ELSEVIER

European Journal of Pharmacology 342 (1998) 217–224

ejp

Halothane attenuates nitric oxide relaxation of rat aortas by competition for the nitric oxide receptor site on soluble guanylyl cyclase

Ming Jing [a,*], Geoffrey S.F. Ling [a], Saiid Bina [a], Jayne L. Hart [b], Sheila M. Muldoon [a]

[a] *Department of Anesthesiology, Uniformed Services, University of the Health Sciences, 4301 Jones Bridge Road, Bethesda, MD 20814-4799, USA*
[b] *Department of Biology, George Mason University, Fairfax, VA, USA*

Received 11 August 1997; revised 14 October 1997; accepted 31 October 1997

Abstract

Endothelial cells play an important role in the regulation of vascular activity through the release of endothelium derived relaxing factor (EDRF) now believed to be nitric oxide (NO). NO and the NO donor drug nitroglycerin relax vascular smooth muscle by stimulating soluble guanylyl cyclase leading to elevation of intracellular levels of cyclic guanosine 3′,5′-monophosphate (cGMP). Halothane has been shown to inhibit the action of NO on blood vessels. This study was designed to further investigate the mechanisms by which halothane attenuates NO-induced vascular relaxations. This was done by examining the effects of halothane on nitroglycerin and NO-induced relaxations in the presence and absence of the inhibitors of soluble guanylyl cyclase, methylene blue and 6-anilino-5,8-quinolinedione (LY 83583). Thoracic aortas from anesthetized male Sprague–Dawley rats were excised and cut into rings and the endothelium was removed. The aortic rings were suspended in organ baths containing Krebs solution and equilibrated at their optimal passive tension. When a stable plateau of contraction was produced by EC_{60} concentrations of norepinephrine, increasing concentrations of nitroglycerin or NO were added to the baths to relax the rings. This contraction–relaxation procedure was repeated three or four times. In some baths halothane was administered by a calibrated vaporizer 10 min before beginning the second procedure. Either methylene blue or LY 83583 was added to the baths 20 min before the third procedure. The combination of halothane, methylene blue or LY 83583 was added before the fourth procedure. Halothane, methylene blue or LY 83583 significantly inhibited nitroglycerin-induced relaxation individually. Halothane and LY 83583 also significantly inhibited NO-induced relaxations (5×10^{-9}–3×10^{-8} M and 5×10^{-9}–3×10^{-5} M, respectively) individually. The combination of halothane and methylene blue or halothane and LY 83583 significantly inhibited nitroglycerin-induced relaxation, also, the combination of halothane and LY 83583 significantly inhibited NO-induced relaxations. Halothane, methylene blue and LY 83583 treatment led to rightward shift in the concentration–effect curves. Halothane, in combination with methylene blue or LY 83583, produced inhibition equivalent to the sum of their individual effects. The present study demonstrates that the halothane, methylene blue and LY 83583 attenuate nitroglycerin and NO-induced relaxations of endothelium-denuded rat aortic rings. This suggests that halothane, methylene blue and LY 83583 may act through competitive antagonism at a common site of action on soluble guanylyl cyclase in the EDRF/NO relaxation pathway. © 1998 Elsevier Science B.V.

Keywords: Halothane; Nitric oxide (NO); Nitroglycerin; Smooth muscle, vascular, rat; Relaxation; Guanylyl cyclase, soluble; Guanylyl cyclase inhibitor, soluble; Methylene blue; LY 83583

1. Introduction

Endothelial cells play an important role in the regulation of vascular activity through the release of endothelium derived relaxing factor (EDRF) now believed to be nitric oxide (NO). NO, and the NO donor drug nitroglycerin, relax vascular smooth muscle by stimulating soluble guanylyl cyclase leading to elevation of intracellular levels of cyclic guanosine 3′,5′-monophosphate (cGMP). Halothane has a variety of systemic and regional hemodynamic effects. Several investigators have reported that halothane attenuated endothelium-dependent and -independent vasodilation in vitro (Muldoon et al., 1988; Stone and Johns, 1989; Toda et al., 1992; Uggeri et al., 1992; Hart et al., 1993; Nakamura et al., 1994; Jing et al., 1995). It is generally agreed that when vascular tissue is exposed to halothane in vitro, halothane decreases vascular relaxation responses to acetylcholine, bradykinin, A 23187, the NO donor drug nitroglycerin, and NO in isolated rabbit, canine

* Corresponding author. Tel.: +1-301-2953021; fax: +1-301-2952200; e-mail: mjing@usuhs.mil

and rat vessels (Muldoon et al., 1988; Hart et al., 1993). However, this activity is not a result of a specific inhibitory effect by halothane of NO synthase, the enzyme which forms NO (Rengasamy et al., 1995). Instead, the mechanism of the action of halothane on vascular smooth muscle is thought to be an inhibition of soluble guanylyl cyclase, the hemoprotein responsible for catalyzing the conversion of GTP to cGMP. Halothane has been reported to be metabolized by the heme containing cytochrome P-450 and can be reduced by hemoglobin (Baker et al., 1983). Therefore, we hypothesized that halothane acts primarily on the heme site of soluble guanylyl cyclase in interfering with the vasodilating actions of NO.

Methylene blue is an inhibitor of soluble guanylyl cyclase and known to affect iron-containing proteins, such as soluble guanylyl cyclase, by oxidizing protein-bound heme ferrous irons (Gruetter et al., 1981; Martin et al., 1985; Kelner et al., 1988; Mayer et al., 1993; Kawada et al., 1994). LY 83583 (6-anilino-5,8-quinolinedione) is a compound which has a chemical structure completely different from that of methylene blue, but has also been reported to be an inhibitor of activation of soluble guanylyl cyclase by NO or NO-producing vasodilators (Diamond, 1986; Malta et al., 1987; Mülsch et al., 1988; Torfgård et al., 1990). Further, methylene blue and LY 83583 have been reported to inhibit nitroglycerin-induced relaxation of rat aortic rings by competitive antagonism (Malta, 1989; Kawada et al., 1994). The suggested mechanism of this inhibition was interference with soluble guanylyl cyclase.

The current studies used the soluble guanylyl cyclase inhibitors methylene blue and LY 83583 to investigate the actions of halothane on the NO pathway in isolated blood vessels.

2. Materials and methods

2.1. Isolated vessel preparation

Male Sprague–Dawley rats (300–450 g) were anesthetized with halothane and thoracic aortas were carefully excised 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_2PO_4, 25.0 $NaHCO_3$, and 5.6 glucose (pH 7.4). The aortas were cleaned of adhering fat and connective tissue and cut into rings 3–4 mm in length. The endothelium was gently removed from all rings by rotating the rings around the tip of a small forceps.

The rings were suspended between two stainless steel wire hangers inserted into the lumen of the aorta in 25 ml water jacketed organ chambers filled with Krebs solution continuously aerated with 95%O_2–5%CO_2 (37°C) The lower hanger was attached to a hook and the upper hanger to a force transducer (Grass FT 03; Quincy, MA), and isometric tension was continuously recorded on Gould recorders (Model RS 3400; Valley Views, OH). Four rings from the same rat were tested simultaneously in separate baths. They were equilibrated for 60 min in Krebs while set at optimal tension of approximately 1.5 g, as determined previously by length tension experiments. The Krebs was changed at 15 min intervals during equilibration.

2.2. Experimental protocol

Following equilibration, cumulative norepinephrine (10^{-9}–10^{-6} M) was added to the baths to establish a concentration–contraction curve. The absence of endothelium was confirmed in each experiment in norepinephrine-contracted rings by lack of relaxation to acetylcholine (10^{-6} M). Following the establishment of a stable baseline, an EC_{60} concentration of norepinephrine was added to the rings to produce an active contraction. When a stable plateau was reached, a range of concentrations of NO (5×10^{-9}–10^{-5} M) or nitroglycerin (10^{-10}–5×10^{-3} M) was added to the endothelium denuded rings to cause relaxation. When maximal relaxation was reached, the organ baths were repeatedly washed with fresh Krebs solution to remove all vasoactive substances and reestablish a stable baseline.

In order to examine the effects of 1 and 2 minimum alveolar concentrations (3.4×10^{-4} M and 7.2×10^{-4} M in Krebs solution, respectively) of halothane on nitroglycerin-induced relaxations, this contraction/relaxation procedure was repeated three times. Halothane was administered 10 min prior to and throughout the second procedure. Halothane was delivered from calibrated vaporizers (Foregoer DR 1, Smithtown, NY) to the O_2/CO_2 mixture aerating the Krebs. The concentrations of halothane in the resulting gas mixtures were monitored by an infrared analyzer (Datax, Model 254; Helsinki) which was calibrated using standard halothane calibration gas mixture. Bath concentrations of halothane in Krebs were confirmed by gas chromatography as previously described (Muldoon et al., 1988).

In protocols examining the soluble guanylyl cyclase inhibitors, methylene blue or LY 83583 was added to the baths 20 min before the third contraction/relaxation procedure. Then a fourth procedure was done in which both halothane (10 min prior to contraction/relaxation procedure) and methylene blue or LY 83583 were administered. Also, superoxide dismutase was added to the baths (300 U/ml) to test its influence on the inhibition of nitroglycerin by LY 83583.

Since halothane decreased contractile responses to norepinephrine, the concentration of norepinephrine was increased to achieve tension development during halothane administration about equal to that before halothane. Simultaneous time control rings were run for all experiments in an identical manner but without halothane, methylene blue or LY 83583.

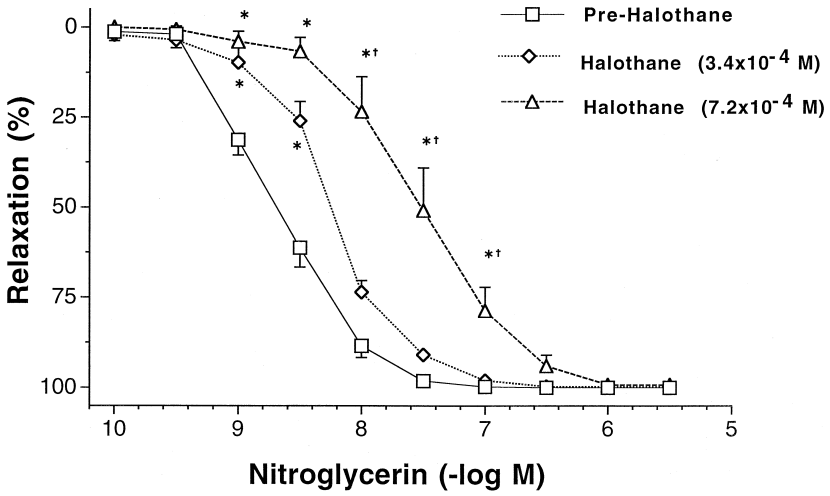

Fig. 1. Effects of halothane on nitroglycerin-induced relaxations of rat aortic rings without endothelium. Concentration–response curve for nitroglycerin on norepinephrine contracted rat aortic rings before halothane treatment (□), in the presence of 3.4×10^{-4} M halothane (◇) and 7.2×10^{-4} M halothane (△). Values are means ± S.E.M. * Significantly different from pre-anesthetic. † Significantly different from 3.4×10^{-4} M halothane treated group ($P < 0.05$, $n = 5$).

The effect of halothane on the response to cGMP analog 8-bromo guanosine 3′:5′-cyclic monophosphate (8-bromo cGMP) was also examined. The response of the EC_{60} norepinephrine contracted rings to 8-bromo cGMP (4×10^{-5} M) were compared in the presence or absence of 1 and 2 minimum alveolar concentrations of halothane.

2.3. Nitric oxide preparation

NO solutions were prepared immediately prior to use, under anaerobic conditions by a modified method of Gillespie and Sheng (1988). To generate NO, sodium nitrite (10 ml, 0.27 g/ml) was added (at a rate of 30 drops per minute) to a 50 ml round bottom flask with a side septum inlet in which $FeCl_2 \cdot 4H_2O$ (4.0 g) was dissolved in deoxygenated 4 M HCl solution (20 ml). To prepare a saturated NO stock solution (1.9×10^{-3} M), 10 ml NO gas was removed from this reaction flask and injected into another gas-tight flask containing 100 ml deoxygenated water. Gas tight syringes were used for transfer of NO solutions to the tissue baths. Final bath concentrations of NO ranged from 5×10^{-9} to 10^{-5} M.

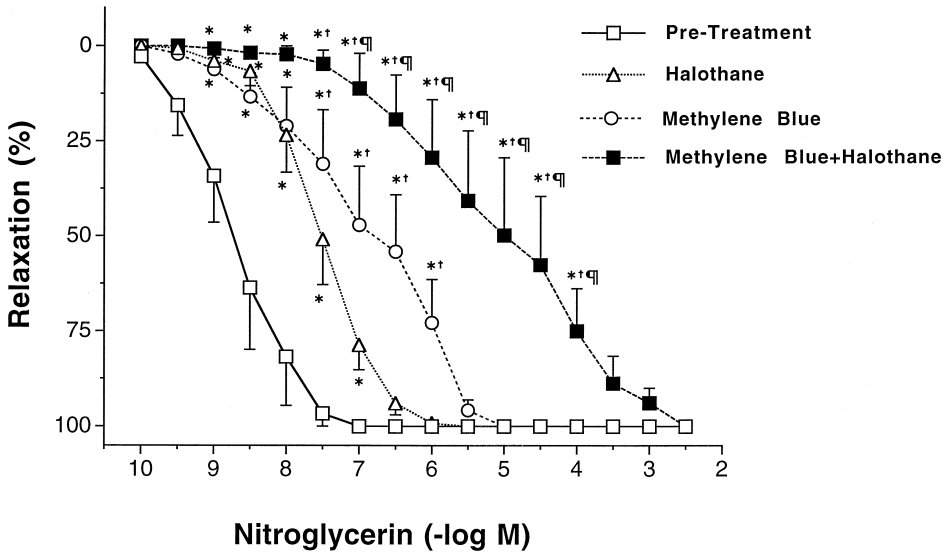

Fig. 2. Effects of halothane and methylene blue on nitroglycerin-induced relaxations of rat aortic rings without endothelium. Concentration–response curve for nitroglycerin on norepinephrine contracted rat aortic rings before drug treatment (□), in the presence of 7.2×10^{-4} M halothane (△), 10^{-5} M methylene blue (○) and combination of 10^{-5} M methylene blue and 7.2×10^{-4} M halothane (■). Values are means ± S.E.M. * Significantly different from control. † Significantly different from halothane treated group. ¶ Significantly different from methylene blue treated group ($P < 0.05$, $n = 5$).

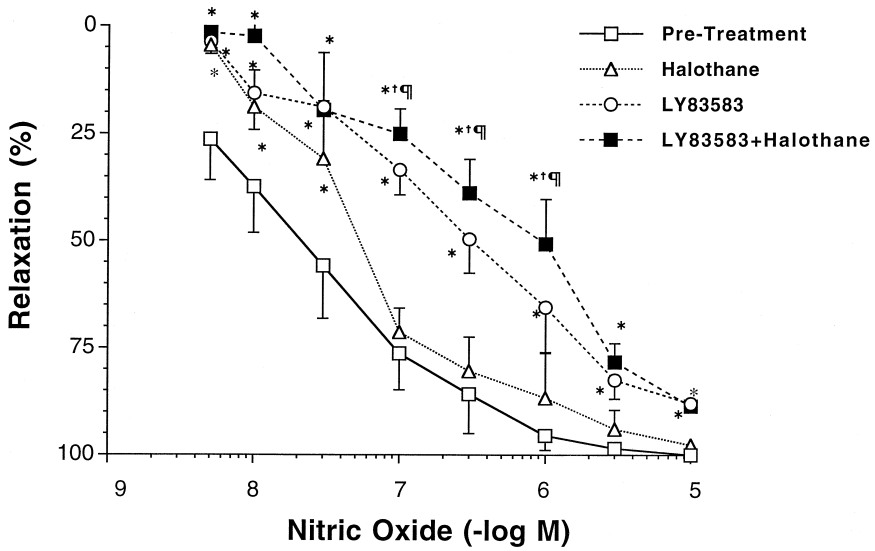

Fig. 3. Effects of halothane and LY 83583 on NO-induced relaxations of rat aortic rings without endothelium. Concentration–response curve for nitric oxide on norepinephrine contracted rat aortic rings before drug treatment (□), in the presence of 7.2×10^{-4} M halothane (△), 5×10^{-7} M LY 83583 (○) and combination of 5×10^{-7} M LY 83583 and 7.2×10^{-4} M halothane (■). Values are means ± S.E.M. * Significantly different from control. † Significantly different from halothane treated group. ¶ Significantly different from LY 83583 treated group ($P < 0.05$, $n = 5$).

2.4. Chemicals and drugs

The following chemicals were used: acetylcholine chloride, norepinephrine HCl, methylene blue, superoxide dismutase (Sigma Chemical, St. Louis, MO); nitroglycerin (Marion Laboratories, Kansas City, MO); 6-anilino-5,8-quinolinedione (LY 83583, Calbiochem, La Jolla, CA); halothane (Halocarbon, N. Augusta, NC).

2.5. Data analyses

Relaxations caused by nitroglycerin or NO are expressed as a percent of the active tension produced by norepinephrine. All data are expressed as means ± standard errors of the means. Statistical analyses of concentration–effect data were performed by repeated measures analysis of the variance between experimental groups followed by Student–Newman–Keuls test when appropriate. A $P < 0.05$ was considered significant. Lines were fit by a least square linear regression model. Comparison of slope and intercept were by analysis of variance.

3. Results

3.1. Effects of halothane on nitroglycerin-induced relaxations

Halothane (1 and 2 minimum alveolar concentrations, 3.4×10^{-4} M and 7.2×10^{-4} M in Krebs solution, re-

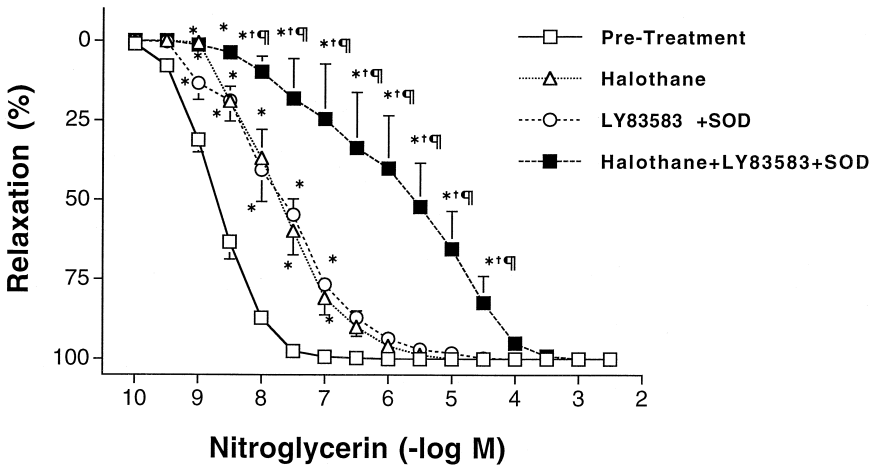

Fig. 4. Effects of halothane and LY 83583 on nitroglycerin-induced relaxations of rat aortic rings without endothelium. (A) Concentration–response curve for nitroglycerin on norepinephrine contracted rat aortic rings before drug treatment (□), in the presence of 7.2×10^{-4} M halothane (△), 5×10^{-7} M LY 83583 (○) and combination of 5×10^{-7} M LY 83583 and 7.2×10^{-4} M halothane (■) in the presence of 300 U/ml superoxide dismutase. Values are means ± S.E.M. * Significantly different from control. † Significantly different from halothane treated group. ¶ Significantly different from LY 83583 treated group ($P < 0.05$, $n = 5$).

spectively) decreased contractions of endothelium-denuded rat aortas in response to norepinephrine. The concentrations of norepinephrine used to contract the aortic rings during halothane (10^{-8} M for 3.4×10^{-4} M and 2×10^{-8} M for 7.2×10^{-4} M) were increased to achieve contractions (1.9 ± 0.3 g) approximately equal to those of the pre-anesthetic group which was contracted (2.0 ± 0.2 g) with 5×10^{-9} M norepinephrine. The increase in norepinephrine concentration has previously been shown not to inference the relaxant responsiveness to NO-dependent vasodilation (Jing et al., 1996). There were no significant differences in norepinephrine-induced contractions between pre-treatment, anesthetic and LY 83583 or methylene blue treatment groups, nor with the time control group. Nitroglycerin (range 10^{-10}–10^{-6} M) produced concentration dependent relaxations ($EC_{50} = 2 \pm 0.2 \times 10^{-9}$ M). At 3×10^{-7} M, nitroglycerin caused 100% relaxation. The concentration–response curves to nitroglycerin in the control rings were reproducible. Halothane (3.4×10^{-4} M) antagonized nitroglycerin-induced relaxations and caused a 3.2-fold parallel shift in the curve of the log concentration response profile without a decrease of maximal effect. Halothane (7.2×10^{-4} M) also caused a parallel shift (16 fold) in the nitroglycerin dose–response curve. (Fig. 1)

3.2. Effects of halothane on NO and nitroglycerin-induced relaxations in the presence and absence of methylene blue and LY 83583

The concentrations of norepinephrine (3.2×10^{-9} M) which produced 60% of maximal contraction of control groups and methylene blue or LY 83583 treated groups were not significantly different. The concentration–response curves of nitroglycerin and NO in the control rings were reproducible. Methylene blue (10^{-5} M) pretreatment antagonized nitroglycerin-induced relaxation causing a parallel rightward shift (100 fold) in the concentration–response curve. The methylene blue rightward shift was parallel to the halothane curve. The combination of methylene blue (10^{-5} M) and 7.2×10^{-4} M halothane led to an even more significant parallel rightward shift of the curve. However, this combination did not affect the maximal response to nitroglycerin. The shift in the nitroglycerin concentration–response curve caused by a combination of methylene blue and halothane was about equal to the logarithmic sum of their individual logarithmic EC_{50} value (Fig. 2).

NO caused concentration dependent relaxations between 5×10^{-9} M and 10^{-5} M. Halothane (7.2×10^{-4} M) significantly attenuated relaxations from 5×10^{-9} M to 3×10^{-8} M and led to a parallel 2.8-fold rightward shift in the NO concentration response profile. LY 83583 (5×10^{-7} M) caused a parallel 14.1-fold rightward shift. The combination of LY 83583 and halothane had greater inhibitory effects than either agent alone, and produced effects that were about equal to the sum of each antagonist alone by comparing the logarithmic EC_{50} concentrations of relaxant in the absence and presence of inhibitors (Fig. 3).

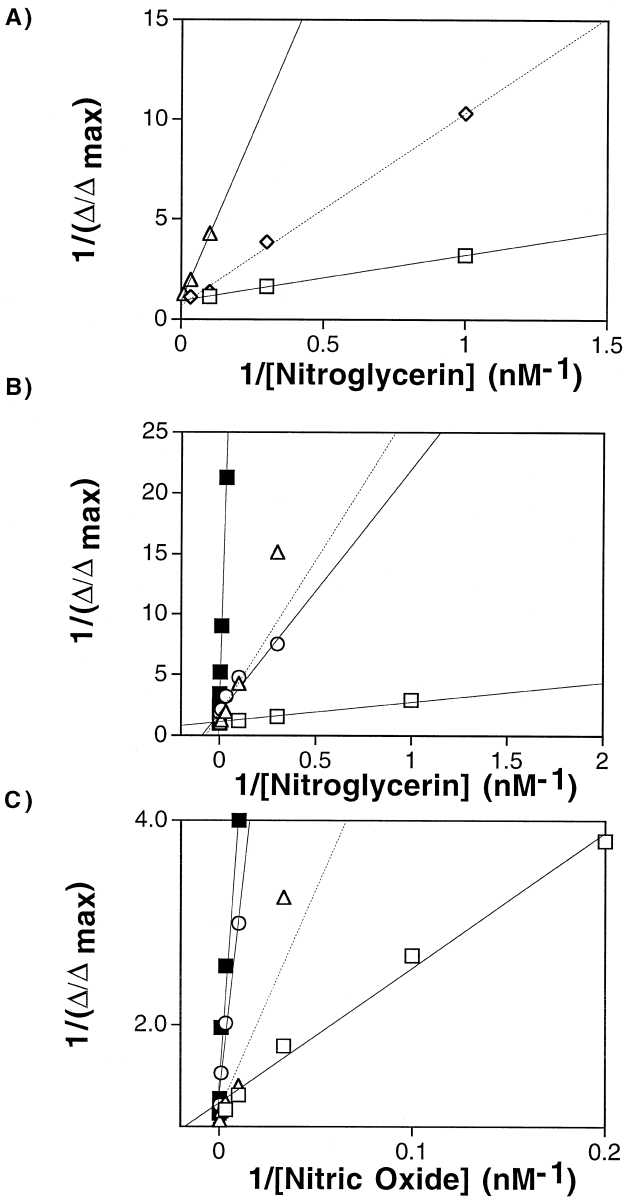

Fig. 5. (A) The double-reciprocal plots inhibition of nitroglycerin-induced relaxations of rat aortic rings before and during 3.4×10^{-4} M and 7.2×10^{-4} M halothane. Data and symbols are same as in Fig. 1. (B) The double-reciprocal plots of nitroglycerin-induced relaxations of rat aortic rings before and during 7.2×10^{-4} M halothane, 10^{-5} M methylene blue, and 7.2×10^{-4} M halothane plus 10^{-5} M methylene blue. Data and symbols are same as in Fig. 2C. The double-reciprocal plots of nitric oxide-induced relaxations of rat aortic rings before and during 7.2×10^{-4} M halothane, 5×10^{-7} M LY 83583, and 7.2×10^{-4} M halothane plus 5×10^{-7} M LY 83583. Data and symbols are same as in Fig. 3. The data points are mean values of five separate determinations. Ordinate is the reciprocal of response/max response (e.g. Δ/Δ_{max}). Abscissa represents reciprocal of concentrations of nitroglycerin or NO (e.g. 1/nM).

In the presence of superoxide dismutase, LY 83583 (5×10^{-7} M) still inhibited nitroglycerin-induced relaxation but did not alter the maximal response to nitroglycerin. The combination of LY 83583 and halothane had greater inhibitory effects than either agent alone but also did not alter the maximal response to nitroglycerin. The combination of LY 83583 and halothane caused a rightward shift in the nitroglycerin concentration–response curve (Fig. 4).

3.3. Analysis of antagonism of halothane, LY 83583 and methylene blue on NO or nitroglycerin-induced relaxations

The effects of halothane, LY 83583 and methylene blue on NO or nitroglycerin-induced relaxations were evaluated by double reciprocal plots (Lineweaver–Burke relationship) as shown in Fig. 5. Fig. 5A shows that 3.4×10^{-4} M and 7.2×10^{-4} M halothane had the same maximal effects (e.g. E_{max}), as seen by the common intercept on the ordinate on nitroglycerin-induced relaxation. This indicates that halothane has a competitive inhibition pattern with respect to nitroglycerin in its site of action. 7.2×10^{-4} M halothane was more potent than 3.4×10^{-4} M halothane. Methylene blue (10^{-5} M) and a combination of methylene blue (10^{-5} M) and 7.2×10^{-4} M halothane also had the same E_{max} which is consistent with similar competitive antagonistic effects on nitroglycerin-induced relaxation (Fig. 5B). There was also competitive antagonism by LY 83583 (5×10^{-7} M) on NO-induced relaxations. Halothane (7.2×10^{-4} M), and the combination of halothane and LY 83583 (5×10^{-7} M) also inhibited NO-induced relaxation through a competitive antagonism (Fig. 5C).

3.4. Effects of halothane on 8-bromo cGMP-induced relaxations

The cGMP analog 8-bromo cGMP (4×10^{-5} M) caused relaxation of norepinephrine-contracted rat aortic rings (88 $\pm$ 4.5%). Halothane (3.4×10^{-4} M and 7.2×10^{-4} M) did not significantly inhibit the relaxation (69 $\pm$ 11.3% and 86 $\pm$ 6.6%, respectively).

4. Discussion

Clinically relevant concentrations of halothane attenuated NO and nitroglycerin-induced relaxations of rat aortic rings. The halothane-induced shifts in the vasodilator concentration–response curves were parallel, without reductions in the maximal effects. These effects of halothane were similar to, and additive with, those of the soluble guanylyl cyclase inhibitors methylene blue and LY 83583, agents which have been reported to interact competitively with NO at its binding site on soluble guanylyl cyclase within the smooth muscle. These results suggest that halothane acts at the same soluble guanylyl cyclase site as methylene blue and LY 83583 to inhibit the actions of NO by a competitive mechanism.

A number of investigators have reported that halothane, and other halogenated hydrocarbon anesthetics, interfere with the important EDRF pathway in blood vessels from various species, as well as with the actions of NO and NO donors (such as nitroglycerin) on these blood vessels (Muldoon et al., 1988; Stone and Johns, 1989; Toda et al., 1992; Uggeri et al., 1992; Hart et al., 1993; Nakamura et al., 1994; Jing et al., 1995). This vasodilator pathway involves a sequence of steps. Nitric oxide is formed in the endothelium by the activation of the enzyme NO synthase which acts on L-arginine. Once formed, NO diffuses out of the endothelium with some entering the underlying vascular smooth muscle where it binds to and activates soluble guanylyl cyclase. This enzyme catalyzes the conversion of GTP to cGMP. The rise in cGMP initiates reactions that result in relaxation of the smooth muscle. Phosphodiesterase then terminates the action of the cGMP (Furchgott and Vanhoutte, 1989; Nathan, 1992). Where in this sequence of steps halothane may be acting has not been clearly established.

We have suggested that halothane is acting at the level of the soluble guanylyl cyclase within the vascular smooth muscle, where it competes with NO for its binding site. Originally, this hypothesis was based on reports that the halogenated hydrocarbons have a high affinity for ferrous heme proteins and that these anesthetics are metabolized by a variety of heme proteins including cytochrome P-450 (Baker et al., 1983). Both NO synthase (in the endothelium) and soluble guanylyl cyclase (in the smooth muscle) are heme-containing proteins (Ignarro, 1994; Masters et al., 1996), and therefore potential sites for halothane effects. However, we had observed that the endothelium was not required for the attenuating effects of halothane on the relaxing actions of NO. Additionally, the actions of NO on a partially purified liver soluble guanylyl cyclase were inhibited by halothane (Jing et al., 1995). Thus, the most likely site for these actions of halothane is at the ferrous heme of the soluble guanylyl cyclase, the same site for NO binding.

In our studies, we found some differences in magnitudes of nitroglycerine and NO responses during halothane. However, responses to 10^{-7} M and higher concentrations of NO and responses to 3×10^{-7} M and higher concentrations of nitroglycerine in the absence and presence of halothane were not significantly different, these results may reflect the different potencies and durations of action between NO and nitroglycerine. The fact that higher concentrations of NO and nitroglycerine could overcome the inhibition by halothane demonstrated the competition between halothane and NO or nitroglycerine. The most important point, however, is not the difference in magnitudes of responses, but in the apparent similar mechanisms of inhibitory effects of halothane on both NO and nitroglycerine relaxations.

The current results with methylene blue and LY 83583 add another line of evidence to support this hypothesis. Although chemically very different, there is strong evidence that the primary action of both methylene blue and LY 83583 is soluble guanylyl cyclase inhibition, and that they both act by competing with NO for the ferrous heme binding site on this enzyme. Craven and De Rubertis (1978) observed that soluble guanylyl cyclase was only effective in the ferrous, but not the ferric state and that methylene blue effectively oxidized the ferrous to the ferric form as the basis for its inhibitory effect. Malta (1989) reported that both methylene blue and LY 83583 significantly inhibited nitroglycerin-induced relaxation of rat aortic rings by competitive antagonism, with LY 83583 being the more selective antagonist. Likewise, Kawada et al. (1994) also reported that LY 83583 and methylene blue inhibited nitroglycerin as well as NO induced relaxation of rat, rabbit and guinea pig aortas in a competitive manner, and that they both decreased cGMP accumulation. The parallel shifts in the NO and nitroglycerin concentration–response curves caused by methylene blue and LY 83583 that we observed, support the findings of these previous reports.

Although most evidence indicates that the primary action of methylene blue and LY 83583 is the inhibition of soluble guanylyl cyclase, these agents may have other actions as well, including inhibition of NO synthase (Brandt and Conrad, 1991). However, in the present study, only endothelial denuded vessels were used. Therefore, NO synthase inhibition was not a factor. There have also been several reports (Gillespie and Sheng, 1990; Hobbs et al., 1991; Rajanayagam et al., 1993) that LY 83583 may inhibit NO-induced relaxation of non-vascular smooth muscle by the generation of superoxide anions, as this effect was blocked by superoxide dismutase (Barbier and Lefebvre, 1992). In our study, the inhibition of LY 83583 of nitroglycerin-induced relaxation was not influenced by superoxide dismutase. In addition, in previous studies we found that the inhibitory effects of halothane on acetylcholine-induced relaxations of the rat aorta were not changed by superoxide dismutase pretreatment (Hart et al., 1993). Therefore, it is unlikely that generation of superoxide anions by LY 83583 played a role in our current findings.

In addition to providing evidence for soluble guanylyl cyclase being the site of action of halothane, our results also indicate that the mechanism of action of halothane is by competitively inhibiting the actions of NO. This is supported by the parallel double-reciprocal plots with similar E_{max} of the NO concentration–response curves in the presence of the combinations of halothane and methylene blue or LY 83583.

In order to further investigate the mechanism of halothane on the NO–cGMP pathway, we used the cGMP analog 8-bromo cGMP, which can mimic the action of cGMP intracellularly, to produce relaxation. Halothane did not cause significant inhibition of 8-bromo cGMP-induced relaxation. This result suggests that halothane acts on a site prior to cGMP in the NO–cGMP pathway. Because we used endothelium-denuded rings to eliminate influences on nitric oxide synthase, this indicates that the most likely site of halothane's action may be on the sGC.

In summary, the current studies using the soluble guanylyl cyclase inhibitors methylene blue and LY 83583, provide additional evidence that halothane interferes with the NO pathway in vascular smooth muscle by competitively inhibiting the actions of NO at the ferrous heme site of soluble guanylyl cyclase. This action can account for the reduced responses of vessels to NO and NO-donating drugs in the presence of halothane.

Acknowledgements

This work was supported by USUHS Grant CO-8038. Presented in part at the meetings of Experimental Biology 93, New Orleans, LA, March 1993 and American Society of Anesthesiologists, Washington, DC, Oct. 1993. The authors gratefully acknowledge the helpful suggestions of Dr. Russell A. Van Dyke.

References

Baker, M.T., Nelson, R.N., Van Dyke, R.A., 1983. The release of inorganic fluoride from halothane and halothane derivative by cytochrome P-450, hemin, and hemoglobin. Drug Metab. Dispos. 11, 303–311.

Barbier, A.J.M., Lefebvre, R.A., 1992. Effect of LY 83583 on relaxation induced by non-adrenergic non-cholinergic nerve stimulation and exogenous nitric oxide in rat gastric fundus. Eur. J. Pharmacol 219, 331–334.

Brandt, M.A., Conrad, K.P., 1991. In vivo and in vitro studies of a putative inhibitor of cyclic guanosine 3′,5′-monophosphate production. Proc. Soc. Exp. Bio. Med. 196, 30–35.

Craven, P.A., De Rubertis, F.R., 1978. Restoration of the responsiveness of purified guanylate cyclase to nitrosoguanidine, nitric oxide, and related activators by heme and hemeproteins. J. Biol. Chem. 253, 8433–8443.

Diamond, J., 1986. Effect of quinacrine, NDGA and LY 83583 on acetylcholine induced elevation of cyclic GMP and relaxation of rabbit aortic rings. Proc. West. Pharmacol. Soc. 29, 105–109.

Furchgott, R.F., Vanhoutte, P.M., 1989. Endothelium-derived relaxing and contracting factors. FASEB J. 3, 2007–2017.

Gillespie, J.S., Sheng, H., 1988. Influence of haemoglobin and erythrocytes on the effects of EDRF, a smooth muscle inhibitory factor, and nitric oxide on vascular and non-vascular smooth muscle. Br. J. Pharmacol. 95, 1151–1156.

Gillespie, J.S., Sheng, H., 1990. The effect of pyrogallol and hydroquinone on the response to NANC nerve stimulation in the rat anococcygeus and the bovine retractor penis muscle. Br. J. Pharmacol. 99, 194–196.

Gruetter, C.A., Kadowitz, P.J., Ignarro, L.J., 1981. Methylene blue inhibits coronary arterial relaxation and guanylate cyclase activation by nitroglycerin, sodium nitrite, and amyl nitrite. Can. J. Physiol. Pharmacol. 59, 150–156.

Hart, J.L., Jing, M., Bina, S., Freas, W., Van Dyke, R.A., Muldoon, S.M., 1993. Effects of halothane on EDRF/cGMP-mediated vascular smooth muscle relaxations. Anesthesiology 79, 323–331.

Hobbs, A.J., Tucker, J.F., Gibson, A., 1991. Differentiation by hydroquinone of relaxation induced by exogenous and endogenous nitrates in-vascular smooth muscle: Role of superoxide anion. Br. J. Pharmacol. 104, 645–650.

Ignarro, L.J., 1994. Regulation of cytosolic guanylyl cyclase by prophyrins and metalloporphyrins. Adv. Pharmacol. 26, 35–47.

Jing, M., Hart, J.L., Masaki, E., Bina, S., Muldoon, S.M., 1995. Vascular effects of halothane and isoflurane: cGMP dependent and independent actions. Life Sci. 56, 19–29.

Jing, M., Hart, J.L., Verma, A., Bina, S., Muldoon, S.M., 1996. Effects of halothane and isoflurane on carbon monoxide induced relaxation in the rat aorta. Anesthesiology 85, 347–354.

Kawada, T., Ishibashi, T., Sasage, H., Kato, K., Imai, S., 1994. Modification by LY 83583 and methylene blue of relaxation induced by nitric oxide, glyceryl trinitrate, sodium nitroprusside and atripeptin in aortae of the rat, guinea-pig and rabbit. Gen. Pharmacol. 25, 1361–1371.

Kelner, M.J., Bagnell, R., Hale, B., Alexander, N.M., 1988. Methylene blue competes with paraquat for reduction by flavo-enzymes resulting in decreased superoxide production in the presence of heme proteins. Arch. Biochem. Biophys. 262, 422–426.

Malta, E., 1989. Biphasic relaxant curve to glyceryl trinitrate in rat aortic rings. Naunyn–Schmiedeberg's Arch. Pharmacol. 339, 236–243.

Malta, E., Macdonald, P.S., Dusting, G.J., 1987. Effect of LY 83583 on relaxant responses and levels of cyclic cGMP in rat aorta. Blood Vessels 24, 213–218.

Martin, W., Villani, G.M., Jothianandan, D., Furchgott, R.F., 1985. Selective blockade of endothelium-dependent and glyceryl trinitrate-induced relaxation by hemoglobin and by methylene blue in the rabbit aorta. J. Pharmacol. Exp. Ther. 232, 708–716.

Masters, B.S., McMillan, K., Sheta, E.A., Nishimura, J.S., Roman, L.J., Martasek, P., 1996. Neuronal nitric oxide synthase, a modular enzyme formed by convergent evolution: Structure studies of a cysteine thiolate-liganded heme protein that hydroxylates L-arginine to produce NO as a cellular signal. FASEB J. 10, 552–558.

Mayer, B., Brunner, F., Schmidt, K., 1993. Inhibition of nitric oxide synthesis by methylene blue. Biochem. Pharmacol. 45, 367–374.

Muldoon, S.M., Hart, J.L., Bowen, K.A., Freas, W., 1988. Attenuation of endothelium-mediated vasodilation by halothane. Anesthesiology 67, 31–37.

Mülsch, A., Busse, R., Liebauand, S., Förstermann, U., 1988. LY 83583 interferes with the release of endothelium-derived relaxing factor and inhibits soluble guanylate cyclase. J. Pharmacol. Exp. Ther. 247, 283–288.

Nakamura, K., Terasako, K., Toda, H., Miyawaki, I., Kakuyama, M., Nishiwada, M., Hatano, Y., Mori, K., 1994. Mechanisms of inhibition of endothelium-dependent relaxation by halothane, isoflurane, and sevoflurane. Can. J. Anaesth. 41, 340–346.

Nathan, C., 1992. Nitric oxide as a secretory product of mammalian cells. FASEB J. 6, 3051–3064.

Rajanayagam, M.A.S., Li, C.G., Rand, M.J., 1993. Differential effects of hydroxocobalammin on NO-mediated relaxation in rat aorta and anococcygeus muscle. Br. J. Pharmacol. 108, 3–5.

Rengasamy, A., Ravichandran, L.V., Reikersdorfer, C.G., Johns, R.A., 1995. Inhalational anesthetics do not alter nitric oxide synthase activity. J. Pharmacol. Exp. Ther. 273, 599–604.

Stone, D.J., Johns, R.A., 1989. Endothelium-dependent effects of halothane, enflurane and isoflurane on rat aortic vascular rings. Anesthesiology 71, 126–132.

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

Torfgård, K., Ahlner, J., Axelsson, K.L., 1990. Relaxation of bovine mesenteric arteries by glyceryl trinitrate and other nitro-compounds: Evidence for partly different mechanisms of action. Pharmacol. Toxicol. 67, 216–221.

Uggeri, M.J., Proctor, G.J., Johns, R.A., 1992. Halothane, enflurane, and isoflurane attenuate both receptor- and non-receptor-mediated EDRF production in rat aorta. Anesthesiology 76, 1012–1017.