic rings.[14]

Apart from the inhibition of endothelium-dependent relaxation, both halothane and isoflurane are also shown to have vasorelaxant effects in this isolated vessel

§ Uhl C, Sill JC, Nelson R, Johnson ME, Blaise G: Isoflurane and halothane and responses of cultured pig coronary artery endothelial cells (abstract). ANESTHESIOLOGY 1990; 73:A621.

preparation because the phenylephrine EC60 tension in the presence of 3% halothane or 3% isoflurane was significantly less than in controls. Consistent with previous reports, this vasorelaxation was endothelium-independent.[28]

In summary, both halothane and isoflurane produced a reversible inhibition of agonist-stimulated, nitric oxide-mediated vasorelaxation of rat aortic rings. Neither halothane nor isoflurane, at the tested concentrations, affected the basal endothelial nitric oxide synthase or inducible nitric oxide synthase vasorelaxation, isolated inducible nitric oxide synthase activity, or the increase of cGMP caused by inducible nitric oxide synthase in the LPS-treated rings. Therefore, the receptor activation and/or downstream signaling events that lead to increases in intracellular calcium and nitric oxide synthase activation or interactions with other cofactors or regulatory mechanisms of nitric oxide synthase activity may be primary sites for inhalational anesthetics to inhibit the nitric oxide-guanylyl cyclase signaling pathway.

References

1. Furchgott RF, Zawadzki JV: The obligatory role of endothelial cells in the relaxation of arterial smooth muscle by acetylcholine. Nature 1980; 288:373–6

2. Moncada S, Palmer RMJ, Higgs EA: Nitric oxide: Physiology, pathophysiology, and pharmacology. Pharmacol Rev 1991; 43:109–42

3. Rapoport M, Murad F: Agonist-induced endothelium-dependent relaxation in rat thoracic aorta may be mediated through cGMP. Circ Res 1983; 52:352–7

4. Knowles RG, Palacios M, Palmer RMJ, Moncada S: Formation of nitric oxide from L-arginine in the central nervous system: A transduction mechanism for stimulation of the soluble guanylate cyclase. Proc Natl Acad Sci U S A 1989; 86:5159–62

5. Knowles RG, Moncada S: Nitric oxide synthases in mammals. Biochem J 1994; 298:249–58

6. Fleming I, Gray GA, Julou-Schaeffer G, Parratt JR, Stoclet J: Incubation with endotoxin activates the L-arginine pathway in vascular tissue. Biochem Biophys Res Commun 1990; 171:562–8

7. Griscavage JM, Rogers NE, Sherman MP, Ignarro LJ: Inducible nitric oxide synthase from a rat alveolar macrophage cell line is inhibited by nitric oxide. J Immunol 1993; 151:6329–37

8. White KA, Marletta MA: Nitric oxide synthase is a cytochrome P450 type hemoprotein. Biochemistry 1992; 31:6627–31

9. Gross SS, Jaffe EA, Levi R, Kolbouren RG: Cytokine-activated endothelial cells express an isotype of nitric oxide synthase which is tetrahydrobioptein-dependent, calmodulin-independent and inhibited by arginine analogs with a rank-order of potency characteristic of activated macrophages. Biochem Biophys Res Commun 1991; 173: 823–9

10. Garthwaite J, Charles SL, Chess-William R: Endothelium-derived relaxing factor release on the activation of NMDA receptors suggest role as intercellular messenger in the brain. Nature 1988; 336:385–8

11. Bredt DS, Synder SH: Nitric oxide mediated glutamate-linked enhancement of cGMP levels in the cerebellum. Proc Natl Acad Sci U S A 1989; 86:9030–3

12. Garthwaite J, Southam E, Anderton M: A kainate receptor linked to nitric oxide synthesis from arginine. J Neurochem 1989; 53:1952–4

13. Johns RA, Moscicki JC, DiFazio CA: Nitric oxide synthase inhibitor dose-dependently and reversibly reduces the threshold for halothane anesthesia. ANESTHESIOLOGY 1992; 77:779–84

14. Uggeri MJ, Proctor GJ, Johns RA: Halothane, enflurane, and isoflurane attenuate both receptor- and non-receptor-mediated EDRF production in the rat thoracic aorta. ANESTHESIOLOGY 1992; 76:1012–7

15. Toda H, Nakamura K, Hatano Y, Nishiwada M, Kakuyama M, Mori K: Halothane and isoflurane inhibit endothelium-dependent relaxation elicited by acetylcholine. Anesth Analg 1992; 75:198–203

16. Yoshida K, Okabe E: Selective impairment of endothelium-dependent relaxation by sevoflurane: Oxygen free radicals participation. ANESTHESIOLOGY 1992; 76:440–7

17. Nakamura K, Mori K: Nitric oxide and anesthesia. Anesth Analg 1993; 77:877–9

18. Muldoon SM, Hart JL, Bowen KA, Freas W: Attenuation of endothelium-mediated vasodilation by halothane. ANESTHESIOLOGY 1988; 68:31–7

19. Stone DJ, Johns RA: Endothelium-dependent effects of halothane, enflurane, and isoflurane on isolated rat aortic vascular rings. ANESTHESIOLOGY 1989; 71:126–32

20. Tobin JR, Martin LD, Breslow MJ, Traystman RJ: Selective anesthetic inhibition of brain nitric oxide synthase. ANESTHESIOLOGY 1994; 81:1264–9

21. Blaise G, To Q, Parent M, Lagarde B, Asenjo F, Sauve R: Does halothane interfere with the release, action, or stability of endothelium-derived relaxing factor/nitric oxide? ANESTHESIOLOGY 1994; 80: 417–26

22. Hart JL, Jing M, Bina S, Freas W, Van Dyke RA, Muldoon SM: Effects of halothane on EDRF/cGMP-mediated vascular smooth muscle relaxations. ANESTHESIOLOGY 1993; 79:323–31

23. Jing M, Hart JL, Masaki E, Van Dyke RA, Bina S, Muldoon SM: Vascular effects of halothane and isoflurane: cGMP dependent and independent actions. Life Sci 1995; 56:19–29

24. Zuo Z, Johns RA: Halothane, enflurane and isoflurane do not affect the basal or agonist-stimulated activity of partially isolated soluble and particulate guanylyl cyclase of rat brain. ANESTHESIOLOGY 1995; 83:395–404

25. Rengasamy A, Ravichandran LV, Reikersdorfer CG, Johns RA: Inhalational anesthetics do not alter nitric oxide synthase activity. J Pharmacol Exp Ther 1995; 273:599–604

26. Johns RA, Tichotsky A, Muro M, Spaeth JP, Le Cras TD, Rengasamy A: Halothane and isoflurane inhibit endothelium-derived relaxing factor-dependent cyclic guanosine monophosphate accumulation in endothelial cell-vascular smooth muscle co-cultures independent of an effect on guanylyl cyclase activation. ANESTHESIOLOGY 1995; 83:824–834

27. Zelenkov P, Mcloughlin T, Johns RA: Endotoxin enhances hypoxic constriction of rat aorta and pulmonary artery through induction of EDRF/NO synthase. Am J Physiol 1993; 265:L346–L54

28. Brendel JK, Johns RA: Isoflurane does not vasodilate rat thoracic aortic rings by endothelium-derived relaxing factor or other cyclic GMP-mediated mechanisms. ANESTHESIOLOGY 1992; 77:126–31

29. Bradford M: A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 1976; 72:248–54

30. Vulliemoz Y, Verosky M, Alpert M, Triner L: Effect of enflurane on cerebellar cGMP and on motor activity in the mouse. Br J Anaesth 1983; 55:79–84

31. Pearce RA, Stringer JL, Lothman EW: Effect of volatile anesthetics on synaptic transmission in the rat hippocampus. ANESTHESIOLOGY 1989; 71:591–98

32. Julou-Schaeffer G, Gray GA, Fleming I, Schott C, Parratt JR, Stoclet J: Loss of vascular responsiveness induced by endotoxin involves L-arginine pathway. Am J Physiol 1990; 259:H1038–H43

33. Radomski MW, Palmer RMJ Moncada S: Glucocorticoids inhibit the expression of an inducible, but not the constitutive, nitric oxide synthase in vascular endothelial cells. Proc Natal Acad Sci U S A 1990; 87:10043–7

34. Tagliente TM: Halothane effects on rat brain nitric oxide synthase enzyme kinetics (abstract). ANESTHESIOLOGY 1994; 81:A806

35. Nakamura K, Terasalo K, Toda H, Miyawaki I, Kakuyama M, Nishiwada M, Hatano Y, Mori K: Mechanisms of inhibition of endothelium-dependent relaxation by halothane, isoflurane, and sevoflurane. Can J Anaesth 1994; 41:340–6

36. Eskinder H, Hillard CJ, Flynn N, Bosnjak ZJ, Kampine JP: Role of guanylyl cyclase-cGMP system in halothane-induced vasodilation in canine cerebral arteries. ANESTHESIOLOGY 1992; 77:482–7

37. Nagao T, Vanhoutte PM: Endothelium-derived hyperpolarizing factor and endothelium-dependent relaxations. Am J Respir Cell Mol 1993; 8:1–6

38. Su JY, Zhang CC: Intracellular mechanisms of halothane's effect on isolated aortic strips of the rabbit. ANESTHESIOLOGY 1989; 71:409–17

39. Klip A, Britt BA, Elliott ME, Walker D, Ramlal T, Pegg W: Changes in cytoplasmic free calcium caused by halothane. Role of the plasma membrane and intracellular Ca^{++} stores. Biochem Cell Biol 1986; 64:1181–9

40. Loeb AL, Longnecker DE, Williamson JR: Alteration of calcium mobilization in endothelial cells by volatile anesthetics. Biochem Pharmacol 1993; 45:1137–42

41. Lechleiter J, Gruener R: Halothane shortens acetylcholine receptor channel kinetics without affecting conductance. Proc Natl Acad Sci U S A 1984; 81:2929–33

42. Brett RS, Dilger JP, Yland KF: Isoflurane causes flickering of the acetylcholine receptor channel: Observations using patch clamp. ANESTHESIOLOGY 1988; 69:157–60

43. Anthony BL, Dennison RL, Aronstam RS: Disruption of muscarinic receptor-G protein coupling is a general property of liquid volatile anesthetics. Neurosci Lett 1989; 99:191–6

44. Durieux M: Halothane inhibits signaling through m1 muscarinic receptors expressed in Xenopus oocytes. ANESTHESIOLOGY 1995; 82:174–82

45. Minami K, Yanagihara N, Toyohira Y, Tsutsui M, Shigematsu A, Wada A, Izumi F: Isoflurane inhibits nicotinic acetylcholine receptor-mediated $^{22}Na^{+}$ influx and muscarinic receptor-evoked cyclic GMP production in cultured bovine adrenal medullary cells. Naunyn-Schmiedeberg Arch Pharmacol 1994; 349:223–9