Isoflurane inhibits endothelium-mediated nitric oxide relaxing pathways in the isolated perfused rabbit lung

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Purpose: The role of volatile anaesthetics on nitric oxide (NO)-dependent relaxation is unclear in the pulmonary circulation. We examined the effects of isoflurane on NO-dependent relaxation in isolated perfused rabbit lungs.

Methods: Eighteen rabbit lungs were perfused in a constant-flow recirculation manner. In study 1 ($n=12$), acetylcholine (ACh, 4×10^{-10} – 10^{-8} M) or nitroglycerine (NTG, 6×10^{-10} – 10^{-8} M) was cumulatively injected into the pulmonary artery in the absence or presence of isoflurane (1, 2 MAC). In study 2 ($n=6$), ACh was injected as in study 1 in the presence or absence of N ω -nitro-L-arginine methyl ester (L-NAME, 100 μ M), an NO synthesis blocker. In all experiments, indomethacin was administered to prevent formation of vasoactive prostanoid metabolites, and the pulmonary vessels were precontracted with prostaglandin F $_{2\alpha}$ (PGF $_{2\alpha}$) infused before ACh or NTG injection. The ACh- or NTG-induced relaxation was expressed as % decrease in PGF $_{2\alpha}$ precontraction.

Results: Isoflurane at 2 MAC attenuated the dose-dependent relaxation to ACh at doses of 4×10^{-9} M and 4×10^{-8} M from $27.8 \pm 4.3\%$ and $38.8 \pm 5.3\%$ to $17.0 \pm 3.5\%$ and $25.5 \pm 4.9\%$, respectively ($P < 0.05$). Isoflurane did not change the dose-dependent relaxation to NTG and L-NAME abolished the ACh-induced relaxation.

Conclusion: Isoflurane inhibited NO-dependent relaxation in the pulmonary circulation at a site distal to the endothelial cell receptor-mediated responses but proximal to guanylate cyclase activation of vascular smooth muscle. Acetylcholine-induced relaxation in isolated perfused rabbit lungs was regulated primarily by NO.

Objectif : Nous connaissons mal l'influence des anesthésiques volatils sur la relaxation de la circulation pulmonaire dépendante du monoxyde d'azote (NO). Cette étude analyse les effets de l'isoflurane sur la relaxation NO-dépendante de poumons perfusés de lapins.

Méthodes : Dix-huit poumons de lapins ont été perfusés par recirculation à débit constant. Dans l'étude 1 ($n=12$), on a injecté de l'acétylcholine (ACh, 4×10^{-10} – 10^{-8} M) ou de la nitroglycérine (NTG, 6×10^{-10} – 10^{-8} M) avec ou sans isoflurane (1,2 MAC). Dans l'étude 2, ($n=6$), l'ACh a été injectée seule comme dans l'étude 1 ou associée au N ω -nitro-L-arginine méthyl ester (L-NAME, 100 μ M), un inhibiteur de la synthèse du NO. Toutes les expériences ont comporté l'administration d'indométhacine pour prévenir la formation de métabolites protanoïdes vasoactifs ainsi que la constriction préalable avec de la prostaglandine F $_{2\alpha}$ (PGF $_{2\alpha}$) administrée en perfusion avant l'injection de l'ACh et de la NTG. La relaxation induite par ACh ou NTG était exprimée en fonction de la diminution du pourcentage de constriction provoquée par la PGF $_{2\alpha}$.

Résultats : L'isoflurane à 2 MAC atténuait la relaxation à l'ACh proportionnellement aux doses de 4×10^{-9} et 4×10^{-8} M respectivement de $27,8 \pm 4,3\%$ et $38,8 \pm 5,3\%$ à $17,0 \pm 3,5\%$ et $25,5 \pm 4,9\%$ ($P < 0,05$). L'isoflurane ne modifiait la relaxation de la circulation pulmonaire NO-dépendante à la NTG ; le L-Name abolissait la relaxation induite par l'ACh.

Conclusion : L'isoflurane inhibe la relaxation NO-dépendante dans la circulation pulmonaire à un site distal à celui des réponses transmises par médiation du récepteur endothélial cellulaire mais proximal à l'activation par le guanylate cyclase du muscle vasculaire lisse. Le NO est le régulateur primaire de la relaxation induite par l'ACh dans le poumon de lapin perfusé.

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VASCULAR endothelium appears to play an important role in the regulation of vascular smooth muscle tone by releasing vasoactive substances such as nitric oxide (NO) in response to various stimuli.¹ Acetylcholine (ACh) activates NO synthesis, releases NO and then increases the cGMP concentration in vascular smooth muscles to cause vasodilatation.¹ Volatile anaesthetics have marked effects on vascular control and haemodynamics. Volatile anaesthetics inhibit endothelium-dependent relaxation induced by ACh in systemic vessel rings such as the aorta, femoral artery, carotid artery and mesenteric artery.²⁻⁹ This inhibition may seem somewhat counterintuitive to anaesthetists who have thought of volatile anaesthetics as pure vasodilators. In the pulmonary circulation, however, the role of volatile anaesthetics on endothelium-dependent relaxation is less clear. The systemic circulation is a high pressure, high resistance system. On the contrary, the pulmonary circulation is characterized by a low pressure system and an ability to constrict in response to hypoxia. This response to hypoxia, hypoxic pulmonary vasoconstriction (HPV), is the opposite of the effects observed in the systemic circulation, where hypoxia produces dilatation rather than constriction. Volatile anaesthetics are often considered to be pulmonary vasodilators because they have generally been shown to inhibit the magnitude of HPV.^{10,11}

Furthermore, ACh-induced relaxation that is resistant to NO synthase inhibitors has recently been reported, and such relaxation may be mediated by an endothelium-derived hyperpolarizing factor (EDHF) that is distinct from NO.¹² The relative importance of NO and EDHF in the endothelium-dependent relaxation in various types of vessels has been actively investigated. Nitric oxide primarily exerts its action on ACh-induced relaxation in the large, conduit arteries,^{12,13} while EDHF acts predominantly in small arterial beds such as the mesenteric artery.^{13,14}

In the present study, using isolated perfused rabbit lungs, we examined whether isoflurane affected ACh-induced endothelium-dependent relaxation in the pulmonary circulation. Second, we examined which factor, NO or EDHF, was the primary determinant of vascular calibre under ACh-induced relaxation in the pulmonary circulation. These findings are relevant because of the extensive clinical and investigational use of isoflurane anaesthesia.

Methods

This study conformed to Guidelines for Animal Experimentation in Faculty of Medicine, Tottori University. A standard *in vitro* perfused rabbit lung preparation^{15,16} was modified as described in the following.

Preparation of isolated rabbit lungs

Adult female Japanese white rabbits weighing 2.3 ± 0.1 kg (mean $\pm$ SEM) were anaesthetized with 20 mg·kg⁻¹ pentobarbital *iv* and 35 mg·kg⁻¹ ketamine *im*, and anticoagulated with 500 IU·kg⁻¹ heparin *iv*. To prevent formation of vasoactive prostanoid metabolites, 10 mg·kg⁻¹ indomethacin *iv* was administered. The neck and sternal regions were infiltrated with xylocaine 1%. A tracheostomy was performed and the lungs were mechanically ventilated with a Harvard ventilator (model 681, Harvard Apparatus, South Natick, MA) at a rate of 40 breaths·min⁻¹ and a tidal volume of 10 ml·kg⁻¹ with a gas mixture consisting of O₂ 21%, CO₂ 5% and balance nitrogen. Positive end-expiratory pressure of 2 cm H₂O was applied. A sternotomy was performed, and the ribs were retracted exposing the heart and lungs. An incision was made into the right ventricle, and then the rabbits were killed by rapid withdrawal of blood. A cannula was inserted into the main pulmonary artery via the incision in the right ventricle and secured with an umbilical tape. The ligature around the pulmonary artery cannula also included the aorta, preventing loss of perfusate into the systemic circulation and effectively isolating the pulmonary circulation. An incision was made in the apex of the left ventricle and a cannula was inserted into the left atrium and secured by a ligature around the apex of the heart. The pulmonary circulation was first perfused in a nonrecirculating manner with bovine serum albumin 3% dissolved in physiological salt solution (PSS) until the effluent was clear. Then, autologous blood was added to the PSS, and the lungs were perfused in a recirculating manner with a peristaltic pump (model 1215, Harvard Apparatus) at a flow rate of 30 ml·kg⁻¹ body weight·min⁻¹ monitored with an electromagnetic blood flowmeter (MF-1200, Nihon Kohden, Tokyo, Japan). The PSS contained 119 mM NaCl, 4.7 mM KCl, 1.17 mM MgSO₄, 22.61 mM NaHCO₃, 1.18 mM KH₂PO₄ and 3.2 mM CaCl₂, and to each 100 ml of this stock solution were added 100 mg dextrose 20 mU insulin, 1 ml NaHCO₃ 8% and 3 g bovine serum albumin. The final perfusate contained 50 ml PSS and 20 ml autologous blood, with a haematocrit of about 8%. The perfusate reservoir temperature was kept at 38°C with a heated water bath. The pulmonary artery pressure (PAP), left atrial pressure and airway pressure were measured continuously via side holes in the cannula with pressure transducers (P23 ID, Gould, Oxnard, CA) and recorded on a polygraph (type 361, NEC San-ei, Tokyo, Japan). All pressures referred to the level of the left atrium. The level of the drainage tube from the left atrium was adjusted to maintain the left atrial pressure at 0 mmHg. The isolated lung preparation remained in the thoracic cavity, which was covered with transparent vinyl

wrapping material to maintain the humidity surrounding the lungs, and an infrared light was applied to maintain the chest cavity temperature at approximately 38°C. After institution of the perfusion circuit, additional indomethacin producing the final circulating concentration of 20 μM was added to the reservoir, and the animals were allowed to stabilize for 30 min before proceeding to the experimental protocol. In studies 1 and 2, perfusate haematocrit, pH, PCO_2 and PO_2 were measured at the beginning and during experiments, from reservoir samples, with a blood gas analyser (JBA-7, Jookoo, Tokyo, Japan). Perfusate pH was maintained between 7.30 and 7.50 by adding small volumes of NaHCO_3 .

Study 1: effects of isoflurane on vasoreactivity

Twelve perfused lungs were divided into two groups treated with ACh and isoflurane (ACh-ISO group, $n = 6$) or NTG and isoflurane (NTG-ISO group, $n = 6$). The ACh- or NTG-induced relaxation was examined during ventilation with randomized concentrations of isoflurane (0, 1 and 2 MAC). Prostaglandin $\text{F}_{2\alpha}$ ($\text{PGF}_{2\alpha}$) was continuously infused at a rate of 5.0–40 $\text{nmol}\cdot\text{min}^{-1}$ into the circuit proximal to the pulmonary artery until the PAP was increased to 30 mmHg. After a stable increase in the PAP was achieved, the drug vehicle (0.5 ml saline) and ACh or NTG dissolved in 0.5 ml saline were injected into the circuit proximal to the pulmonary artery in a cumulative fashion in three doses, which produced final circulating concentrations of 4×10^{-10} M, 4×10^{-9} M and 4×10^{-8} M for ACh or 6×10^{-10} M, 6×10^{-9} M and 6×10^{-8} M for NTG, respectively. Each dose was injected after the maximal effect of the previous dose was achieved. $\text{PGF}_{2\alpha}$ was discontinued after the maximal effect of ACh or NTG was achieved by the dose of 4×10^{-8} M or 6×10^{-8} M, respectively. After the baseline pressure had returned to the control level by discontinuation of $\text{PGF}_{2\alpha}$, 15 min were allowed for equilibration at every new inspiratory concentration of isoflurane. The minimum alveolar concentration (MAC) for rabbits was assumed to be 2.05% for isoflurane,¹⁷ and a vaporizer (type I MK III, Acoma, Tokyo, Japan) was used to deliver isoflurane vapour. The end-tidal concentration of isoflurane was measured continuously with an anaesthetic gas monitor (type 1304, Brüel & Kjær, Naerum, Denmark). The anaesthetic concentration in the perfusate was equilibrated with that in the lungs within 10 min after changing the concentration.¹⁰ At the end of the study and immediately after completion of perfusion, the lungs were removed. After weighing the wet weight, the whole lungs were dried for 72 hr and reweighed. The simple, blood-inclusive, wet-to-dry lung weight ratio was

then calculated by (wet lung weight – dry lung weight)/dry lung weight ($\text{ml}\cdot\text{g}^{-1}$).

Study 2: effects of Nω-nitro-L-arginine methyl ester (L-NAME) on vasoreactivity

Six perfused lungs were studied. As in study 1, the pulmonary vasculature was precontracted with $\text{PGF}_{2\alpha}$, and then drug vehicle and ACh (4×10^{-10} M, 4×10^{-9} M and 4×10^{-8} M) were injected cumulatively into the pulmonary artery after the maximal effect of the previous dose had been achieved. $\text{PGF}_{2\alpha}$ was discontinued after the maximal effect of ACh at the dose of 4×10^{-8} M was achieved. After the baseline pressure had returned to the control level by discontinuation of $\text{PGF}_{2\alpha}$, L-NAME producing a final circulating concentration of 100 μM was added to the reservoir. After 30-min equilibration, the above protocol of ACh injections was repeated.

Statistics

The data are shown as mean $\pm$ SEM. The values within a group were compared with one-way analysis of variance and, if a statistical difference was observed, a multiple-comparison test was performed by Scheffé's F-test. The values between groups were compared by unpaired t test. Differences were considered significant at $P < 0.05$.

Results

The general conditions did not differ among the three groups throughout experiments. Average data were as follows: pH 7.361 ± 0.008 , PaCO_2 35.3 ± 0.6 mmHg, PaO_2 146.5 ± 2.8 mmHg, base excess -2.9 ± 0.5 and perfusate haematocrit $7.9 \pm 0.2\%$. Peak airway pressures (data not shown) were not altered by the protocol and administration of isoflurane or L-NAME. The relaxant response to ACh or NTG was expressed as % decrease in $\text{PGF}_{2\alpha}$ precontraction, which was calculated with the following formula:

$$(\text{PAP}_{\text{PGF}_{2\alpha}} - \text{PAP}_{\text{ACh or NTG}}) / (\text{PAP}_{\text{PGF}_{2\alpha}} - \text{baseline PAP}) \times 100\%.$$

Study 1

When isoflurane was at 0, 1 and 2 MAC, the baseline PAP before infusing $\text{PGF}_{2\alpha}$ was 11.3 ± 1.0 mmHg, 11.6 ± 1.4 mmHg and 11.4 ± 1.6 mmHg in the ACh-ISO group; and 12.3 ± 1.6 mmHg, 11.7 ± 1.1 mmHg and 12.2 ± 2.3 mmHg in the NTG-ISO group, respectively. There were no differences in baseline PAP between groups with and without administration of isoflurane. The wet-to-dry lung weight ratio ($\text{ml}\cdot\text{g}^{-1}$) was 5.27 ± 0.23 in the ACh-ISO group, and 5.06 ± 0.30 in the NTG-ISO group; there was no difference between the two groups.

After administration of 0 MAC isoflurane, dose-dependent relaxation to ACh at doses of 4×10^{-10} M, 4×10^{-9} M and 4×10^{-8} M was $13.3 \pm 2.4\%$, $27.8 \pm 4.3\%$ and $38.8 \pm 5.3\%$, respectively: when isoflurane was at 2 MAC, the dose-dependent relaxation to ACh at doses of 4×10^{-10} M, 4×10^{-9} M and 4×10^{-8} M was $8.2 \pm 2.0\%$, $17.0 \pm 3.5\%$ and $25.5 \pm 4.9\%$, respectively. These showed that 2 MAC isoflurane ($P < 0.05$) decreased the dose-dependent relaxation to ACh at doses of 4×10^{-9} M and 4×10^{-8} M (Figures 1B, 2A). Dose-dependent relaxation to

FIGURE 1 Representative tracings of responses to acetylcholine (ACh) at doses from 4×10^{-10} to 10^{-8} M in perfused lungs. A: Responses to ACh without isoflurane: volume loading with drug vehicle (0.5 ml saline) increases pulmonary arterial pressure (PAP) transiently, and ACh decreases the PAP in a dose-dependent manner. B: Responses to ACh with isoflurane at 2 MAC: dose-dependent relaxation to ACh is attenuated. C: Responses to ACh with 100 μM L-NAME: the dose-dependent relaxations to ACh are abolished, and ACh at the dose of 4×10^{-8} M is turned to constrict pulmonary arteries.

NTG (6×10^{-10} – 10^{-8} M) was not changed by the presence of isoflurane (Figure 2B).

FIGURE 2 Dose-response curves to acetylcholine (ACh) (A) or nitroglycerine (NTG) (B) treated with isoflurane (ISO) at 0 to 2 MAC. The relaxant response to ACh or NTG is expressed as percentage of contractions induced by prostaglandin F₂α (PGF₂α). * $P < 0.05$ compared with 0 MAC. $n = 6$.

Study 2

The baseline PAP before infusing PGF2 was 13.4 ± 0.6 mmHg and 13.3 ± 0.7 mmHg without and with administration of L-NAME, respectively; there was no statically significant difference.

After administration of L-NAME, ACh-induced relaxation was abolished and an ACh dose of 4×10^{-8} M increased the PAP (Figures 1C, 3).

Discussion

There were two major findings in the present experiments with isolated perfused rabbit lungs. First, isoflurane at 2 MAC inhibited ACh-induced relaxation in the pulmonary circulation, but not NTG-induced relaxation. Second, by the administration of the NO synthesis blocker L-NAME, ACh-induced vascular relaxation was abolished and high-dose ACh caused constriction of the pulmonary arteries.

FIGURE 3 Dose-response curves to acetylcholine (ACh) with and without N ω -nitro-L-arginine methyl ester (L-NAME). The relaxant response to ACh is expressed as percentage of contractions induced by prostaglandin F2 α (PGF2 α).

* $P < 0.01$ compared with L-NAME (-). $n = 6$.

In larger vessels such as the aorta, NO plays the dominant role in ACh-induced endothelial relaxation.^{12,13} From previous studies on vascular rings of conduit arteries, volatile anaesthetics exerting endothelium-dependent relaxation have different inhibitory mechanisms: isoflurane inhibits NO synthesis mainly in the endothelium; halothane inhibits NO action in vascular smooth muscles; and sevoflurane produces oxygen-free radicals that deactivate NO action.^{4,7,8} Nitric oxide is formed from the amino acid L-arginine by NO synthase and activation is calcium-dependent.¹ When administered with the fluorescent Ca²⁺ indicator fura-2, halothane and isoflurane decrease intracellular calcium in bovine aortic endothelial cells stimulated by bradykinin.¹⁸ The resultant decrease in Ca²⁺ availability is likely to interfere with NO production by modulating NO synthase activity.

In the mesenteric resistance arteries, EDHF acts preferably on ACh-induced relaxation than does NO.^{13,14} Volatile anaesthetics inhibit the vasodilating actions not only of NO but also of EDHF.^{9,19} However, when NO is not deactivated, the inhibitory action of volatile anaesthetics on ACh-induced relaxation is weak in the mesenteric resistance arteries.²⁰⁻²²

In the pulmonary circulation, volatile anaesthetics may attenuate endothelium-dependent relaxation, although the results are conflicting. When pulmonary vascular pressure-flow plots are generated in chronically instrumented dogs, halothane anaesthesia (1.2% end-tidal concentration) abolished the pulmonary vasodilating actions of bradykinin, an endothelium-dependent vasodilator, and of nitroprusside, an endothelium-independent vasodilator.²³ In the case of isoflurane anaesthesia, an end-tidal concentration of 1.9% attenuated the pulmonary vasodilator response of bradykinin, but enhanced the pulmonary vasodilator response of SIN-1, an endothelium-independent vasodilator.²⁴ On the other hand, in vascular rings of the rat main pulmonary artery, halothane at 2 MAC did not inhibit exogenous NO-induced relaxation.²⁵ In the present study with isolated perfused rabbit lungs, 2 MAC isoflurane inhibited ACh-induced relaxation in the pulmonary circulation, but did not change NTG-induced relaxation. Acetylcholine activates NO synthesis, releases NO and then activates guanylate cyclase, while NTG, an endothelium-independent vasodilator, directly activates guanylate cyclase. Both agents increase the cGMP concentration in vascular smooth muscles to cause vasodilation.¹ We speculate that 2 MAC isoflurane blocks endothelium-dependent relaxation in the pulmonary circulation as well as in the conduit arteries such as the aorta, through inhibiting NO synthesis mainly in the endothelium of pulmonary vessels. When examined under the bioassay system using bovine aorta, both halothane and isoflurane interfere with the stability of

NO rather than with its secretion, release or action.^{26,27} Direct measurement of NO would resolve the speculation that isoflurane decreases NO synthesis in the endothelium of pulmonary vessels.

Endothelium-derived hyperpolarizing factor is believed to cause hyperpolarization and results in relaxation by opening K⁺ channels.¹⁴ According to experimental studies on vascular rings of pulmonary vessels, ACh-induced relaxation of the main pulmonary artery is reduced to 20–25% in the presence of haemoglobin and methylene blue, which are blockers of NO action.¹² Furthermore, the ACh-induced relaxation disappears in the presence of another blocker, L-NAME.¹³ It seems logical to assume that endothelium-dependent relaxation which is resistant to inhibitors of the L-arginine-NO pathway is mediated by EDHF. This interpretation is supported by the observation that L-NAME-resistant relaxation was not observed in high-K⁺ solutions, where endothelium-dependent hyperpolarization is absent.²⁸ In vascular smooth muscle cells of the rat, the ACh-induced hyperpolarizing response is 100-fold weaker in the main pulmonary artery than in the aorta, and negligibly small in the intralobular small pulmonary artery.²⁸ In the present experiments, the administration of NO synthesis blocker L-NAME abolished the ACh-induced vascular relaxation, and the action of high-dose ACh was converted from dilator to constrictor. These results suggest that ACh-induced vascular relaxation is regulated primarily by NO in isolated perfused rabbit lungs, although the membrane potential of the smooth muscle cell was not directly measured.

Models *in vivo* include a number of factors that can modify pulmonary vascular tone indirectly, and these preparations are useful in evaluating the net effects of the drugs on both pulmonary and systemic haemodynamics. We selected the constant-flow *in vitro* model in the present study. An *in vitro* constant-flow perfused lung preparation is suitable for examining the direct effect of anaesthetics on pulmonary vascular tone because a high concentration of anaesthetic may be administered without the confounding effects of circulatory depression. Because of the constant perfusion and constant left atrial pressure (0 mmHg), pulmonary vascular resistance was directly proportioned to and increased together with the PAP. Each experiment lasted for up to 2.5 hr, but no oedema was observed (dry/wet lung weight determinations). High-dose ACh causes a rapid increase in PAP in rabbit pulmonary circulation and induces pulmonary oedema. This process is prevented by indomethacin,¹⁶ because ACh stimulates the release of thromboxane A₂ from the vascular endothelium, which causes constriction of the intrapulmonary resistance arteries.²⁹ The endothelial cells can release several vasodilators such as prostacyclin, NO and

EDHF. Halothane increases prostacyclin release in bovine pulmonary artery endothelial cells,³⁰ while inhibits bradykinin-stimulated prostacyclin production in bovine aortic endothelial cells.³¹ Our experimental setup involves the testing NO and EDHF, because prostaglandin synthesis is inhibited by indomethacin. In addition, in experiments of pulmonary vascular rings, the action of isoflurane in blocking bradykinin-induced vasorelaxation is abolished without indomethacin treatment.³²

In conclusion, 2 MAC isoflurane inhibited NO-dependent relaxation in the pulmonary circulation at a site distal to the endothelial cell receptor-mediated responses but proximal to guanylate cyclase activation of vascular smooth muscle. Endothelium-dependent relaxation induced by ACh was regulated primarily by NO in the isolated perfused rabbit lungs. The observed effects have important clinical implications in regulating pulmonary vascular tone during surgery and anaesthesia.

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