Anesthesiology
76:440–447, 1992

# *Selective Impairment of Endothelium-dependent Relaxation by Sevoflurane: Oxygen Free Radicals Participation*

*Kazu-ichi Yoshida, D.D.S.,* Eiichiro Okabe, D.D.S., Ph.D.†*

**To determine whether sevoflurane alters endothelium-mediated vasodilation of vascular smooth muscle, isolated ring preparations of canine mesenteric arteries were suspended for isometric tension recordings in modified Krebs-Ringer bicarbonate solution at 37° C. Following contraction with norepinephrine, cumulative concentration-response curves were generated using endothelium-dependent vasodilators (acetylcholine, bradykinin, and calcium ionophore A23187) or nitroglycerin. The relaxation produced by acetylcholine, bradykinin, or A23187 was impaired by sevoflurane (2.3 and 4.6 vol%); sevoflurane did not affect relaxation caused by nitroglycerin, which, in these vessels, acts by an endothelium-independent mechanism. Under the same experimental conditions as those used for the concentration–response relationship, electron spin resonance spin-trapping with 5,5-dimethyl-1-pyrroline N-oxide verified generation of hydroxyl radical from the sevoflurane-delivered bathing media; the generation of hydroxyl radical was inhibited by superoxide dismutase, a scavenger of superoxide anion radical, or by the powerful iron chelator deferoxamine. Furthermore, sevoflurane-induced impairment of the relaxation caused by the endothelium-dependent vasodilators used was significantly decreased by superoxide dismutase. These results indicate that superoxide anion radical and/or closely related species of oxygen free radicals, possibly hydroxyl radical, are involved in the observed effect of sevoflurane. We propose that sevoflurane selectively impairs endothelium-dependent relaxation in canine mesenteric arteries by an oxygen free radical mechanism, mainly due to inactivation of endothelium-derived relaxing factor. (Key words: Anesthetics, volatile: sevoflurane. Artery: mesenteric. Endothelium: endothelium-derived relaxing factor. Measurement technique: electron spin resonance. Oxygen free radicals: hydroxyl radical; superoxide anion. Pharmacology: acetylcholine; bradykinin; calcium ionophore A23187; deferoxamine; nitroglycerin.)**

SEVOFLURANE is a fluorinated volatile anesthetic agent that provides rapid induction and recovery consistent with its low blood solubility.[1–3] The drug appears to be similar to the other potent halogenated inhalational anesthetics in its ability to depress the cardiovascular system.[2] With regard to the effects of volatile anesthetics, Muldoon *et al.*[4] suggested an effect of halothane on the synthesis, release, or transport of the endothelium-derived relaxing factor (EDRF) in rabbit aorta and canine femoral and carotid arteries, and proposed that halothane interferes with EDRF-mediated relaxation of vascular smooth muscle. Furthermore, Stone and Johns[5] have shown that at concentrations up to 4%, enflurane and isoflurane cause vasoconstriction through inhibition of basal EDRF production and/or stimulation of the release of an endothelium-derived constricting factor.

Endothelial cells control or modify the reactivity of vascular smooth muscle in mammalian blood vessels.[6–8] Furchgott and Zawadzki[8] reported that acetylcholine (ACh) induces the endothelial production of a diffusible potent smooth muscle relaxant, termed EDRF, in isolated *in vitro* preparations of arteries. Subsequent *in vitro* studies have demonstrated that EDRF production may also be induced by many other vasoactive substances, such as bradykinin, calcium ionophore A23187, ADP, and ATP[9,10] not only in arteries but also in veins.[11] The mechanism by which EDRF causes the vascular relaxation is associated with increased levels of guanosine 3′,5′-cyclic monophosphate (cGMP) in vascular smooth muscle resulting from activation of soluble guanylate cyclase.[12]

The superoxide anion radical ($\cdot O_2^-$) is capable of inhibiting the action of EDRF, as observed by Griffith *et al.*[13] that EDRF inhibitors such as phenidone, BW755C, dithiothreitol, and hydroquinone inhibit the action of EDRF released from the endothelial cells; the inhibitory action of these compounds is attenuated by concomitant application of superoxide dismutase (SOD). This suggests that EDRF inhibitors used can inactivate EDRF *via* generation of $\cdot O_2^-$. Furthermore, this postulate was confirmed by demonstrating that another generator of $\cdot O_2^-$, pyrogallol, inhibits the action of EDRF and that cytochrome c, an $\cdot O_2^-$ scavenger, potentiates the action of EDRF.[14]

Although sevoflurane has only recently been used clinically in Japan, no reports have been made concerning the mechanism of action of sevoflurane on endothelium-dependent vascular relaxation. Therefore, we designed the following study in an attempt to gain insight into the effect of sevoflurane on EDRF-dependent relaxation (produced by ACh, bradykinin, and calcium ionophore A23187) and on EDRF-independent relaxation (produced by nitroglycerin). Attempts were also made using electron spin resonance (ESR) spectrometry, a technique permitting the direct study of oxygen free radicals, to test the hypothesis that oxygen free radical mechanism contributes

* Instructor of Anesthesiology.

† Associate Professor of Pharmacology.

Received from the Departments of Anesthesiology and Pharmacology, Kanagawa Dental College, Yokosuka, Kanagawa, Japan. Accepted for publication November 11, 1991. Supported in part by a Grants-in-Aid for Science Research from Ministry of Education, Science and Culture of Japan and by Kanagawa Odontological Society Academic Development Fund.

Address reprint requests to Dr. Okabe: Department of Pharmacology, Kanagawa Dental College, 82 Inaoka-Cho, Yokosuka, Kanagawa 238, Japan.

to the effect of sevoflurane. In this paper, we report that the action of EDRF released from endothelial cells is inhibited directly by sevoflurane due to oxygen free radicals generated from sevoflurane itself.

## Materials and Methods

### Vessel Collection, Preparation, and Isometric Tension Recordings

In accordance with our institutional Animal Care Committee guidelines, mesenteric arteries were taken from mongrel dogs of either sex (7–15 kg) after exsanguination during anesthesia with sodium pentobarbital (30 mg/kg intravenously). Fat and other nonvascular tissues were gently dissected off of the blood vessels, which were cut into rings (2–3 mm in length, 1–3 mm intimal diameter) without disturbing the intimal layer after placement in cold modified Krebs-Ringer solution (control solution) of the following composition (in millimolar): 0.05 indomethacin to prevent volatile anesthetics-induced release of a vasodilating prostanoid from endothelium,[5] 128.0 NaCl, 4.9 KCl, 1.2 $MgCl_2$, 1.6 $CaCl_2$, 14.8 $NaHCO_3$, 1.18 $NaH_2PO_4$, 10.0 dextrose, and 0.026 calcium disodium ethylenediaminetetraacetic acid (*p*H 7.4). Rings with endothelium were prepared from adjacent segments of the same vessel.

The rings were suspended in a 20-ml water-jacketed tissue bath (37° C) and equilibrated for 120 min in control solution continuously aerated with 95% $O_2$–5% $CO_2$. The solution was changed at 15-min intervals during equilibration. During this time, the rings were stretched to a final tension of 2.0 g.[15] Tension development was measured with an isometric force transducer (Nihon Kohden, TB-612-T, Tokyo) and recorded using an amplifier (Nihon Kohden, AP-601-G, Tokyo) attached to a recorder (Nihon Kohden, PJ-691-G, Tokyo).

Isolated blood vessels have been shown to exhibit little or no active tension; therefore, for the study of smooth muscle relaxation, tension first needs to be induced with a vasoconstrictor. The concern raised is whether or not norepinephrine, when used, is inactivated by sevoflurane. However, sevoflurane at 2.3 and 4.6 vol% 1 h after initiation of this anesthetic delivery attenuated the phenylephrine ($10^{-7}$ to $10^{-3}$ M)-evoked contraction of the ring preparations, but not norepinephrine ($10^{-7}$ to $10^{-4}$ M)-evoked contraction, with rightward shift of the concentration–contraction curves. This ensures that norepinephrine is not inactivated by sevoflurane in our system, and suggests that the differential inhibition by sevoflurane, like enflurane,[15] may be a consequence of its preferential interference with postjunctional $\alpha_1$ adrenoceptors in the vascular smooth muscle of mesenteric arteries, on the one hand, and to the relatively higher affinity of phenylephrine for $\alpha_1$ compared to $\alpha_2$ adrenoceptors,[16] on the other. Thus, relaxation responses to ACh, bradykinin, calcium ionophore A23187, and nitroglycerin were determined in rings contracted to a stable plateau tension by the addition of norepinephrine at concentrations that elicited approximately 50% of the maximum tension that develops in response to norepinephrine. Concentration–response curves were determined by the method of stepwise cumulative addition of ACh ($10^{-7}$ to $10^{-4}$ M), bradykinin ($10^{-7}$ to $10^{-4}$ M), A23187 ($10^{-7}$ to $10^{-5}$ M), and nitroglycerin ($10^{-8}$ to $10^{-5}$ M) to the bathing media in the presence or absence of sevoflurane. Three ring preparations (for control and 2.3 and 4.6 vol% sevoflurane) obtained from the same vessel were studied in parallel, and one concentration–response curve was made per ring preparation; different rings taken from the same dog were used for different drugs. Preliminary experiments demonstrated that norepinephrine-induced contractions and the relaxations produced by the vasodilators used in the present study were stable for 5 h in endothelium-intact and denuded vessels in the presence or absence of indomethacin in our experimental system.

### Sevoflurane Delivery

Sevoflurane was delivered from a vaporizer (Ohmeda, Sevotec 3, Steeton, England) in the $O_2$–$CO_2$ mixture aerating the bathing media; the gas was humidified prior to entering the four-serial tissue baths. The concentration in the resulting gas mixture was monitored continuously by an anesthetic gas monitor (WTI, AG101, Amsterdam) calibrated daily with a sevoflurane mixture. The tissue bath was covered with plastic to prevent the aerating gas from immediately escaping into the atmosphere. To determine the time of equilibration of sevoflurane, the concentration of sevoflurane in bathing media was measured by gas chromatography (Shimazu, GC-9A, Kyoto).[17] It was found that equilibration of the bathing media with sevoflurane was complete within 30 min and that stable bath concentrations were achieved at a sevoflurane–$O_2$–$CO_2$ mixture flow rate of 300 ml/min of gas flow through the fritted glass disks at the bottom of the four-serial bath chambers. The bath anesthetic concentrations after a 30-min equilibration for 2.3 and 4.6 vol% sevoflurane were 4.43 ± 0.36 and 9.02 ± 0.77 mM (mean ± SEM, n = 3), respectively.

On the basis of this, the preincubation conditions used to assess the effect of sevoflurane on the vessel preparations were chosen. We used 1 h of preincubation; all experiments in the presence of sevoflurane were carried out 1 h after the initiation of the anesthetic delivery. Time-matched control studies in the absence of sevoflurane were also performed.

### Spin Trapping and Electron Spin Resonance Spectrometry

The spin trapping agent that has been most often used to detect oxygen-centered free radicals has been 5,5-dimethyl-1-pyrroline N-oxide (DMPO) both because of the relative efficiency with which DMPO is capable of trapping $\cdot O_2^-$ and hydroxyl radical ($\cdot$OH) and because of the stability of the resulting spin adducts.[18-20] Therefore, the ESR spin trapping studies were performed using DMPO as the spin trap. Desired reaction mixtures (0.2 ml) were prepared in glass tubes and transferred to a flat quartz ESR cuvette (0.3 mm thickness), which was in turn fixed to the cavity of the ESR spectrometer (JEOL JES FE-1X with 100-kHz field modulation, X-band, Tokyo). Sequential ESR scans were started 45s after the addition of 15 $\mu$l DMPO to sevoflurane (4.6 vol%)-saturated bathing media. The ESR spectra of DMPO-OH, the spin-trapped adduct of $\cdot$OH, was identified from the hyperfine parameters. ESR spectrometer settings were modulation amplitude 0.1 mT (100 kHz), receiver gain 5 × 100, scan range 5 mT, scanning time 2 min, time constant 0.1 s, microwave power 8 mW, and magnetic field 334.9 mT, at room temperature. To quantitate the DMPO spin adducts detected, the manganese oxide standard ESR spectrum was obtained.

Inasmuch as it appears that the rather long experimental period designed in the present study causes some inactivation of SOD, the stability of added SOD (120 U/ml) during the experiments was investigated. The SOD activity in the sevoflurane (4.6 vol%)-saturated media in the presence of the ring preparations was determined by an ESR spin-trapping technique as described previously.[21] The activity was stable for 5 h (range 130–151 SOD U/ml), indicating that our experimental condition is a valid means for assessing the effect of SOD.

### Drugs

The following drugs were used: sevoflurane (Maruishi Pharmaceutical, Osaka), DL-norepinephrine hydrochloride (Sigma, St. Louis, MO), acetylcholine chloride (Sigma), bradykinin (Sigma), calcium ionophore A23187 (Sigma), nitroglycerin (Nihon Kayaku, Tokyo), indomethacin (Sigma), SOD (from bovine blood, 2,800 U/mg protein, Sigma), and deferoxamine (Desferal mesylate, Ciba-Geigy). All of these drugs except sevoflurane, indomethacin, and A23187 were dissolved in pure water and diluted in the Krebs-Ringer solution gassed with a mixture of 95% $O_2$–5% $CO_2$ before being added to the tissue bath. Indomethacin stock solution was prepared by dissolving three parts indomethacin and one part sodium bicarbonate in distilled water. A23187 was dissolved in dimethyl sulfoxide ($2 \times 10^{-3}$ M final concentration). The addition of 100 $\mu$l of this stock solution gives $10^{-5}$ M final concentration in the tissue bath. We have confirmed that this amount of dimethyl sulfoxide has no detectable effect in our experimental system. DMPO (Mitsui Toatsu Chemicals, Tokyo, 99–100% pure) was used as the spin trap.

### Analysis of Data

All data are expressed on the basis of arterial weights of 8.0 mg to correct for variations in tissue weight. The mean (± SEM) weight of the tissue was 8.21 ± 3.95 mg. Two sets of statistical comparisons were made. Student's $t$ test for paired samples were used when comparing two populations to each other. Comparisons of subsequent intervention to controls were made using a one-way analysis of variance, followed by a Duncan's multiple-range test.[22] Differences were considered significant when $P < 0.05$.

## Results

We first determined whether or not endothelium-dependent relaxation induced by the vasodilators used is altered by sevoflurane. In order to determine the effect of sevoflurane, it is necessary to allow the endothelium-intact vessels to be exposed to sevoflurane for > 30 min, as stated in "Materials and Methods." The final experimental condition chosen was 1 h of preincubation of sevoflurane. The effect of sevoflurane 1 h after the initiation of this anesthetic delivery on endothelium-dependent relaxations is shown in figures 1 and 2. In time-matched control experiments (fig. 1A), ACh, bradykinin, and A23187 relaxed the norepinephrine-contracted ring preparations in a concentration-dependent manner. Sevoflurane (2.3 and 4.6 vol%) significantly attenuated these responses with rightward shift of the concentration–relaxation curves and with a decrease in the maximum relaxation (figs. 2A–2C).

Nitroglycerin, an endothelium-independent vasodilator, caused vascular relaxation of norepinephrine-contracted ring preparations; nitroglycerin-induced relaxation was concentration-dependent, and sevoflurane (2.3 and 4.6 vol%) did not significantly alter this relaxation (fig. 2D). ACh, bradykinin, A23187, or nitroglycerin produced relatively rapid relaxation of the ring preparations in a concentration-dependent manner; thus, the experiments for the concentration–response relationship in the presence of sevoflurane were completed within 30 min. The endothelium-dependent vasodilators used produced maximal responses of about the same magnitude, in the absence of sevoflurane, before and after this series of experiments (data not shown).

Recently, oxygen free radicals have been shown to se-

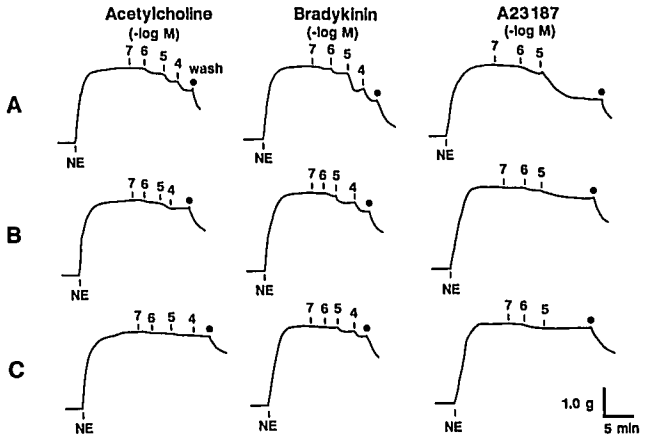


FIG. 1. Representative recordings of relaxation of precontracted (3 × $10^{-6}$ M norepinephrine) endothelium-intact mesenteric artery rings induced by acetylcholine, bradykinin, and calcium ionophore A23187 in time-matched control experiments (*A*) and 1 h after the initiation of 2.3 vol% (*B*) or 4.6 vol% sevoflurane delivery (*C*). After a stable response to norepinephrine-induced contraction occurred, the vessel was relaxed with increasing concentrations of the endothelium-dependent vasodilators used in the presence or absence of sevoflurane delivery.

lectively impair endothelium-dependent relaxation as opposed to endothelium-independent relaxation in intact vessels.[13,14,23] If it is correct that sevoflurane impairs endothelium-dependent relaxation of the ring preparations in part by a mechanism dependent on oxygen free radicals, the effect of sevoflurane would be inhibited by radical scavengers. We tested this hypothesis by adding SOD, a scavenger of $\cdot O_2^-$, to our experimental system. Figure 3 shows the results of this study. As expected, SOD, when added to the tissue bath before the initiation of the sevoflurane (2.3 and 4.6 vol%) delivery, significantly inhibited the sevoflurane-induced impairment of the relaxation of the ring preparations to $10^{-6}$ M ACh (fig. 3A) or $10^{-6}$ M bradykinin (fig. 3B), or to 3 × $10^{-6}$ M A23187 (fig. 3C), a concentration that produces approximately 50% of the maximum relaxation. SOD had no significant effect on the concentration–response curves of the ring preparations to the endothelium-dependent vasodilators used (fig. 4). Furthermore, heat-inactivated SOD (inactivated by boiling at 100° C for 20 min) had no effect on the observed sevoflurane-induced impairment of the relaxation (data not shown), suggesting that $\cdot O_2^-$ and/or a closely related species of oxygen free radical are involved in the effect of sevoflurane.

The identification of the oxygen free radicals that are responsible for the effect of sevoflurane rests entirely on the use of SOD. Therefore, the spin-trapped adduct(s) produced by sevoflurane were examined, by using a highly sensitive ESR spectroscopy and the spin trap DMPO. The 1:2:2:1 quartet (the hyperfine splittings were $A_N = A_H$ = 1.49 mT), characteristic of DMPO-OH,[24] was observed under the conditions in which 4.6 vol% sevoflurane was delivered from a vaporizer in the $O_2$–$CO_2$ mixture aerating the bathing media for 0.5–5 h in the absence of the ring preparations (fig. 5), and its intensity increased, in a time-dependent manner (fig. 5A). We next examined the effects of SOD and deferoxamine on DMPO-OH adduct produced 5 h after the initiation of the sevoflurane delivery (fig. 5B). The addition of SOD (120 U/ml) or deferoxamine (0.05 mM) before the sevoflurane delivery in-

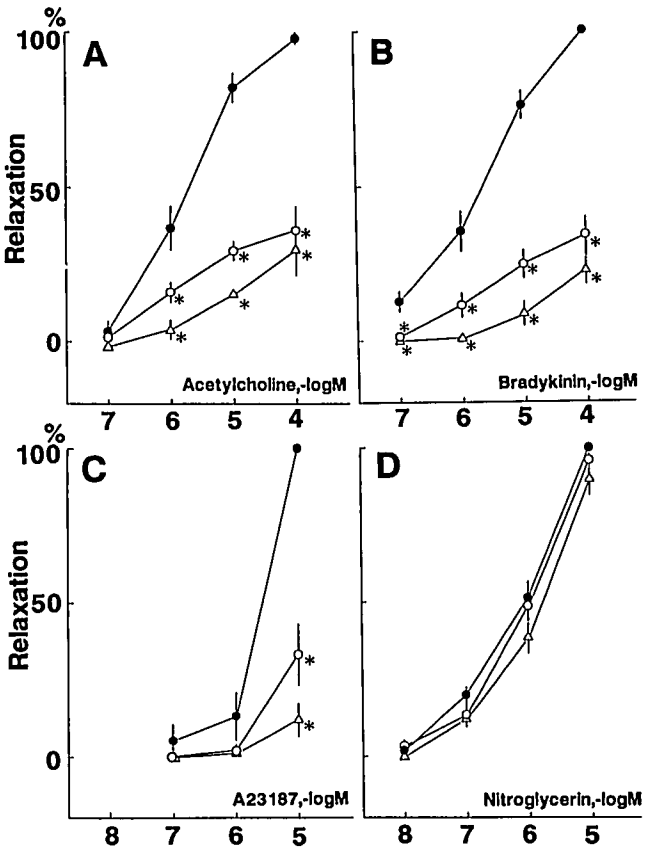


FIG. 2. Effects of sevoflurane (● = time-matched control; ○ = 2.3 vol%; △ = 4.6 vol%) on the relaxing response to acetylcholine (*A*), bradykinin (*B*), A23187 (*C*), or nitroglycerin (*D*) in the endothelium-intact mesenteric artery rings. Mean values of the precontractile tensions produced by 3 × $10^{-6}$ M norepinephrine in these experiments were 2.85 ± 0.13 g (*A*, n = 6), 2.91 ± 0.21 g (*B*, n = 6), 2.64 ± 0.29 g (*C*, n = 5), and 2.89 ± 0.31 g (*D*, n = 6), respectively; n refers to the number of dogs from which the mesenteric artery was taken. The maximum relaxations induced by $10^{-4}$ M acetylcholine (1.14 ± 0.31 g), $10^{-4}$ M bradykinin (1.26 ± 0.41 g), $10^{-5}$ M A23187 (0.89 ± 0.31 g), and $10^{-5}$ M nitroglycerin (1.57 ± 0.49 g) in time-matched control experiments are taken as 100%, and other data are plotted in relation to it. The points represent the mean and vertical lines show SEM. *Significantly ($P < 0.05$) different from the corresponding value for control.

hibited the formation of an observable DMPO-OH adduct (fig. 5B, c and d). Deferoxamine by itself had no detectable effect on this system (fig. 5C).

## Discussion

Rubanyi and Vanhoutte[25] suggested that the depression of the ACh-induced relaxation of the canine coronary artery by $\cdot O_2^-$ could be explained by inactivation of the EDRF during its diffusion toward the vascular smooth muscle, and demonstrated that the inactivation of the EDRF may not be caused solely by $\cdot O_2^-$, but other radicals may also contribute to it. Further evidence in support of the possibility that $\cdot O_2^-$ destroys EDRF comes from observations in which the effluent from chromatographic columns, packed with microcarrier beads covered with cultured endothelial cells from the porcine aorta, was bioassayed with strips (without endothelium) of the thoracic aorta of the rabbit.[26] This study demonstrated that SOD stabilizes the EDRF(s) released in response to bradykinin.

The major findings of the present study are as follows. First, sevoflurane, when delivered to the bathing media under the conditions used, produced DMPO-OH adduct, in a time-dependent manner (0.5–5 h); the adduct obtained 5 h after the initiation of the sevoflurane delivery was altered by SOD or deferoxamine (fig. 5). In addition, sevoflurane impaired the endothelium-dependent relaxation of the ring preparations, which was SOD-inhibitable (fig. 3). It has been suggested that the chemical reactivity of $\cdot O_2^-$ is quite low[27,28] but others[29] argue that reaction

$$\cdot O_2^- + H_2O_2 \rightarrow O_2 + \cdot OH + OH^- \quad (1)$$

is too slow to compete with the dismutation reaction

$$\cdot O_2^- + \cdot O_2^- + 2H^+ \xrightarrow{SOD} H_2O_2 + O_2 \quad (2)$$

Therefore, our results suggest that $\cdot O_2^-$ is involved in a mechanism that may cause the impairment produced by sevoflurane of the endothelium-dependent relaxations, inasmuch as the effect of sevoflurane was inhibited by SOD, which is a very specific enzyme.[30] Also, a strong possibility exists for the formation of $\cdot OH$ *via* Fenton and Haber-Weiss reactions, due to contaminant iron salts as shown below:

$$\cdot O_2^- + Fe^{3+} \rightarrow O_2 + Fe^{2+} \quad (3)$$

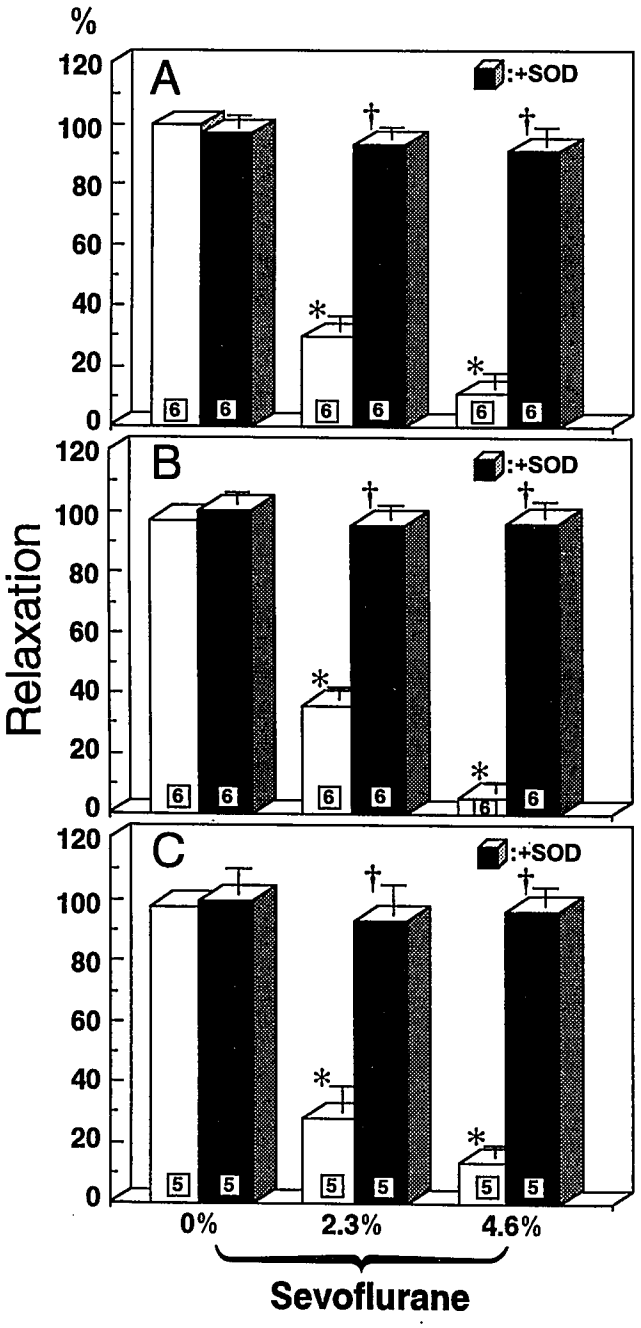


FIG. 3. Modification by sevoflurane of the relaxing response to acetylcholine (*A*), bradykinin (*B*), or A23187 (*C*) in the endothelium-intact mesenteric artery rings. Mean values of the precontractile tensions produced by $3 \times 10^{-6}$ M norepinephrine in these experiments were 2.58 ± 0.16 g (*A*, n = 6), 2.48 ± 0.21 g (*B*, n = 6), and 2.81 ± 0.24 (*C*, n = 5), respectively; n refers to the number of dogs from which the mesenteric artery was taken. Relaxations induced by $10^{-6}$ M acetylcholine (0.51 ± 0.11 g), $10^{-6}$ M bradykinin (0.59 ± 0.19 g), and $3 \times 10^{-6}$ M A23187 (0.56 ± 0.13 g) in the time-matched control experiments in the absence of SOD (0% sevoflurane) are taken as 100%. SOD (120 U/ml) was added to the tissue bath before the initiation of the sevoflurane (0, 2.3, and 4.6 vol%) delivery. Column heights are means; brackets indicate ± SEM. *Significantly ($P < 0.05$) different from the corresponding value measured under time-matched standard conditions in the absence of sevoflurane. †Significantly ($P < 0.05$) different from the corresponding value for sevoflurane alone.

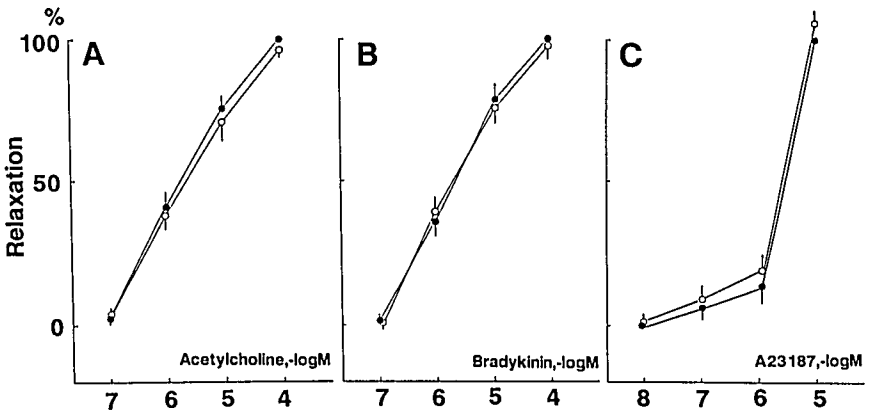


FIG. 4. Concentration–response curves to acetylcholine (*A*), bradykinin (*B*), and calcium ionophore A23187 (*C*) in the presence (O) or absence (●) of SOD. Mean values of the precontractile tensions produced by $3 \times 10^{-6}$ M norepinephrine in these experiments were 2.98 ± 0.21 g (*A*, n = 5), 2.81 ± 0.25 g (*B*, n = 6), and 3.00 ± 0.31 g (*C*, n = 5), respectively; n refers to the number of dogs from which the mesenteric artery was taken. The maximum relaxations induced by $10^{-4}$ M acetylcholine (1.42 ± 0.25 g), $10^{-4}$ bradykinin (1.61 ± 0.32 g), and $10^{-5}$ M A23187 (1.01 ± 0.29 g) in the absence of SOD are taken as 100%, and other data are plotted in relation to it. Experimental conditions were identical to those of time-matched control shown in figure 1A, except that SOD (120 U/ml) was added 1 h before the precontraction. The points represent the mean and vertical lines show SEM.

$$Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + \cdot OH \quad (4)$$

If $H_2O_2$ were directly involved in the effect of sevoflurane, SOD should not have protected as the main product of SOD is $H_2O_2$.[31]

Hence, the protective effect of SOD may be due to prevention of $\cdot O_2^-$-dependent reduction of $Fe^{3+}$ to $Fe^{2+}$ and thus inhibition of ·OH radical formation. Strong evidence for this possibility is provided by deferoxamine, which inhibited the sevoflurane-induced formation of DMPO-OH adduct (fig. 5B, d). In this regard, SOD has been shown to depress ·OH radical formation.[32,33] Furthermore, it is known that deferoxamine is a powerful iron chelator and has poor reactivity with ·OH radical at concentrations below 1 mM.[34,35] It tightly binds to Fe(III) and, therefore, cannot be reduced to Fe(II) by $\cdot O_2^-$ for ·OH radical production.[36] It is well established that SOD will enhance basal, ACh, and bradykinin EDRF production and their subsequent relaxation of vascular smooth muscle.

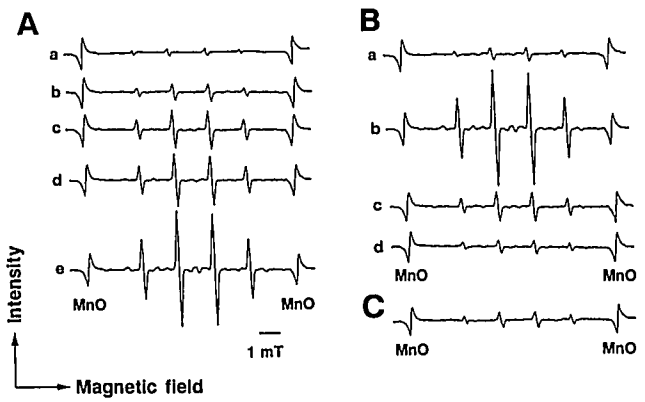


FIG. 5. Electron spin resonance (ESR) spectra of 5,5-dimethyl-1-pyrroline N-oxide (DMPO) spin adducts obtained in the bathing media. *A*: Time course experiment of oxygen free-radical generation before (a) and after sevoflurane (4.6 vol%) was delivered to the bathing media aerated with 95% $O_2$–5% $CO_2$ for 0.5 h (b), 1 h (c), 2 h (d), and 5 h (e). Experimental conditions are as described under "Materials and Methods." *B*: ESR spectra of DMPO spin adducts observed at 5 h of sevoflurane (4.6 vol%) delivery in the absence (b) or presence of SOD (120 U/ml, c) and deferoxamine (0.05 mM, d). The ESR spectra formed under the time-matched standard conditions was also recorded (a). Experimental conditions are as described in *A* except that SOD or deferoxamine was added to the bathing media before the sevoflurane delivery. *C*: Time-matched (5 h) deferoxamine (0.05 mM)-control without sevoflurane. MnO = manganese oxide standard ESR spectrum.

Although we have not eliminated the possibility that the observed reversal of sevoflurane inhibition of the effect of endothelium-dependent vasodilators by the addition of SOD may merely reflect an enhanced release of EDRF independent of the $\cdot O_2^-$ produced by sevoflurane, this appears unlikely: when the ring preparations had been relaxed by ACh, bradykinin, or A23187, the relaxation was not enhanced by the addition of SOD under the experimental conditions of this study (fig. 4). The observed effect of sevoflurane on endothelium-dependent relaxations of ring preparations is thus due to the ability of this anesthetic to inactivate EDRF released from endothelial cells in response to ACh, bradykinin, or A23187 *via* the generation of $\cdot O_2^-$ and/or $\cdot O_2^-$-dependent formation of ·OH radical. If oxygen free radicals were the mechanism one might expect nitroglycerin relaxation to be affected, since nitroglycerin is broken down to nitric oxide, which would be inactivated by $\cdot O_2^-$. The nitric oxide formation from nitroglycerin occurs within the vascular smooth muscle cells but does not require the presence of endothelial cells.[37,38] Although evidence exists[39,40] to support this biochemical mechanism for nitrates, the subcellular location of the metabolic activation process has not been identified. Interpretation of our experimental data that sevoflurane had no effect on the relaxations

produced by nitroglycerin (fig. 2D) is that the subcellular site for nitroglycerin metabolism to nitric oxide in the smooth muscle cells may not be sensitive to oxygen free radicals generated from sevoflurane itself. This postulate is inferred from the observation that the generation of oxygen free radicals from the system consisting of autoxidizing dihydroxyfumarate/$Fe^{3+}$–ADP or of $H_2O_2/Fe^{2+}$ has no effect on nitroglycerin-induced relaxations of coronary vessels.[41]

The second major finding of this study is that sevoflurane selectively impaired endothelium-dependent relaxation produced by ACh and bradykinin to a similar degree (figs. 2A and 2B) but not endothelium-independent relaxation produced by nitroglycerin (fig. 2D). Since the effect of bradykinin is endothelium-dependent but is not mediated by muscarinic receptors, this effect of sevoflurane is not specific to muscarinic receptor activation. The present study was extended to include another endothelium-dependent vasodilator, the calcium ionophore A23187, which causes relaxation without acting *via* receptors. The A23187-induced relaxation is also impaired by sevoflurane (fig. 2C). Thus, it is possible that sevoflurane acts at a site distal to the ACh and bradykinin receptors, and that it interferes with non–receptor-mediated generation of EDRF. Both EDRF and nitroglycerin are reported to produce vascular relaxation by increasing the production of cGMP which inhibits the smooth muscle contractile process.[12] Since sevoflurane inhibits the action only of ACh, bradykinin, and A23187, and not that of nitroglycerin, its action may be at some site(s) between the endothelial site of interaction and the smooth muscle production of cGMP, possibly a released EDRF. In the present study, the experiments for the concentration–response relationship were completed 1–1.5 h after the initiation of the sevoflurane delivery (see "Results"), the experimental conditions sufficient to detect the DMPO-OH adducts (fig. 5). The interpretation of these findings is complicated by the assumption that $\cdot O_2^-$ and $\cdot OH$ generated by sevoflurane in the absence of DMPO will have an extremely short half-life and will not accumulate over time. However, the intensity of DMPO-OH adduct observed 45 s after the addition of DMPO to the sevoflurane-delivered bathing media at 0.5, 1, 2, and 5 h of the anesthetic delivery (DMPO accumulation of sevoflurane-induced oxygen free radicals for 45 s at each time) was increased, in a time-dependent fashion (fig. 5A), suggesting that the oxygen free radical production rate may be increased during sevoflurane delivery under the conditions used. Thus, it seems safe to conclude that the observed effect of sevoflurane on endothelium-dependent relaxations can be interpreted as indicative that oxygen free radical mechanism is involved. Inasmuch as the concentration of $\cdot O_2^-$ or $\cdot OH$ necessary to produce the inhibition of endothelium-dependent relaxations is unknown, we cannot rule out that a very small amount of oxygen free radicals not detectable by DMPO at earlier time points may be sufficient for EDRF inactivation.

Our laboratory has provided evidence that a major target organelle attacked by oxygen free radicals is the system that regulates calcium delivery (sarcoplasmic reticulum and sarcolemma) to the contractile proteins and not the contractile proteins *per se* in cardiac muscle.[42–44] In the endothelial cells, an increase in intracellular free calcium has been reported to be necessary for the release and/or production of EDRF. In this regard, Elliott and Schilling[45] have shown that the membrane-permeant oxidant *t*-butylhydroperoxide inhibits bradykinin-stimulated calcium flux pathway of pulmonary vascular endothelial cells, and postulated that the changes in calcium-dependent signal transduction may be the initial events associated with ultimate cell death. However, the depressed effect of endothelium-dependent vasodilators used was reversible after the experiments in the absence of sevoflurane, suggesting that oxygen free radicals, in the concentrations generated from sevoflurane in bathing media, may be able to destroy only released EDRF without damaging the endothelial cells.

In conclusion, these data suggest that sevoflurane selectively impairs endothelium-dependent relaxation as opposed to endothelium-independent relaxation in canine mesenteric arteries *via* inactivation of EDRF, and that the effect of sevoflurane is mediated by the generation of $\cdot O_2^-$ and/or a closely related species of oxygen free radicals, possibly $\cdot OH$. This action may contribute in part to the hemodynamic effects seen clinically during administration of sevoflurane. Clearly, our results raise the question of determining the sevoflurane-induced chemical reaction sequence leading to the generation of oxygen free radicals. There appears to be no obvious answer, and in the current literature, no consideration has been given to this problem.

The authors thank Professor M. Noguchi (Department of Anesthesiology), Professor H. Ito (Department of Pharmacology), and Professor E. Sugaya (Department of Physiology), Kanagawa Dental College, for useful discussions during the preparation of this paper.

## References

1. Bernard J-M, Wouters PF, Doursout M-F, Florence B, Chelly JE, Merin RG: Effects of sevoflurane and isoflurane on cardiac and coronary dynamics in chronically instrumented dogs. ANESTHESIOLOGY 72:659–662, 1990
2. Wallin RF, Regan BM, Napoli MD, Stern IJ: Sevoflurane: A new inhalational anesthetic agent. Anesth Analg 54:758–765, 1975
3. Holaday DA, Smith FR: Clinical characteristics and biotransformation of sevoflurane in healthy human volunteers. ANESTHESIOLOGY 54:100–106, 1981
4. Muldoon SM, Hart JL, Bowen KA, Freas W: Attenuation of endothelium-mediated vasodilation by halothane. ANESTHESIOLOGY 68:31–37, 1988

5. Stone DJ, Johns RA: Endothelium-dependent effects of halothane, enflurane, and isoflurane on isolated rat aortic vascular rings. ANESTHESIOLOGY 71:126–132, 1989
6. Van De Voorde J, Leusen I: Role of the endothelium in the vasodilator response of rat thoracic aorta to histamine. Eur J Pharmacol 87:113–120, 1983
7. Furchgott RF: Role of endothelium in responses of vascular smooth muscle. Circ Res 53:557–573, 1983
8. Furchgott RF, Zawadzki JV: The obligatory role of endothelial cells in the relaxation of arterial smooth muscle by acetylcholine. Nature 288:373–376, 1980
9. Chand N, Altura BM: Endothelial cells and relaxation of vascular smooth muscle cells: Possible relevance to lipoxygenases and their significance in vascular diseases. Microcirculation 1:297–317, 1981
10. Burnstock G, Kennedy C: A dual function for adenosine 5'-triphosphate in the regulation of vascular tone. Circ Res 58:319–330, 1986
11. Cross KS, EL-Sanadiki MN, Murray JJ, Mikat EM, McCann RL, Hagen P-O: Functional abnormalities of experimental autogenous vein graft neoendothelium. Ann Surg 208:631–638, 1988
12. Rapoport RM, Murad F: Agonist-induced endothelium-dependent relaxation in rat thoracic aorta may be mediated through cGMP. Circ Res 52:352–357, 1983
13. Griffith TM, Edwards DH, Lewis MJ, Newby AC, Henderson AH: The nature of endothelium-derived vascular relaxant factor. Nature 308:645–647, 1984
14. Moncada S, Palmer RMJ, Gryglewski RJ: Mechanism of action of some inhibitors of endothelium-derived relaxing factor. Proc Natl Acad Sci USA 83:9164–9168, 1986
15. Kobayashi Y, Yoshida K-I, Noguchi M, Wakasugi Y, Ito H, Okabe E: Effect of enflurane on contractile reactivity in isolated canine mesenteric arteries and veins. Anesth Analg 70:530–536, 1990
16. Polonia JJ, Paiva MQ, Guimaraes S: Pharmacological characterization of postsynaptic $\alpha$-adrenoceptor subtypes in five different dog arteries in vitro. J Pharm Pharmacol 37:205–208, 1985
17. Sato S: Determination of inhalation anesthetics in blood by equilibration method. Masui 14:461–471, 1965
18. Britigan BE, Rosen GM, Chai Y, Cohen MS: Do human neutrophils make hydroxyl radical? J Biol Chem 261:4426–4431, 1986
19. Cohen MS, Britigan BE, Hassett DJ, Rosen GM: Do humans neutrophils form hydroxyl radical? Evaluation of an unresolved controversy. Free Radic Biol Med 5:81–88, 1988
20. Ueno I, Kohno M, Mitsuta K, Mizuta Y, Kanegasaki S: Reevaluation of the spin-trapped adduct formed from 5,5-dimethyl-1-pyrroline-1-oxide during the respiratory burst in neutrophils. J Biochem (Tokyo) 105:905–910, 1989
21. Taga R, Okabe E: Hydroxyl radical participation in the in vitro effects of Gram-negative endotoxin on cardiac sarcolemmal Na,K-ATPase activity. Jpn J Pharmacol 55:339–349, 1991
22. Walpole RE: Introduction to Statistics. New York, Macmillan, 1974, pp 277–281
23. Rubanyi GM, Vanhoutte PM: Superoxide anions and hyperoxia inactivate endothelium-derived relaxing factor. Am J Physiol 250:H822–H827, 1986
24. Finkelstein E, Rosen GM, Rauckman EJ: Spin trapping of superoxide and hydroxyl radical: Practical aspects. Arch Biochem Biophys 200:1–16, 1980
25. Rubanyi GM, Vanhoutte PM: Oxygen-derived free radicals, endothelium, and responsiveness of vascular smooth muscle. Am J Physiol 250:H815–H821, 1986
26. Gryglewski RJ, Palmer RMJ, Moncada S: Superoxide anion is involved in the breakdown of endothelium-derived vascular relaxing factor. Nature 320:454–456, 1986
27. Freeman BA, Crapo JD: Biology of disease: Free radicals and tissue injury. Lab Invest 47:412–426, 1982
28. Halliwell B, Gutteridge JMC: Oxygen radicals and tissue damage, Advances in Studies on Heart Metabolism. Edited by Caldarera CM, Harris P. Bologna, Cooprativa Libraria Universitaria Editrice, 1982, pp 403–411
29. Thomas MJ, Mehl KS, Pryor WA: The role of the superoxide anion in the xanthine oxidase-induced autoxidation of linoleic acid. Biochem Biophys Res Commun 83:927–932, 1978
30. Fridovich I: The biology of oxygen radicals. Science 201:875–880, 1978
31. Okabe E, Kuse K, Sekishita T, Suyama N, Tanaka K, Ito H: The effect of ryanodine on oxygen free radical-induced dysfunction of cardiac sarcoplasmic reticulum. J Pharmacol Exp Ther 256: 868–875, 1991
32. Motohashi N, Mori I: Superoxide-dependent formation of hydroxyl radical catalyzed by transferrin. FEBS Lett 157:197–199, 1983
33. Kukreja RC, Okabe E, Schrier GM, Hess ML: Oxygen radical-mediated lipid peroxidation and inhibition of $Ca^{2+}$-ATPase activity of cardiac sarcoplasmic reticulum. Arch Biochem Biophys 261:447–457, 1988
34. Hoe S, Rowley DA, Halliwell B: Reactions of ferrioxamine and desferrioxamine with the hydroxyl radical. Chem Biol Interact 41:75–81, 1982
35. Halliwell B: Use of desferrioxamine as a "probe" for iron-dependent formation of hydroxyl radicals. Evidence for a direct reaction between desferal and the superoxide radical. Biochem Pharmacol 34:229–233, 1985
36. Gutteridge JMC, Richmond R, Halliwell B: Inhibition of the iron-catalysed formation of hydroxyl radicals from superoxide and lipid peroxidation by desferrioxamine. Biochem J 184:469–472, 1979
37. Ignarro LJ, Lippton H, Edwards JC, Baricos WH, Hyman AL, Kadowitz PJ, Gruetter CA: Mechanism of vascular smooth muscle relaxation by organic nitrates, nitrites, nitroprusside and nitric oxide: Evidence for the involvement of s-nitrosothiols as active intermediates. J Pharmacol Exp Ther 218:739–749, 1981
38. Murad F: Cyclic guanosine monophosphate as a mediator of vasodilation. J Clin Invest 78:1–5, 1986
39. Feelisch M, Noack EA: Correlation between nitric oxide formation during degradation of organic nitrates and activation of guanylate cyclase. Eur J Pharmacol 139:19–30, 1987
40. Brien JF, McLaughlin BE, Kobus SM, Kawamoto JH, Nakatsu K, Marks GS: Mechanism of glyceryl trinitrate-induced vasodilation. I. Relationship between drug biotransformation, tissue cyclic GMP elevation and relaxation of rabbit aorta. J Pharmacol Exp Ther 244:322–327, 1988
41. Todoki K, Okabe E, Ito H: Oxygen free radicals inactivate endothelium function of canine coronary artery (abstract). Heart Vessels 4(suppl):68, 1990
42. Okabe E, Hess ML, Oyama M, Ito H: Characterization of free radical-mediated damage of canine cardiac sarcoplasmic reticulum. Arch Biochem Biophys 225:164–177, 1983
43. Okabe E, Odajima C, Taga R, Kukreja RC, Hess ML, Ito H: The effect of oxygen free radicals on calcium permeability and calcium loading at steady state in cardiac sarcoplasmic reticulum. Mol Pharmacol 34:388–394, 1988
44. Okabe E, Sugihara M, Tanaka K, Sasaki H, Ito H: Calmodulin and free oxygen radicals interaction with steady-state calcium accumulation and passive calcium permeability of cardiac sarcoplasmic reticulum. J Pharmacol Exp Ther 250:286–292, 1989
45. Elliott SJ, Schilling WP: Oxidative stress inhibits bradykinin-stimulated $^{45}Ca^{2+}$ flux in pulmonary vascular endothelial cells. Am J Physiol 260:H549–H556, 1991