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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

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Background: **Interaction of inhalational anesthetics with the nitric oxide signaling pathway and the mechanism of such effects are controversial. The aim of this study was to clarify the sites and mechanism of inhalational anesthetic interaction with the vascular nitric oxide and guanylyl cyclase signaling pathway.**

Methods: **To specifically study the mechanism of anesthetic interaction with the nitric oxide-guanylyl cyclase pathway, cultured vascular smooth muscle and endothelial cell-vascular smooth muscle (EC-VSM) co-culture models were chosen. Monolayer cultures of VSM with or without cultured endothelial cells grown on microcarrier beads were preequilibrated with anesthetic and stimulated with agonists. The effect of inhalational anesthetics on cyclic guanosine monophosphate (GMP) content of unstimulated VSM and of VSM in which soluble guanylyl cyclase had been activated by the endothelium-independent nitrovasodilators, sodium nitroprusside, nitroglycerin, or nitric oxide was determined. Experiments were also performed to assess the effect of inhalational anesthetics on unstimulated endothelial cell-vascular smooth muscle co-cultures and on co-cultures in which nitric oxide synthase and subsequent cyclic GMP production had been activated by the receptor-mediated agonists bradykinin and adenosine triphosphate and by the non-receptor-mediated calcium ionophore A23187.**

Results: **Increasing concentrations of halothane and isoflurane from 0.5 to 5% had no effect on basal cyclic GMP concentrations in cultured VSM alone or in endothelial cell-vascular smooth muscle co-cultures, and had no effect on sodium nitroprusside, nitroglycerin, or nitric oxide stimulated cyclic GMP accumulation in cultured VSM. In agonist-stimulated co-cultures, however, halothane and isoflurane significantly ($P < 0.05$) inhibited increases in cyclic GMP concentration in response to both receptor- and non-receptor-mediated nitric oxide synthase activating agents.**

Conclusions: **Inhalational anesthetics do not stimulate or inhibit basal cyclic GMP production in co-cultures or VSM, suggesting that inhalational anesthetics do not activate soluble or particulate guanylyl cyclase and do not activate nitric oxide synthase. Inhalational anesthetics do not inhibit nitrovasodilator-induced cyclic GMP formation, suggesting a lack of interference with soluble guanylyl cyclase activation. Inhalational anesthetics inhibit both agonist and calcium ionophore-stimulated nitric oxide-dependent cyclic GMP accumulation in endothelial cell-vascular smooth muscle co-cultures. Consistent with previous vascular ring studies, anesthetics appear to inhibit nitric oxide-guanylyl cyclase signaling distal to receptor activation in the endothelial cell and proximal to nitric oxide activation of guanylyl cyclase. (Key words: Anesthetics, inhalational. Co-culture. Endothelium. Endothelium-derived relaxing factor. Nitric oxide. Nitric oxide synthase. Vascular smooth muscle.)**

NITRIC oxide plays a major role in modulating vascular tone under both normal and pathophysiologic conditions. Several studies have examined the role of the endothelium in mediating the vascular response of anesthetics or the effects of anesthetics on endothelium-dependent responses. Blaise *et al.* have demonstrated

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that isoflurane impairs the contractile response of canine coronary arteries induced by phenylephrine in an endothelium-dependent manner and proposed that this might be due to isoflurane-induced release of endothelium-derived relaxing factor (EDRF).[1] Consistent with this observation, in a report by Greenblatt *et al.*, the microsphere technique was used to measure tissue-specific blood flow, suggesting indirectly that isoflurane may stimulate EDRF/nitric oxide production in certain vascular beds.[2] Several laboratories, however, provide strong direct evidence that anesthetics are not capable of stimulating EDRF release.[3,4] Rather, inhalational anesthetics appear to be potent inhibitors of EDRF-dependent vascular relaxation at clinically relevant doses. Muldoon *et al.* have demonstrated that halothane inhibits endothelium-dependent vasodilation in response to the receptor-mediated agonists acetylcholine and bradykinin.[5] Stone and Johns reported that a small vasoconstricting response observed with low concentrations of isoflurane, enflurane, and halothane required an intact endothelium and may be due to the inhibition of EDRF production or action.[6] Uggeri *et al.* demonstrated that these three volatile anesthetics can inhibit both receptor and non-receptor-mediated EDRF/nitric oxide dependent vasodilation.[7] Toda *et al.* have presented similar results with halothane and isoflurane.[8]

The site of anesthetic inhibition of nitric oxide-dependent vasodilation has been highly controversial. In contrast to the early work by Muldoon[5] and studies by Uggeri *et al.*[7] and Toda *et al.*,[8] recent studies[9,10] have suggested that vasodilation induced by sodium nitroprusside, nitroglycerin, or nitric oxide is inhibited by halothane, and that halothane causes this inhibition through interference with guanylyl cyclase activation. Blaise *et al.*, however, suggest that halothane does not interfere with endothelial cell release of EDRF/nitric oxide or with the activation of guanylyl cyclase, but rather halothane impairs either EDRF/nitric oxide half-life or its activated redox form.[11]

To specifically study the mechanisms of anesthetic interaction with this pathway, we chose a cultured endothelial cell-vascular smooth muscle (EC-VSM) co-culture model that avoided complicating factors in isolated vascular ring studies such as degree of baseline resting tone, type of agonist used to achieve active tone, and viability of the tissue as well as complications in intact preparations owing to flow-dependent dilation or central modulation of vascular tone by anesthetics.

Methods

Cell Culture

Bovine aorta was obtained from a slaughterhouse and the aortic endothelial cells were prepared for culture by lightly scraping the intimal surface of the aorta. The cells adhering to the blade were rinsed into a 35-mm tissue culture dish and grown in medium 199 supplemented with 20% fetal calf serum, penicillin (100 IU/ml), and streptomycin (100 μg/ml). A pure culture of endothelial cells was obtained by fluorescent-activated cell sorting using Dil-acyl-low density lipoprotein as the fluorescent marker. Identity of the endothelial cells was further confirmed by immunostaining for Factor VIII antigen and for endothelial cell actin, by demonstrating by Northern blot a single mRNA for actin in endothelium (cultures of VSM express two actin mRNAs) and by observation of a characteristic cobblestone appearance. The endothelial cells were subsequently placed into microcarrier culture by seeding 2 $\times$ 10^7 cells onto 0.6 g Cytodex 3 microcarrier beads (Pharmacia, Uppsala, Sweden) (4600 cm^2 surface area/g beads) in 200 ml medium 199 containing 20% fetal calf serum and maintained in 2-L roller bottles. Endothelial cells used in experiments were 2–6 days postconfluent and in passages 8–15. Rat aortic smooth muscle was isolated, cultured, and maintained as previously described.[12] Vascular smooth muscle cells were cultured on 24-well plates (2 cm^2 per well) in monolayer culture and studied between 5–8 days postconfluency.

Co-culture Experiments

Endothelial cells on microcarrier beads were washed with serum-free medium and placed in monolayer culture wells containing VSM that also had been washed with serum-free medium. The number of each cell type was calculated to provide a one-to-one ratio of endothelial to smooth muscle cells in each co-culture. Co-cultures were incubated at 37°C for 3–4 hr before experimentation, after which time the medium was replaced with phosphate-buffered saline (pH 7.4), which in some experiments contained isobutylmethyl xanthine (2×10^{-4} M) to inhibit phosphodiesterase metabolism of cyclic guanosine monophosphate (GMP).

Validation of Cell Culture Models

To characterize and confirm the validity of our VSM and EC-VSM co-culture preparations as models for studying the effect of inhalational anesthetics on the

nitric oxide-guanylyl cyclase signaling pathway, we studied the ability of agents that activate nitric oxide synthase and/or soluble guanylyl cyclase to stimulate increases in cyclic GMP production by our endothelial and VSM cell lines alone and in co-culture. Preliminary time course and concentration response studies for each agent to be used in these experiments were performed to determine conditions for optimal response. Agents studied included the receptor-mediated activators of nitric oxide synthase, bradykinin (1×10^{-6} M), and adenosine triphosphate (1×10^{-4} M) as well as the receptor-independent activator of nitric oxide synthase, the calcium ionophore A23187 (1×10^{-6} M). In addition, the direct activators of soluble guanylyl cyclase, sodium nitroprusside (10^{-7} to 10^{-3} M), nitroglycerin (10^{-3} to 10^{-7}), and nitric oxide in phosphate-buffered saline solution (10^{-12} to 10^{-8} M) were evaluated.

To further confirm the selective expression of nitric oxide synthase in endothelium but not VSM and of soluble guanylyl cyclase in VSM but not endothelium, total RNA was purified from bovine aortic endothelial cells and rat VSM cells using TRIREAGENT (Molecular Research Center Inc., Cincinnati, OH) and the method of Chomczynski.[13] Poly A+ mRNA was purified by oligo-dT affinity chromatography (oligo-dT columns—5′ Prime-3′ Prime Inc., Boulder, CO). Poly A+ mRNA was quantitated by absorbance at 260 nm, and 3 μg per cell type was analyzed using standard Northern blot and hybridization techniques with cDNA probes.[14] cDNA probes to the bovine endothelial nitric oxide synthase[15] and to the 70 kd subunit of rat soluble guanylyl cyclase[16] as well as probes to the constitutive "housekeeping" genes β-actin and glyceraldehyde-3-phosphate dehydrogenase were labeled with 32P by random primed labeling. High stringency washing conditions were as elucidated in Sessa *et al.*[15]

Finally, to confirm the presence of functional particulate guanylyl cyclase in both endothelial and vascular smooth muscle cell lines, studies were performed to demonstrate the ability of these cell lines to produce cyclic GMP in response to atrial natriuretic peptide.

Extraction and Measurement of Cyclic Guanosine Monophosphate

Forty seconds after the addition of agonists (shown in preliminary studies to be the optimal time for detecting cyclic GMP changes under the current experimental conditions), the media in the culture well was aspirated, and 0.5 ml of 0.1 N HCl was added to each well to extract cyclic GMP. Cyclic GMP concentrations were determined by radioimmunoassay of the acetylated HCl extract by the methods of Harper and Brooker,[17] using an automated radioimmunoassay. Values were expressed as picomoles per well of cultured cells.

Administration and Equilibration of Inhalational Anesthetics with Cell Cultures

Individual culture wells of VSM alone or of endothelium and VSM in co-culture were sealed with tightly fitting methyl methacrylate polymer plugs. Each plug had two holes, one for administration of the carrier gas with or without anesthetic and the other serving as a combination vent and passageway for administration of drugs, removal of media, and addition of HCl to stop the reaction. Air, serving as a carrier gas, was delivered through halothane and isoflurane vaporizers at 3 L/min, and subsequently passed through a warmed (37°C) humidification chamber. Gas entry into each cell culture well was valve regulated and maintained at 35 ml/min. Studies of VSM alone or in co-culture with endothelium were performed in the presence and absence of varying concentrations of each anesthetic. Culture wells containing 1 ml phosphate-buffered saline were equilibrated with anesthetic for 5 min before experimentation. Extensive preliminary studies determined that equilibration occurred within 3 min of exposure of the wells to the anesthetic. Plates were continuously maintained at 37°C in a water bath, and no significant evaporation of the culture well buffer occurred during the course of the experiment due to humidification of the gases. Factory calibration of vaporizers was confirmed using infrared spectroscopy (Ohmeda "Rascal," Liberty Corner, NJ) and the media in individual wells was randomly sampled throughout the course of the experiments to confirm concentrations by using standard gas chromatography methods.[7] Vapor concentration after equilibration of a buffer aliquot with a measured volume of air was compared to a standard curve of known concentrations obtained by vaporizing measured volumes of liquid anesthetic in a measured volume of air.

Preparation of Nitric Oxide Solutions

Phosphate-buffered saline was deoxygenated by gassing with N_2 for 2 hr; then it was anaerobically transferred to an in-line series of 7-ml, rubber-stoppered glass vacuum tubes previously flushed with N_2 for 30 min. Fifty milliliters of pure nitric oxide gas (Roberts Oxygen Supply Co., Rockville, MD) was drawn through

potassium hydroxide pellets and into a gastight syringe and subsequently slowly bubbled through one of the 7-ml glass tubes containing 2 ml deoxygenated phosphate-buffered saline (N_2 headspace) under continuous positive pressure and vented with a 23-G needle to yield a stock nitric oxide solution, the concentration of which ranged from 1 to 1.5 mM (confirmed by chemiluminesence measurement). A series of tenfold dilutions was then prepared anaerobically using the same 7-ml glass tubes with deoxygenated phosphate-buffered saline. Each tube was used for withdrawal of only a single specimen for experimental use, and 100 μl of the appropriate concentration of nitric oxide was added to the experimental culture well.

Specific Experimental Protocols

Effect of Anesthetics on Guanylyl Cyclase Activation in Vascular Smooth Muscle Alone. The dose-dependent effects of halothane and isoflurane (0, 0.5, 1, 2, 3, 4, and 5%) on both basal and sodium nitroprusside (10^{-6} M) stimulated wells of VSM were determined. In addition, the effect of preequilibration of VSM cultures with 2% halothane and 2% isoflurane on the dose-dependent increases in cyclic GMP stimulated by sodium nitroprusside (10^{-7} to 10^{-3} M), nitroglycerin (10^{-4} to 10^{-0} M), and nitric oxide in solution (10^{-12} to 10^{-8} M) were determined. These studies were performed in the presence and absence of isobutylmethyl xanthine 2×10^{-4} M, a nonspecific phosphodiesterase inhibitor to rule out the possibility of a phosphodiesterase-stimulating effect of the anesthetic masking an anesthetic depression of guanylyl cyclase activity.

Effect of Anesthetics on Nitric-oxide-stimulated Cyclic Guanosine Monophosphate in Endothelial Cell-Vascular Smooth Muscle Co-cultures. The effect of halothane and isoflurane (0, 0.5, 1, 2, 3, 4, and 5%) on unstimulated EC-VSM co-cultures and on co-cultures in which nitric oxide synthase (and subsequent cyclic GMP production) had been activated by the receptor-mediated agonists bradykinin (10^{-6} M) and adenosine triphosphate (10^{-4} M) and by the nonreceptor-mediated calcium ionophore A23187 (10^{-6} M) were determined.

In additional control experiments to demonstrate the reversibility of anesthetic effects on cyclic GMP accumulation in VSM and in EC-VSM co-cultures, anesthetic-exposed cultures were gassed with air for 45 min before administration of stimulants and extraction of cyclic GMP.

Data Analysis

Each data point represents a mean of 3–4 experiments each performed in quadruplicate. Data were analyzed by one-way analysis of variance with Neuman Keul's test when appropriate, unless otherwise noted. For the dose-response studies to sodium nitroprusside, nitroglycerin, and nitric oxide, comparisons at each dose in the presence and absence of anesthetic were made by unpaired *t* tests. Values are reported as the mean ± standard error of the mean and $P < 0.05$ was accepted as significant.

Materials and Chemicals

Bradykinin, adenosine triphosphate, A23187, sodium nitroprusside, atrial natriuretic peptide (1–28), and isobutylmethyl xanthine were obtained from Sigma Chemical Co. (St. Louis, MO). These agents were prepared and diluted in distilled and deionized water. Calcium ionophore A23187 was also obtained from Sigma, and was initially dissolved in dimethyl sulfoxide and subsequently diluted in distilled and deionized water. Preliminary studies demonstrated that the final concentration of dimethyl sulfoxide had no effect on our experimental preparations. Halothane was obtained from Halocarbon Laboratories (Hackensack, NJ) and isoflurane from Anaquest, BOC Healthcare (Madison, WI). The following fragments were used for cDNA probes: 4091bp *Eco* RI eNOS cDNA fragment (donated by Dr. W.C. Sessa); 2600bp *Eco* RI 70 kd subunit of soluble guanylyl cyclase cDNA fragment (a gift from Dr. M. Nakane); 550bp *Eco* RI *Hind* III β-actin cDNA fragment (American Type Culture Collection); and 780bp *Xba* I *Pst* glyceraldehyde-3-phosphate dehydrogenase cDNA fragment (American Type Culture Collection).

Results

Cell Culture Model Validation

The endothelium-dependent, nitric oxide synthase stimulating agents, bradykinin and adenosine triphosphate, had no effect on cyclic GMP content of endothelial or VSM cells alone but markedly increased cyclic GMP content in EC-VSM co-cultures ($P < 0.01$; fig. 1). The direct-acting soluble guanylyl cyclase stimulating agent sodium nitroprusside had no effect on endothelial cell cyclic GMP content but markedly stimulated cyclic GMP production in VSM and in EC-VSM co-cultures ($P < 0.01$; fig. 1). Thus, our endothelial cell line was

functionally shown to express nitric oxide synthase but not to express soluble guanylyl cyclase and our rat aortic VSM cell line was shown to express soluble guanylyl cyclase but not nitric oxide synthase.

Atrial natriuretic peptide, 10^{-7}M, which activates particulate but not soluble guanylyl cyclase stimulated cyclic GMP production in both endothelial and VSM cells (figs. 2A and 2B), demonstrating the presence of a functional particulate guanylyl cyclase in both cell types.

Northern blotting of poly A+ selected mRNA from endothelium and from VSM demonstrated that endothelial cells express mRNA for nitric oxide synthase but not for soluble guanylyl cyclase and that the VSM cell line expresses mRNA for soluble guanylyl cyclase but not for endothelial nitric oxide synthase (fig. 3).

Anesthetic Effects on Basal and Stimulated Guanylyl Cyclase

Increasing concentrations of halothane from 0.5 to 5% had no significant effect on basal cyclic GMP content of VSM and had no inhibitory or stimulatory effect on the cyclic GMP content of VSM stimulated by a 10^{-6}M solution of nitroprusside (figs. 4A and 4B). Similarly, isoflurane 0.5 to 5.0% had no significant effect on cyclic GMP concentration in unstimulated VSM and had no positive or negative effect on 10^{-6}M sodium nitroprusside-stimulated cyclic GMP production in VSM (figs. 5A and 5B). To further evaluate the possibility of subtle interactions of inhalational anesthetics with soluble guanylyl cyclase activation, the effect of 2% halothane and 2% isoflurane on dose-dependent increases in cyclic GMP content in response to sodium nitroprusside (table 1), nitroglycerin (Table 2), and nitric oxide (figs. 6A and B) were determined. Neither halothane nor isoflurane had a significant effect on sodium nitroprusside- (table 1) or nitroglycerin-stimulated (table 2) increases in VSM cyclic GMP content in the presence or absence of isobutylmethyl xanthine. Because of the highly reactive and rapid acting effects of nitric oxide, studies using nitric oxide were performed in the presence of superoxide dismutase (10 units/ml) to prevent degradation of nitric oxide by its interaction with superoxide radical and in the presence of isobutylmethyl xanthine (2×10^{-4}M) to prevent cyclic GMP degradation. There was no difference in cyclic GMP content in response to nitric oxide at any nitric oxide concentration in the presence or absence of 2% halothane or 2% isoflurane (figs. 6A and B). In recovery experiments in which anesthetic was washed out for 45 min before stimulation of VSM or co-cultures, cyclic GMP responses were within 5% of initial control responses and not significantly different.

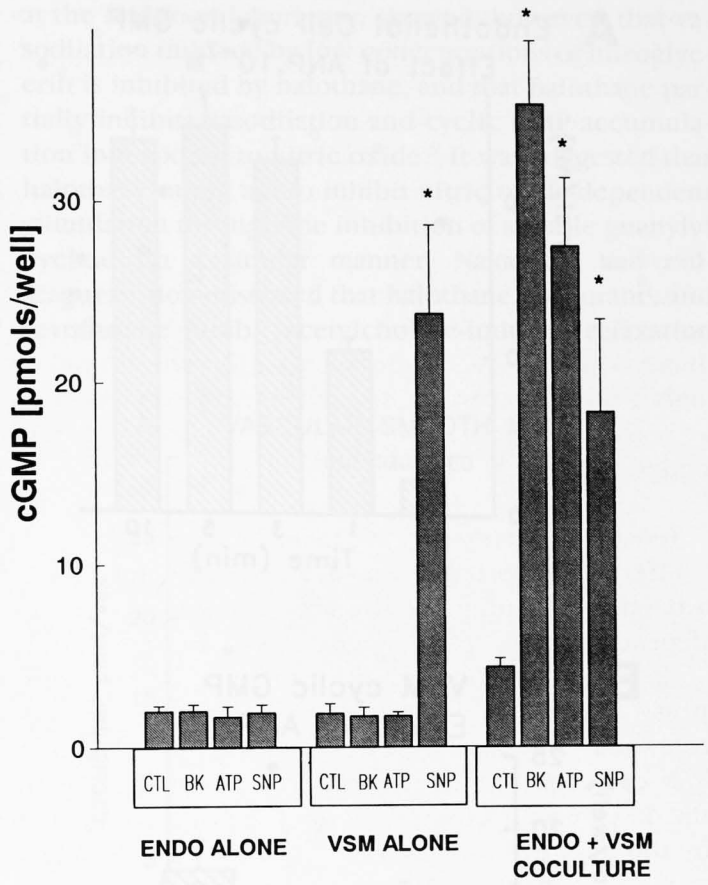
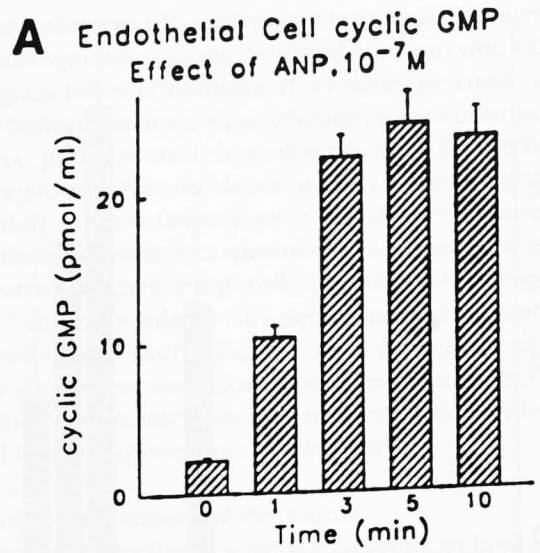


Fig. 1. Validation of endothelial cell-vascular smooth muscle co-culture model for assessment of agent actions on nitric oxide synthase and/or soluble guanylyl cyclase. ATP = adenosine triphosphate 10^{-4}M; BK = bradykinin 10^{-6}M; CTL = control; ENDO = endothelium; SNP = sodium nitroprusside 10^{-6}M; VSM = vascular smooth muscle. *Significantly different from control ($P < 0.01$).

Anesthetic Effects on Basal and Stimulated Co-cultures

In agonist-stimulated co-cultures, both halothane and isoflurane significantly inhibited increases in cyclic GMP concentrations in response to the receptor-mediated nitric oxide synthase activating agents adenosine triphosphate and bradykinin, as well as increases in cyclic GMP concentration caused by the receptor-independent nitric oxide synthase activator, calcium ionophore A23187, at the concentrations of anesthetic indicated in figures 7A–7D and 8A–8D. Neither halothane nor isoflurane exerted significant effects on cyclic GMP content in co-cultures stimulated with control buffer.



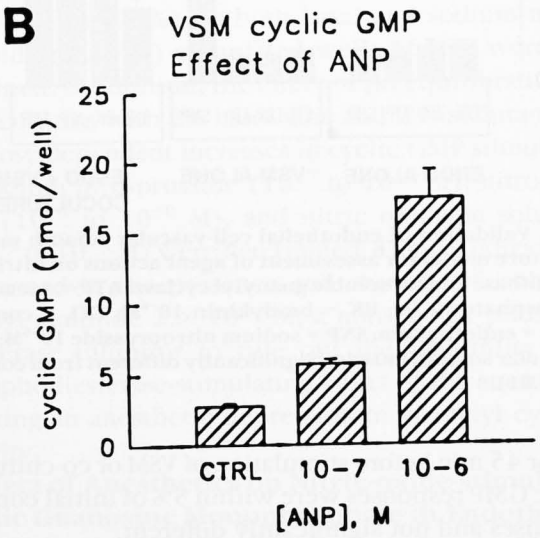


Fig. 2. Activation of particulate guanylyl cyclase by atrial natriuretic factor. (*A*) Time course of atrial natriuretic factor induced cyclic guanosine monophosphate accumulation in endothelial cells. (*B*) Stimulation of cyclic guanosine monophosphate in vascular smooth muscle by atrial natiuretic peptide.

Discussion

It is generally accepted that inhalational anesthetics inhibit nitric oxide-mediated, endothelium-dependent vasodilation; however, the site at which this inhibition takes place has been a subject of controversy. The current study using cultured VSM and EC-VSM co-cultures as models for assessing the nitric oxide synthase-soluble guanylyl cyclase signaling system, investigated the sites at which halothane and isoflurane interact with this pathway. In this model, inhalational anesthetics do not stimulate or inhibit basal cyclic GMP production in EC-VSM co-cultures or in VSM alone, suggesting that inhalational anesthetics do not activate soluble or particulate guanylyl cyclase and do not activate nitric oxide synthase. Inhalational anesthetics do not inhibit cyclic GMP formation in VSM in response to the nitrovasodilators sodium nitroprusside and nitroglycerin or in response to nitric oxide itself, strongly suggesting a lack of interference with soluble guanylyl cyclase activation. Conversely, inhalational anesthetics inhibit both agonist and calcium ionophore stimulated nitric oxide dependent cyclic GMP accumulation in endothelial cell and EC-VSM co-cultures. Consistent with previous vascular ring studies, anesthetics appear to inhibit the nitric oxide-guanylyl cyclase signaling pathway distal to receptor activation in the endothelial cell and proximal to nitric oxide activation of guanylyl cyclase. This of course does not rule out an effect on receptor activation (inhalational anesthetics have been shown to impair some agonist-stimulated calcium responses in endothelial cells[18] and to impair muscarinic receptor activation,[19–21] however, it cannot be the only site of action, because the calcium ionophore response was also inhibited.

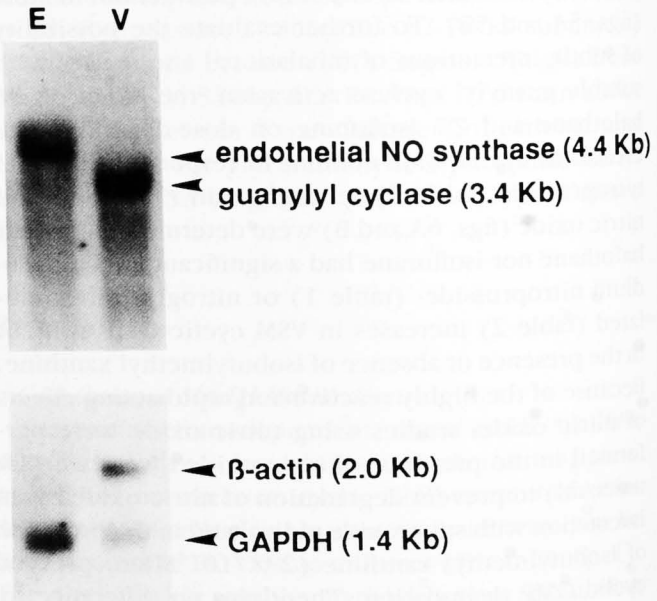


Fig. 3. Northern blot of poly A+ mRNA extracted from bovine aortic endothelial cells (E) and rat vascular smooth muscle (V) hybridized with the complete cDNA's for bovine endothelial nitric oxide synthase and for the 70 kd subunit of rat soluble guanylyl cyclase.

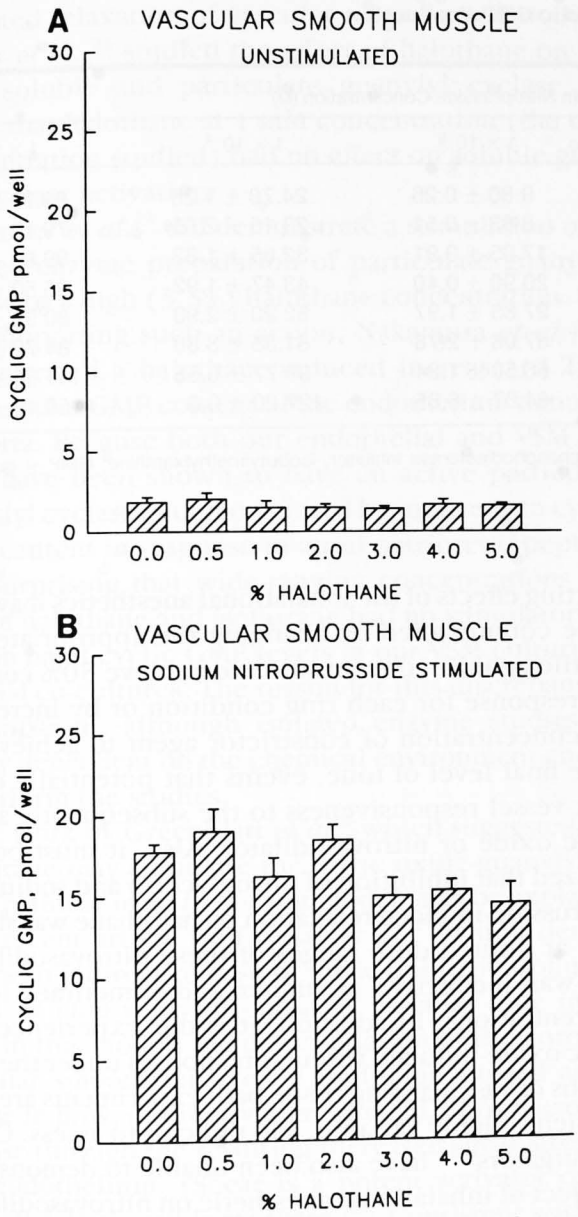


Fig. 4. Effect of increasing concentrations of halothane on unstimulated vascular smooth muscle (*A*) and sodium nitroprusside 10^{-6}M stimulated vascular smooth muscle (*B*).

In initial studies by Muldoon *et al.*,[5] Uggeri *et al.*,[7] and Toda *et al.*,[8] demonstrating inhalational anesthetic inhibition of EDRF-dependent vasodilation, it was strongly suggested that this inhibition was caused by an effect on the production, release, or transport of EDRF and independent of an effect on guanylyl cyclase activation in the VSM. The evidence for this was that vasodilation induced by nitroglycerin and sodium nitroprusside, which is mediated by a direct activation of VSM soluble guanylyl cyclase, was not affected by any of the anesthetics. A recent article, also generated at the Muldoon laboratory, showed, however, that vasodilation induced by low concentrations of nitroglycerin is inhibited by halothane, and that halothane partially inhibits vasodilation and cyclic GMP accumulation in response to nitric oxide.[9] It was suggested that halothane might act to inhibit nitric oxide-dependent stimulation through the inhibition of soluble guanylyl cyclase. In a similar manner, Nakamura and colleagues[10] demonstrated that halothane, isoflurane, and sevoflurane inhibit acetylcholine-induced relaxation

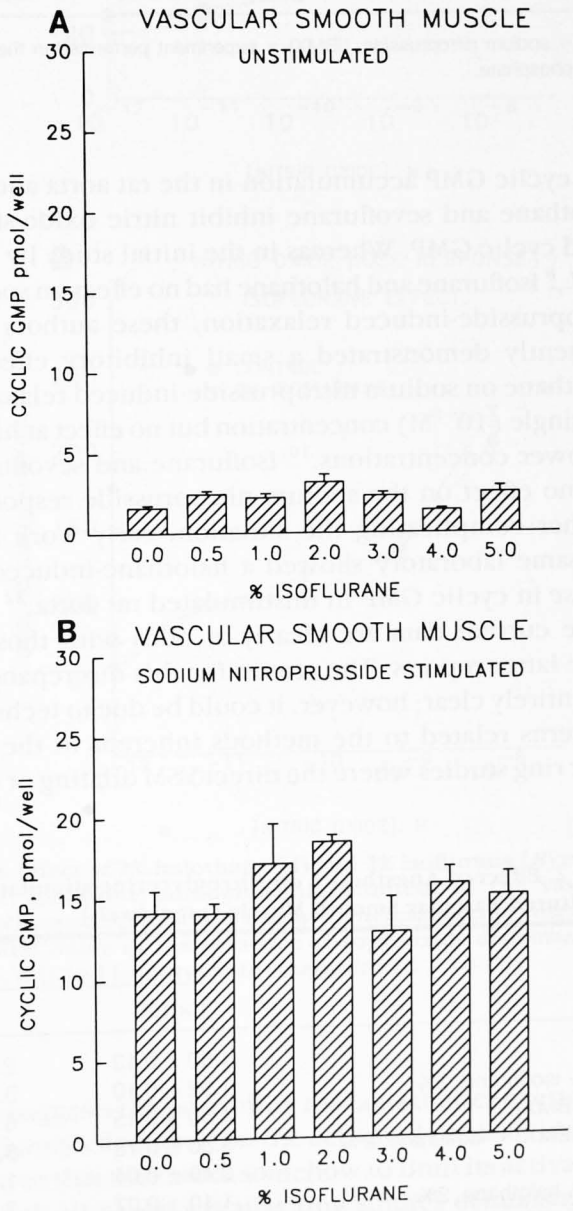


Fig. 5. Effect of increasing concentrations of isoflurane on cyclic GMP content of unstimulated vascular smooth muscle (*A*) and sodium nitroprusside, 10^{-6}M stimulated vascular smooth muscle (*B*).

Table 1. Effect of Anesthetics on Sodium Nitroprusside-stimulated Cyclic GMP Production in Cultured Vascular Smooth Muscle (pmol/well)

	Sodium Nitroprusside Concentration (M)				
	1×10^{-7}	1×10^{-6}	1×10^{-5}	1×10^{-4}	1×10^{-3}
SNP	2.10 ± 0.24	4.02 ± 0.08	9.80 ± 0.26	24.78 ± 1.26	88.77 ± 5.75
SNP + isoflurane, 2%	2.45 ± 0.05	3.92 ± 0.03	8.03 ± 0.51	23.15 ± 2.55	79.13 ± 5.97
SNP (IBMX)	5.47 ± 0.07	8.30 ± 0.45	17.05 ± 0.91	32.65 ± 1.82	99.80 ± 7.84
SNP (IBMX) + isoflurane, 2%	6.72 ± 0.07	8.95 ± 0.33	20.90 ± 0.40	43.47 ± 1.92	95.50 ± 9.81
SNP	1.40 ± 0.33	6.00 ± 0.72	27.85 ± 1.97	82.20 ± 2.90	80.95 ± 6.62
SNP + Halothane, 2%	2.05 ± 0.17	8.75 ± 0.85	37.08 ± 2.78	81.35 ± 3.80	84.78 ± 11.8
SNP (IBMX)	4.85 ± 0.19	10.48 ± 0.67	66.50 ± 1.84	99.77 ± 6.55	141.50 ± 6.4
SNP (IBMX) + halothane, 2%	5.88 ± 0.19	16.45 ± 0.94	64.07 ± 6.85	127.20 ± 9.0	159.15 ± 18.

SNP = sodium nitroprusside; (IBMX) = experiment performed in the presence of the phosphodiesterase inhibitor, isobutylmethylxanthine; GMP = guanosine monophosphate.

and cyclic GMP accumulation in the rat aorta and that halothane and sevoflurane inhibit nitric oxide-stimulated cyclic GMP. Whereas in the initial study by Toda *et al.*,[8] isoflurane and halothane had no effect on sodium nitroprusside-induced relaxation, these authors subsequently demonstrated a small inhibitory effect of halothane on sodium nitroprusside-induced relaxation at a single (10^{-8}M) concentration but no effect at higher or lower concentrations.[10] Isoflurane and sevoflurane had no effect on the sodium nitroprusside responses. Further complicating the situation, early work from the same laboratory showed a halothane-induced *increase* in cyclic GMP in unstimulated rat aorta.[22]

The current data are clearly at odds with those in these latter reports. The reason for this discrepancy is not entirely clear; however, it could be due to technical concerns related to the methods inherent in the vascular ring studies where the direct VSM dilating or constricting effects of the inhalational anesthetics may need to be counteracted by changing the appropriate contractile agonist concentration to achieve 50% contractile response for each ring condition or by increasing the concentration of constrictor agent to achieve the same final level of tone, events that potentially could alter vessel responsiveness to the subsequently added nitric oxide or nitrovasodilator. Also, it must be recognized that inhibition of nitroglycerin and sodium nitroprusside-induced relaxation by halothane was shown only at limited dose ranges of these nitrovasodilators and was ineffective at higher and sometimes lower concentrations. In addition, it is our experience that nitric oxide-induced vascular responses under the conditions of vascular ring tissue bath experiments are very transient, highly variable, and difficult to assess. Other investigators[8,11] have also been unable to demonstrate an effect of inhalational anesthetic on nitrovasodilator-

Table 2. Effect of Anesthetics on Nitroglycerine-stimulated Cyclic GMP Production in Cultured Vascular Smooth Muscle (pmol/well)

	Nitroglycerin Concentration (M)				
	1×10^{-7}	1×10^{-6}	1×10^{-5}	1×10^{-4}	1×10^{-3}
NTG	1.60 ± 0.13	2.82 ± 0.09	8.33 ± 0.62	17.15 ± 1.50	36.77 ± 2.41
NTG + isoflurane, 2%	1.92 ± 0.10	3.47 ± 0.27	7.35 ± 0.22	15.13 ± 0.22	39.57 ± 1.49
NTG (IBMX)	4.50 ± 0.15	6.88 ± 0.24	17.92 ± 0.17	36.45 ± 1.12	85.70 ± 2.28
NTG (IBMX) + isoflurane, 2%	5.30 ± 0.18	8.00 ± 0.48	21.05 ± 1.03	38.13 ± 0.50	83.10 ± 3.26
NTG	0.40 ± 0.04	1.33 ± 0.17	3.08 ± 0.30	7.93 ± 0.62	23.95 ± 4.02
NTG + halothane, 2%	1.10 ± 0.07	2.10 ± 0.28	3.47 ± 0.29	8.38 ± 0.55	20.03 ± 1.23
NTG (BMX)	2.40 ± 0.08	4.73 ± 0.17	11.65 ± 0.88	20.85 ± 1.83	44.50 ± 3.89
NTG (IMBX) + halothane, 2%	2.72 ± 0.52	5.02 ± 0.09	12.05 ± 0.55	25.18 ± 2.97	36.67 ± 2.37

NTG = nitroglycerin; (BMX) = experiment performed in the presence of the phosphodiesterase inhibitor, isobutylmethylxanthine; GMP = guanosine monophosphate.

mediated relaxation of vascular rings. In addition, Eskinder *et al.*,[23] studied the effect of halothane on isolated soluble and particulate guanylyl cyclase and found that halothane at 1 mM concentration (the only concentration studied) had no effect on soluble guanylyl cyclase activation.

Eskinder *et al.*[23] did demonstrate a stimulation of an isolated enzyme preparation of particulate guanylate cyclase by a high (3.3%) halothane concentration. Further supporting such an action, Nakamura *et al.*,[22,24] demonstrated a halothane-induced increase (2.25%) in the cyclic GMP content of the endothelium denuded rat aorta. Because both our endothelial and VSM cell lines have been shown to have an active particulate guanylyl cyclase as demonstrated by increases in cyclic GMP content in response to atrial natriuretic peptide, it is surprising that wide-ranging concentrations (0–5%) of halothane and isoflurane had no stimulatory effect on basal cyclic GMP levels in our VSM cultures or EC-VSM co-cultures. The reason for this discrepancy is not apparent, although isolated enzyme studies are highly dependent on the chemical environment chosen to perform the studies.

The work by Greenblatt *et al.*[2] which suggested that isoflurane may stimulate the nitric oxide-guanylyl cyclase pathway in an *in vivo* rat model, may be explained by a recent article by Crystal *et al.*[25] which demonstrates an indirect release of nitric oxide by isoflurane in an *in vivo* study of coronary vascular tone in the dog. In this model, the direct dilation of the coronary vascular smooth muscle by isoflurane caused an increase in flow, indirectly increasing EDRF/nitric oxide release through the resulting increased shear stress on the endothelium. (Shear is a potent activator of endothelial nitric oxide synthase.[26]) Consistent with the current data, an *in vivo* study by Wang *et al.*[27] provided evidence suggesting inhibition of the nitric oxide-guanylyl cyclase pathway in the vasculature of the intact animal implying an important role for inhibition of this pathway in the hemodynamic response to halothane. In their study, halothane prevented the pressor response to L-NAME and to LNMMA (inhibitors of nitric oxide synthase) but not to the nitric oxide-independent vasoconstrictors angiotensin II or norepinephrine. This could be interpreted as prior inhibition of nitric oxide synthase (NOS) by halothane preventing further inhibition by the NOS inhibitor L-NAME.

In the current study, inhalational anesthetics inhibited agonist- and A23187-stimulated co-cultures but had no apparent effect on basal nitric oxide production.

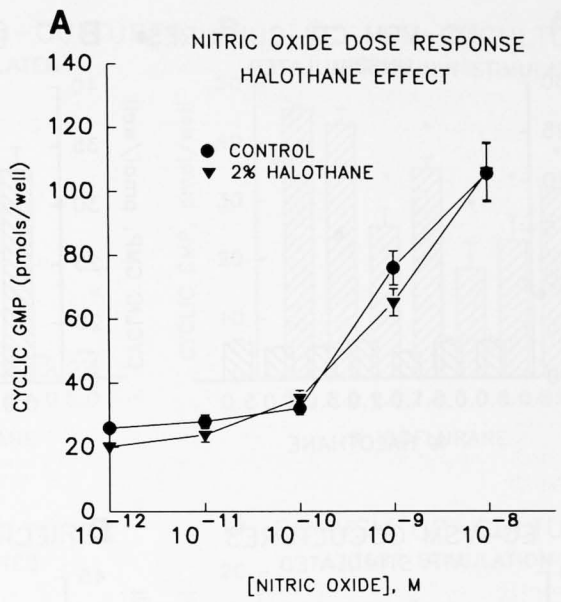


Fig. 6. Effect of 2% halothane (*A*) and 2% isoflurane (*B*) on the cyclic guanosine monophosphate content of the vascular smooth muscle cells stimulated with increasing concentrations of nitric oxide in the presence of superoxide dismutase (10 units/ml) and isobutylmethylxanthine (2×10^{-4}M).

This suggests the possibility that anesthetics exert their inhibitory effect only on the activated nitric oxide synthase or that they work somehow to limit its activation. Indeed, all of the vascular ring studies demonstrating inhibition of EDRF/nitric oxide dependent vasodilation have studied agonist- or calcium-ionophore-activated nitric oxide synthase and not basal nitric oxide release

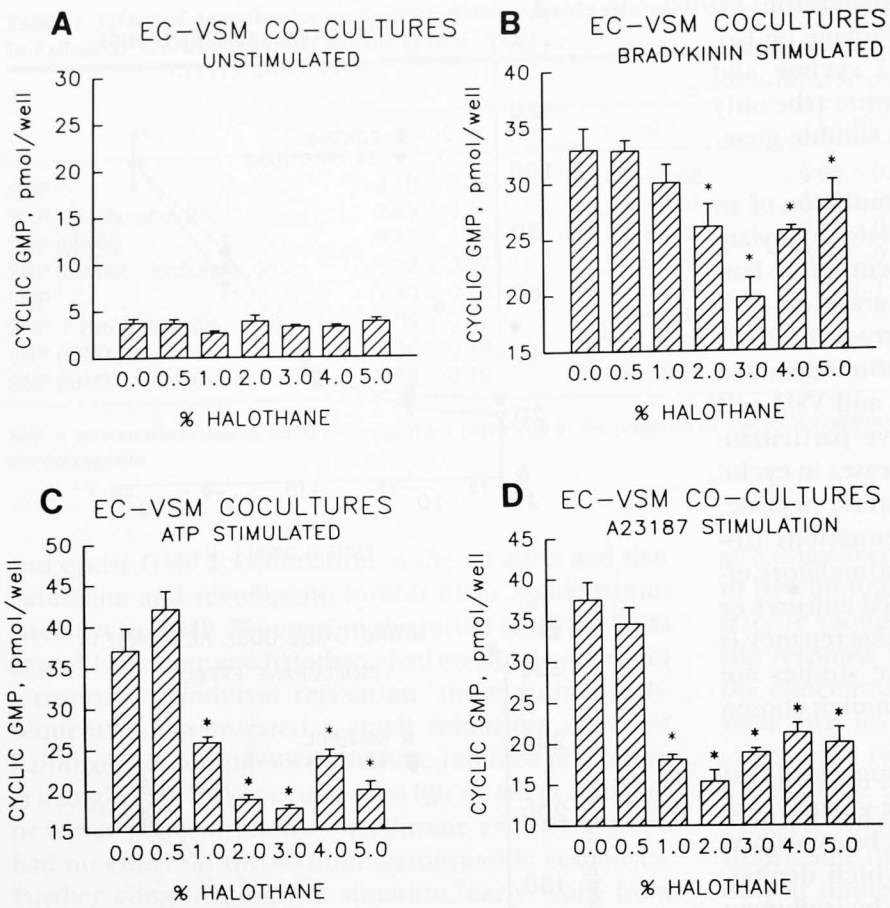


Fig. 7. Effect of increasing concentrations of halothane on cyclic guanosine monophosphate content of endothelial cell-vascular smooth muscle co-cultures stimulated with control buffer (*A*), bradykinin 10^{-6}M (*B*), adenosine triphosphate 10^{-4}M (*C*), or the calcium ionophore A23187 10^{-6}M (*D*). *$P < 0.05$.

from unstimulated vessels. At a minimum, some degree of NOS activation was provided in such studies by contractile agonists such as phenylephrine or norepinephrine, which also release EDRF/nitric oxide in addition to their direct contractile effects. Interference with receptor-activated increases in calcium are reported for anesthetics in multiple cell types but cannot completely account for their ability to inhibit NOS, as the EDRF/nitric oxide dilation in response to the receptor-independent calcium ionophore A23187 was also inhibited. A direct effect on NOS activity is a possible mechanism and has been suggested,[28] although we[29] and others[30] have been unable to demonstrate such an effect in homogenate or purified isolated brain or endothelial NOS activity assays. Anesthetic interference with co-factor availability, however, cannot be ruled out in isolated enzyme studies where cofactors are added in large quantities. A recent report by Blaise *et al.*[11] suggests that anesthetics inactivate nitric oxide after its production, but if this were the case one might have expected anesthetics to have interfered with the exogenous nitric oxide or nitric oxide donors used in the current study.

Possible sites at which inhalational anesthetics may interfere with EDRF/nitric oxide-dependent vasodilation have recently been reviewed.[31] These include interference with receptor activation, a decrease in availability of calcium for activation of NOS, inhibition of calmodulin or other cofactors required for NOS activation, direct interaction with nitric oxide, generation of superoxide or other free radicals that interact with nitric oxide, or inhibition of the activation of soluble guanylyl cyclase. It is possible that anesthetics will have multiple sites of interaction with the nitric oxide-guanylyl cyclase signaling pathway, not necessarily the same for each anesthetic or for different tissue types or isoforms of NOS and not necessarily at the same concentration of anesthetic at each site/tissue.

There has been a great deal of interest in the interactions of inhalational anesthetics with protein kinase C activity. Whereas some studies have shown a decrease in protein kinase C activity by these anesthetics,[32] there

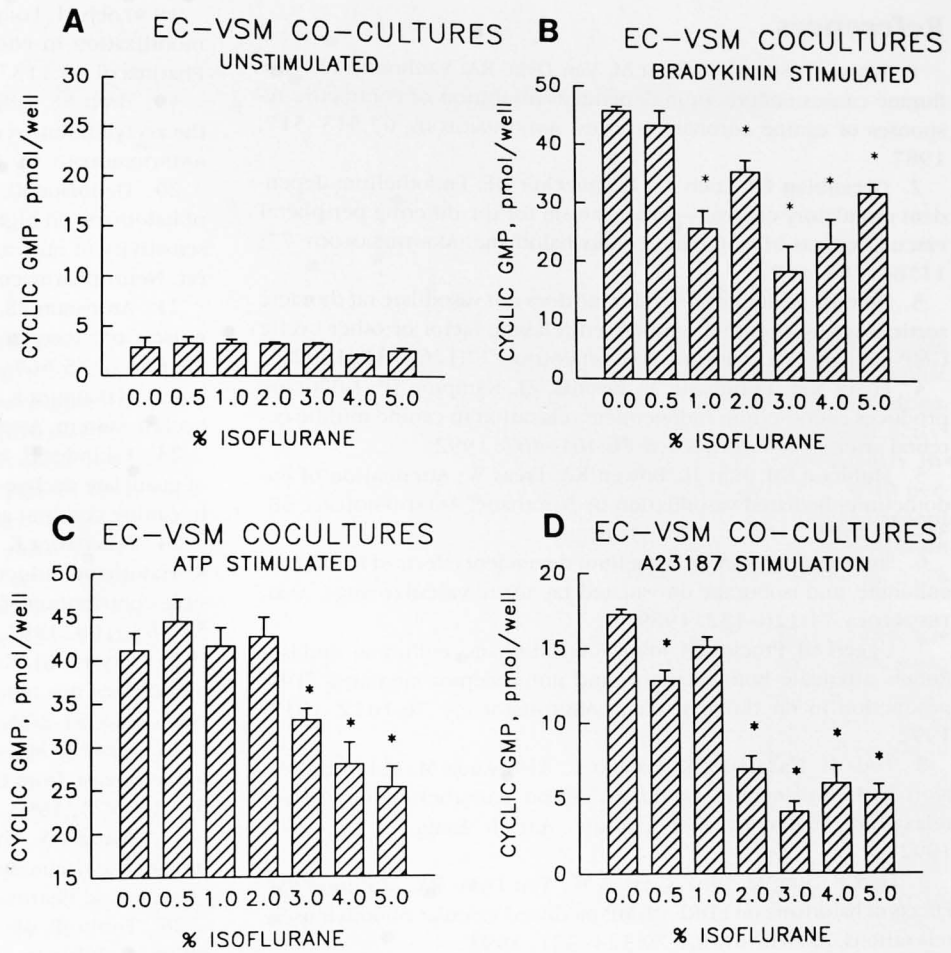


Fig. 8. Effect of increasing concentrations of isoflurane on cyclic guanosine monophosphate content of endothelial cell-vascular smooth muscle cocultures stimulated with control buffer (*A*), bradykinin 10^{-6}M (*B*), adenosine triphosphate 10^{-4}M (*C*), or the calcium ionophore A23187 10^{-6}M (*D*). $^{*}P < 0.05$.

is an increasing consensus that protein kinase C is activated by inhalational anesthetics.[33–35] As activation of protein kinase C in the endothelium has been shown to lead to phosphorylation of endothelial nitric oxide synthase, resulting in removal of the enzyme from its membrane association and a decrease in activity,[36,37] this is another site to consider for anesthetic inhibition of nitric oxide-mediated, endothelium-dependent vasodilation.

The current studies in cultured cells have avoided many of the potentially complicating factors associated with vascular ring studies and with *in vivo* pharmacologic assessment. The variable alteration of vascular tone by different anesthetic agents, which is due to effects not involving the nitric oxide-guanylyl cyclase pathway, are avoided in this model. Thus, there is no need to use a different EC50 that could alter subsequent vascular responses or to add additional vasoconstrictor to bring anesthetic-exposed vessels back to the desired degree of isometric tone. At the same time, the cellular milieu of the components of the nitric oxide-guanylyl cyclase pathway is maintained, unlike the more artificial situation of isolated enzyme preparations.

Using VSM and EC-VSM co-cultures, we have shown that inhalational anesthetics do not stimulate or inhibit basal cyclic GMP production, suggesting that they do not activate either soluble or particulate guanylyl cyclase and do not activate nitric oxide synthase. Inhalational anesthetics do not inhibit nitrovasodilator-induced cyclic GMP formation, suggesting a lack of interference with soluble guanylyl cyclase activation. Inhalational anesthetics do, however, inhibit both agonist- and calcium-ionophore-stimulated nitric oxide-dependent cyclic GMP accumulation in EC-VSM cocultures. Consistent with previous vascular ring studies, inhalational anesthetics appear to inhibit nitric oxide-guanylyl cyclase signaling distal to receptor activation in the endothelial cell and proximal to nitric oxide activation of guanylyl cyclase.

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83:835–843, 1995

Chest Wall Re
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David O. Warner, M.D.,† Mich

Background: The patter
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