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Isoflurane Does Not Vasodilate Rat Thoracic Aortic Rings by Endothelium-derived Relaxing Factor or Other Cyclic GMP–Mediated Mechanisms

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Endothelium-derived relaxing factor (EDRF) is a potent endogenous vasodilator that has been indirectly suggested to play a role in isoflurane-mediated vasodilation. To examine directly the possible role of EDRF in isoflurane-mediated vasodilation, isolated rat thoracic aortic rings were suspended for isometric tension measurements, equilibrated to a resting tension of 2 g, and constricted with a 50% maximal concentration (EC_{50}) dose of phenylephrine or KCl. Three groups of rings were studied: endothelium-intact, endothelium-denuded, and endothelium-intact rings treated with nitro-L-arginine methyl ester (L-NAME), a specific inhibitor of EDRF synthase. Isoflurane was then added at 1, 2, and 3% in a cumulative manner, allowing 10 min for each concentration to equilibrate. Indomethacin was present in all experiments to prevent the formation of vasoactive prostanoid metabolites. Since EDRF causes vascular relaxation by stimulating soluble guanylyl cyclase and increasing cyclic GMP, the effect of isoflurane on vascular ring cyclic GMP content was determined as an additional indicator of EDRF-mediated dilation. Rings with intact and denuded endothelium were isolated as described above, constricted with phenylephrine, and challenged with methacholine (positive control) or 1, 2, or 3% isoflurane. After 8 min exposure, the rings were flash-frozen in dry-ice–cooled acetone and homogenized in 1 N HCl for subsequent analysis of cyclic GMP content by radioimmunoassay. Isoflurane caused dose-dependent vasodilation of both KCl- and phenylephrine-constricted rings. In the phenylephrine group, at 2% and 3% isoflurane, endothelium-denuded and L-NAME–treated rings relaxed to a greater extent than endothelium-intact rings ($P < 0.01$). There were no differences in isoflurane-induced relaxation of any of the KCl-constricted groups. Methacholine, an endothelium-dependent vasodilator, increased cyclic GMP concentration of endothelium-intact vascular rings significantly above control ($P < 0.001$). Isoflurane 1, 2, and 3% had no effect on cyclic GMP content of either endothelium-intact or endothelium-denuded vessels. Vasodilation of the rat aorta by isoflurane is due to a direct effect on vascular smooth muscle and is independent of the stimulation of EDRF or other cyclic GMP–mediated mechanisms. (Key words: Anesthetics, volatile: isoflurane. Artery, vascular smooth muscle: vasodilation. Endothelium: 3′,5′-cyclic guanosine monophosphate; endothelium-derived relaxing factor; guanylyl cyclase.)

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RESEARCH IN THE FIELD of vascular physiology has yielded an improved understanding of the cellular pathways of communication between endothelium and smooth muscle.[1–3] Endothelium-derived relaxing factor (EDRF) is a potent endogenous vasodilator that is likely to be nitric oxide or a similar nitrogen oxide compound. It is produced by the endothelial cell both basally and in response to a variety of hormones and chemicals.[2–6] These include acetylcholine, bradykinin, calcium ionophore (A23187), and adenosine triphosphate, as well as vasoconstrictors such as phenylephrine, serotonin, and norepinephrine, which release EDRF in addition to their direct constricting effects.[7–8] These agents cause an increase in endothelial cell cytosolic calcium which, with the cofactors NADPH and calmodulin, activates EDRF synthase which, in turn, metabolizes L-arginine to citrulline and EDRF.[3,6,9–11] Once produced, EDRF activates vascular smooth muscle soluble guanylyl cyclase to convert guanosine triphosphate to 3′,5′-cyclic guanosine monophosphate (cyclic GMP).[12–14] Cyclic GMP accumulation has been shown in numerous investigations to correlate with vascular smooth muscle relaxation, probably through the extrusion of calcium from the vascular smooth muscle cytosol.[12–14] The understanding of this pathway for EDRF production has allowed for the development of specific inhibitors of EDRF synthase. These inhibitors are analogues of L-arginine, the substrate for EDRF synthase, and include L-NAME and N^G-mono-methyl-L-arginine.[15–17]

There are conflicting reports in the literature on the interactions of isoflurane with endothelium and the role of EDRF in isoflurane-mediated vascular responses. It has been suggested that in canine cerebral arteries,[18,19] isoflurane causes an endothelium-independent relaxation. Stone and Johns[20] also provided evidence that isoflurane vasodilates rat aortic rings independently of endothelium and that at low concentrations it may inhibit EDRF production. Conversely, Blaise *et al.*[21] reported that 2.3% isoflurane attenuated vasoconstrictor-induced canine coronary artery contraction only when the endothelium was present and suggested that isoflurane may stimulate the release of an endothelium-dependent dilator, possibly EDRF.

The purpose of this investigation was to clarify the conflicting literature regarding the role of EDRF in iso-

flurane-mediated vasodilation using the highly specific EDRF inhibitor L-NAME and by measuring changes in EDRF-stimulated vascular smooth muscle cyclic GMP.

Materials and Methods

In accordance with institutional Animal Care Committee standards, male Sprague-Dawley rats (300–350 g) were killed and the descending thoracic aorta gently removed and placed in modified Krebs' buffer (NaCl 111 mM, KCl 5 mM, NaH_2PO_4 1 mM, $MgCl_2$ 0.5 mM, $NaHCO_3$ 25 mM, $CaCl_2$ 2.5 mM, Dextrose 11.1 mM). The aorta was then dissected clean of fat and extraneous tissue and divided into 2.5–3.0-mm ring segments. The rings were either left intact (*i.e.*, with endothelium) or denuded of endothelium by gentle rotation on a forceps.

Isometric Tension Measurements

The vessel rings were suspended in 37° C water-jacketed 25-ml tissue baths containing 10 ml modified Krebs' solution gassed with 95% O_2, 5% CO_2 at 4 l/min and connected to Grass FT-03® force transducers (Quincy, MA) for isometric tension measurement. The optimal resting tension of 2 g was predetermined by length–tension relationship experiments. The buffer was changed every 15 min during a 60-min equilibration period.

Endothelial cell status was confirmed by constricting the rings with phenylephrine (1×10^{-7} M) followed by methacholine 1×10^{-6} M, known to be an endothelium-dependent vasodilator of vascular smooth muscle. Rings relaxing more than 60% to methacholine were considered intact. Denuded rings showed no relaxation. Rings were washed, reequilibrated to baseline tension, and constricted with a 50% maximal concentration (EC_{50}) of phenylephrine, 1×10^{-7} M for intact vessels and 5×10^{-8} M for denuded/L-NAME–treated endothelium-intact vessels, or with KCl 4×10^{-2} M. The EC_{50} for phenylephrine was different between endothelium-intact and -denuded or L-NAME–treated vessels because phenylephrine releases EDRF from the endothelium in addition to effecting constriction directly.[22-23] Studies were also performed with KCl-constricted vessels because KCl has no effect on EDRF. Preliminary experiments demonstrated stable constriction for phenylephrine and KCl for the duration of our experiments.

Three groups of rings were studied for each constrictor: 1) endothelium-intact, 2) endothelium-denuded, and 3) L-NAME–treated endothelium-intact rings. L-NAME (10^{-4} M) had no effect on resting tone nor did L-NAME alter the vasodilator response to sodium nitroprusside (10^{-6} M). We have demonstrated previously that L-NAME maximally inhibits EDRF release from rat aortic ring endothelium and from endothelial cell–vascular smooth muscle cocultures at a concentration equal to that used in the current study.[17] To confirm L-NAME inhibition of EDRF in the current studies, rings were tested for their response to the EDRF-dependent dilator methacholine. None of the L-NAME–treated rings vasodilated to methacholine. Indomethacin 2.8×10^{-5} M (an inhibitor of cyclooxygenase metabolism of arachidonic acid) was present in all baths throughout all experiments in order to prevent formation of vasoactive prostanoid metabolites. At maximal plateau constriction, isoflurane was administered at 1, 2, and 3% concentrations for 10 min each, in a cumulative manner. This was achieved by adding isoflurane to the 95% O_2/5% CO_2 gas bubbling the tissue baths using an in-line Fortec® vaporizer (Orchard Park, NY).

Cyclic GMP Analysis

Soluble guanylyl cyclase determinations were made from rings prepared and equilibrated as described above. Rings were divided into two groups: endothelium-denuded and endothelium-intact. After preconstriction with phenylephrine (1×10^{-7} M) for 5 min, the rings were given either methacholine (1×10^{-6} M) as positive control or 1%, 2%, or 3% isoflurane for 8 min. The rings were then flash-frozen in dry-ice–cooled acetone and stored at −90° C for subsequent total protein and cyclic GMP determinations.

Cyclic GMP was extracted by homogenization of each ring in 1 ml 1 N HCl (0–5° C). After centrifugation at 2,200 rpm for 10 min, the supernatant was analyzed for cyclic GMP content by radioimmunoassay (I^{125} kit, Advanced Magnetics Inc., Cambridge, MA). Protein content was determined by dissolving the remaining pellet in 2 N NaOH and analyzing the total dissolved protein with a BioRad® Protein Assay kit (Richmond, CA).

Chemicals/Drugs

Indomethacin, L-phenylephrine HCl, L-NAME HCl, and acetyl-β-methylcholine HCl (methacholine) were obtained from Sigma Chemical Co. (St. Louis, MO). Sodium nitroprusside was obtained from Fischer Scientific (Pittsburgh, PA). All drugs were dissolved in buffer except indomethacin, which was dissolved in $NaHCO_3$ (150 mM, *p*H 8.3). Isoflurane was obtained from Anaquest (Madison, WI). Accurate vaporizer calibration was confirmed using Raman spectroscopy (RASCAL, Albion Labs, Salt Lake City, UT). Isoflurane concentrations in the tissue bath buffer were determined by gas chromatography. Vapor concentration after equilibration of a bath aliquot with a measured volume of air was compared to a standard curve of known concentrations obtained by vaporizing a measured volume of isoflurane (liquid) in a measured volume of air. We achieved 99.8% agreement comparing

the dialed vaporizer concentration to the bath concentration as determined by using the partition coefficient of isoflurane in Krebs' solution.[24]

Statistical Analysis

The data were plotted as mean ± SEM. For isometric tension studies, each point represents 18 rings from each of six animals (n = 6). Percent relaxation was determined by dividing isoflurane-induced relaxation (grams) from the stable KCl or phenylephrine plateau constriction by KCl or phenylephrine plateau constriction (grams) and multiplying by 100. For cyclic GMP assays, each point represents four rings (n = 4). The data were analyzed by analysis of variance with multiple-range testing (Newman Keuls test) where needed. $P < 0.05$ was accepted as significant.

Results

Isometric Tension Measurements

The absolute tension in grams after phenylephrine (EC_{50}) and KCl preconstriction is given in table 1. The EC_{50} concentration of phenylephrine was less for the endothelium-denuded and L-NAME–treated rings (5×10^{-8} M) than for the endothelium-intact rings (1×10^{-7} M). There were no significant differences in total tension among the phenylephrine-constricted rings among the three groups. In the KCl-constricted rings there was a small but significant ($P < 0.05$) difference in absolute tension generated in the endothelium-intact rings *versus* the endothelium-denuded but not the L-NAME–pretreated rings. In the KCl-constricted rings, 1%, 2%, and 3% isoflurane produced a dose-dependent relaxation ($P < 0.05$). There were no significant differences in the isoflurane-induced relaxation of any of the KCl-constricted groups (endothelium-intact, endothelium-denuded, or L-NAME–treated) at any concentration of isoflurane (fig. 1). In the phenylephrine-constricted rings, isoflurane also produced a dose-dependent relaxation among all groups studied ($P < 0.05$). In addition, isoflurane 2% and 3% caused significantly ($P < 0.01$) less relaxation in the intact *versus* both the denuded and L-NAME–treated groups (fig. 2).

TABLE 1. Absolute Active Tension (g) of Vascular Rings Prior to Isoflurane Administration

Drug	Endothelium-intact	Endothelium-denuded	L-NAME-treated
Phenylephrine (1×10^{-7} M)	1.01 ± 0.16	—	—
Phenylephrine (5×10^{-8} M)	—	1.30 ± 0.14	1.32 ± 0.17
KCl (4×10^{-2} M)	1.92 ± 0.12*	1.40 ± 0.17	1.61 ± 0.17

Drug concentrations are those that provided the 50% maximal contractile response (EC_{50}) for each vessel condition. Values are expressed as mean ± SEM; n = 6 animals (18 rings).

* $P < 0.05$ for KCl intact *versus* KCl denuded.

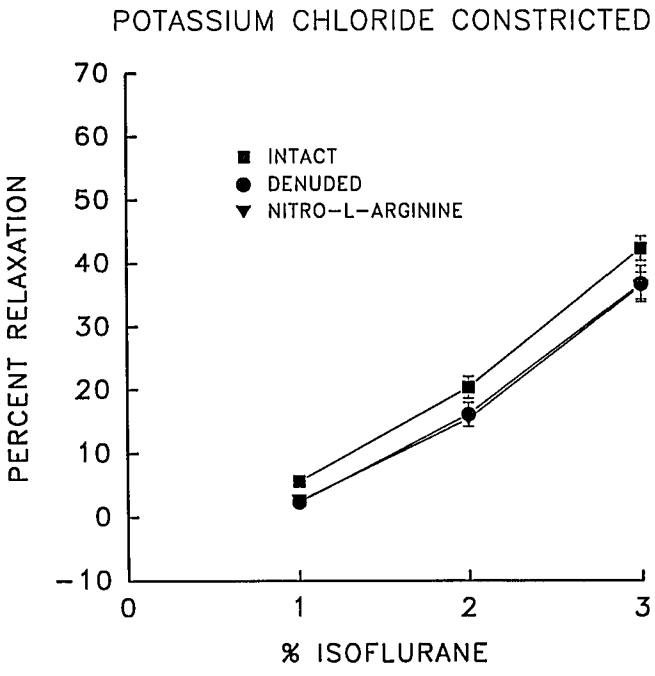

FIG. 1. Dose-dependent effect of isoflurane on isometric tension measurements in the potassium chloride–constricted rings. Intact = endothelium present; denuded = no endothelium; nitro-L-arginine = L-NAME–treated endothelium intact. Values expressed are mean ± SEM. Each point represents 18 rings from each of six animals (n = 6).

Cyclic GMP Data

Isoflurane 1%, 2%, and 3% had no effect on cyclic GMP content of endothelium-intact or endothelium-denuded vessels (fig. 3). In comparison, methacholine (1×10^{-6} M), an endothelium-dependent vasodilator, increased cyclic GMP concentration of endothelium-intact vascular rings significantly ($P < 0.001$) above control (fig. 3).

Discussion

Isoflurane vasodilates most vascular beds, but the mechanism of vasodilation has been unclear. The current data provide evidence for isoflurane as a direct vascular smooth muscle vasodilator independent of endothelium-related factors. By using the specific inhibitor of EDRF synthase, L-NAME, to block EDRF production as well as by mechanically denuding the endothelium, we created a model for differentiating EDRF-dependent relaxation from that of direct vascular smooth muscle relaxation. If isoflurane stimulates EDRF production, isoflurane would be expected to cause a greater vasodilation in endothe-

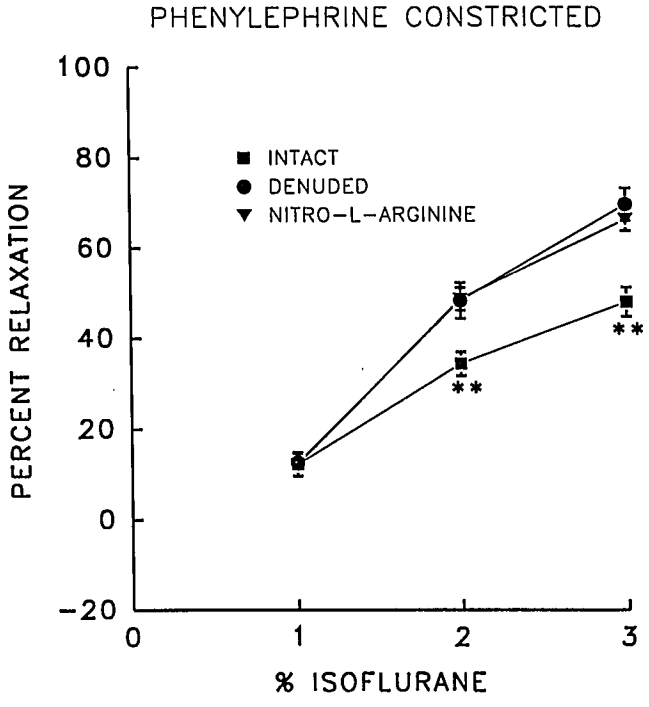

FIG. 2. Dose-dependent effect of isoflurane on isometric tension measurement in the phenylephrine constricted rings. Intact = endothelium present; denuded = no endothelium; nitro-L-arginine = L-NAME–treated endothelium intact.* $P < 0.05$ (endothelium-intact response compared to endothelium-denuded and compared to L-NAME–treated endothelium-intact rings). Values are expressed as mean ± SEM. Each point represents 18 rings from each of six animals (n = 6).

lium-intact rings than in rings in which the endothelium has been removed or in rings in which the ability to produce EDRF has been eliminated by the specific EDRF synthase inhibitor L-NAME. The removal of endothelium or the inhibition of EDRF had no effect on the vasodilation induced by 1%, 2%, or 3% isoflurane on KCl-preconstricted rings. However, in phenylephrine-preconstricted rings, 2% and 3% isoflurane induced a greater degree of relaxation in the endothelium-denuded and L-NAME–treated rings compared to the endothelium-intact rings. This effect is opposite of that expected if isoflurane stimulated the release of an endothelium-dependent dilator. Rather, this action is consistent with the stimulation of an endothelium-derived vasoconstrictor or the inhibition of an endothelium-derived vasodilator.

We believe the latter to be most likely. It is probable that the decreased relaxation in endothelium-intact rings is due to an isoflurane-induced inhibition of phenylephrine-stimulated EDRF release. Phenylephrine, unlike KCl, has been shown to modulate its direct vascular smooth muscle constriction with the simultaneous production of EDRF, a vasodilator, from the endothelium.[22-23] Inhibition of this phenylephrine-stimulated EDRF production by isoflurane in endothelium-intact rings would cause a relative constriction compared to endothelium-denuded or L-NAME–treated rings, where no EDRF is present. The fact that rings treated with L-NAME, a specific EDRF inhibitor, responded to isoflurane in the same manner as endothelium-denuded rings strongly suggests that isoflurane is inhibiting EDRF production or action and is not acting to inhibit an endothelium-derived vasoconstrictor. Consistent with this explanation, we have observed that isoflurane, like halothane,[25-26] is a potent inhibitor of EDRF production.

Because EDRF activates soluble guanylyl cyclase, and because it has been demonstrated that cyclic GMP accumulation directly correlates with vascular smooth muscle relaxation by EDRF,[12,14,16] we examined isoflurane's ability to stimulate vascular smooth muscle cyclic GMP accumulation. Methacholine, an EDRF-dependent dilator, caused significant increases in vascular smooth muscle content of cyclic GMP. Isoflurane 1%, 2%, and 3% did not cause accumulation of cyclic GMP in either endothelium-intact or endothelium-denuded rings, demonstrating that vasodilation by isoflurane does not involve cyclic GMP–mediated mechanisms.

Flynn *et al.*[18] evaluated endothelium-denuded and N^G-mono-methyl-L-arginine–treated canine cerebral vessels preconstricted with serotonin and challenged with isoflurane. They found that the dose-dependent vasodilation

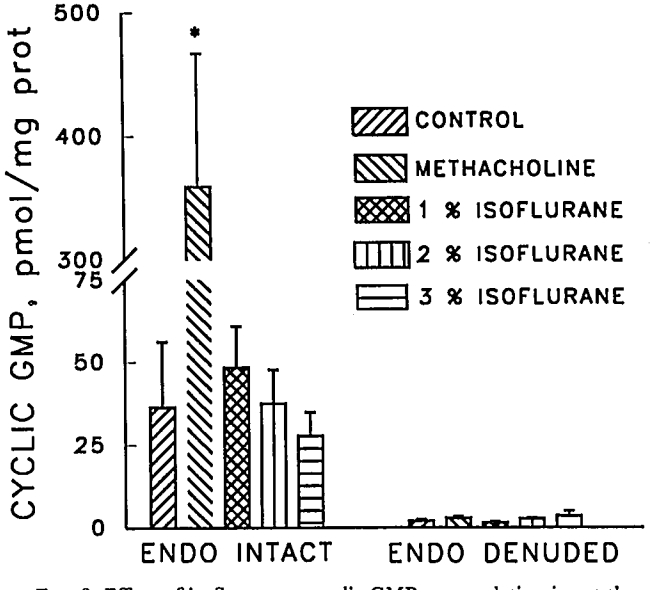

FIG. 3. Effect of isoflurane on cyclic GMP accumulation in rat thoracic aorta. Endo intact = endothelium intact; endo denuded = endothelium denuded; prot = protein. Values expressed as mean ± SEM; n = 6. *$P < 0.001$ *versus* control.

by isoflurane was endothelium independent. Jensen *et al.*[19] also found dose-dependent, non–endothelium-mediated vasodilation by isoflurane in rabbit cerebral arteries. Stone and Johns[20] investigated the vasodilating response to isoflurane in phenylephrine-preconstricted rat thoracic aorta. When administered slowly at concentration increments of 0.5%, isoflurane at lower concentrations produced vasoconstriction in endothelium-intact vessels but vasodilation in denuded vessels. Once higher concentrations were achieved, they noted endothelium-independent vasodilation in both groups. In the current studies, when anesthetics were administered in larger increments, we observed no overall vasoconstriction at any concentration but did note occasional transient vasoconstriction in the endothelium-intact rings at 1% and 2% isoflurane.

In contrast to the above studies, Blaise *et al.*[21] indirectly suggested that isoflurane may stimulate the release of an endothelium-dependent dilator, possibly EDRF. They reported that isoflurane, studied at 2.3% concentration only, attenuated phenylephrine, prostaglandin $F_2\alpha$, and serotonin-induced dose-dependent vasoconstriction of dog coronary arteries to a greater degree when the endothelium was intact than when it was denuded. The extent of this difference between endothelium-intact and endothelium-denuded rings varied widely with the specific vasoconstrictor studied.[21]

The discrepancy between our results and those of Blaise *et al.*[21] could relate to differences in the species or vascular bed studied or to the indirect manner in which their studies were performed. The current experiments, which failed to demonstrate an endothelial component of isoflurane-induced vasodilation, studied the direct vasodilating actions of isoflurane over a wide range of concentrations. In addition to studying endothelium-intact and endothelium-denuded vessels, we clearly ruled out EDRF production by isoflurane through the use of the specific EDRF inhibitor L-NAME and by the measurement of cyclic GMP. Because the pathway for EDRF production is highly conserved across species and vascular beds, it is unlikely that differences in species or vessel type[27–28] account for the discrepancies between the data of Blaise *et al.*[21] and those of Jensen *et al.*,[19] Flynn *et al.*,[18] Stone and Johns,[20] and our own. We chose the rat thoracic aorta as our model because it has been studied extensively and has been well demonstrated to contain all components of the EDRF pathway.

In summary, isoflurane produced dose-dependent vasodilation of endothelium-intact, endothelium-denuded, or L-NAME–treated rat aortic vascular rings preconstricted with either phenylephrine or KCl. Vasodilation by isoflurane is not mediated by EDRF, and the process does not involve the production of cyclic GMP.

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