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Volatile Anesthetics Do Not Alter Bradykinin-induced Release of Nitric Oxide or L-Citrulline in Crystalloid Perfused Guinea Pig Hearts

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Background: **Nitric oxide (NO) and L-citrulline (L-cit) are released by endothelial NO synthase (eNOS) to induce vasodilation *via* guanylyl cyclase and cyclic guanosine monophosphate (cGMP). Volatile anesthetics directly reduce vascular muscle tone, but their effects on the eNOS cGMP pathway is controversial. The aim of this study was to examine the effects of anesthetics on bradykinin-induced increases in flow, NO, and L-cit in isolated hearts.**

Methods: **Guinea pig hearts were isolated, perfused at 55 mmHg with a crystalloid or erythrocyte perfusate at 37°C, and heart rate, left ventricular pressure, coronary flow (CF), effluent *p*H, and oxygen tension were monitored. Effluent [NO] was measured by a Clark-type electrode (sensitivity ≥1 nM = 3 pA) with a selectively permeable membrane. Effluent [L-cit] was measured by chromatography. Before, during, and after exposure to halothane, isoflurane, or sevoflurane, hearts were infused with as much as 100 nM bradykinin to induce increases in CF and effluent release of NO and L-cit.**

Results: **In crystalloid-perfused hearts, 10 nM bradykinin produced maximal concentration-dependent increases in CF (87 ± 2%), [NO] (24 ± 4 nM), NO release (128 ± 18 pmol·g·$^{-1}$ min·$^{-1}$), and [L-cit] (58 ± 8 nM). Isoflurane slightly increased CF but not NO. Anesthetics did not alter the bradykinin-induced CF, NO slope relationship, or change [L-cit]. In erythrocyte-perfused hearts, isoflurane also did not alter the bradykinin-induced increase in CF and decrease in percentage of oxygen extracted.**

Conclusions: **This is the first study to simultaneously measure CF with bradykinin-induced changes in [NO] and [L-cit] in the presence of halothane, isoflurane, and sevoflurane in intact hearts. The study shows for the first time that volatile anesthetics do not alter the CF to NO relationship and suggests that NO production, NO release, and NO vasodilatory effects mediated by the eNOS cGMP pathway are not significantly affected by anesthetics in crystalloid or erythrocyte-perfused guinea pig hearts. (Key words: Coronary; hemoglobin; myocardium.)**

SINCE the discovery of endothelium-derived relaxing factor[1] and its identification as nitric oxide (NO),[2,3] it has been shown that NO gas is released from the coronary endothelium and modulates local coronary vascular tone.[4] Nitric oxide is generated by stimulation of endothelial NO synthase (eNOS) or by metabolism of nitrosyl compounds at soluble guanylyl cyclase (sGC),[5,6] which in turn promotes vasodilation by increasing smooth muscle cyclic guanosine monophosphate (cGMP).[6] Nitric oxide contributes importantly to coronary vasodilation elicited by various physiologic and pharmacologic stimuli.[7]

It is possible that some of the vascular effects of anesthetics may be mediated through the endothelium. The role of volatile anesthetics on vascular endothelium has come under intense investigation, but most studies have been done *in vitro* in endothelial-vessel strip or coculture preparations in the absence and presence of eNOS antagonism.[8-19] There are several reports that volatile anesthetics alter vascular endothelial function, or the effects of endothelial factors on vascular smooth muscle activity, yet there is no consensus that anesthetics significantly alter the NO signaling pathway.[10,13,20-25]

We proposed to test in the intact heart if volatile anesthetics significantly alter endothelium-dependent vasodilation induced by bradykinin and if they correspondingly alter the rate of NO and L-citrulline (L-cit)

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production and release by eNOS. We reasoned that NO release could be monitored continuously on-line from the intact coronary endothelium in crystalloid-perfused hearts because much less luminally released NO is metabolized in the absence of hemoglobin. Coronary flow (CF) measured at constant perfusion pressure assesses global coronary vascular resistance; simultaneous monitoring of the percentage of oxygen extracted assesses vascular tone independent of cardiac metabolism. Only endothelium-dependent vasodilator agents that stimulate NO production should cause both an increase in effluent NO and L-cit concentrations and CF.[26] To assess the possible pathways involved, we measured changes in coronary effluent NO concentration ([NO]), NO release, and L-cit concentration ([L-cit]) with increasing concentrations of an eNOS stimulator, bradykinin, with and without halothane, isoflurane, or sevoflurane. We also determined if volatile agents also significantly alter endothelium-mediated increases in CF in intact, erythrocyte perfused intact hearts.

Materials and Methods

Langendorff Heart Preparation

The investigation conformed to the *Laboratory Animal Care and Use Standards* (National Institutes of Health publication 85-23, revised 1995) and was approved by the Medical College of Wisconsin animal studies committee. Hearts were harvested for study after 25 mg ketamine and 1,000 units heparin were injected intraperitoneally into 52 albino English short-haired guinea pigs (250–300 g). Description of the surgical preparation for this model has been reported in detail.[26,27] Each heart was perfused in a retrograde manner through the aorta at 55 mmHg with a 95% oxygenated, modified Krebs-Ringer's solution as described before.[26,27] Seventeen of these hearts were perfused with erythrocytes. Isovolumetric left ventricular (LV) pressure, CF, and spontaneous heart rate were measured continuously, as described previously.[26,27] Maximal CF was elicited with adenosine (0.2 ml of 200 μM stock solution) injected directly into the aortic root cannula during the initial control period and after the last control reading.

Nitric Oxide, Oxygen, pH, and Anesthetic Measurements

Coronary sinus effluent was collected by placing a small gas-impermeable cannula into the right ventricle through the pulmonary artery after ligating the superior and inferior vena cavae. Coronary outflow (coronary sinus) oxygen tension and *p*H were measured continuously on-line with a miniature thermostabile Clark oxygen electrode (model 203B; Instech Laboratories, Plymouth Meeting, PA) and a temperature-compensated *p*H electrode (microcomputer pro-vision *p*H meter, model 05669-20; *p*H electrode PHE 2121; Cole Palmer Instruments, Vernon Hills, IL). Perfusate, bath, oxygen, *p*H, and NO electrode temperatures were maintained tightly at 37.2 ± 0.1°C using a thermostatically controlled water circulator to jacketted glass tubing, bath, and aluminum heat exchangers. Coronary sinus and NO electrode temperature was tightly controlled because NO electrode current is affected by the artifact of temperature alone (equivalent to +24.5 nM/°C).[26]

Coronary inflow and effluent *p*H and oxygen and carbon dioxide tensions were measured during each maneuver off-line at 37°C with an intermittently self-calibrating analyzer system (Radiometer ABL-2; Medtron Chicago, Des Plaines, IL). Coronary effluent carbon dioxide tension was calculated from the on-line *p*H signal with bicarbonate assumed to be constant at 15 mM; calculated effluent carbon dioxide tension at a given *p*H was verified off-line with the gas analyzer. The NO electrode was slightly sensitive to changes in carbon dioxide induced by changing the percentage of carbon dioxide flow in the gas mixing chamber in the absence of hearts.[26] The effect of changes in effluent carbon dioxide to alter the *p*H of the internal electrolyte solution was minimized by using a two-part buffered electrolyte solution (World Precision Instruments, Sarasota, FL, catalog #7521). The small effect of a change in effluent H^+ concentration (−0.22 nM/mV), or partial carbon dioxide pressure (−0.50 nM/mmHg) was adjusted mathematically at each data point based on the coronary venous *p*H. The maximal decrease in coronary effluent carbon dioxide tension from control CF (6.3 ± 0.2 $ml \cdot^{-1} g \cdot min^{-1}$) to a maximal steady state increase in CF (13.3 ± $ml \cdot^{-1} g \cdot min^{-1}$) was 4 mmHg, an apparent NO increase of about 2 nM. The concentrations of each anesthetic used did not alone alter NO current.

The NO concentration was measured as the change in redox current (pA) generated by a gas-permeable, water-impermeable NO electrode (ISO-NOP 2 mm; World Precision Instruments, Sarasota, FL). The Clark-type electrode measures dissolved NO gas concentration in aqueous solutions polarographically.[28] An electric current is generated as NO diffuses through the membrane and becomes oxidized at the platinum elec-

trode. Generated current is proportional to the diffusion of NO through the membrane, and diffusion is based on its partial pressure, which, in turn, is proportional to [NO] at the probe tip. Current is measured with a sensitive amperometer during zero voltage suppression to expand the response range. The membrane selectivity for NO over other gases is determined by the potential applied to the electrode. The presence of dissolved NO_2 gas ($2NO + O_2 = 2NO_2$) could generate a current, but NO_2 is highly unstable in aqueous solution and reacts at *p*H >7 with alkali ($2NO_2 + 2OH- = NO_2- + NO_3- + H_2O$) to form nitrite and nitrate ions, which are not permeable.

The NO calibration curves were generated by graded chemical production of NO, where KI and H_2SO_4 are in excess: $2NaNO_2 + 2KI + 2H_2SO_4 = 2NO + I_2 + 2H_2O + K_2SO_4 + Na_2SO_4$. Calibration was done 1 day after the gas-permeable membrane and internal filling solution were changed (they were changed weekly). There was no significant change in NO electrode sensitivity (≥1 nM) during a 1-week period. Calibrations, conducted at the beginning and end of a 5-day use period, were unchanged over time. The average change in current generated for a given concentration of chemically generated NO (*i.e.*, the slope [$P \leq 0.0001$]) amounted to 2.92 pA/nM ± 0.17 (95% CI), so that for every pA increase in current, the NO concentration was increased by 0.34 nM. The Y intercept was not significantly different from zero. Transient time for perfusate from the heart to the NO electrode *via* the coronary sinus and pulmonary artery tubing was between 1 to 1.5 s, as assessed by the appearance of methylene blue at a CF rate of 20 ml/min. Nitric oxide release ($pmol \cdot g^{-1} \cdot min^{-1}$) was calculated as CF (ml/min) times NO concentration (nM) divided by heart wet weight (average 1.80 ± 0.06 g [SEM]). The basal NO concentration cannot be measured directly by this method, but we have shown that perfusion of hearts with 100 μM N^G nitro L-arginine methyl ester (L-NAME) decreases [NO] by about 12 ± 3 nM, which suggests that this is the basal effluent [NO] in this preparation.[26]

Anesthetics were delivered by calibrated agent-specific vaporizers, and anesthetic concentrations were measured in the effluent by gas chromatography, as described before.[27] Effective anesthetic fractions, in volume percentage (vol %), were determined as noted previously[27] and are shown in table 1. The percentage of oxygen extracted was calculated as the inflow and outflow tension difference multiplied by 100 and divided by inflow oxygen tension. The percentage of O_2 extraction assesses a direct vasodilatory response separate from that caused by an autoregulatory response resulting from altered contractility; this measurement assumes that local metabolites are produced in proportion to myocardial oxygen consumption and that local metabolites are important factors in controlling CF autoregulation. Inflow perfusate oxygen tension was kept constant by maintaining the reservoir pressure 5 mmHg above atmospheric pressure. Heart rate, inflow, and outflow oxygen (mmHg), *p*H (in mV), CF, systolic and diastolic isovolumetric LV pressure, and NO electrode current (pA) were displayed continuously on a fast-writing (3 kHz), high-resolution, eight-channel chart recorder (Astro-Med Inc., West Warwick, RI). The NO (pA) and *p*H (mV) electrode signals were zero suppressed and amplified for continuous display.

Measurement of L-Citrulline

L-Citrulline was measured by high-performance liquid chromatography in 250 coronary effluent samples from 32 hearts before and during exposure to each anesthetic in the absence and presence of 0.1, 1, 10, and 100 nM bradykinin. The high-performance liquid chromatography system consisted of a Laboratory Data Control constametric III G pump (Rivera Beach, FL), a Gilson Automatic Sampler model 231 (Middletown, WI), and an electrochemical detector (BAS LC-4B; Bioanalytical Systems, West Lafayette, IN). The column (Beckman Ultrasphere ODS 5 μ, 4.6 mm × 25 cm, Fullerton, CA) was perfused at a mobile phase flow rate of 1.5 ml/min. The detector potential was set at +0.7 V. The mobile phase consisted of 800 ml 0.1 M sodium acetate, adjusted to *p*H 5.7, plus 260 ml acetonitrile. L-Citrulline was detected electrochemically as the o-phthaldialdehyde derivative. The o-phthaldialdehyde reagent consisted of 25 ml 0.1 M borate buffer (*p*H 9.5), 50 μl 2-methyl 2-propanethiol, 2.5 ml methanol, and 135 mg o-phthaldialdehyde. All chemicals were high-performance liquid chromatography grade. The chromatographic data were collected on a Hewlett Packard 3393A integrator and stored on a Hewlett Packard 9122 disc drive (Wilmington, DE). Coronary venous effluent was collected (2 ml) during drug-free control periods and during the last 30 s of bradykinin infusion and was frozen at −15°C. Samples were later prepared and analyzed as follows: To each 0.5-ml sample was added 25 μl methyl-L-arginine (2 μg/ml) as an internal standard. Three milliliters of ethanol was added to each sample, mixed, and centrifuged. The supernatant was transferred to a clean tube and evaporated to dryness under a stream of air at 40°C. The dried

Table 1. Effluent Anesthetic Concentration, Effective Anesthetic Vapor Pressure (effluent vol %), and Effects of Anesthetics Alone on Isovolumetric Left Ventricular Pressure (LVP), Heart Rate, Coronary Flow, and Oxygen Extraction in Isolated Hearts

Volatile Anesthetic	Inflow (vol %)	Effluent (mM)	Effective (vol %)	LVP (mmHg)	Heart Rate (bpm)	Coronary Flow ($ml \cdot g^{-1} \cdot min^{-1}$)	O_2 Extraction (%)
Halothane	Control	0	0	80 ± 4	241 ± 3	6.6 ± 0.3	66 ± 3
n = 7	0.5	0.11 ± 0.01	0.34 ± 0.03	69 ± 4*	229 ± 4*	7.2 ± 0.4	57 ± 3*
	Control	0	0	77 ± 2	244 ± 2	6.7 ± 0.4	69 ± 3
n = 7	1.0	0.26 ± 0.01	0.84 ± 0.03	62 ± 3*	228 ± 3*	7.2 ± 0.3	55 ± 4*
Isoflurane	Control	0	0	80 ± 4	239 ± 2	6.3 ± 0.3	77 ± 3
n = 7	1.0	0.20 ± 0.01	0.87 ± 0.03	75 ± 4*	230 ± 4*	7.3 ± 0.4*	66 ± 4
	Control	0	0	80 ± 3	242 ± 3	6.5 ± 0.4	73 ± 3
n = 7	2.0	0.34 ± 0.01	1.47 ± 0.03	69 ± 5*	230 ± 3*	8.3 ± 0.3*	54 ± 2*
Sevoflurane	Control	0	0	83 ± 4	241 ± 4	6.3 ± 0.4	77 ± 5
n = 7	2.0	0.22 ± 0.01	1.51 ± 0.03	77 ± 4*	227 ± 4*	6.9 ± 0.5	65 ± 5

* $P < 0.05$ *versus* control for each anesthetic concentration.

residue was redissolved in 2 ml mobile phase and 400 μl was mixed with 40 μl of the o-phthaldialdehyde reagent for exactly 2 min before injection of 100 μl into the high-performance liquid chromatograph. The L-cit concentration was calculated from the standard curve of the respective peak height ratio *versus* concentration. The standard curve data were derived using perfusate that did not pass through the isolated heart. The L-cit standard curves were linear over the concentration range studied. The limit of detection was 1 ng/ml perfusate. The absolute retention time for L-cit was 10.3 min.

Protocol

Crystalloid-perfused hearts were randomized to three treatments, halothane (HAL), isoflurane (ISO), or sevoflurane (SEV), at several concentrations. Fourteen hearts were exposed to 0.5% HAL or to 1% HAL by vaporizing HAL in random order into Krebs-Ringer's solution. Similarly, 14 other hearts were exposed to 1% or 2% ISO, and seven hearts were exposed only to 2% SEV because effects on NO did not appear to be concentration dependent. Each heart was infused with 0, 0.1, 1, and 10 nM bradykinin given in random order. After a period of stabilization, adenosine was bolus injected to measure maximal CF. A given bradykinin concentration was then infused for 1 min. This was followed 10 min later by a 15-min exposure to a given anesthetic concentration, while the same bradykinin concentration was again infused for 1 min; then the anesthetic was discontinued, bradykinin alone was again infused for 1 min, and this was followed by a 15-min drug-free control period. This protocol was repeated twice again in the same heart for the other two concentrations of bradykinin. Adenosine was again bolus injected at the end of each experiment at the same concentration into the aortic cannula to observe any change in maximal CF reserve. For all groups (table 1), basal CF was 6.4 ± 0.2 $ml \cdot g^{-1} \cdot min^{-1}$. Beginning and ending CF responses to adenosine were 16.2 ± 0.9 and 12.7 ± 1.3 $ml \cdot g^{-1} \cdot min^{-1}$, respectively, for all groups combined ($P < 0.05$). Measurements and L-cit samples were obtained during the peak steady state change in CF during exposure to each concentration of an anesthetic or bradykinin. The CF and NO current returned approximately to the pre-drug level after a 15-min washout of bradykinin, anesthetic, or both. Drug-free control periods were interspersed between treatment. The basal NO levels can be determined only indirectly,[26] so NO concentration and NO release were calculated and expressed as changes from the preceding control.

Seventeen additional hearts were perfused with oxygenated Krebs-Ringer's solution containing erythrocytes. One liter of freshly obtained and heparin-processed canine whole blood was washed in saline and centrifuged three times and the buffy coat discarded. The resulting packed erythrocyte suspension was filtered (40-μm pore size) and diluted to a hematocrit of about 9% immediately before use. Ionized Na^+, K^+, Ca^{2+}, and *p*H were within normal limits and not different in erythrocyte and erythrocyte-free Krebs-Ringer's perfusate. Using the same randomized protocol as described before, bradykinin-induced changes in CF and oxygen extraction were measured in the absence and presence of 2% and 4% isoflurane delivered by vaporizer.

Statistics

All data were expressed as mean ± SEM. Each heart served as its own control. Individual drug responses

were compared with the preceding control by paired Student's t tests. The effects of anesthetic concentrations on CF, NO release, and NO and L-cit concentrations during increasing concentrations of bradykinin were compared by Tukey's comparison of means' tests after analysis of variance for repeated measures (Super Anova 1.11® software for Macintosh; Abacus Concepts, Berkeley, CA). The change in CF induced by increasing concentrations of bradykinin was plotted as a function of the change in NO concentration for each coordinate, and data were fitted by linear regression analysis to determine slopes, y intercepts, correlation coefficients, significance of slopes, and 95% confidence intervals. Differences in slopes in the absence (control) and presence of an anesthetic concentration were determined by slope heterogeneity comparison tests. Differences among means were considered significant when $P \leq 0.05$.

Results

Table 1 displays the effects of each volatile anesthetic concentration alone on LV pressure, heart rate, CF, and percent oxygen extraction. Anesthetic concentration is expressed by the inflow vaporizer setting (vol %), coronary effluent concentration (mM), and effective vapor pressure (vol %). Each concentration of each anesthetic significantly decreased LV pressure and heart rate. The decrease in LV pressure by HAL, but not by ISO, was concentration dependent. Only ISO increased CF, and both HAL and ISO decreased oxygen extraction. The increase in CF by ISO was concentration dependent.

Figure 1 shows a typical response to 2% ISO alone and bradykinin plus 2% ISO. Bradykinin increased CF, effluent oxygen tension, LV pressure, and [NO], and it decreased the RR wave interval and pH (mV). The time course of the NO response closely matched that of the CF response. Although ISO slightly increased CF, it had no appreciable effect on other responses to bradykinin.

Figure 2A to 2C summarizes the effect of bradykinin on increasing CF in the presence and absence of HAL, ISO, and SEV in crystalloid-perfused hearts. In the absence of bradykinin, ISO and SEV, but not HAL, alone significantly increased CF. Bradykinin alone significantly increased CF in each anesthetic group in a concentration-dependent manner. The presence of any of the three volatile anesthetics did not significantly alter the bradykinin-induced increase in CF, with the single exception of a significant increase in CF by 2% ISO with 0.1 nM bradykinin. Bradykinin had no significant effect on LV pressure or heart rate (data not shown).

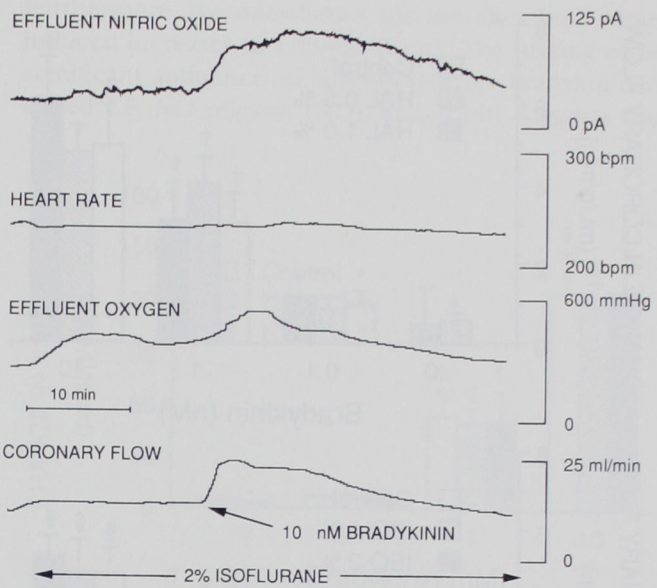

Fig. 1. Graphic recording of one experiment showing the effect of 2% isoflurane alone and with a 1-min infusion of 10 nM bradykinin on nitric oxide current (in picoamperes), heart rate, effluent oxygen tension (in millimeters of mercury), and coronary flow (in milliliters per minute). Not shown is the equivalent increases in nitric oxide and coronary flow with bradykinin in the absence of isoflurane.

Figure 3A to 3C summarizes the effect of increasing concentrations of bradykinin on increasing coronary effluent [NO] in the presence and absence of HAL, ISO, and SEV in crystalloid-perfused hearts. None of the anesthetics altered [NO] from the drug-free control. Bradykinin alone significantly increased [NO] in each anesthetic group in a concentration-dependent manner. The presence of any of the three anesthetics did not significantly alter the increase in [NO].

Figure 4A to 4C summarizes the effect of increasing concentrations of bradykinin on changing coronary effluent NO release in the presence and absence of HAL, ISO, and SEV in crystalloid-perfused hearts. None of the anesthetics altered NO release from the drug-free control. Bradykinin alone significantly increased NO release in each anesthetic group in a concentration-dependent manner. The presence of any of the three anesthetics did not significantly alter the increase in NO release, with the single exception of a significant increase in NO release by 2% ISO in the absence of bradykinin, an effect likely caused by the increase in CF by ISO. None

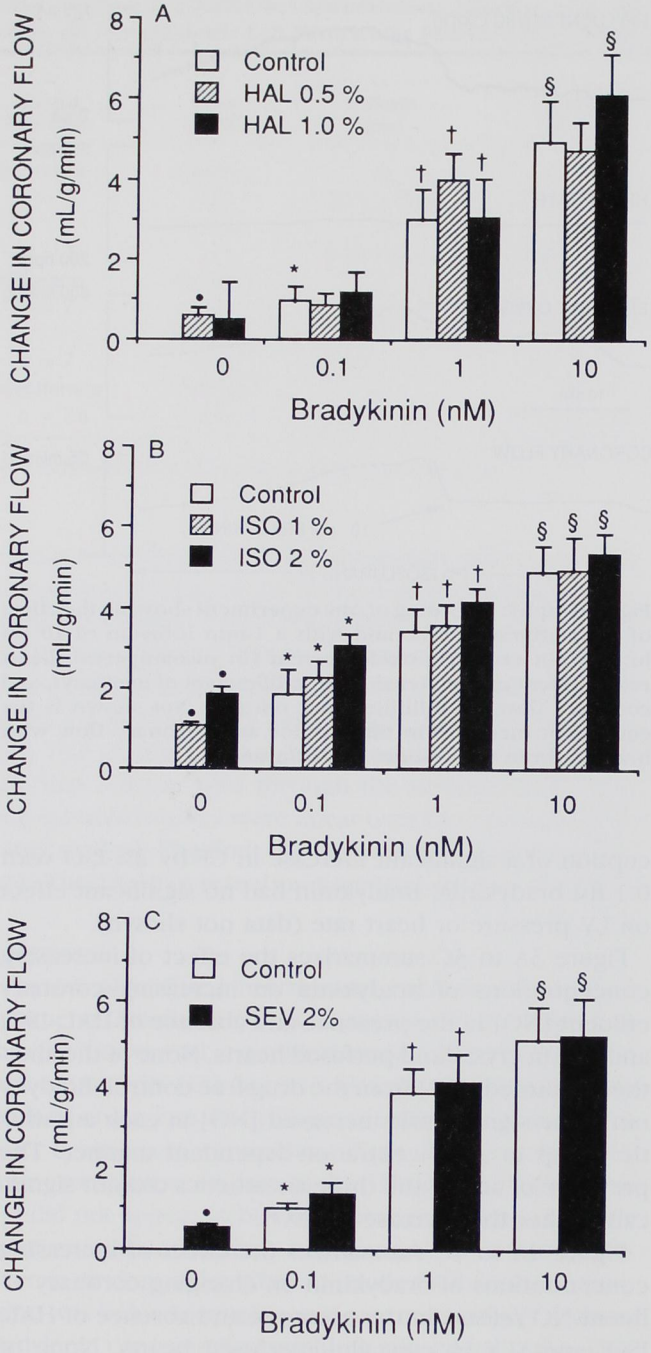

Fig. 2. The effect of increasing concentrations of bradykinin on increasing coronary flow (CF) in the absence and presence of halothane (HAL, *A*), isoflurane (ISO, *B*), and sevoflurane (SEV, *C*). For $P < 0.05$: •anesthetic *versus* control; *0.1 nM bradykinin *versus* 0 bradykinin; †1 nM *versus* 0.1 nM bradykinin; §10 *versus* 1 nM bradykinin. Isoflurane alone increased flow in a concentration-dependent manner ($P < 0.05$). There was no concentration of any anesthetic that significantly altered the bradykinin-induced increase in CF. Table 1 shows effective effluent concentrations for each anesthetic.

of the anesthetics significantly altered the effect of bradykinin to increase effluent NO release.

Figures 5A and 5B, 6A and 6B, and 7 show increases in CF as a linear correlate function of the increase in effluent [NO] induced by 0.1, 1, and 10 nM bradykinin in the absence and presence of lower (A panels) and higher (B panels) concentrations of HAL and ISO and of SEV (2% only) in crystalloid-perfused hearts. All slopes are significant, which indicates that the increases in [NO] were associated with the increases in CF. There were no differences in slopes among the three anesthetic controls or between a concentration of any anesthetic and its control. Only the y intercepts for 1% and 2% ISO were significantly different from controls.

Figure 8 displays bradykinin-induced increases in [L cit] in the presence and absence of each anesthetic in crystalloid-perfused hearts. Significant increases in [L-cit] were induced by 1–100 nM bradykinin. The responses to 0.1 to 10 nM bradykinin were not altered significantly by the given concentrations of each anesthetic.

Figure 9A and 9B summarizes data in 12 erythrocyte-perfused hearts on the effect of two concentrations of bradykinin on altering CF and the percentage of oxygen extracted in the presence and absence of 2% and 4% isoflurane. Although ISO alone did not alter CF, it decreased the percentage of oxygen extracted significantly. Bradykinin (10 nM) increased CF and decreased the percentage of oxygen extracted significantly. Whereas ISO had no added effect on the increase in CF by 10 nM bradykinin, it accentuated bradykinin's effect to decrease the percentage of oxygen extracted. In five additional erythrocyte-perfused hearts, 4% isoflurane did not alter CF (4.8 ± 0.6 to 4.9 ± 0.5 ml · g^{-1} · min^{-1}) but significantly increased effluent oxygen tension (200 ± 5 to 251 ± 6 mmHg), whereas 100 μM L-NAME slightly decreased basal CF (3.8 ± 0.6 ml · g^{-1} · min^{-1}) and oxygen tension (158 ± 8 mmHg); 4% isoflurane had no added effects (3.6 ± 0.4 ml · g^{-1} · min^{-1}; 142 ± 9 mmHg) when combined with L-NAME.

Discussion

This is the first study to measure simultaneously bradykinin-induced CF and NO release in the presence of a

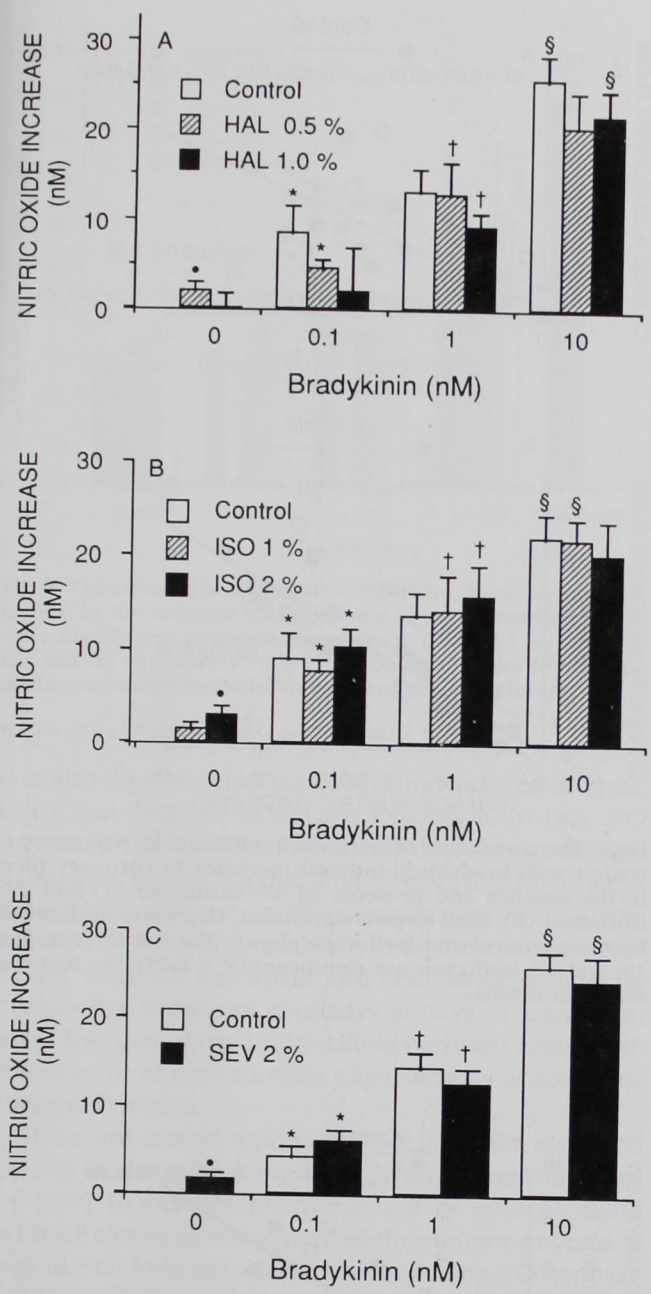

Fig. 3. The effect of increasing concentrations of bradykinin on increasing the concentration of nitric oxide (NO) in the absence and presence of halothane (*A*), isoflurane (*B*), and sevoflurane (SEV, *C*). See the legend for figure 2 for explanations of symbols. There was no concentration of any anesthetic that significantly altered the nitric oxide concentration.

volatile anesthetic in intact, crystalloid-perfused hearts. The study shows, first, a significant correlation between increases in coronary effluent [NO] and CF induced by the eNOS dependent drug bradykinin; and, second, that this relationship is not altered by volatile anesthetics. Furthermore, the anesthetics did not alter bradykinin-induced increases in effluent [L-cit]. The finding of no significant influence of anesthetics on bradykinin-induced CF, NO release, [NO], and [L-cit] suggests that

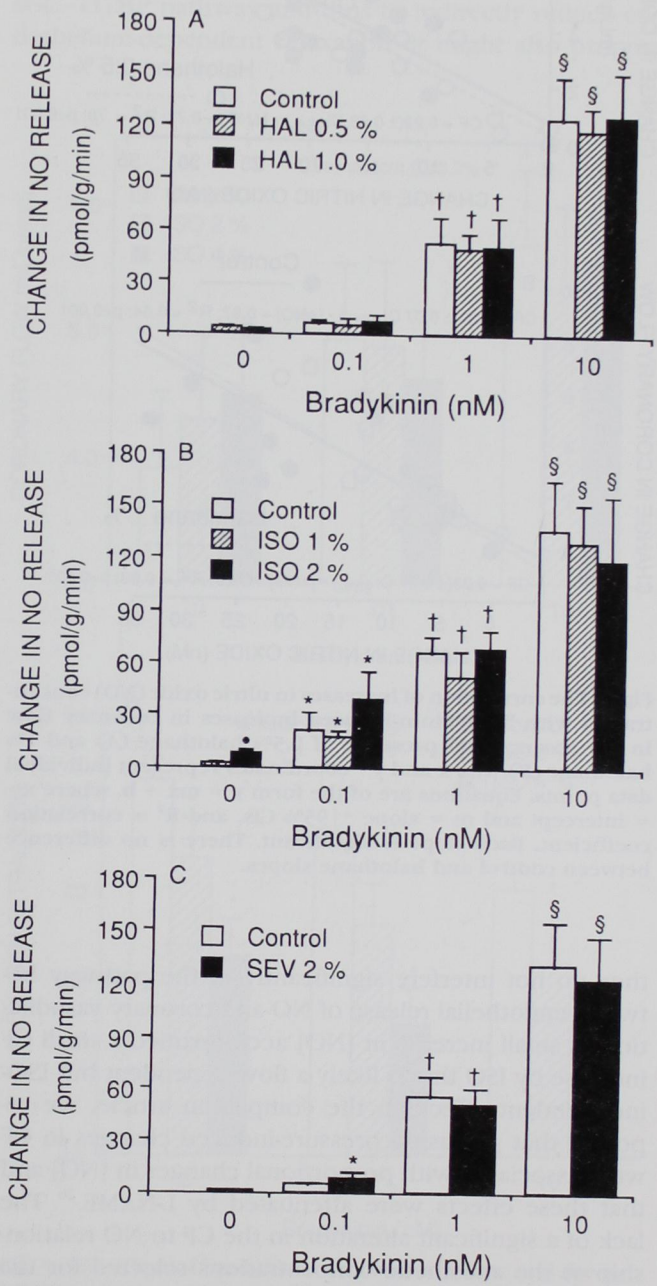

Fig. 4. The effect of increasing concentrations of bradykinin on increasing coronary effluent release of nitric oxide (NO) in the absence and presence of halothane (*A*), isoflurane (*B*), and sevoflurane (SEV, *C*). See the legend for figure 2 for explanations of symbols. There was no concentration of any anesthetic that significantly altered the release of nitric oxide.

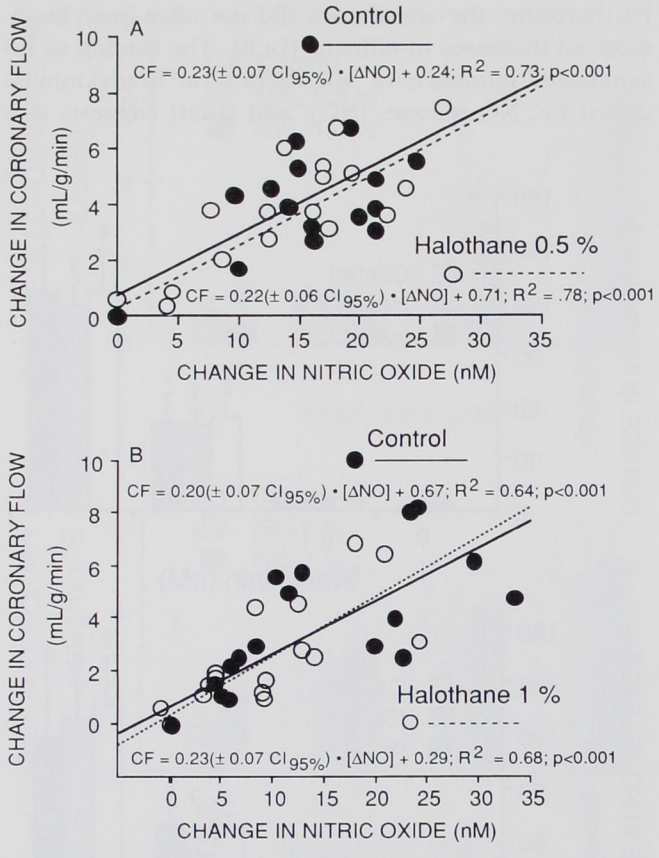

Fig. 5. The correlation of increases in nitric oxide (NO) concentration with bradykinin-induced increases in coronary flow in the absence and presence of 0.5% halothane (*A*) and 1% halothane (*B*). The x and y− coordinates represent individual data points. Equations are of the form y = mx + b, where x− = intercept and m = slope ± 95% CIs, and R^2 = correlation coefficient. Each slope is significant. There is no difference between control and halothane slopes.

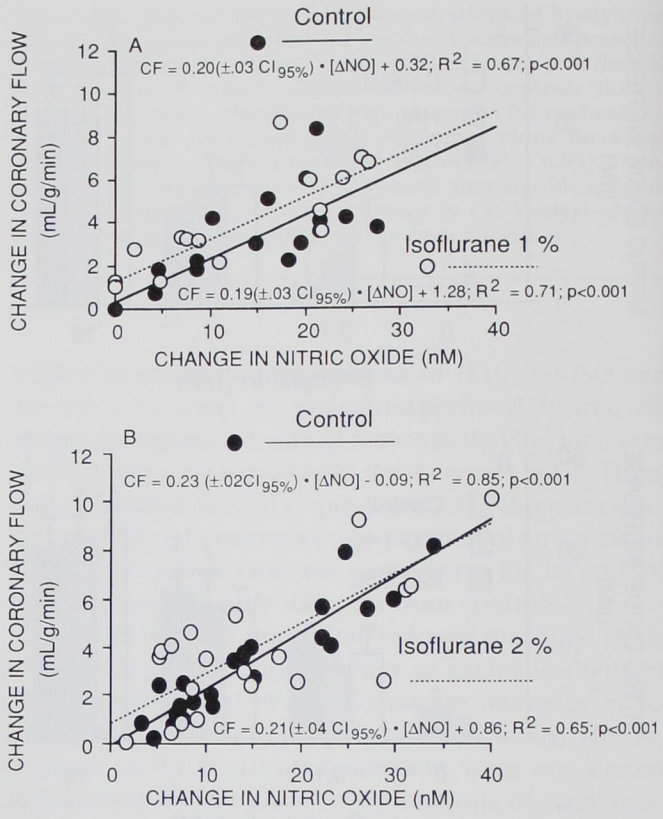

Fig. 6. The correlation of increases in nitric oxide (NO) concentration with bradykinin-induced increases in coronary flow in the absence and presence of 1% isoflurane (*A*) and 2% isoflurane (*B*). Each slope is significant. There is no difference between control and isoflurane slopes. The Y intercepts for 1% and 2% isoflurane are significant ($P < 0.05$). See figure 4 for other details.

they do not interfere significantly in the pathway between endothelial release of NO and coronary vasodilation. A small increase in [NO] accompanied a small CF increase by ISO that is likely a flow-dependent but ISO-independent effect. In the companion article, we reported that perfusion pressure-induced changes in CF were associated with proportional changes in [NO] and that these effects were attenuated by L-NAME.[26] The lack of a significant alteration in the CF to NO relationship at the anesthetic concentrations selected for the crystalloid-perfused heart studies does not preclude that higher concentrations may have an inhibitory effect on the NO signaling pathway between coronary endothelium and vasculature. However, the erythrocyte-perfused heart studies confirm that a higher ISO concentra-

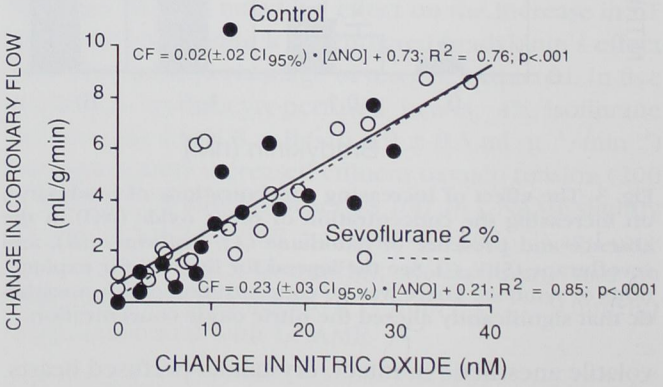

Fig. 7. The correlation of increases in nitric oxide (NO) concentration with bradykinin-induced increases in coronary flow in the absence and presence of 2% sevoflurane. Each slope is significant. There is no difference between the control and sevoflurane slopes. See figure 4 for other details.

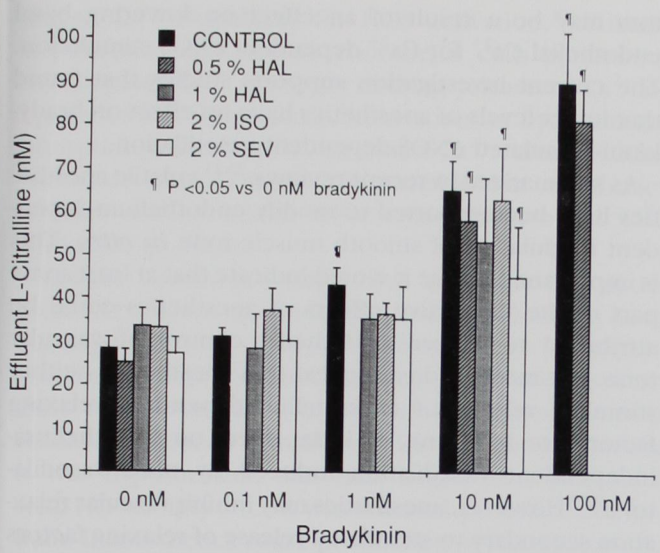

Fig. 8. The effect of bradykinin on effluent L-citrulline concentration in the absence and presence of each anesthetic. Data were insufficient to assess responses to 0.1 and 1 nM bradykinin during exposure to 0.5% HAL and to 100 nM bradykinin during exposure to 2% isoflurane and 2% sevoflurane.

tion also does not alter the bradykinin-induced increase in CF and decrease in oxygen extraction. In fact, ISO enhanced the bradykinin-induced decrease in oxygen extraction, suggesting that after normalizing for an autoregulatory influence on CF, vascular tone is additively reduced by bradykinin and ISO. In erythrocyte-perfused hearts, basal CF was lower and bradykinin-induced increases in CF, but not decreases in oxygen extraction, were less than those in crystalloid-perfused hearts. Differences in oxygen-carrying capacity may account for these differences.

There are several reports that CF is locally regulated by NO in the guinea pig heart.[4,29,30] A direct measure of [NO] in hemoblobin-free coronary effluent along with CF allows an assessment of the amount and rate of vascular endothelial cell NO production by NO synthase (eNOS) and the coronary vasodilation produced by NO. Volatile anesthetics could alter specific endothelial receptor responses (*e.g.*, B_2-kinin receptor subtype),[31] modulate NO production or release, or alter the effects of NO on the sGC cGMP system that promotes vascular relaxation. Until now, most studies on NO synthesis, NO release, and NO effects have been based inferentially on antagonism of eNOS by L-arginine analogs such as N^G-monomethyl L-arginine or L-NAME. These antagonists may not completely inhibit eNOS.[26] Our conclusions are based on a lack of effect by anesthetics to alter the correlation between coronary effluent [NO] and CF rather than on antagonism by L-arginine analogs. Although unlikely, this could be an oversimplification. For example, if an anesthetic agent were to decrease the effect of a given amount of NO released on the sGC-cGMP pathway and thus to indirectly reduce endothelium-dependent relaxation, it might also propor-

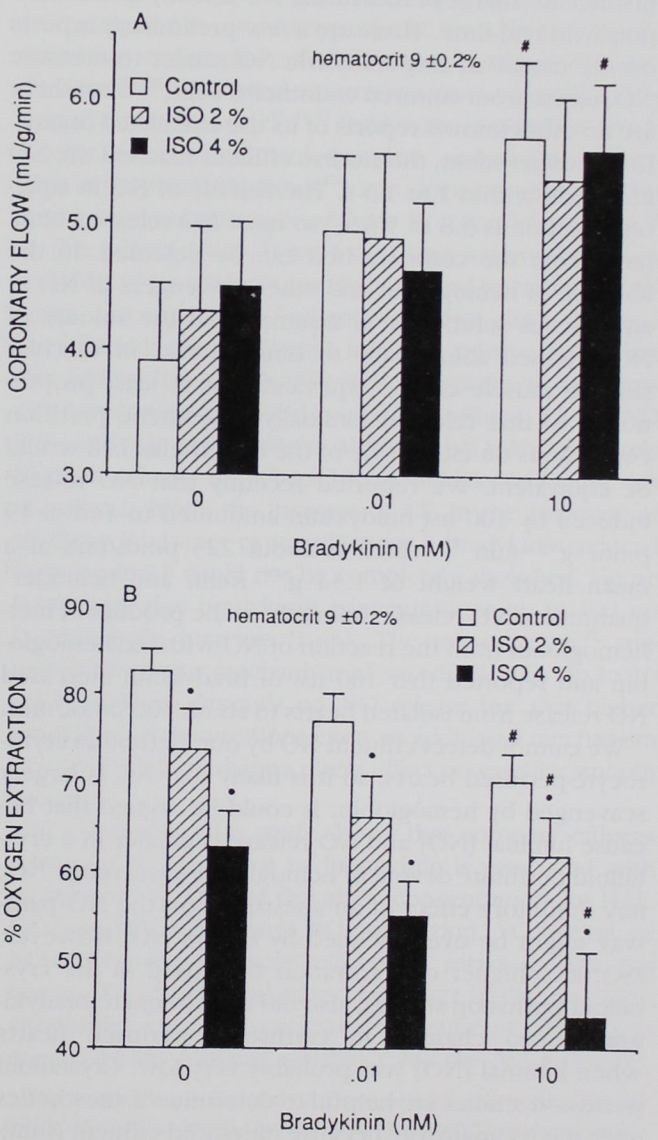

Fig. 9. The effect of bradykinin on coronary flow and the percentage of oxygen extracted in the absence and presence of isoflurane. Hearts were perfused with canine erythrocytes. Isoflurane alone did not increase coronary flow ($P > 0.1$) but decreased the percentage of oxygen extraction in a concentration-dependent manner ($\bullet P < 0.05$); the bradykinin-induced increase in coronary flow and decreases in the percentage of oxygen extracted ($\#P < 0.05$) were not altered by isoflurane.

tionally enhance relaxation by another endothelium-dependent effect.

Several methods are reported to quantitate NO release from endothelial cells. Techniques to measure NO release directly or indirectly from tissue include methemoglobin,[4] bioassay,[32,33] chemoluminescence,[34] and electron paramagnetic response spectra.[35] Compared with these techniques, an NO-sensing electrode has the distinct advantage of measuring NO directly and continuously in real time. There are a few preliminary reports on the use of an amperometric NO sensor to measure NO release from cultured endothelial cells,[36,37] but there are no other known reports of its use in isolated organs. In our experiment, the cardiac effluent reached the NO electrode within 1 to 1.5 s. The half-life of NO in aqueous solution is 3.8 to 5.6 s,[4] so most NO released luminally from the coronary bed can be detected. In the absence of hemoglobin and other scavengers of NO in an aqueous solution, it is assumed that the amount of NO released abluminally to bind to sGC in vascular smooth muscle cells is equivalent, or at least proportional, to that released luminally. Anesthetic partition coefficients on either side of the endothelial cell would be equivalent. We reported recently that NO release induced by 100 nM bradykinin amounted to 146 ± 19 $pmol \cdot g^{-1} \cdot min^{-1}$, which is about 225 pmol/min at a mean heart weight of 1.54 g.[26] Kelm and Schrader[4] quantitated NO release indirectly as the product of methemoglobin from the reaction of NO with oxyhemoglobin and reported that 100 nM of bradykinin increased NO release from isolated hearts to about 300 pmol/min.

We cannot detect effluent NO by our method in erythrocyte-perfused hearts, so it is likely that NO is largely scavenged by hemoglobin. It could be argued that because luminal [NO] and NO release is greater in a crystalloid perfusate devoid of hemoglobin to scavenge NO, any inhibitory effect of an anesthetic on the NO pathway might be overwhelmed by excess NO. However, ISO, at a higher concentration than used in the crystalloid perfusion studies, also did not attenuate bradykinin-induced changes in erythrocyte-perfused hearts when luminal [NO] was probably very low. Crystalloid perfusion studies are helpful to determine if anesthetics alter the NO pathway because measured effluent (luminal) [NO] is more likely to be proportional to abluminal (interstitial) [NO], where an anesthetic might be expected to alter NO signaling between abluminal release of NO and NO activation of vascular smooth muscle sGC and cGMP. The inhibitory effect of anesthetics on the NO vasodilator pathway noted by some investigators may be a result of an effect on lowering basal endothelial Ca^{2+} for Ca^{2+}-dependent eNOS stimulation. The current investigation supports studies that found that lower levels of anesthetics have no effect on bradykinin-stimulated eNOS-dependent vasodilation.

As summarized in recent reviews,[22,23] volatile anesthetics have been reported to modify endothelium-dependent modulation of smooth muscle tone *in vitro*. This is important because it would indicate that at least some part of the vasoactive effects of anesthetics could be attributed to altered endothelial control of vascular tone. But most studies suggest that anesthetics neither stimulate release of endothelium-dependent relaxing factors nor have any, or little, effect on endothelium-independent vasodilation induced by nitro vasodilators.[9,10] However, anesthetics may inhibit vascular relaxation secondary to stimulated release of relaxing factors by endothelial muscarinic receptors, bradykinin receptors, thrombin, and Ca^{2+} inophores.[22,23]

Several indirect studies[12-16,25,38] suggest that anesthetics modify responses to endothelium-dependent vasodilators. HAL may inhibit vasodilation induced by NO and nitroglycerin,[19] but HAL did not inhibit measured sGC,[39] and Johns *et al.*[13] reported that >2% HAL or 1% ISO depressed cGMP formation independent of sGC activation when endothelial and vascular smooth muscle cell co-cultures were treated with bradykinin. Chelly *et al.*,[38] using L-NAME-treated intact dogs, reported that 1.2% HAL may interfere with NOS activity in carotid, mesenteric, and renal vascular beds, but not in the coronary vascular bed. Sigmon *et al.*[25] also suggested that HAL impairs NO-mediated regulation of some vascular beds, but not the coronary vascular bed, because it largely eliminated systemic responses of eNOS inhibition by L-NAME in rats.

Most investigations suggest that anesthetic-induced vasodilator effects are independent of vascular smooth muscle sGC activation, but anesthetics could attenuate endothelium-dependent relaxation by inhibiting the production, transport, or release of relaxing factors, primarily NO. They could alter activation of endothelial receptor sites, modify second messengers, or affect intracellular Ca^{2+} flux in the cascade of events leading to stimulation of eNOS.[22] In one study it was suggested that a receptor mechanism is not the primary site of anesthetic inhibition of NO signaling, because anesthetics attenuated non-receptor-mediated NO production, an effect that bypasses the receptor.[12] Much as anesthetic effects on Ca^{2+} have been examined in cardiac and smooth muscle cells, it is possible that anesthetics de-

crease Ca^{2+} availability for NOS activation.[40] Volatile anesthetics might act on endothelial cells by altering sarcolemmal Ca^{2+} entry, by altering release of Ca^{2+} or uptake of Ca^{2+} from intracellular stores, or by inhibiting phospholipase C and IP_3-mediated Ca^{2+} release.[18] Also within the endothelium, it is possible that anesthetics interact with or inhibit other cofactors involved with activation of eNOS.

Volatile anesthetics might also inactivate NO or reduce NO efficacy once released from eNOS. It has been reported that HAL attenuates NO-mediated relaxation, but that it does not interfere with endothelial cell release of NO, based on a bioassay technique, or with relaxation induced by nitroprusside.[16] From this finding it was hypothesized that HAL might have interfered with NO stability once it was released from the endothelium. If anesthetics cause production of free radicals by reductive metabolic pathways (*e.g.*, during ischemia), this could enhance inactivation of NO.[41] Nakamura *et al.*[15] reported that 4% SEV reduces relaxation of intact rat aortas by acetylcholine and suggested that SEV inactivates NO or the vasodilator effect of NO. Yoshida *et al.*[14] reported that SEV impairs isolated canine mesenteric artery relaxation induced by a high concentration of bradykinin and suggested that SEV functions as a free radical to interact with NO, also a free radical, because superoxide dismutase attenuated the decrease in bradykinin-induced relaxation caused by SEV. However, it is doubtful that any volatile anesthetic is metabolized by a free radical-producing reductive pathway in the absence of ischemia. With ischemia, their effect on NO-dependent control of vascular tone could be very different.

Just as there are many reports that anesthetics interfere in endothelial vascular muscle signaling, there are others that anesthetics do not alter NO-induced vasodilation.[9,10,13,21] Flynn *et al.*[9] showed that ISO relaxes isolated cerebral arteries rings independently of the endothelium. Brendel and Johns[10] reported that ISO-induced relaxation of isolated aortic rings is independent of endothelium-derived relaxing factor, or NO, and cGMP-mediated mechanisms. Crystal *et al.*[21] reported that coronary vasodilation induced by 1.4% ISO in open-chest dogs was not affected by L-NAME and suggested that NO does not mediate or modify this response. Johns *et al.*[13] furnished data that volatile anesthetics do not stimulate or inhibit cGMP production in endothelial-vascular co-cultures and suggested that they do not activate eNOS or GC. The present results concur with the conclusions made from these later studies that anesthetics have no significant effect on NO-mediated relaxation. Differences in methods, organ specificity, or type of preparation may account for different results and conclusions in various studies.

A possible limitation of our study is that only bradykinin was given to induce NO production by eNOS. Other drugs, that stimulate NO release, such as acetylcholine and serotonin, cause direct vasoconstriction at high doses or in the presence of L-arginine antagonism, and so they were not used to test our hypothesis. Bradykinin primarily stimulates endothelial cell Ca^{2+} influx to trigger NO production, but it has been reported to cause vasodilation by several mechanisms.[17,31,41,42] Bradykinin may induce prostacyclin production[43] and stimulate endothelium-dependent hyperpolarizing factor[44] to produce vasodilation. This factor may endogenously activate K^+ channel opening.[45] In the related study,[26] we found that indomethacin, an inhibitor of prostacyclin production, and tetraethylammonium, a nonspecific K^+ channel blocker, did not alter a bradykinin-induced increase in CF or release of NO but that adding 100 μM L-NAME with indomethacin and tetraethylammonium inhibited increases in effluent CF and NO induced by 1 nM bradykinin. Although L-NAME completely blocked release of NO to 10 nM bradykinin, it did not completely block the increase of CF. In the presence of all three inhibitors of vasodilation, a bradykinin-induced increase in CF could not be completely abolished. Figure 8 indicates that bradykinin concentrations up to 100 nM incrementally increase [L-cit]. The related study[26] suggested the major mechanism of vasodilation by up to 10 nM bradykinin depends on NO release but that higher bradykinin concentrations exert an additional mechanism of vasodilation, perhaps a direct effect on vascular smooth muscle.

In conclusion, this study shows that coronary effluent release of NO and L-cit by bradykinin is associated with an increase in CF, and the given concentrations of HAL, ISO, and SEV do not alter NO production, NO release, or NO-mediated vasodilator effects in intact guinea pig hearts. We question whether the lower levels of volatile anesthetics exert a physiologically significant effect to reduce coronary vascular resistance *via* the NO pathway.

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