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Does Halothane Interfere with the Release, Action, or Stability of Endothelium-derived Relaxing Factor/Nitric Oxide?

Gilbert Blaise, M.D., Quy To, B.Sc.,* Martin Parent, B.Sc.,* Benoit Lagarde, M.Sc.,† Francisco Asenjo, M.D.,* Remy Sauvé, Ph.D.,‡*

Background: Halothane attenuates endothelium-dependent relaxation. To differentiate halothane's effect on endothelium-derived relaxing factor/nitric oxide (EDRF/NO) production from its effect on nitric oxide action on vascular smooth muscle, halothane's effect on endothelium-dependent relaxation was studied in a bioassay system.

Methods: Indomethacin-treated, bovine aortic endothelial cells (BAEC) grown on microcarrier beads, continuously perfused by oxygenated and carbonated (95% O_2, 5% CO_2) Krebs-Ringer solution served as nitric oxide donors while an isolated denuded rabbit aortic ring directly superfused by the effluent of the BAEC and precontracted with phenylephrine was used to detect EDRF/NO release. The effect of basal and bradykinin-stimulated EDRF release on the tension of the vascular ring was measured. In the bioassay, it was possible to treat either the vascular denuded ring alone or the vascular ring plus the BAEC with halothane by adding it to the perfusate either upstream or downstream from the BAEC. Halothane (final concentration 2.2%) was added to the perfusate at these two positions, and its effect on the relaxation induced by EDRF/NO was determined. In some experiments, the preparations were treated with hemoglobin or L-monomethyl-L-arginine to attenuate the relaxation induced by the EDRF/NO pathway. Finally, halothane's effect on vascular relaxation induced by an increasing concentration of sodium nitroprusside was measured. Halothane's concentration in the perfusate was determined by gas chromatography using electron capture for anesthetic measurement.

Results: EDRF/NO released by the BAEC was responsible for the relaxation of the vascular ring. Halothane added to the perfusate potentiated the tension induced by phenylephrine (7.1 ± 1.89%) and attenuated the relaxation induced by the release of EDRF/NO. This effect was reversible after discontinuation of halothane. Halothane's effect was present even when the anesthetic was added to the perfusate downstream to the perfusion of the endothelial cells. Halothane had no effect on the vascular relaxation induced by sodium nitroprusside.

Conclusions: The authors' data demonstrate that halothane does not interfere with endothelial cell release of EDRF/NO and its smooth muscle cell relaxation but seems to modify either EDRF/NO half-life or its activated redox form. (Key words: Anesthetics, volatile: halothane. Endothelium: endothelium-derived relaxing factor; nitric oxide. Relaxation, bioassay.)

HALOTHANE, a volatile anesthetic agent, is a vasoactive drug. *In vivo,* it decreases arterial blood pressure, partially by decreasing peripheral vascular resistance.[1] The mechanism of halothane's induced vasodilation is multifactorial. It has a direct effect on neurotransmitter release,[2] smooth muscle calcium homeostasis,[3] and endothelium-dependent relaxation.[4–6] Furthermore, halothane reduces platelet aggregation,[7] a source of potent vasoconstrictors; and it reduces coronary arterial ring contractile response to 5-hydroxytryptamine.[8]

In rat aortic rings precontracted with norepinephrine, a biphasic response has been shown with increasing concentrations of halothane. An initial increase is followed by a subsequent decrease in tension in rings with and without endothelium.[9] The same biphasic response has been shown to occur in rabbit aortic rings.[10] Muldoon *et al.* have shown that halothane inhibits the relaxation induced by acetylcholine and bradykinin in isolated contracted rabbit and canine vascular rings. This effect was reversible after cessation of halothane.[4] Uggeri *et al.*[5] and Toda *et al.*[6] have confirmed these findings. Acetylcholine and bradykinin induce the release of endothelium-derived relaxing factor/nitric oxide (EDRF/NO) from the endothelial cells, EDRF/NO diffuses to stimulate the soluble guanylate cyclase and increases cyclic GMP in smooth muscles.[11] The mechanism by which halothane affects EDRF/NO remains speculative. The possible site of action of halothane

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Address reprint requests to Dr. Blaise: Department of Anesthesia, Notre-Dame Hospital, C.P. 1560, Succursale C, Montreal, Quebec, Canada H2L 4K8.

could be on endothelial cells (decreased EDRF/NO production), on smooth muscle cells (decreased EDRF/NO action), or on EDRF/NO itself (decreased half-life, decreased affinity of EDRF/NO for the soluble guanylate cyclase). All these effects would reduce the endothelium-dependent relaxation. Using a bioassay system, it was possible to treat either the endothelial cells or the effluent of these cells by halothane and to determine the effect of halothane on EDRF/NO production or on EDRF/NO itself (EDRF/NO stability, EDRF/NO action).

Material and Methods

This research protocol was approved by the Research and Animal Welfare committee of our institution; the animals were treated according to guidelines set by the Canadian Protective Animal committee.

Experiments were performed in a bioassay system where cultured bovine aortic endothelial cells (BAEC) seeded on microcarrier beads were used as the donor of EDRF, and a contracted rabbit aortic ring without endothelium was used as the EDRF detector. Endothelial cells and denuded rings were perfused continuously with an oxygenated and carbonated (95% O_2, 5% CO_2) Krebs-Ringer solution of the following composition (mM): NaCl 119, KCl 4.8, $CaCl_2 \cdot H_2O$ 2.6, KH_2PO_4 1.2, $MgSO_4 \cdot 7H_2O$ 1.2, $NaHCO_3$ 25, and glucose 15, maintained at 37° C and also containing indomethacin (10^{-5} M) to block the synthesis of prostaglandins.[12]

Endothelial Cell Preparation

Microcarrier beads and endothelial cells were prepared in a sterilized environment, including labware and solutions. All solutions, including the serum, were kept at 37° C. The incubator was maintained at 37° C with 5% CO_2. The cells bathing solution was replaced every two days with new DMEM (Dulbecco's modified Eagle medium) solution, which contained either 10% or 20% newborn calf serum.

Bovine endothelial aortic cells were obtained from the Physiology Department of the University of Montreal. The presence of factor VIII-related antigen was tested to confirm the endothelial nature of the cells.[13] The cells were subcultured in 75-cm^2 flask (no. 3024, Falcon, Oxnard, CA) with 15 ml of Dulbecco's modified Eagle medium (no. 430-1600eb, Gibco, Grand Island, NY) supplemented with 3.7 g/l $NaHCO_3$, 100 U/ml penicillin, 100 μg/ml streptomycin (no. 600-6010, Gibco), and 10% of newborn calf serum (no. 230-6010, Gibco). The *p*H was maintained at 7.2 in a 5% CO_2 atmosphere. Once they were confluent, the cells were removed from the flask with an Earle-like solution containing 0.05% trypsin (no. 840-7250, Gibco) plus 0.02% EDTA (no. 890-1267, Gibco) washed and resuspended in the culture medium to obtain a fourfold dilution. Cells were counted by hemocytometer before being placed in contact with collagen-coated microcarrier beads (Cytodex 3, Pharmacia). Microcarrier beads were placed in Dulbecco's phosphate buffered saline without Ca^{++} and Mg^{++}. Saline medium was discarded and beads washed three times with DMEM solution before the cells in their culture medium were added to the beads suspension. The suspension of cells and beads initially was placed in a 100-ml spinner bottle and stirred intermittently at 30 rpm for 2 min every 30 min for the first 7 h and then continuously to allow optimal seeding of cells onto the beads. More culture medium was added after 5 and 7 h. The cells confluence on the beads (80 cells/ bead), which usually occurred 3–4 days after seeding, was verified by microscopy. When the cells were ready for the study, they were perfused in the bioassay system.

The Perfusion System

Two 3-ml glass cylinders filled with either the microcarrier beads or the BAEC seeded on beads were perfused continuously by 4 ml/min of an oxygenated and carbonated (95% O_2, 5% CO_2) Krebs-Ringer solution containing 10^{-5} M indomethacin at 37° C (fig. 1). One denuded rabbit aortic ring was suspended between two L-shaped hooks, one of which was connected to a metallic support and the other to an isometric force transducer. The transducer was linked to a recorder to measure the tension imposed or generated by the vascular tissue. The isometric transducer and support were fixed to the same metallic frame to allow the vascular ring to be displaced. The ring was perfused by either the effluent from the microcarrier beads alone or the effluent of microcarrier beads covered by endothelial cells.

Vascular Ring Preparation and Perfusion

Forty New Zealand white rabbits weighing between 1.5 and 2 kg were anesthetized with intraperitoneal nembutal. The abdominal aorta was removed by laparotomy, cleaned of fat and fibrotic tissue, and cut into rings with a length of 5 mm. The endothelium was removed by rolling each ring on a wet paper towel with the tip of small surgical forceps for 15 s. The ring

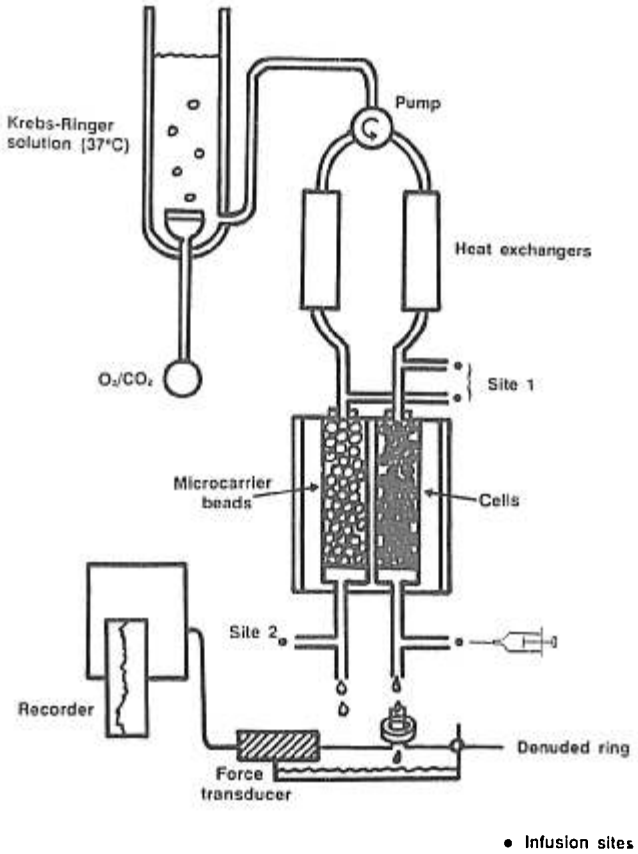

Fig. 1. Bioassay system. The vascular denuded ring can be perfused or superfused directly by the effluent of the endothelial cells by moving the holding tray. The perfusate is an oxygenated and carbonated solution of Krebs-Ringer solution. Medication can be added to the perfusate or to the perfusion line at site 1 (upstream to the perfusion of the endothelial cells) or at site 2 after the endothelial cells have been perfused.

was suspended between two L-shaped hooks and first perfused by the effluent of the beads (direct line). The ring was first perfused for 30 min to let it recover from the surgical and ischemic stress; and then progressively stretched by steps of 2–10 g. The tension that gives the maximal active response to 20 mM of KCl was considered to be the optimal passive tension for isolated rabbit aortic rings. The optimal passive tension was kept at this level for the duration of the experiment. The rings were contracted with 3×10^{-7} M phenylephrine to induce a stable contraction. After the plateau was reached, 10^{-9} M bradykinin was added to the perfusate to verify the removal of the endothelium from the vascular ring. Any ring relaxing to bradykinin while being directly perfused was discarded. Bradykinin was discontinued, and the holding tray was moved under the BAEC column. The relaxation induced by the basal release of EDRF/NO was recorded and measured; EDRF production was stimulated by bradykinin added to the perfusate upstream to the perfusion of the endothelial cells (site 1, fig. 1), and the resulting relaxation was recorded and measured.

Addition of Halothane to the Perfusate

We prepared a concentrated solution of halothane in Krebs-Ringer solution equivalent to a solution equilibrated with a gas containing 50% of halothane (based on halothane solubility coefficient in saline solution of 0.825,[14,15] this solution contains 3,260 μg halothane/ml, or 16.3 mM). Eighty-six microliters of halothane in the liquid phase were added to the Krebs-Ringer solution in a 50-ml glass gas-tight syringe. This amount is the theoretical limit of the solubility of halothane in saline solution.[14,15] Halothane was mixed with Krebs-Ringer solution with a stirring magnet for 30 min. The concentrated solution of halothane was perfused by an automatic pump in the bioassay system at site 1 (upstream to the perfusion of the BAEC) or at site 2 (downstream to the perfusion of the endothelial cells and upstream to the perfusion of the aortic ring). Halothane was added to the perfusate in the bioassay at a rate of 0.2 ml/min and yielded a final halothane concentration of 2.5% in the bioassay (diluted 20 times). Compared to the basal flow in the bioassay (0.2 ml/min *vs.* 4 ml), this low flow coming from the automatic syringe had no significant diluting effect on the phenylephrine or EDRF/NO concentration; the small increase in flow (0.2 ml/min) was insufficient to increase the shear stress on the BAEC and to increase EDRF secretion. Halothane concentration in the bioassay system was measured by gas chromatography.

Experimental Design

The BAEC were stimulated only once with bradykinin, and a series of three experiments was performed the same day. A different ring from the same animal was used in each experiment, and the BAEC used in each of these experiments came from the same culture cycle. In one experiment, no anesthetic was used (control); in the others, halothane was perfused either at site 1 or at site 2. The effect of halothane on the tension induced by phenylephrine and on the relaxation induced by the basal or bradykinin-stimulated release of EDRF was measured. As halothane added to the perfusate increased the tension of phenylephrine contracted rings and to match the tension between control and halo-

thane treated rings, in some experiments, phenylephrine concentration was reduced in halothane-treated rings. To verify whether the BAEC stimulated by bradykinin produced EDRF/NO, experiments were performed in the presence of hemoglobin (10^{-6} M) perfused at site 2 or L-monomethyl-L-arginine (L-NMMA) (10^{-4} M) perfused at site 1. Hemoglobin binds nitric oxide with high affinity,[16] and L-NMMA is an analog of L-arginine, the substrate for nitric oxide synthase and the precursor of EDRF/NO.[17]

Preparation of Oxyhemoglobin. A tenfold molar excess of the reducing agent sodium dithionite (Na_2SO_4) was added to a 10^{-3} M solution of commercial hemoglobin in distilled water. Sodium dithionite was removed by dialysis in 15 l of distilled water for 2 h at room temperature, as described by Martin *et al.*[18]

Organ Chamber Experiments

Rabbit aortic denuded rings were prepared as previously described. Each ring was suspended between two metallic stirrups and introduced into organ chambers filled with a carbonated and oxygenated (5% CO_2, 95% O_2) Krebs-Ringer solution. The lower stirrup was fixed at the bottom of the organ chamber, the upper stirrup was connected to an isometric force transducer (Harvard Isometric Force Transducer 52-9503, Ealing Scientific, St. Laurent, Quebec, Canada), which in turn was connected to an amplifier (Hewlett Packard 8802A, Kirkland, Quebec, Canada) and a recorder (Gould Brush 480, Cleveland, OH) to measure the tension imposed on or generated by the ring when stimulated by different agonists. After being placed in organ chambers, the rings were progressively stretched to their optimum passive tension (10 g). This level of passive tension was maintained throughout the experiments. Each ring's response to 40 mM KCl was measured; the absence of endothelium was detected by the lack of relaxation to 10^{-6} M acetylcholine in KCl-contracted rings. Any ring showing relaxation to acetylcholine was discarded. After this initial preparation, each organ chamber was washed several times with fresh Krebs-Ringer solution, and the rings were allowed to relax for 30 min.

Each organ chamber was treated with 3×10^{-7} M phenylephrine. At the plateau of α-adrenergic contraction, half of the organ chambers were treated with 2% halothane added to the gas mixture bubbling the saline solution. The halothane was delivered to the gas mixture by a calibrated vaporizer. Its concentration in the gas phase was measured by a gas analyzer (Siemens Servo Gas monitor 120, Pointe-Claire, Quebec, Canada). Fifteen minutes after halothane's introduction, the relaxation induced by increasing the sodium nitroprusside concentration from 10^{-9} to 10^{-6} M was measured in control and halothane-treated organ chambers.

Drugs and Chemicals

The following drugs were used: phenylephrine, bradykinin, L-NMMA, hemoglobin, indomethacin (Sigma, Mississauga, Ontario, Canada), and halothane (Ayerst, St.-Laurent, Montreal, Quebec).

The drugs were dissolved daily in fresh distilled water, except indomethacin, which was dissolved in an equimolar solution of Na_2CO_3.

Calculation and Statistics

The contraction to phenylephrine is presented in grams and expressed as mean ± SEM. The relaxation is expressed in percent of previous tension induced by phenylephrine, which was considered to be 100%. In experiments where halothane's effect on endothelium-dependent relaxation was measured, 100% tension was the tension induced by phenylephrine either before or after the introduction of halothane. Control rings tension was compared to the halothane treated rings tension. Data are expressed as mean ± SEM; Student paired *t* tests or a two-tailed analysis of variance was used when appropriate to analyze the results, and $P < 0.05$ was accepted as significant.

Results

We were able to dissolve 86 μl liquid halothane in 50 ml Krebs-Ringer solution. The anesthetic concentration measured in Krebs-Ringer solution after the addition of halothane (2,937 ± 114 μg/ml or 16.3 mM) was close to the calculated concentration based on halothane coefficient solubility in saline (0.825). The concentration of halothane was maintained throughout the perfusion system; there was no halothane absorption by Teflon extension tubing (table 1). When halothane was added to the perfusate at site 2, an approximate concentration of 2% halothane was regularly obtained. When halothane was added to the perfusate at site 1, the concentration at the end of the bioassay system was slightly less than expected (128 ± 5 *vs.* 96 ± 5 μg/ml, $P < 0.05$). This could be due to a leak of halothane, the absorption and/or the metabolism of halothane by BAEC. Table 1 shows the concentrations of halothane in Krebs-Ringer solution at several levels of the perfusion system.

Table 1. Halothane's Concentrations in the Bioassay System

Site	Annotation	n	Mean ± SEM (μg/ml)	Mean ± SEM (mM)
Syringe	A	48	2,937 ± 115	14.9 ± 0.6
Syringe plus tip	B	48	2,805 ± 116	14.2 ± 0.6
Site 2 infusion				
Before ring	C	48	128.6 ± 5.8 *	0.65 ± 0.03 *
After ring	D	48	113.6 ± 4.8 *	0.58 ± 0.02 *
Site 1 infusion				
Before ring	C′	8	96.4 ± 5.5	0.49 ± 0.03
After ring	D′	8	81.9 ± 3.8	0.42 ± 0.02

* $P < 0.05$.

Phenylephrine (3×10^{-7} M) induced a stable contraction in denuded rabbit aortic rings. The tensions induced by phenylephrine were 11.72 ± 3.22 g and 13.42 ± 3.74 g for the control and the halothane-treated groups (not statistically significant), respectively (n = 24). The absence of endothelium was demonstrated by the lack of relaxation to bradykinin when the ring was not superfused by the effluent of the BAEC. Halothane, when added to the perfusate at site 2 at the plateau of phenylephrine contraction, induced an increase of 7.1 ± 1.89% ($P < 0.05$) in tension that was stable until the next step of the experiment.

Moving the vascular ring from direct-line perfusion to endothelial superfusion induced a partial reduction in tension because of the basal release of EDRF. Bradykinin (10^{-9} M) added to the perfusate upstream to the endothelial cells induced further release of EDRF/NO and further relaxation. Halothane perfused at site 2 decreased the relaxation induced by the basal release of EDRF/NO ($P < 0.05$; fig. 2) and the relaxation induced by bradykinin (fig. 3) ($P < 0.05$). The maximum relaxation induced by the basal and bradykinin-stimulated release of EDRF/NO was, respectively, 24.14 ± 6.44% and 68.75 ± 1.78% in control experiments, and 5.15 ± 3.02% and 44.72 ± 2.10% in halothane-treated rings. Discontinuation of halothane was followed by further relaxation (fig. 3).

Figure 4 shows the effect of halothane when added to the perfusate at site 1 or 2 on the relaxation of the phenylephrine precontracted aortic rings induced by an increasing concentration of bradykinin. Halothane was added to the perfusate 20 min before the first dose of bradykinin. Switching halothane perfusion from site 2 to site 1 had no effect on the tension. Halothane treatment of both endothelial and vascular smooth muscle cells had no more effect on the tension than treatment

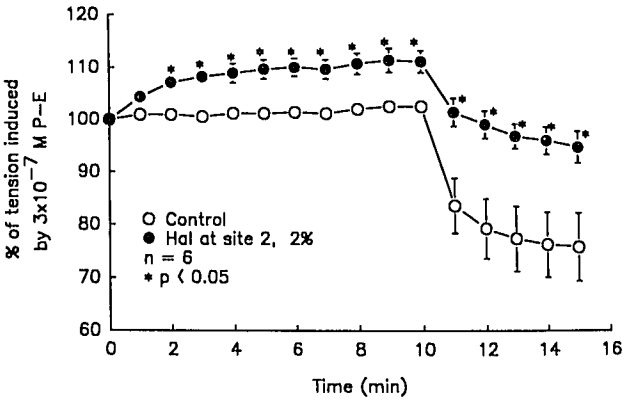

Fig. 2. Effect of halothane perfused at site 2 on tension induced by phenylephrine and on relaxation induced by basal release of endothelium-derived relaxing factor. Ten minutes after the addition of halothane to the perfusate, the holding tray was moved to superfuse the vascular ring by the effluent of the endothelial cells. Halothane potentiated the phenylephrine-induced contraction and attenuated the relaxation induced by the basal release of endothelium-derived relaxing factor/nitric oxide.

of vascular smooth muscle alone. Halothane discontinuation was followed by further relaxation. Because halothane potentiates the tension induced by phenylephrine, the tension was matched between control and halothane-treated preparations to induce relaxation

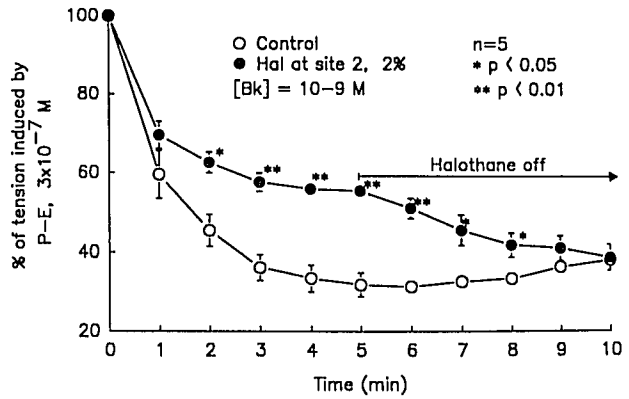

Fig. 3. Time course (0–10 min) of the relaxation induced by bradykinin (10^{-9} M) in endothelium-superfused phenylephrine-contracted denuded rings. For rings that have been treated by halothane, the latter was added to the perfusate at site 2 and bradykinin at site 1. Five minutes after the introduction of bradykinin, halothane's perfusion was stopped. Halothane perfused downstream to the endothelial cells attenuated the relaxation. This effect was reversible after halothane discontinuation. Results are expressed as percent (mean ± SEM) of tension recorded before the relaxation induced by bradykinin.

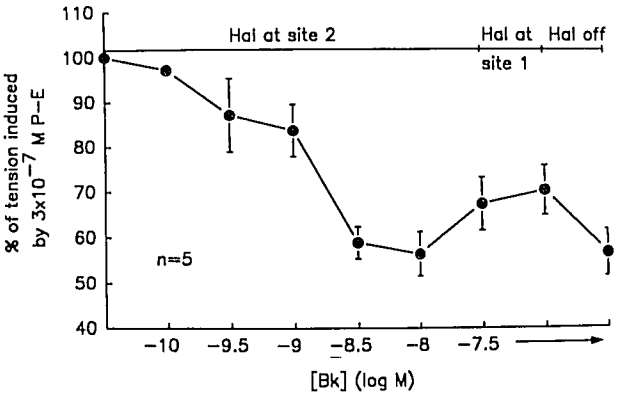

Fig. 4. Effect of halothane perfused at site 1 or 2 on the relaxation induced by an increasing concentration of bradykinin in endothelial superfused denuded rings. Halothane perfusion was started at site 2, 20 min before the first dose of bradykinin. After the last concentration of bradykinin was added to the perfusate, halothane perfusion was changed from site 2 to site 1. Site change had no effect on tension. Discontinuation of halothane was followed by further relaxation.

from the same level of tension. After the tension had been matched, the effect of halothane administered at site 2 had a greater influence on relaxation induced by basal release of EDRF than halothane perfused at site 1 (fig. 5). Perfusion of halothane at site 1 or 2 had the same inhibitory effect on vasodilation induced by a dose response from 10^{-10} to 3×10^{-8} M of bradykinin. Bradykinin added to the bioassay at site 1 induced a dose-dependent relaxation ($EC_{50} = 3 \times 10{-}9$ M). The maximum relaxation induced by bradykinin was 79.62 ± 7.79% in control rings, and 67.98 ± 5.11% and 60.06 ± 4.97% in halothane treated rings at site 2 and site 1, respectively ($P < 0.05$; fig. 6).

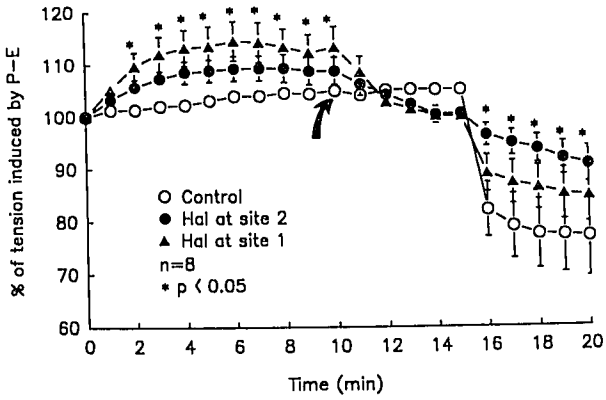

Fig. 5. Effect of 2% halothane perfused at site 1 or 2 on relaxation induced by basal release of endothelium-derived relaxing factor/nitric oxide. Denuded rings directly perfused were contracted by phenylephrine, which induced a tension taken as 100%. Halothane added to the perfusate at site 1 or 2 increased the tension. To match the tension between halothane-treated and control rings, phenylephrine concentration was reduced in halothane-treated rings (↗). Five minutes later, rings were superfused by the effluent of the endothelial cells, and tension was recorded for 5 min.

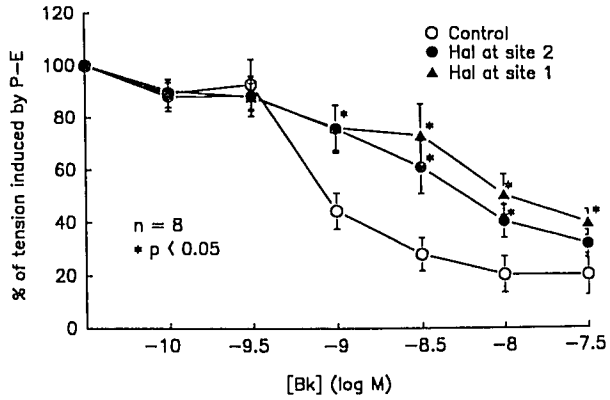

Fig. 6. Effect of 2% halothane perfused at site 1 or 2 on relaxation induced by an increasing concentration of bradykinin in denuded endothelium-superfused vascular rings. Initial tensions were matched between control and halothane-treated rings.

Oxygenated hemoglobin (10^{-5} M final concentration) added to the perfusate at site 2 reversed the endothelium-dependent relaxation (n = 2 data not shown). L-NMMA (10^{-4} M final concentration in the perfusate) added at site 1 decreased the endothelium-dependent relaxation in control and halothane-treated rings (fig. 7). In organ chamber experiments, 2% halothane had no effect on relaxation induced by an increasing con-

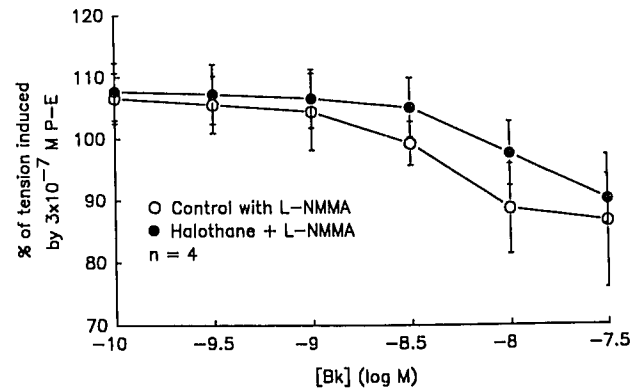

Fig. 7. Effect of L-monomethyl-L-arginine (10^{-4} M) on relaxation to bradykinin of control and halothane-treated denuded rabbit aortic rings superfused by the effluent of the endothelial cells in the bioassay system.

centration of sodium nitroprusside in phenylephrine precontracted denuded rabbit aortic rings, as shown in figure 8.

Discussion

Halothane introduced into the bioassay increased the tension of precontracted rings with phenylephrine by 7.1 ± 1.8% ($P < 0.05$). This increase in tension was stable until the next step of the experimentation. We previously showed that 2% halothane has a biphasic time-dependent effect on the tension of isolated preconstricted rabbit aorta rings where an increase in tension was followed by a progressive reduction.[10] Halothane introduced into organ chambers before phenylephrine had no effect on basal tension and decreased the tension evoked by the α agonist.[19] The effect of halothane on the tension depends on the order of drug administration and the time elapsed after the introduction. The vascular smooth muscle contraction induced by the α-agonist stimulation is biphasic, an initial fast contraction due principally to intracellular Ca^{++} release is followed by a sustained contraction, which is due to extracellular Ca^{++} entry.[20,21] The mechanism by which halothane increases the tension is not well understood. It is possible that halothane increases intracellular free Ca^{++} by potentiating Ca^{++} release from intracellular stores as it does in other cell types, such as heart,[22] liver,[23] skeletal muscle,[24] blood,[25] and brain.[26] The relaxation induced by halothane is due to an inhibition of Ca^{++} entry through receptors[27] or voltage-operated Ca^{++} channels[28] and a reduction of the Ca^{++} stores.

The endothelial cells can release several vasodilators such as prostacyclin, EDRF/NO, and endothelium-derived hyperpolarizing factor.[29] Recently it was demonstrated that endothelial cells can produce C-type natriuretic peptide.[30] Our experimental setup involves only the testing of EDRF/NO, because prostaglandin synthesis was inhibited by indomethacin. The relaxation was blocked by L-NMMA, a blocker of nitric oxide synthase,[17] and by hemoglobin, a nitric oxide scavenger that binds nitric oxide with a very high affinity.[16] These observations strongly suggest that nitric oxide was the mediator involved. EDHF was not involved, because the relaxation was blocked by L-NMMA and BAEC release mainly EDRF/NO. It also was demonstrated previously that halothane does not interfere with vasomotion induced by substances such as EDHF acting on the K channel.[31,32] C-type natriuretic peptide is a neuropeptide and its vascular effect is not well known.[33]

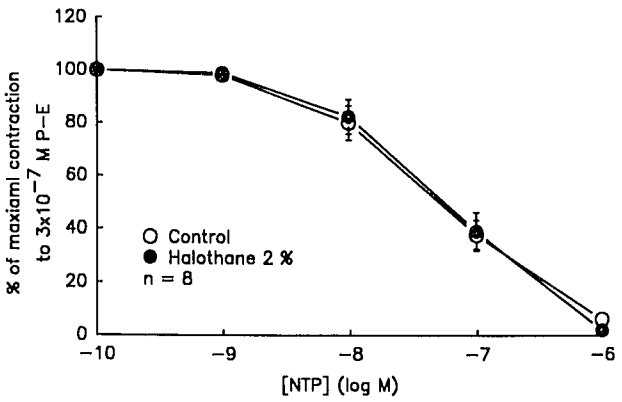

Fig. 8. Effect of halothane on relaxation induced by increasing concentrations of sodium nitroprusside in isolated denuded rabbit aortic rings contracted with phenylephrine (3×10^{-7} M, experiments were performed in organ chambers).

Our data are in agreement with several previous publications[4-6] that halothane attenuates the relaxation induced by EDRF/NO. Halothane has a direct effect on vascular smooth muscle tone of precontracted vessels as shown by an initial potentiation of the tension followed by a slow, continuous decrease in tension.[10] Since the relaxation induced by the action of halothane on smooth muscles is opposite its inhibition of endothelium-dependent relaxation, the difference in endothelium-dependent relaxation between the control and halothane-treated rings is even larger than can be measured by differences in tension in the bioassay system.

Our data support the hypothesis that halothane interferes with the EDRF/NO pathways even after EDRF/NO has been secreted by the endothelial cells. The site of halothane inhibition of endothelium-dependent relaxation is not on the EDRF/NO synthesis or release; indeed, halothane added to the perfusate downstream from the endothelial cells inhibits the endothelium-dependent relaxation due to the basal release of EDRF/NO even more than when it is added to the perfusate upstream from the endothelial cells. We do not have a definitive explanation for this difference. It may be that, when halothane is added to the perfusate at site 1, the small decrease in halothane concentration at the end of the perfusion system may explain the smaller effect of halothane on relaxation. Halothane, perfused at site 1 could increase EDRF/NO production by the endothelial cells *via* an increase in intracellular free Ca^{++} as it does in several other cells.[22-26] The constitutive nitric oxide synthase present in BAEC is Ca^{++}-

and calmodulin-dependent, and its activity is regulated by free intracellular Ca^{++}.[34]

Because relaxation depends on the initial tension, adjustment in tension between control and halothane-treated rings in some experiments was mandated to exclude any effect of different levels of initial tension on evoked relaxation. In experiments with and without matched tension, halothane attenuated the relaxation.

The inability to find any effect of halothane on sodium nitroprusside-induced relaxation in phenylephrine-contracted rabbit denuded aortic rings is in agreement with several previous publications.[4–6] Therefore, it can be inferred that halothane does not interfere with the activation of soluble guanylate cyclase, the production of cyclic GMP, the activation of cyclic GMP-dependent kinase, and the resulting relaxation. It also has been shown that, in canine cerebral arteries, halothane induces a vascular relaxation by activation of the particulate form of the guanylate cyclase that causes an increase in cyclic GMP.[35] These data also confirm that halothane does not interfere with the relaxation induced by cyclic GMP. However, we previously showed that halothane reduces the relaxation of preconstricted rabbit aortic rings to EDRF/NO.[10] It has been shown that nitric oxide half-life in a bioassay system is very short and can vary from a few seconds to as long as 50 s.[36] The half-life can be increased by superoxide dismutase, which catalyzes the metabolism of superoxide anion (O_2^{--}) to hydrogen peroxide. The EDRF/NO half-life is reduced *in vitro* by pyrrogalol, which enhances O_2^{--} production.[37] Xanthine and xanthine oxydase combined in organ chambers produce superoxide anions and other oxygen-derived radicals, which inhibit endothelium-dependent relaxation.[38] Oxygen-derived compounds can then react with EDRF/NO. Indeed, Beckmann *et al.* have suggested that nitric oxide reacts with O_2^{--} to produce peroxynitrite $OONO^{--}$, which decays to hydroxyl radical OH- and nitrite radical NO_2^{--}.[39]

Halothane interferes with EDRF/NO stability, but what is the mechanism? Can halothane produce free radicals that interfere with EDRF/NO? Power and McCay have shown that halothane's metabolism in the liver produces two types of radicals by abstraction of one chloride or one bromide.[40] It is not known whether the halothane-derived free radicals decrease nitric oxide half-life. Moreover, halothane's effect on endothelium-dependent relaxation does not require endothelial cell treatment with halothane. If halothane-derived radicals inhibit relaxation in the bioassay system, they are produced either spontaneously or by halothane's metabolism in smooth muscles. These halothane-derived radicals would have a direct effect on EDRF/NO without interaction with the mechanism of relaxation, because nitroprusside's relaxation is not attenuated by halothane. Nitroglycerin and sodium nitroprusside stimulate soluble guanylate cyclase *via* the release of EDRF/NO intracellularly. How can EDRF/NO produced from the metabolism of nitroglycerin or sodium nitroprusside be protected from halothane action? We do not know the answer to this question. It has been shown that sevoflurane, an ether-volatile anesthetic very close to isoflurane, attenuates endothelium-dependent relaxation. Its effect on EDRF/NO pathways is suppressed by superoxide dismutase, which suggests that O_2^{--} is involved in sevoflurane attenuation of endothelium-dependent relaxation. The source of the free radical is not established.[41]

EDRF/NO has a major function in cardiovascular physiology and pharmacology.[42] EDRF/NO is also a central and peripheral neurotransmitter.[43,44] Is halothane's interaction with the nitric oxide pathway one of the mechanisms of anesthesia? A recent paper demonstrated that L-NMMA decreases the minimum alveolar concentration of halothane in rats, suggesting that EDRF/NO in the brain plays a role in maintaining wakefulness.[45] Halothane has been shown to have a marked inhibitory effect on cyclic GMP concentrations in specific rat brain regions.[46] The decreased EDRF/NO stability by halothane could be one of the mechanisms of anesthesia induced by this anesthetic.

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References

1. Merin RG: Are the myocardial functional and metabolic effects of isoflurane really different from those of halothane and enflurane? ANESTHESIOLOGY 55:398–408, 1981

2. Rorie DK, Tyce GM, Mackenzie RA: Evidence that halothane inhibits norepinephrine release from sympathetic nerve endings in dog saphenous vein by stimulation of presynaptic inhibitory muscarinic receptors. Anesth Analg 63:1059–1064, 1984

3. Sill JC, Uhl C, Eskuri S, Van Dyke R, Tarara J: Halothane inhibits agonist-induced inositol phosphate and Ca^{2+} signaling in a7r5 cultured vascular smooth muscle cells. Mol Pharmacol 40:1006–1013, 1991

4. Muldoon SM, Hart JL, Bowen KA, Freas W: Attenuation of endothelium-mediated vasodilation by halothane. ANESTHESIOLOGY 68: 31–37, 1988

5. Uggeri MJ, Proctor GJ, Johns RA: Halothane, enflurane, and isoflurane attenuate both receptor-and non-receptor-mediated EDRF

production in rat thoracic aorta. ANESTHESIOLOGY 76:1012–1017, 1992

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

7. Ueda I: The effects of volatile general anesthetics on adenosine diphosphate-induced platelet aggregation. ANESTHESIOLOGY 34:405–408, 1971

8. Blaise GA, Dumont L, Buluran J, Girard D, Still C, De Lean A: Mechanism of halothane attenuation of isometric induced by serotonin in isolated coronary artery rings. J Cardiovasc Pharmacol 20: 445–450, 1992

9. Stone DJ, Johns RA: Endothelium-dependent effects of halothane, enflurane, and isoflurane on isolated rat aortic vascular rings. ANESTHESIOLOGY 71:126–132, 1989

10. Blaise GA: Effect of volatile anesthetic agent on endothelium dependent relaxation, Mechanisms of Anesthetic Action in Skeletal, Cardiac and Smooth Muscle. Volume 301, part 3. Edited by Blanck T, Wheeler D. New York, Plenum, 1991, pp 229–235

11. Ignarro LJ, Harbison RG, Wood KS, Kadowitz PJ: Activation of purified soluble guanylate cyclase by endothelium-derived relaxing factor from intrapulmonary artery and vein: Stimulation by acetylcholine, bradykinin and arachidonic acid. J Pharmacol Exp Ther 237: 893–900, 1986

12. Moncada S, Gryglewski R, Bunting S, Vane JR: An enzyme isolated from arteries transforms prostaglandin endoperoxides to an unstable substance that inhibits platelet aggregation. Nature 263:663–665, 1976

13. Sauvé R, Parent L, Simoneau C, Roy G: External ATP triggers a biphasic activation process of a calcium-dependent K^+ channel in cultured bovine aortic endothelial cells. Pflugers Arch 412:469–481, 1988

14. Bollen BA, Tinker JH, Hermsmeyer K: Halothane relaxes previously constricted isolated porcine coronary artery segments more than isoflurane. ANESTHESIOLOGY 66:748–752, 1987

15. Lerman J, Willis M, Gregory G, Eger E. Osmolarity determines the solubility of anesthetics in acqueous solutions at 37° C. ANESTHESIOLOGY 59:554–558, 1983

16. Martin W, Smith JA, White DG: The mechanisms by which haemoglobin inhibits the relaxation of rabbit aorta induced by nitrovasodilators, nitric oxide, or bovine retractor penis inhibitory factor. Br J Pharmacol 89:563–571, 1986

17. Johns RA, Peach MJ, Linder J, Tichotsky A: N^G-monomethyl L-arginine inhibits endothelium-derived relaxing factor stimulated cyclic GMP accumulation in cocultures of endothelial and vascular smooth muscle cells by an action specific to the endothelial cell. Circ Res 67:979–985, 1990

18. Martin W, Villani GM, Jothianandan D, Furchgott RF: 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, 1985

19. Hatano Y, Nakamura K, Yakushiji T, Nishiwada M, Mori K: Comparison of the direct effects of halothane and isoflurane on large and small coronary arteries isolated from dogs. ANESTHESIOLOGY 73: 513–517, 1990

20. Bolton TB: Mechanisms of action of transmitters and other substances on smooth muscle. Physiol Rev 59:606–718, 1979

21. Godfraind T, Dieu D: The inhibition by flunarizine of the norepinephrine-evoked contraction and calcium influx in rat aorta and mesenteric arteries. J Pharmacol Exp Ther 217:510–515, 1981

22. Wheeler DM, Rice T, Hansford RG, Lakatta EG: The effect of halothane on the free intracellular calcium concentration of isolated rat heart cells. ANESTHESIOLOGY 69:578–583, 1988

23. Iaizzo PA, Olsen RA, Seewald MJ, Powis G, Stier A, Van Dyke RA: Transient increases of intracellular Ca^{2+} induced by volatile anesthetics in rat hepatocytes. Cell Calcium 11:515–524, 1990

24. Klip A, Hill M, Ramlal T: Halothane increases cytosolic Ca^{2+} and inhibits Na^+/H^+ exchange in L6 muscle cells. J Pharmacol Exp Ther 254:552–559, 1990

25. Klip A, Mills GB, Britt BA, Elliott ME: Halothane-dependent release of intracellular Ca^{2+} in blood cells in malignant hyperthermia. Am J Physiol 258:C495–C503, 1990

26. Daniell LC, Harris RA: Neuronal intracellular calcium concentrations are altered by anesthetics: Relationship to membrane fluidization. J Pharmacol Exp Ther 245:1–7, 1988

27. Yashima N, Wada A, Izumi F: Halothane inhibits the cholinergic-receptor-mediated influx of calcium in primary culture of bovine adrenal medulla cells. ANESTHESIOLOGY 64:466–472, 1986

28. Bollen BA, McKlveen RE, Stevenson JA: Halothane Relaxes Previously Constricted Human Epicardial Coronary Artery Segments More than Isoflurane. Anesth Analg 75;4–8, 1992

29. Vane JR, Anggard EE, Botting RM: Regulatory functions of the vascular endothelium. N Engl J Med 323:27–36, 1990

30. Suga S, Nakao K, Itoh H, Komatsu Y, Ogawa Y, Hama N, Imura H: Endothelial production of C-type natriuretic peptide and its marked augmentation by transforming growth factor-β: Possible existence of "vascular natriuretic peptide system." J Clin Invest 90:1145–1149, 1992

31. Chen G, Suzuki H: Some electrical properties of the endothelium-dependent hyperpolarization recorded from rat arterial smooth muscle cells. J Physiol (Lond) 410:91–106, 1989

32. Chen G, Yamamoto Y, Miwa K, Suzuki H: Hyperpolarization of arterial smooth muscle induced by endothelial humoral substances. Am J Physiol 260:H1888–H1892, 1991

33. Sudoh T, Minamino N, Kangawa K, Matsuo H: C-type natriuretic peptide (CNP): A new member of natriuretic peptide family identified in porcine brain. Biochem Biophys Res Commun 168:863–870, 1990

34. Moncada S, Palmer RMJ, Higgs EA: Nitric oxide: Physiology, pathophysiology and pharmacology. Pharmacol Rev 43:109–142, 1991

35. Eskinder H, Hillard CJ, Flynn N, Bosnjak ZJ, Kampine JP: Role of guanylate cyclase-cGMP systems in halothane-induced vasodilation in canine cerebral arteries. ANESTHESIOLOGY 77:482–487, 1992

36. Rubanyi GM, Vanhoutte PM: Superoxide anion and hyperoxia inactivate endothelium-derived relaxing factor. Am J Physiol 250: H822–H827, 1986

37. Ignarro LJ, Byrns RE, Buga GM, Wood KS, Chaudhuri G: Pharmacological evidence that endothelium-derived relaxing factor is nitric oxide: Use of pyrogallol and superoxide dismutase to study endothelium-dependent and nitric oxide-elicited vascular smooth muscle relaxation. J Pharmacol Exp Ther 244:181–189, 1988

38. Rubanyi GM, Vanhoutte PM: Oxygen-derived free radicals, endothelium and responsiveness of vascular smooth muscle. Am J Physiol 250:H815–H821, 1986

39. Beckman JS, Beckman TW, Chen J, Marshall PA: Apparent hydroxyl radical production by peroxynitrite: Implications for endothelial injury from nitric oxide and superoxide. Proc Natl Acad Sci USA 87:1620–1624, 1990

40. Poyer JL, McCay PB, Weddle CC, Downs PE: In vivo spin-trapping of radicals formed during halothane metabolism. Biochem Pharmacol 30:1517–1519, 1981

41. Yoshida K, Okabe E: Selective impairment of endothelium-dependent relaxation by sevoflurane: Oxygen free radicals participation. ANESTHESIOLOGY 76:440–447, 1992

42. Searle NR, Sahab P: Endothelial vasomotor regulation in health and disease. Can J Anaesth 39:838–857, 1992

43. Snyder SH: Nitric oxide: First in a new class of neurotransmitters? Science 257:494–496, 1992

44. Hoffman M: A new role for gases: Neurotransmission. Science 252:1788, 1991

45. Johns RA, Moscicki JC, DiFazio CA: Nitric oxide synthase inhibitor dose-dependently and reversibly reduces the threshold for halothane anesthesia. ANESTHESIOLOGY 77:779–784, 1992

46. Kant GJ, Muller TW, Lenox RH, Meyerhoff JL: In vivo effects of pentobarbital and halothane anesthesia on levels of adenosine 3′,5′-monophosphate and guanosine 3′, 5′-monophosphate in rat brain regions and pituitary. Biochem Pharmacol 29:1891–1896, 1980