Effects of Halothane on EDRF/cGMP-mediated Vascular Smooth Muscle Relaxations

J. L. Hart, Ph.D.,* M. Jing, M.D.,† S. Bina, Ph.D.,‡ W. Freas, Ph.D.,§ R. A. Van Dyke, Ph.D.,¶ S. M. Muldoon, M.D.#

Background: Halothane has been reported to inhibit endothelium-dependent relaxation in a variety of vessels. These studies were done to determine whether this inhibition is caused by interference with synthesis, release, or action of endothelium-derived relaxing factor (EDRF) on cyclic guanosine monophosphate (cGMP) levels within the vascular smooth muscle.

Methods: Rat aortic rings were suspended in aerated Krebs solution (37° C) and were contracted to a stable plate with ECA-70 norepinephrine (NE). Relaxations caused by acetylcholine (ACH; 1 × 10^-8 - 1 × 10^-4 M), nitric oxide (NO; 5 × 10^-9 - 1 × 10^-6 M), or nitroglycerin (NG; 2 × 10^-9 - 3 × 10^-7 M) in rings contracted with NE were compared in the presence and absence of halothane. Tissue cGMP contents were measured using a radioimmunoassay method.

Results: In the presence of halothane (0.5, 1.0, and 2.0 MAC), the ACH-induced relaxations were significantly attenuated in a concentration-dependent manner, an effect that was reversible. Halothane (2 MAC) significantly attenuated NO-induced relaxations at all concentrations and NG-induced relaxations at low concentrations (5 × 10^-7 - 3 × 10^-8 M) but not at higher concentrations (1 × 10^-8 - 3 × 10^-7 M) in denuded vessels. Nitric oxide-stimulated (5 × 10^-8 - 5 × 10^-6 M) cGMP content was significantly attenuated by halothane (2 MAC) at NO concentrations between 1 × 10^-7 and 5 × 10^-6 M.

Conclusions: Nitric oxide, either endogenous or exogenous, interacts with the enzyme guanylate cyclase to stimulate the production of cGMP. Halothane interfered with the relaxations caused by NO (in rings without endothelium) and decreased the NO-stimulated cGMP content. These results suggest that the site of action of halothane in attenuating endothelium-dependent relaxation in the rat aorta is within the vascular smooth muscle, rather than on the synthesis, release, or transit of the EDRF from the endothelium and that its action may involve an interference with guanylate cyclase activation. (Key words: Anesthetics, volatile halothane. Artery: rat aorta. Endothelium: endothelium-derived relaxing factor; nitric oxide. Pharmacology: acetylcholine; cyclic guanosine monophosphate; nitric oxide; nitroglycerin; norepinephrine.)

THE vascular effects of volatile anesthetics have been the subject of investigations for many years. Halothane has been reported to have a variety of effects on vascular smooth muscle, both direct and indirect. The indirect effects occur through interactions of the anesthetics with sympathetic nerve transmission at the neuroeffector junction as well as at ganglia and within the central nervous system, effects that may vary between species and between vascular beds within a species.1 The significant role of endothelium-derived nitric oxide (NO) in modulating vascular tone2 has made the effect of anesthetics on endothelium and endothelium-derived vasoactive factors the focus of recent investigations.3-9

In a previous study on the dog carotid and femoral arteries and rabbit aorta, we observed that halothane attenuated relaxation caused by acetylcholine (ACH) and bradykinin but not by nitroglycerin (NG). We concluded from this study that "halothane is not interfering with cyclic guanosine monophosphate [cGMP]-mediated relaxation of vascular smooth muscle, but may interfere with the synthesis, release, or transport of the endothelium-derived relaxing factor [EDRF]."15 However, recent studies in our laboratory on the rat aorta suggest a different conclusion; that halothane may have its effect on the endothelium-dependent relaxation of...
the smooth muscle by interfering with the EDRF receptor on guanylate cyclase.\textsuperscript{10}

To investigate this possible mechanism in more detail, the rat aorta was used to compare the effects of halothane on endothelium-dependent, ACh-induced, and endothelium-independent, exogenous NO-induced, relaxations. In addition, the cGMP content of vascular smooth muscle during halothane exposure was measured. These results in the rat aorta suggest that the site of action of the halothane attenuation of endothelium-dependent relaxation is within the vascular smooth muscle.

**Methods and Materials**

These studies were carried out with the approval of the Uniformed Services University of the Health Sciences Laboratory Animal Review Board.

**Aortic Ring Preparation**

Thoracic aortas were removed rapidly from halothane-anesthetized male Sprague Dawley rats and placed in Krebs solution of the following composition (mm): 118.2 NaCl, 4.7 KCl, 2.5 CaCl\(_2\), 1.2 MgSO\(_4\), 1.2 KH\(_2\)PO\(_4\), 25.0 NaHCO\(_3\), and 5.6 glucose (pH 7.4). After removal of adhering connective tissue, rings (2–3 mm) were cut and mounted on 0.6-G stainless steel wire hangers, with care being taken to neither stretch the vessels nor disrupt the endothelium.

Endothelium removal was accomplished when necessary by rotating rings around a small metal blade. Vessels were attached to isometric force transducers (Grass FT 03, Quincy, MA) in 25 ml water jacketed Krebs-filled organ baths (37°C), which were continuously aerated with 95% O\(_2\)/5% CO\(_2\). Isometric tension was recorded on a Gould recorder (model 2400, Valley View, OH). Four rings from adjacent segments were tested simultaneously after 60–90 min of equilibration at a passive tension of 1.5 g, determined in preliminary studies to be the optimal passive tension for this vessel. Presence or absence of endothelium was confirmed in each experiment by the use of ACh: lack of relaxation to ACh (1 × 10\(^{-6}\) M) of vessels contracted with norepinephrine (NE) confirmed the absence of functional endothelium, and a minimum of 50% relaxation confirmed the presence of functional endothelium.

**NO Preparation**

For the ring studies, NO solutions were freshly prepared immediately before use, under anaerobic conditions by the method of Menon et al.\textsuperscript{11} and Kelm and Schrader.\textsuperscript{12} Deoxygenated water (0°C) was saturated with NO by bubbling NO gas through the solution in glass containers for 10 min. This resulted in a calculated concentration of NO of 3.3 mm.\textsuperscript{13} The presence of NO in the dilutions used for testing was verified by bubbling them with either 100% O\(_2\) (n = 3) to convert NO to its oxides or with 100% N\(_2\) (n = 5) to purge the solution of NO, and then testing for their vasorelaxant effects. In five rings, purging with nitrogen decreased biologic activity to 17 ± 4% of pretreatment value. Therefore, 83 ± 4% of the vasoactivity was due to NO and not to NO metabolites.\textsuperscript{13} In the more recent experiments, NO was prepared by the method of Gillespie and Sheng.\textsuperscript{14} This later procedure produced stock concentrations of NO of 1.9 mm at room temperature (20°C). Appropriate serial dilutions were made from these saturated solutions by removing aliquots with gas-tight syringes. These aliquots were added to sealed containers that had been purged of oxygen with 100% N\(_2\).

**Halothane Delivery and Analysis**

Halothane was delivered from a calibrated vaporizer to give appropriate concentrations in the oxygen/carbon dioxide mixture aerating the Krebs solution. The concentration in the resulting gas mixture was monitored by an infrared halothane analyzer (model LB-2, Sensor Medics, Anaheim, CA), which was calibrated using a standard halothane calibration gas mixture (Scott Medical, Plumsteadville, PA). Concentrations of halothane in Krebs solution were confirmed by gas chromatography as previously described.\textsuperscript{5}

**Experimental Protocols for Contraction Studies**

For all of the following protocols, an initial, cumulative NE-induced contraction response test was done. Pairs of aortic rings from the same rat were examined simultaneously. These parallel time controls were used to accommodate any time-dependent changes in agonist sensitivity.

**Effects of Halothane on NE-induced Contractions.** Three consecutive NE concentration-response tests were performed on each ring. Ten minutes before the second test, halothane (2 MAC) was added to the aerating mixture and continued throughout the second test. The responses of the halothane-treated ring were compared to those of the time control.

**Effects of Halothane on ACh-induced Relaxations.** Both the control rings and those to be exposed to halothane were contracted using the EC\(_{50}\) of acetylcholine (ACh) in each experiment. The ACh was washed out, and the ring was then exposed to NO (100% NO) for 10 min. The NO was then washed out, and the response to the NO-NO sequence was compared to that of the time control.
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Concentration of NE as determined from the initial NE concentration response test. When a stable plateau was reached, increasing concentrations of ACh (1 × 10^{-9} – 1 × 10^{-5} M) were added to the bath until no further relaxation occurred. After washing and return to baseline, this procedure was repeated twice. In the halothane-exposed vessels, 10 min before the second procedure, halothane was begun and continued throughout the second procedure. The NE concentration was increased during halothane to achieve contraction equivalent to that in the first NE-induced contraction. Three concentrations of halothane were tested in separate experiments: 0.56% (0.5 MAC for the rat, 0.18 mm), 1.13% (1 MAC, 0.37 mm), and 2.26% (2 MAC, 0.74 mm).^{15,16}

Effects of Halothane on NO-induced Relaxations. Rings without endothelium were contracted with EC_{60-70} NE until a stable plateau was reached. Nitric oxide was added cumulatively to the baths in concentrations from 5 × 10^{-9} to 1 × 10^{-6} M. The rings were washed with Krebs, and when they returned to the baseline tension, this procedure was repeated twice. Ten minutes before the second procedure, 2 MAC halothane was begun and continued throughout the second procedure. Norepinephrine concentrations during halothane were increased to achieve contractions equivalent to those before halothane exposure.^{2}

Effects of Halothane on NG-induced Relaxations. Vessels without endothelium were used for the experiments with NG. This protocol was identical to that in which NO was used except that NG (3 × 10^{-9} – 3 × 10^{-7} M) was used to induce relaxations.

cGMP Analysis
Thoracic aortic rings without endothelium were placed in separate organ baths containing aerated Krebs solution at 37°C for 60–90 min. Norepinephrine (EC_{60-70}) was then added to all of the baths for 10 min. One set of aortic rings was removed and frozen in liquid nitrogen. This group served as the untreated control. The second set of aortic rings was exposed to 2 MAC halothane for 15 min and then frozen. The remaining rings were paired and maintained in aerated Krebs with or without 2 MAC halothane for 15 min, and then stimulated for 15 s with one of five different concentrations of NO (5 × 10^{-8} to 5 × 10^{-6} M). In a separate series of experiments, rat aortic rings were stimulated with a higher concentration of NO (1.6 × 10^{-4} M) and exposed to 2 MAC halothane. A 15-s exposure to NO was chosen as this corresponded to the time of maximal relaxations induced by NO. The frozen tissues were analyzed for cGMP content using an Amersham cGMP radioimmunoassay kit (Arlington Heights, IL). Results were expressed as nanomoles/gram of tissue wet weight.

Drugs
The following drugs and chemicals were used: NE HCl and ACh chloride (Sigma, St. Louis, MO), NG (Sterling Drug, McPherson, KS), halothane (Halocarbon, North Augusta, NC), and NO (Aldrich, Milwaukee, WI).

Data Analyses
Relaxations caused by ACh, NO, or NG were expressed as a percent of the active tension produced by EC_{60-70} NE, designated as percent relaxation of NE contraction on the graphs. Time-control and treated rings from adjacent sections of the aorta from the same rat were used. The number of rats used for each experiment is indicated in the figure legends. All data are expressed as mean ± SEM. Unless indicated otherwise, responses from halothane-treated vessels were compared to those from the appropriate time control. Data were analyzed by analysis of variance for repeated measures within groups. Where significant effects were determined, multiple comparisons were made using the Student-Newman-Keuls test. P ≤ 0.05 was considered significant.

Results

Effects of Halothane on NE-induced Contractions
The NE concentration-response curve was shifted significantly to the right by 2 MAC halothane (fig. 1). Because of this decreased responsiveness, the concentration of NE used to contract the vessels during halothane exposure was increased to achieve contractions approximately equal to those of the time controls. The mean concentration of NE used before halothane was 3.7 (±3.0) × 10^{-8} M and that during 2 MAC halothane was 5.7 (±3.0) × 10^{-8} M (n = 23). This was necessary because it is known that the amount of relaxation caused by ACh is inversely related to the amount of preexisting tension.^{2}

Effects of Halothane on ACh-induced Relaxations
There were no significant differences in the ACh-induced relaxations of the time controls contracted with NE (fig. 2A). Relaxations caused by all concentrations

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of ACh were attenuated significantly by 2 MAC halothane (fig. 2B). For the time controls, the mean relaxation at $1 \times 10^{-7}$ M ACh was $66.0 \pm 4.2\%$, whereas that for the halothane-exposed vessels was $26.3 \pm 2.9\%$ (n = 21). Relaxations induced by ACh following halothane were not significantly different from time controls (fig. 2C). Norepinephrine-induced tensions before ACh-induced relaxations were not significantly different between time controls and halothane-exposed vessels (fig. 2, vertical bars).

The concentration-related effects of halothane on ACh-induced relaxations were determined. As the concentration of halothane was increased from 0.56% (0.5 MAC) to 1.1% (1 MAC) to 2.3% (2 MAC), the amount of inhibition of ACh-induced relaxations also increased (fig. 3).

**Effects of Halothane on NO-Induced Relaxations**

In NE-contracted vessels without endothelium, NO caused concentration-related relaxations, responses that were reproducible over time (fig. 4A). Halothane (2 MAC) significantly inhibited NO-induced relaxations

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**Fig. 1.** Increases in tension in response to norepinephrine (NE) of time controls and 2 MAC halothane-treated rat aortic rings. Tension is expressed as a percent of the maximum NE-induced tension during the first NE concentration-response procedure. (100% = 2.28 ± 0.6 g). *Significant difference from control. $P < 0.05$ and $n = 3$.

**Fig. 2.** Effect of halothane on acetylcholine (ACh)-induced relaxations of rat aortic rings. (A) Acetylcholine-induced relaxations of vessels contracted with norepinephrine (NE, EC$_{50}$) during three consecutive treatments (line tracings) and the NE-induced tension just before the first ACh administration (vertical bars). (B) Comparison of ACh-induced relaxations of vessels in the presence and absence of 2 MAC halothane (line tracings) and NE-induced tension before the first ACh administration (vertical bars). *Significant difference from time control. $P < 0.05$ and $n = 21$. 

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**Fig. 3.** Acetylcholine-induced relaxations of vessels contracted with norepinephrine before (control) and during increasing concentrations of halothane (0.5, 1, and 2 MAC). Significant difference from pre-halothane value. $P < 0.05$ and $n = 5$.

(fig. 4B). Nitric oxide-induced relaxations ($1 \times 10^{-7}$ M) were $73.5 \pm 6.3\%$ ($n = 5$) for time controls and $20.7 \pm 6.8\%$ ($n = 5$) for halothane-exposed rings. The effect of halothane was reversible (fig. 4C).

**Effects of Halothane on NG-induced Relaxations**

In vessels without endothelium, NG caused concentration-dependent relaxations of vessels contracted with NE. Halothane (2 MAC) significantly inhibited the NG-induced relaxations at all concentrations of NG below but not at or above $1 \times 10^{-7}$ M (fig. 5).

**Effects of Halothane on cGMP Content**

Untreated control rings had a mean cGMP content of $0.05 \pm 0.02$ nm/g wet weight. Halothane (2 MAC) alone did not significantly change cGMP content ($0.12 \pm 0.08$ nm/g). Nitric oxide ($5 \times 10^{-8}$ to $5 \times 10^{-6}$ M) caused a concentration related stimulation of cGMP up to $1 \times 10^{-6}$ M. Halothane (2 MAC) significantly decreased NO stimulation of cGMP at NO concentrations of $10^{-7}$ M and above (fig. 6). In the presence of $1.6 \times$

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Studies by Ignarro et al.\textsuperscript{17} have demonstrated that endothelial cells produce NO, or a closely related compound, and that the activity of these compounds is indistinguishable from that of EDRF. EDRF/NO is formed from the amino acid L-arginine by NO synthase, and activation of this enzyme is calcium-dependent. Since halothane is known to decrease calcium influx in some cells,\textsuperscript{18} it is possible that the effects of halothane may depend in part on an inhibition of EDRF/NO synthesis at the endothelial cell level. This appears unlikely, however, because when the endothelial cell production of EDRF/NO is bypassed by adding exogenous NO or NO generating agents (in endothelial denuded vessels), halothane still inhibited relaxation. The magnitude of the inhibition was similar between these endothelium-dependent and independent relaxants. This suggests that halothane is acting within the vascular smooth muscle at a site in the cGMP pathway that is common to all of these relaxants. This site of action also is supported by Blaise, who reported that halothane inhibited NO-induced relaxations of the rabbit aorta.\textsuperscript{19}

In our previous study on the canine carotid artery, we did not observe inhibition of NG-induced relaxations by halothane; whereas in the current study on the rat aorta, halothane inhibited relaxations by NG at concentrations below $1 \times 10^{-7}$ M. The rat aorta is about 100 times more sensitive to NG, achieving 100% relaxation with $3 \times 10^{-7}$ M NG, whereas a higher concentration of NG ($5 \times 10^{-7}$ M) caused only a 35% reduction in the canine carotid artery. Since the highest

\[ 10^{-4} \text{ M NO, cGMP values were inhibited by 2 MAC halothane by 33 \pm 11\%.} \]

**Discussion**

We have previously reported that halothane inhibited endothelium-mediated relaxation of isolated canine and rabbit blood vessels possibly by interfering with EDRF synthesis, release, or transit.\textsuperscript{6} The present study was designed primarily to gain further insight into the nature of this inhibition. In these studies, halothane significantly inhibited vascular relaxation induced by ACh, NO, and the nitrovasodilator NG in rat aortic ring preparations. Because the relaxant responses to ACh, NO, and nitrovasodilators are mediated by activation of soluble guanylate cyclase and production of cGMP in smooth muscle cells, we propose that halothane exerts its inhibitory action at a site or sites in this pathway. Our observation that halothane decreased NO stimulation of guanylate cyclase is consistent with this hypothesis.

\[ \text{Nitric Oxide (NO)} \]
\[ \text{NO+Halothane (2MAC)} \]

**Fig. 6.** The effects of halothane, nitric oxide (NO), and NO plus halothane on cGMP content (nmol/g wet weight) of rat aortic rings without endothelium. *Significant difference from NO-treated vessels. $P < 0.05$ and $n = 4-5$.\]

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concentration of NG used on the rat aorta (3 × 10⁻⁷ m) caused nearly 100% relaxation and was not significantly inhibited by halothane, higher concentrations of NG were not investigated. The differences, therefore, between the current studies using rat vessels and previous results obtained with canine vessels, may reside in species and/or regional vascular heterogeneity in responses to NG and to halothane.

Nitroglycerin and nitroprusside are NO donor drugs, and both increase cGMP levels. However, the biotransformation pathways by which they release NO differ significantly. In their studies, Uggeri et al.⁹ and Toda et al.²⁰ confirmed that halothane inhibited ACh-induced relaxation in the rat aorta, but they did not find inhibition of nitroprusside responses by halothane. Toda also used NG in his studies and did not report inhibition by halothane. This may depend on differences in the agonists used (phenylephrine vs. NE), endothelial status (intact vs. denuded), or initial active tension during halothane (less than pre-halothane vs. equal to pre-halothane). In contrast to these findings are those of Su and Zhang,⁸ who reported that halothane (1%, 2%, and 3%) reversed the relaxing effects of nitroprusside on isolated rat aortic rings contracted with NE. Further studies would be necessary to clarify the reasons for these differences. Because mechanisms by which organic nitrates and other compounds donate NO are as yet unclear, we used exogenous NO in contraction and cGMP studies, and halothane caused an inhibition of both.

A likely site for halothane to interfere with the generation of cGMP is at the NO binding site on guanylate cyclase. Soluble guanylate cyclase is a ferrous (Fe⁺⁺) iron heme protein. According to Ignarro,²¹ removing the heme moiety from the protein inactivates the enzyme. Removing only the iron from guanylate cyclase while retaining the heme maximally activates the enzyme, and in this form it is unresponsive to NO. Thus, only the intact ferrous-iron-heme containing guanylate cyclase is responsive to NO. Nitric oxide tightly binds to the ferrous iron to form a nitrosylheme complex. With this binding, NO alters the conformation of heme by pulling the Fe⁺⁺ away from the enzyme and out of the plane of the porphyrin ring, while retaining the heme protein association.

The mechanism of the possible interference of NO activation by halothane is unclear, but it is known that certain halogenated hydrocarbons are readily attracted to and reduced by heme proteins. For example, halothane, because of its unique configuration of electronegative halogens, is reduced by cytochrome P450, hemoglobin, and hemin, provided the heme iron is in the reduced state in each case.²²,²³ It would be logical, therefore, for halothane to have an affinity for the reduced iron in guanylate cyclase, and in the process, interfere with the activation of this enzyme by NO.

Changes in cGMP concentration affect the activity of cGMP-dependent protein kinases and the subsequent phosphorylation of a number of cellular proteins.²⁴ In a manner not fully understood, this translates into a decreased intracellular calcium concentration, which results in vascular relaxation. In the present experiments, activation of the guanylate cyclase by NO increased vascular cGMP levels, over a range of NO concentrations from 5 × 10⁻⁸ to 5 × 10⁻⁶ m. In the presence of halothane (2 MAC) the NO-stimulated cGMP was attenuated between 27% and 45%. This supports the hypothesis that halothane is interfering with NO activation of guanylate cyclase. This is also consistent with the most recent report of Nakamura et al.²⁵ and that of Toda et al.²⁰ in which halothane (2%) significantly depressed ACh-stimulated cGMP levels of the rat aorta.²⁵ These data, however, do not rule out the possibility that halothane is also acting at a site(s) proximal to the guanylate cyclase.

The tonic phase of an NE-induced contraction in isolated blood vessels has been shown to be due to an influx of extracellular calcium.²⁶ Halothane has been reported to decrease calcium influx through receptor-operated channels in vascular smooth muscle.¹⁹ This effect may be the basis for the decreased NE contractile responses observed during halothane. However, tensions of the halothane-exposed vessels were equalized with those of controls by increasing the NE concentrations to take into account the observations of Furchgott relative to the importance of initial tension in EDRF-induced relaxation.² Therefore, the differences in relaxations between controls and halothane-exposed vessels did not depend on differences in the amount of initial active tension.

The possibility that halothane also may change post-cGMP events should be considered. Recently Su and Zhang,⁸ in studies on the intracellular actions of halothane in skinned rabbit aortic smooth muscle preparations contracted with KCl, found that halothane caused marked increases in calcium release and decreases in calcium accumulation by the sarcoplasmic reticulum. Both of these effects would increase, at least initially, cytosolic calcium and could add to the effects of the decreased cGMP levels in attenuating the relaxing actions of NO.

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The potential significance of the current in vitro results with halothane in whole animals is supported by the recent report of Wang et al. They observed that blocking the enzyme that catalyzes the formation of NO, NO synthase, with N^6-nitro-L-arginine caused a rise in blood pressure of conscious rats but did not cause a significant change in blood pressure in halothane-anesthetized rats. Basal release of EDRF, therefore, is important in the control of blood pressure in the conscious rat, but this basal release is attenuated in the halothane-anesthetized rat, and blood pressure is then not affected by N^6-nitro-L-arginine treatment. Likewise, Murray et al. reported that halothane (but not pentobarbital) abolished cGMP-mediated vasodilation in dogs, when compared to the conscious state. Halothane interference with EDRF activity thus appears to play a role in the whole animal as well as isolated blood vessels. Greenblatt et al. reported that treatment of halothane-anesthetized rats with a different inhibitor of NO synthase (N^6-monomethyl-L-arginine) did result in a rise in mean arterial blood pressure. However, they did not compare the level of basal release of EDRF between conscious and anesthetized rats. Therefore, their results cannot be directly related to the current results.

In conclusion, we have demonstrated a dose-related inhibitory effect of halothane on the guanylate cyclase-mediated relaxation pathway in vascular smooth muscle. These results extend observations reported previously in canines and rabbit vessels and suggest a mechanism of action of halothane in influencing vascular tone not described before. The clinical relevance of this effect of halothane on the cGMP pathway is the subject of continuing investigations, and these may provide a better understanding of the vascular effects of halothane and its interactions with frequently used nitrovasodilators.

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