Laboratory Investigations

Mechanisms of inhibition of endothelium-dependent relaxation by halothane, isoflurane, and sevoflurane

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Volatile anaesthetics inhibit endothelium-dependent relaxation, but the underlying mechanism(s) have not been clarified. In an attempt to elucidate the mechanism(s), we determined the effects of halothane, isoflurane and sevoflurane on relaxation induced by acetylcholine and sodium nitro-prusside (SNP) and the cGMP formation elicited by exogenous nitric oxide (NO) and SNP in rat aortas. Acetylcholine (10^{-7} – 10^{-5} M) – induced relaxation was attenuated by halothane (2%), isoflurane (2%) and sevoflurane (4%). SNP (10^{-8} M) – induced relaxation was reduced by halothane (2%), but not by isoflurane (2%) or sevoflurane (4%). The cGMP level of NO-stimulated aorta was reduced by halothane (2%) and sevoflurane (4%), but not by isoflurane (2%). The cGMP level of SNP (10^{-7} M) – stimulated aorta was reduced by halothane (2%), but not by iso-

flurane (2%) and sevoflurane (4%). We conclude that the mechanisms responsible for the inhibition of endothelium-dependent relaxation differ among anaesthetics. Isoflurane inhibits the relaxation mainly by inhibiting the formation of NO in the endothelium. In contrast, the effect of halothane on endothelium-dependent relaxation may be largely due to the inhibition of action of NO in the vascular smooth muscle and the effect of sevoflurane may be to inactivate NO or to inhibit the action of NO.

Les agents anesthésiques volatils inhibent la relaxation d'origine endothéliale dont le mécanisme sous-jacent n'a pas été éclairci. Dans le but d'en élucider le(s) mécanisme(s), nous avons déterminé sur des aortes de rats les effets de l'halothane, de l'isoflurane et du sévoflurane sur la relaxation induite par l'acétylcholine et le nitroprussiate de sodium (SNP), et la synthèse de cGMP élicitee par l'oxyde nitrique (NO) et le SNP. La relaxation induite par l'acétylcholine (10^{-7} – 10^{-5} M) est atténuée par l'halothane 2%, l'isoflurane 2% et le sévoflurane 4%. La relaxation induite par le SNP (10^{-8} M) est diminuée par l'halothane 2%, mais non par l'isoflurane 2% ou le sévoflurane 4%. Le niveau de cGMP de l'aorte stimulée par le NO est diminué par l'halothane 2% et le sévoflurane 4%, mais non par l'isoflurane 2%. Le niveau de cGMP de l'aorte stimulée par le SNP (10^{-7}) est diminué par l'halothane 2%, mais non par l'isoflurane 2% et le sévoflurane 4%. Nous concluons que les mécanismes responsables de l'inhibition de la relaxation d'origine endothéliale diffèrent selon l'anesthésique. L'isoflurane inhibe la relaxation principalement en inhibant la synthèse endothéliale de NO. Par contre, l'effet de l'halothane sur la relaxation d'origine endothéliale peut être en grande partie due à l'inhibition de l'activité du NO sur le muscle vasculaire lisse et l'effet du sévoflurane peut être dû à l'inactivation du NO ou à l'inhibition de l'activité du NO.

Key words

ANAESTHETICS, VOLATILE: halothane, isoflurane, sevoflurane;

ARTERIES: vasodilatation, endothelium, endothelium-derived relaxing factor;

METABOLISM: guanosine monophosphate.

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Presented in part at the Annual Meeting of American Society of Anesthesiologists, Washington D.C., October 11–13, 1993.

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Accepted for publication 5th December, 1993.

Endothelium releases important regulators of vascular tone, including endothelium-derived relaxing factor (EDRF),¹ which is now believed to be identical to nitric oxide (NO) or related compounds.^{2,3} Nitric oxide is formed from L-arginine by NO synthase in generator cells such as vascular endothelial cells, and then diffuses to target cells such as vascular smooth muscle cells. In vascular smooth muscles, NO activates soluble guanylate cyclase to increase 3',5'-cyclic guanosine monophosphate (cGMP) levels, and induces relaxation.

Halogenated anaesthetics, including halothane,⁴⁻⁶ isoflurane,^{5,6} enflurane⁵ and sevoflurane,⁷ inhibit endothelium-dependent relaxation. However, the mechanism(s) underlying these effects are controversial. Muldoon *et al.*⁴ suggested that halothane inhibits endothelium-dependent relaxation by inhibiting formation or release of NO. In contrast, Blaise *et al.*⁸ demonstrated that halothane inhibits relaxation induced by extrinsic NO and suggested that the effect of halothane on endothelium-dependent relaxation is mediated through the inhibition of action of NO in the smooth muscle. More recently, Hart *et al.*⁹ demonstrated that cGMP formation elicited by extrinsic NO was suppressed by halothane and suggested that halothane interferes with guanylate cyclase activation. Yoshida and Okabe⁷ demonstrated the formation of free radicals by sevoflurane *in vitro*, and suggested that sevoflurane inhibits endothelium-dependent relaxation by inactivation of NO.

We previously demonstrated the inhibitory effect of halothane and isoflurane on endothelium-dependent relaxation and cGMP formation.⁶ In the present study, therefore, we first determined if sevoflurane inhibits endothelium-dependent effects, as do halothane and isoflurane. Secondly, we compared the effects of halothane, isoflurane and sevoflurane on cGMP formation or relaxation elicited by application of NO and sodium nitroprusside (SNP), which activate soluble guanylate cyclase in smooth muscles endothelium-independently. In these experiments, we attempted to determine which of the following mechanisms is responsible for the inhibition of endothelium-dependent relaxation by anaesthetics: (1) inhibition of formation of NO in the endothelium, (2) inactivation of NO during its diffusion from the endothelium to smooth muscle cells, or (3) inhibition of action of NO in the muscle cell.

Methods

The protocol was approved by Kyoto University Animal Use Committee. Wistar rats (250–300 g) were anaesthetized with sodium pentobarbital (50 mg · kg⁻¹, *ip*), and killed by bleeding from the common carotid artery. Descending portions of the thoracic aorta were removed.

They were cut into helical strips (17 mm long) for mechanical testing and cut longitudinally into pairs of strips (30 mm long) for determination of cGMP. Strips were bathed in 10 ml organ baths containing Krebs' bicarbonate solution with the following composition (mM): NaCl 118.2, KCl 4.6, CaCl₂ 2.5, KH₂PO₄ 1.2, MgSO₄ 1.2, NaHCO₃ 24.8, dextrose 10. The bathing fluid was aerated with a mixture of 95% O₂ and 5% CO₂ to keep the pH within the range 7.35–7.45, and it was maintained at 36.5–37.5°C.

Halothane, isoflurane and sevoflurane were introduced into the gas mixture through agent-specific vaporizers (Fluotec 3, Ohmeda, England for halothane; Fortec, Ohmeda for isoflurane and PPV₂, Penlon, England for sevoflurane) as described previously.^{6,10} The concentrations in the resulting gas mixture were monitored and adjusted by using an Atom 303 anaesthetic agent monitor (Atom, Tokyo, Japan). The molar concentrations of halothane (2%), isoflurane (2%) and sevoflurane (4%) in the bathing solution, measured by gas chromatography (Hewlett Packard 5890A Gas Chromatograph, Palo Alto, CA) (*n* = 3, each), were $5.27 \pm 0.09 \times 10^{-4}$ M, $3.63 \pm 0.03 \times 10^{-4}$ M, and $5.20 \pm 0.88 \times 10^{-4}$ M, respectively.

Mechanical experiments

Arterial strips were vertically fixed between two hooks, and the hook anchoring the upper end was connected to the lever of a force-displacement transducer (Toyo Baldwin T7-240, Tokyo, Japan). Changes in isometric tension were recorded on an oscillograph (Rectigraph 8K, Nihondenki-Sanei Co., Tokyo, Japan). Resting tensions were adjusted to 1.0 g, which induced the maximal contraction with KCl (20 mM) in a preliminary study. Then the arterial strips were allowed to equilibrate for 90–120 min in the control bathing fluid, which was replaced every 15 min.

In order to verify the function of the endothelium, after the initial stretching, strips were contracted with phenylephrine, exposed to acetylcholine (10^{-6} M) and then to papaverine (10^{-4} M). The relaxation to acetylcholine was compared to the relaxation to papaverine, and strips whose response to 10^{-6} M acetylcholine was more than 60% of the maximal response to papaverine were considered to have an intact endothelium and were used in the following study.

Fifty-two arterial strips with intact endothelium, obtained from 29 rats, were randomly assigned into nine groups (*n* = 5 to 7, each). Dose-response relationship for an identical relaxant (acetylcholine, 10^{-8} – 10^{-5} M, in five groups and SNP, 10^{-9} – 10^{-6} M, in four other groups) was determined three times in each strip. Before each cumulative exposure to a relaxant, strips were precontracted submaximally with phenylephrine (3×10^{-7} M).

At the end of each cumulative exposure, papaverine (10^{-4} M) was added to the bathing fluid to induce maximal relaxation, and the response to each concentration of relaxant was expressed as the percentage relative to the maximal response to papaverine. Thereafter, strips were washed with fresh bathing fluid more than three times and equilibrated.

To determine the effects of anaesthetics, strips were exposed to anaesthetics 30 min before and during the second exposure to the relaxant, and the response to a relaxant in the second exposure was compared with that in the first (pre-anaesthetic control) and the third (post-anaesthetic control) exposures. Anaesthetics used were halothane (2%), isoflurane (2%), and sevoflurane (2 and 4%) in the acetylcholine groups, and halothane (2%), isoflurane (2%), and sevoflurane (4%), in the SNP groups. Time controls ($n = 5$, each) were run in an identical manner but without exposure to anaesthetics.

Radioimmunoassay of cGMP

Fifty-six pairs of rat aortic strips were suspended in organ baths without tension, and equilibrated for 90 min. One of the two strips obtained from each rat was exposed to halothane (2%), isoflurane (2%) or sevoflurane (2–4%) for 30 min, and the other strip was not exposed to anaesthetics (for control). Subsequently, the strips were exposed to NO (addition of acidified NaNO_2 solution to the final concentration of 10^{-4} M) or SNP (10^{-7} M) for 1 min. The basal cGMP level (without exposure to stimulants) and the acetylcholine (10^{-5} M)-stimulated (for one minute) levels were also measured in strips exposed to sevoflurane (2–4%) for 30 min and control strips. All the strips were quick-frozen in liquid nitrogen and homogenized in 6% trichloroacetic acid. After ether extraction and succinylation,¹¹ the samples were radioimmunoassayed for cGMP with a Yamasa cGMP assay kit (Yamasa Shoyu Co., Chiba, Japan).

Drugs

Drugs used were acetylcholine (Daiichi Pharmaceutical Co., Osaka, Japan), SNP (Nacalai Tesque, Kyoto, Japan), halothane (Takeda Pharmaceutical Co., Osaka), isoflurane (Dainabott, Osaka) and sevoflurane (Maruishi Pharmaceutical Co., Osaka). Acetylcholine and SNP were dissolved in distilled water and added directly to the bathing fluid; the volume added was less than 1% (v/v) of the bathing fluid. Tubes and organ baths containing SNP were covered with black paper to prevent light degradation. Strips were exposed to NO by addition of acidified NaNO_2 solution (pH 2) to the bathing fluid.^{12,13} In a preliminary study, addition of 11 mM HCl (pH 2) to a concentration of 1% (v/v) did not have any effect on tension or cGMP level.

TABLE Phenylephrine-induced precontractions (mg) of rat aortae, untreated or treated with anaesthetics, before being exposed to acetylcholine and SNP

	1st exposure	2nd exposure	3rd exposure
Time/control ($n = 10$)	626 ± 32	510 ± 43	416 ± 54^a
Halothane 2% ($n = 11$)	529 ± 41	330 ± 38^a	328 ± 52^a
Isoflurane 2% ($n = 12$)	618 ± 74	468 ± 44	561 ± 58
Sevoflurane 2% ($n = 6$)	542 ± 83	343 ± 61^a	365 ± 28^a
Sevoflurane 4% ($n = 13$)	605 ± 43	392 ± 41^b	509 ± 53

Aortic strips for time-control were not treated with anaesthetics during three exposures, and those for halothane, isoflurane and sevoflurane groups were treated with anaesthetics 30 min before and during the 2nd exposure. ^a $P < 0.05$, ^b $P < 0.01$ versus first exposure.

Statistical analysis

The cGMP concentrations in control and anaesthetic-treated strips were analyzed by Student's *t* test for paired data. Other data were analyzed by ANOVA and Newman Keuls multiple range test. Data were expressed as means $\pm$ SEM. Differences at $P < 0.05$ were considered significant.

Results

The mean precontraction values induced by phenylephrine are shown in the Table. In the time-controls, phenylephrine-induced contractions were reduced by repetition. Exposure of the strips to 2% halothane and 2–4% sevoflurane significantly attenuated phenylephrine-induced precontractions. In the time controls, responses to acetylcholine or SNP were not altered in the first to third exposures (data not shown).

Acetylcholine (10^{-7} – 10^{-5} M)-induced relaxation was not affected by 2% sevoflurane, but was reduced by 2% halothane, 2% isoflurane and 4% sevoflurane ($P < 0.05$ – 0.01 , Figure 1). Relaxation induced by a low concentration (10^{-8} M) of SNP was reduced by 2% halothane ($P < 0.05$), but not by 2% isoflurane or 4% sevoflurane (Figure 2).

Basal levels of cGMP ($\text{pmol} \cdot \text{g}^{-1}$ wet weight) were 30.3 ± 2.8 and 23.4 ± 4.0 ($n = 5$, each), respectively, in anaesthetic-untreated and sevoflurane (4%)-treated strips. Acetylcholine (10^{-5} M)-stimulated cGMP level in sevoflurane (4%)-treated strips was lower than that of anaesthetic-untreated ($P < 0.05$, Figure 3). The cGMP levels of strips stimulated with NO (acidified NaNO_2 , 10^{-4} M) were reduced by halothane (2%) and sevoflurane (4%) ($P < 0.05$), but not by isoflurane (2%) or sevoflurane (2%) (Figure 4). The cGMP levels of SNP (10^{-7} M)-stimulated strips were reduced by halothane (2%) ($P < 0.05$), and not by isoflurane (2%) or sevoflurane (2–4%) (Figure 5).

FIGURE 1 Modification by halothane (2%), isoflurane (2%) and sevoflurane (2 and 4%) of acetylcholine (10^{-8} – 10^{-5} M)-induced relaxations. The maximum relaxation induced by papaverine was taken as 100%. ^a $P < 0.05$, ^b $P < 0.01$ compared to pre-anaesthetic control. $n = 5$ – 6 , each.

Discussion

In time-control studies, the magnitude of phenylephrine (3×10^{-7} M)-induced contraction was decreased by repeated application. This phenomenon is probably due to desensitization to α -adrenergic agonists, which is commonly observed in the rat aortic preparation with intact endothelium, but not when denuded, and reportedly is caused by a gradual increase in NO synthesis.¹⁴ Halothane and sevoflurane attenuated phenylephrine-induced contraction. However, the attenuation of cGMP-mediated relaxation by these anaesthetics cannot be ascribed to the reduction of phenylephrine-induced precontractions, since cGMP levels, in the absence of phenylephrine, were reduced by these anaesthetics.

The cGMP level of unstimulated aortic strips was insignificantly reduced by sevoflurane (4%) in the present study and by halothane (2%) and isoflurane (2%) in the previous study.⁶ Since it is known that endothelium releases EDRF even in the absence of stimulants and active tension, these findings suggest that these anaesthetics may decrease basal release of EDRF or may suppress its action.

In the present study, the magnitude of inhibition of acetylcholine (10^{-7} – 10^{-5} M)-induced relaxation was in

the order halothane (2%) > isoflurane (2%) = sevoflurane (4%). Sevoflurane (2%) had no effect. The finding that the effect of 2% halothane on acetylcholine-induced tension changes was greater than that of 2% isoflurane is in agreement with our previous finding.⁶ In that study, the increase in cGMP induced by acetylcholine (10^{-5} M) in rat aorta was abolished by isoflurane (2%, ca. 1.7 human MAC¹⁵), and attenuated to approximately 40% by halothane (2%, ca. 2.7 human MAC¹⁶). In the present study, the acetylcholine-stimulated cGMP level was not affected by sevoflurane at 2%, and was reduced to approximately 50% by sevoflurane at 4% (ca. 2.1 human MAC¹⁷). Taking these data together, the inhibitory effect of sevoflurane on acetylcholine-induced cGMP formation is weaker than that of halothane and isoflurane at equivalent concentrations and seems to be weaker than that of isoflurane if compared at equivalent MAC.

Isoflurane (2%), in spite of the strong inhibition of acetylcholine's effect, did not inhibit NO- or SNP-stimulated cGMP formation or SNP-induced relaxation. These results suggest that isoflurane inhibits the formation of NO in the endothelium, possibly by acting on endothelial muscarinic receptor, G protein, Ca^{++} channels of plasmic membrane or intracellular Ca^{++} storage site, or an NO synthase (Figure 6). In contrast, halothane (2%) and sevoflurane (4%) reduced cGMP levels in arterial strips exposed to exogenous NO, suggesting that sevoflurane and halothane, in contrast to isoflurane, inactivate NO or inhibit the action of NO.

Blaise and coworkers recently demonstrated that halothane (2%)¹⁸ and isoflurane (2%)¹⁹ inhibit relaxation of endothelium-denuded rabbit aorta, elicited by the exudate of bradykinin-stimulated bovine endothelial cells. The disagreement between their findings and ours may reflect species differences in the action of isoflurane.

Sodium nitroprusside splits NO or related compounds in vascular smooth muscle to activate soluble guanylate cyclase. We examined further the effect of anaesthetics on SNP-induced relaxation and cGMP formation, and demonstrated that at concentrations which inhibited acetylcholine's effect, halothane reduced cGMP in SNP-stimulated arteries. These findings indicate that halothane acts in vascular smooth muscle to inhibit the action of NO, or facilitate cGMP breakdown.

In contrast, 4% sevoflurane did not affect SNP-induced relaxation or cGMP formation, although it strongly inhibited cGMP formation elicited by externally applied NO. This suggests that, in agreement with the finding by Yoshida and Okabe,⁷ sevoflurane inactivates NO extracellularly (Figure 6). However, it is also likely that exogenous NO added to the bathing fluid is more susceptible to inactivation than endogenous NO/EDRF, which is released from the endothelium and diffuses into the

FIGURE 2 Modification by halothane (2%), isoflurane (2%) and sevoflurane (4%) of SNP (10^{-9} – 10^{-6} M)-induced relaxations. The maximum relaxation induced by papaverine was taken as 100%. ^a $P < 0.05$, ^b $P < 0.01$ compared to preanaesthetic control. $n = 6$ –7, each.

FIGURE 3 The acetylcholine (10^{-5} M)-stimulated cGMP level of anaesthetic-unexposed and sevoflurane (2–4%)-exposed strips. SEV, sevoflurane. ^a $P < 0.05$ compared to anaesthetic-unexposed strip. Values in parentheses indicate the number of aortic strips studied.

FIGURE 4 The NO-stimulated cGMP level of anaesthetic-unexposed and exposed strips. HAL., halothane; ISO, isoflurane; SEV, sevoflurane. ^a $P < 0.05$ compared to anaesthetic-unexposed one. Values in parentheses indicate the number of aortic strips studied.

attached smooth muscle cells. Therefore, the contribution of inactivation of NO to the inhibition of endothelium-dependent relaxation may be less pronounced than thought from *in vitro* findings.

In the present study, although the relaxation induced by SNP at 10^{-8} M was reduced by halothane (2%), the relaxation by SNP at higher concentrations was not attenuated by the anaesthetics, in spite of the marked decrease in the cGMP level. Moreover, in our previous study⁶ and that by others,⁵ halothane (2%) did not inhibit SNP (10^{-9} – 10^{-6} M)-induced relaxation. This discrepancy

could be explained by the assumption that SNP, or NO derived from SNP, acts not only on soluble guanylate cyclase, but also on multiple sites of smooth muscles to induce relaxation.^{20,21} The slight differences in the results in tension changes between our previous study⁶ and the present one may be due to differences in experimental

FIGURE 5 The SNP-stimulated cGMP level of anaesthetic-unexposed and exposed strips. HAL., halothane; ISO., isoflurane; SEV., sevoflurane. * $P < 0.05$ compared to anaesthetic-unexposed one. Values in parentheses indicate the number of aortic strips studied.

FIGURE 6 Possible sites of anaesthetic actions in the vascular endothelium and smooth muscle to inhibit endothelium-dependent relaxation. R, receptor; G, G-protein; NOS, NO synthase; Hal., halothane; Iso., isoflurane; Sev., sevoflurane.

protocol or conditions. In our previous study, the relaxant response in different strips was compared, and in the present study the response in the same arterial strip in the

presence and absence of anaesthetics was compared in an attempt to test the effect more precisely. In addition, the response was observed in indomethacin-treated arteries in the previous study, but in untreated arteries in the present study.

In summary, it is suggested that the main mechanism responsible for the inhibition of endothelium-dependent relaxation differ among anaesthetics. We speculate that the effect of isoflurane is mediated mainly through its ability to inhibit formation of NO in the endothelium, and that halothane inhibits the action of NO in the vascular smooth muscle. The ability of sevoflurane to inhibit endothelium-dependent relaxation is weaker than those of other anaesthetics, and may be due to inactivation of NO or inhibition of the action of NO.

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