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Attenuation of nitric oxide-stimulated soluble guanylyl cyclase from the rat brain by halogenated volatile anesthetics

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Abstract

Purpose. The present study was undertaken to examine whether interaction between halogenated volatile anesthetics and nitric oxide (NO) at soluble guanylyl cyclase (sGC) would occur in rat brain.

Methods. A soluble brain fraction was prepared from extensively perfused Sprague-Dawley rat brains by centrifugation and used as the source of sGC. sGC was incubated with NO and halogenated volatile anesthetics, and cGMP production was determined by enzyme immunoassay in aliquots of the supernatant.

Results. Halothane and sevoflurane produced significant ($P < 0.01$) and dose-dependent inhibition of NO-stimulated sGC activity over a range of NO concentrations (2×10^{-9} to 2×10^{-5} M). Among the anesthetics, halothane tended to have a large inhibitory effect on NO-stimulated sGC activity, which was, however, not significant. sGC activity was also inhibited by both anesthetics ($P < 0.05$) in the absence of NO stimulation. GTP dose-dependently increased both NO-stimulated and -nonstimulated sGC activities. Halothane and sevoflurane decreased these activities ($P < 0.01$), but the inhibition by these anesthetics was not significant at higher GTP concentrations.

Conclusion. These results suggest that halogenated volatile anesthetics can attenuate the activity of NO-stimulated sGC by competing with NO for the NO binding site on the enzyme.

Key words: Nitric oxide, Halogenated volatile anesthetics, Guanylyl cyclase, Ferrous iron

Introduction

Nitric oxide (NO) is a unique, reactive, radical gas that is known to function as an intracellular and intercellular messenger as well as having cytotoxic activity. The production of NO from L-arginine by NO synthase (NOS) has been reported to occur in numerous tissues [1,2] including the central nervous system (CNS) [3]. In target cells, NO exerts its effects by elevating intracellular cyclic guanosine monophosphate (cGMP) levels through activation of the heme protein enzyme, soluble guanylyl cyclase (sGC). The binding site for NO on sGC is a ferrous iron (Fe^{2+}) heme [4]. Because the Fe^{2+} contains an electron that can be shared or transferred, this site is especially attractive to NO with an unpaired electron.

In the CNS, NO is released in response to the stimulation of excitatory amino acids [5,6] and appears to play a role as a neuromodulator or neurotransmitter by increasing cGMP content [7,8]. Although the physiological function of NO remains uncertain, inhibition of the NO signal pathway in the CNS may be implicated in sedative, analgesic, or anesthetic effects. It is well known that volatile halogenated anesthetics decrease the cGMP content in specific brain regions [9,10]. Ketamine, a potent intravenous anesthetic, blocks the *N*-methyl-D-aspartate (NMDA) receptor and inhibits NO synthesis [11]. The administration of specific inhibitors of NOS reduces the minimum alveolar concentration (MAC) of halothane [12] and isoflurane [13,14]. Moreover, methylene blue (MB), an sGC inhibitor, produces antinociception on intracerebroventricular administration [15].

The halogenated volatile anesthetics, as a consequence of their specific halogen content, are attracted to ferrous iron containing heme protein. Among the anesthetics, halothane, because it contains a bromide atom, has the greatest affinity for ferrous iron heme proteins and is reported to be reductively metabolized by heme proteins [16,17] because of this affinity. Thus, a strong similarity exists between halogenated volatile anesthetics and NO in their attraction for ferrous iron heme. Competition between NO and halogenated volatile

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anesthetics has been demonstrated at the ferrous iron heme of cytochrome p-450 [18].

The effects of halogenated volatile anesthetics on sGC in different tissues have been reported. These anesthetics would compete with NO at the ferrous iron heme of sGC and inhibit the activity in the rat aortal [19] and liver [20]. However, in the rat brain, sGC activity was not affected by halogenated volatile anesthetics [21]. The present study was undertaken to examine whether halogenated volatile anesthetics would compete with NO at sGC and affect NO-stimulated sGC activity in a soluble fraction of rat brain.

Materials and methods

Preparation of perfused soluble fraction

Male Sprague-Dawley rats (150–200 g) were anesthetized with sodium pentothal (20 mg·kg^{-1}) i.p. After laparotomy, heparin was injected into the left renal vein. In situ, the brain was transcardially perfused with ice-cold 50 mM Tris containing 0.9% NaCl (pH 7.4), and then the brain was excised and immediately placed in ice-cold 50 mM Tris containing 1 mM DL-dithiothreitol (pH 7.4), finely minced, washed twice with this buffer to remove contaminating erythrocytes and hemoglobin, and homogenized. The homogenate was centrifuged for 10 min at 10000 *g*, and the supernatant was ultracentrifuged for 60 min at 105000 *g*. The resulting supernatant was used as the source of sGC for these experiments. Protein concentration was measured by the method of Bradford [22].

NO preparation

NO solution was prepared by equilibrating 10 ml of pure NO gas and 95 ml of carefully and extensively deoxygenated water, prepared by bubbling with 100% nitrogen for 40 min as described by Moy et al. [23]. The concentration of NO in this saturated solution was reported to average 2.4 mM ± 0.1 mM [24].

Determination of cGMP formation

The incubation was initiated by the addition of guanosine triphosphate (GTP), the substrate of sGC, and NO solution simultaneously and was carried out for 5 min at 37°C using a total volume of 600 µl with 50 mM Tris, 5 mM $MgCl_2$, 1 mM DL-dithiothreitol, 1 mM isobutylmethylxanthine, 0.1 mM GTP, the indicated concentration of halogenated volatile anesthetic, and 0.1 mg protein of the soluble fraction in a capped 10-ml conical glass tube. The preincubation was performed for 3 min at 37°C before GTP and NO solution were added. The indicated anesthetic concentration was achieved by adding saturated anesthetic buffer, and the final concentration of anesthetics in the incubation solution was measured by gas chromatography. The incubation was terminated by the addition of 100 µl of 10% trichloroacetic and cooling on ice. After centrifugation for 10 min at 2500 *g*, cGMP production was determined by enzyme immunoassay in aliquots of the supernatant.

Chemicals

A cyclic GMP enzyme immunoassay kit (RPN 226) was obtained from Amersham Life Sciences, Arlington Heights, IL, USA. NO gas was purchased from Yamato-Sanki, Tokyo, Japan. Halothane and sevoflurane were purchased from Nippon Hoechst Marion Roussel, Tokyo, Japan. Other chemicals were obtained from Sigma Chemical, St. Louis, MO, USA.

Statistical analysis

The data were analyzed by ANOVA with subsequent intragroup comparisons using Scheffé's *F*. A value of $P < 0.05$ was considered significant.

Results

Halothane and sevoflurane clearly inhibited cGMP production stimulated by NO. cGMP production was also inhibited by both anesthetics without NO stimulation (Fig. 1). This inhibition was dose-dependent. Significant differences were found with halothane at 0.222, 0.727, and 1.134 mM and with sevoflurane at 0.812 and 1.165 mM. Among the anesthetics, halothane tended to have a large inhibitory effect on NO-stimulated sGC activity, which was, however, not significant (Fig. 2).

NO increased cGMP production at concentrations as low as 2×10^{-9} M. At each concentration, halogenated volatile anesthetics attenuated this production (Fig. 3). Because both anesthetics had inhibitory effects on sGC in the absence of NO stimulation, the effect of the concentration of GTP was examined. GTP dose-dependently increased both NO-stimulated and -nonstimulated sGC activities. Halothane and sevoflurane decreased these activities, but the inhibition by these anesthetics was not significant at 0.5, 1, and 5 mM GTP in the absence of NO, and at 5 mM GTP in the presence of NO stimulation (Fig. 4a,b).

Discussion

The data presented in this study clearly show that halothane and sevoflurane dose-dependently inhibited NO-stimulated sGC activity. Although there are many

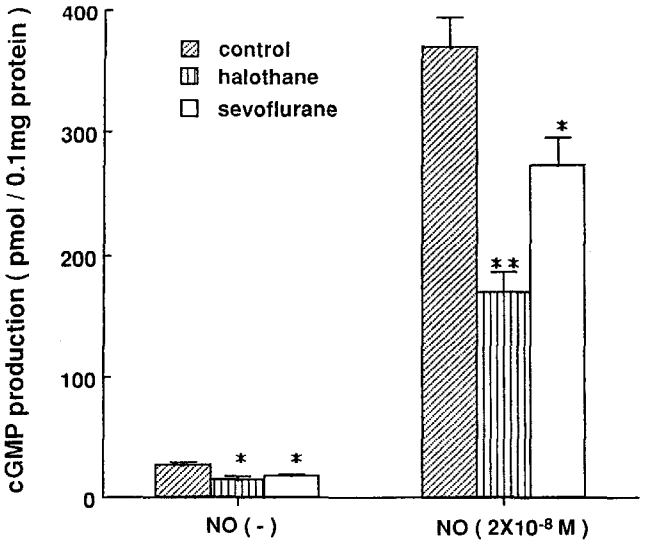

Fig. 1. Effects of halogenated volatile anesthetics on cGMP production in the presence and absence of NO (2×10^{-8}M). The incubations were carried out at 37°C for 5 min with halothane (1.134 mM), with sevoflurane (1.165 mM), and without anesthetics. Data are expressed as means ± SE $n = 10$. $^{**}P < 0.01$, $^{*}P < 0.05$ *vs* control

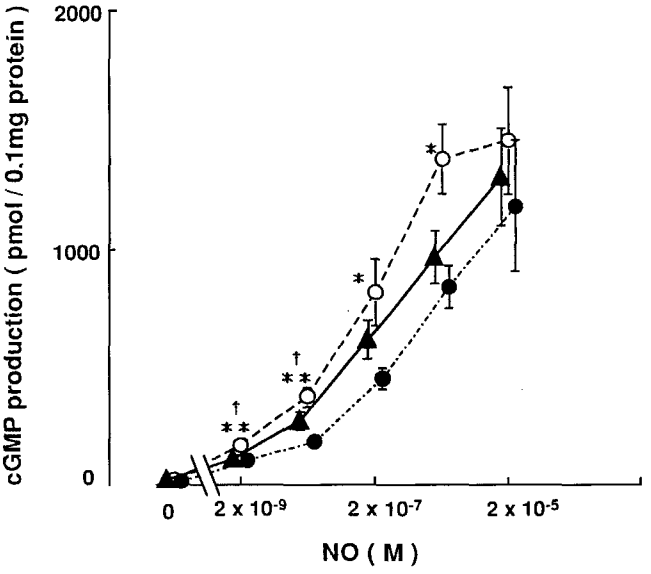

Fig. 3. Dose–response curve of NO. The incubations were carried out at 37°C for 5 min with halothane (1.134 mM) (*filled circles*), with sevoflurane (1.165 mM) (*triangles*), and without anesthetics (*open circles*). Data are expressed as means ± SE. $n = 6$. $^{**}P < 0.01$, $^{*}P < 0.05$ *vs* halothane; $^{\dagger}P < 0.05$ *vs* sevoflurane

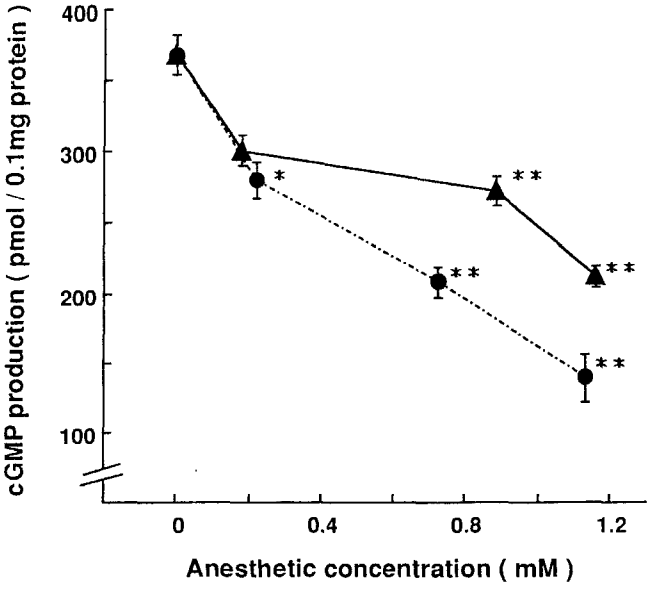

Fig. 2. Dose–response curve of anesthetics. The incubations were carried out at 37°C for 5 min with NO (2×10^{-8}M). *Circles*, halothane; *triangles*, sevoflurane. Data are expressed as means ± SE. $n = 6$. $^{**}P < 0.01$, $^{*}P < 0.05$ *vs* 0 mM

possible ways for halogenated volatile anesthetics to attenuate sGC activity, it is very likely that NO and both anesthetics compete for the NO active site on the enzyme.

Soluble guanylyl cyclase is a ferrous heme-containing enzyme found in most tissues. Although it is not the only target for NO, it is predominantly activated by nitrosation of the ferrous iron atom, as proposed by Ignarro [25]. Recently, carbon monoxide (CO) was also reported to activate this enzyme by the same mechanism as NO [26,27]. Thus, the essential component seems to be the reduced iron atom through which NO can form a coordinate bond. A great affinity of NO for the ferrous iron heme protein is demonstrated by its ease in binding to other heme proteins, such as hemoglobin [28], myoglobin [29], and cytochrome p-450 [18].

Because halogens have extremely high electron affinities, halogenated volatile anesthetics are also strongly attracted to ferrous heme protein. Halothane has been reported to be not only attracted to ferrous iron, but metabolized by them as well. Cytochrome p-450 [16], hemoglobin, and hemin [17] have all been shown to metabolize halothane when the iron is in the ferrous state. Therefore, it would be logical halogenated volatile anesthetics had an affinity for the ferrous iron of sGC and inhibited NO-stimulated sGC activity by this process.

Data are also presented to show that sevoflurane is a relatively poor inhibitor of NO-stimulated sGC when compared with halothane. Sevoflurane is also only poorly oxidatively metabolized by cytochrome p-450 and undergoes no reductive metabolism, as halothane

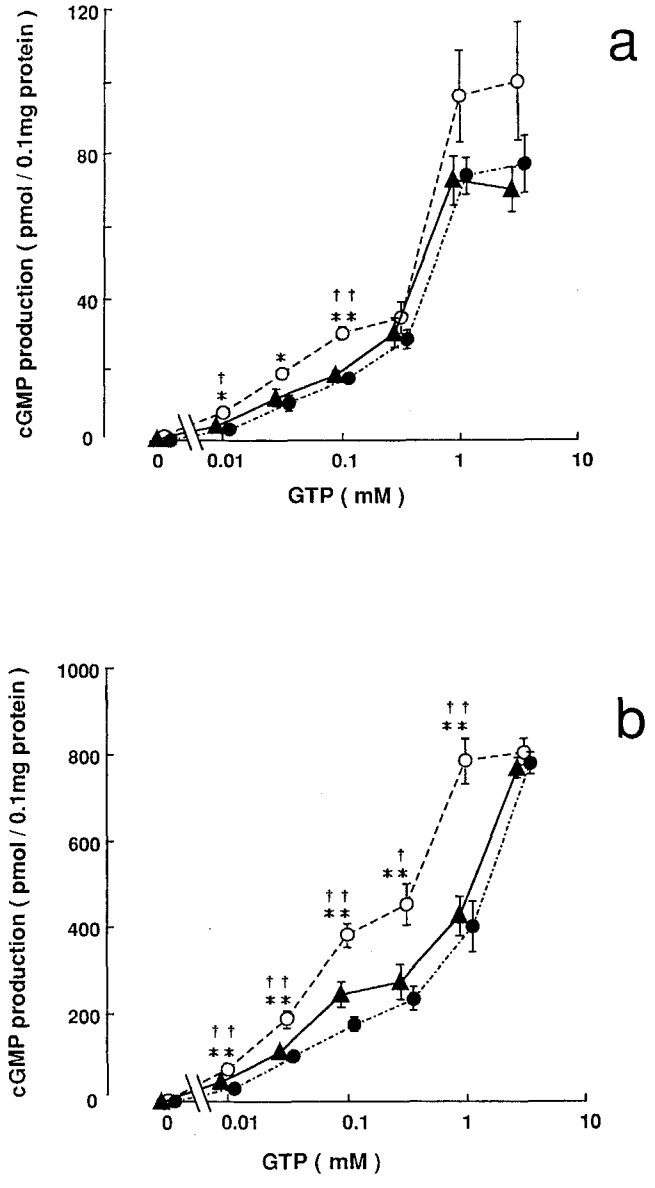

Fig. 4. Dose–response curve of GTP. The incubations were carried out at 37°C for 5 min with halothane (1.134 mM) (*filled circles*), with sevoflurane (1.165 mM) (*triangles*), and without anesthetics. (*open circles*). **a** In absence of NO; **b** in presence of NO (2×10^{-8} M). Data are expressed as means ± SE. $n = 6$. $^{**}P < 0.01$, $^{*}P < 0.05$ *vs* halothane; $^{\ddagger}P < 0.01$, $^{\dagger}P < 0.05$ *vs* sevoflurane

does [30]. The difference is related to the halogen composition of the two compounds: halothane contains bromide, a stronger electrophile than fluorine [31], which is found in sevoflurane. Thus, sevoflurane would have a lower affinity for the ferrous iron of sGC and a smaller effect on this activity than halothane.

Hart et al. [19] demonstrated that halothane (0.5, 1, and 2 MAC) significantly inhibited the relaxation of rat aorta rings induced by NO, acetylcholine, and nitroglycerine (the NO donor drug). Because NO-stimulated cGMP content was also attenuated by halothane and the relaxation responses to these agents are mediated by the production of cGMP through activation of sGC, they suggested that halothane interferes with the activation of sGC by NO in smooth muscle cells. These results are quite consistent with ours. Recently, halothane has been reported to inhibit CO-induced vascular relaxation and cGMP content [32]. Like NO, although less potently, CO exerts its effect by binding the ferrous iron of the heme of sGC to activate the enzyme, with subsequent production of cGMP. CO has a lower affinity for heme protein than NO, and CO-induced vasorelaxation was more effectively inhibited by halothane than NO-induced relaxation. Thus, this report proposed that interaction between CO and halothane occurred at the ferrous iron of sGC and inhibited the effect of CO.

Halogenated volatile anesthetics may affect the stability of NO. Blaise et al. [33] demonstrated that the relaxation of rabbit abdominal aorta, induced by perfusion of the bovine aortic endothelial cells, was attenuated by halothane. This study concluded that halothane seemed to modify either EDRF/NO half-life or its activated redox form. However, Zuo et al. [21], who showed that halogenated volatile anesthetics had no effect on NO-stimulated activity of partially isolated sGC from rat brain, pointed out that inhalation anesthetics can affect arterial ring tone by other mechanisms apart from the NO-guanylyl cyclase pathway. Therefore it is difficult to isolate the mechanisms that contributed to the effect of halothane on vessel tone in the experiment of Blaise et al. Yoshida et al. [34] reported that sevoflurane attenuated endothelium-dependent relaxation. The suppressive effects of added superoxide dismutase on sevoflurane suggest that the inactivation of NO by superoxide is involved in the attenuation of sevoflurane. However, they did not show how sevoflurane produced superoxide in their experimental conditions. In both studies [33,34], free radicals produced by halogenated volatile anesthetics would be implicated in the stability of NO. Although it is not certain whether halothane and sevoflurane generate free radicals in our incubation, whole tissue, like the aorta ring, might be necessary for halogenated volatile anesthetics to produce free radicals.

In the present study, halothane and sevoflurane also inhibited sGC activity without NO stimulation as well as with NO stimulation. Without NO, sGC could be activated by its substrate, GTP. The effects of both anesthetics on sGC activity with various concentrations of GTP were examined. sGC activity was attenuated by these anesthetics at a wide range of concentrations of GTP, but at 0.5, 1, and 5 mM GTP without NO, and at 5 mM GTP with NO stimulation, the inhibitory effects of

halothane and sevoflurane were not significant. These results suggest that halogenated volatile anesthetics interfere with the GTP-binding site of sGC and attenuate its activity. Our previous study demonstrated that halothane decreased NO-stimulated cGMP production in partially purified sGC from rat liver. However, the basal level of cGMP was not affected by halothane [20]. The reason for this difference is not clear, although there might be several isozymes in sGC, depending on the origin of the enzyme, and the effects of halogenated volatile anesthetics on these isozymes might differ.

Terasako et al. [35] reported that the production of cGMP was suppressed by halothane after stimulation by NMDA and D-aspartate, but not sodium nitroprusside, and that isoflurane suppressed the NMDA-stimulated, but not the D-aspartate- and sodium nitroprusside-stimulated, formation of cGMP in rat cerebellar slices. These results indicate that halothane inactivates NOS and isoflurane interacts with the NMDA receptor without any effects on sGC, which disagreed with our results. They administered halothane and isoflurane at only single concentrations. In addition, the sodium nitroprusside used as the NO donor was the maximum dose. Therefore, cGMP production, which was stimulated by a large amount of NO, would not be inhibited by their anesthetic concentrations. Zuo et al. [21] also demonstrated that halothane, isoflurane, and enflurane did not affect the basal or agonist-stimulated activity of partially isolated soluble and particulate GC from rat brain. The exact reason for the discrepancy with the present study is not apparent; however, the enzymes were not equilibrated with anesthetics before the addition of NO in their study. Because of their lower affinity for heme protein than for NO, the anesthetics may need a longer time to exert their effects on sGC. Therefore, it would not be possible to observe anesthetic-induced inhibition of sGC if NO were mixed with the enzymes simultaneously and the assay were conducted for only a short time, as reported by Jing et al. [32]. In our study, the preincubation was performed with anesthetics, prior to the incubation. Moreover, they provided GTP, the substrate for sGC, by a GTP-regenerating system. Although it is not clear how much GTP is produced by this system, halothane and sevoflurane did not have inhibitory effects on sGC at higher GTP concentrations in our experiment.

In summary, the activation of sGC by NO was attenuated by halothane and sevoflurane. The attenuation could be associated with the attraction of halogenated anesthetics to ferrous heme iron and competition for the binding site of NO. Although the inhibitory effects of halogenated volatile anesthetics on sGC suggest a partial explanation for the mechanism of action of these anesthetics, further investigation is needed to determine this matter.

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References

1. Palmer RMJ, Ashton DS, Moncada S (1988) Vascular endothelial cells synthesize nitric oxide from L-arginine. Nature (Lond) 333:664–666
2. Azuma H, Ishikawa M, Sekizawa S (1986) Endothelium-dependent inhibition of platelet aggregation. Br J Pharmacol 88:411–415
3. Bredt DS, Snyder SH (1992) Nitric oxide, a novel neuronal messenger. Neuron 8:3–11
4. Olstein EH, Wood KS, Ignarro LJ (1982) Purification and properties of heme-deficient soluble guanylate cyclase: effects of heme and other factors on enzyme activation of NO, NO-heme and protoporphyrin. Arch Biochem Biophys 218:187–198
5. Vincent SR, Hope BT (1992) Neurons that say NO. Trends Neurosci 15:108–117
6. Garthweite J, Charles SL, Chess-Williams R (1988) Endothelium-derived relaxing factor release on activation of NMDA receptor suggests role as intracellular messenger in the brain. Nature (Lond) 336:385–388
7. Garthweite J, Garthweite G, Palmer RMJ, Moncada S (1989) NMDA receptor activation induces nitric oxide synthesis from arginine in rat brain slices. Eur J Pharmacol 172:413–416
8. Bredt DS, Snyder SH (1989) Nitric oxide mediates glutamate-linked enhancement of cGMP levels in the cerebellum. Proc Natl Acad Sci USA 86:9030–9033
9. Kant GJ, Muller TW, Lenox RH, Meyerhoff JL (1980) In vivo effects of pentobarbital and halothane anesthesia on levels of adenosine 3′, 5′-monophosphate and guanosine 3′, 5′-monophosphate in rat brain regions and pituitary. Biochem Pharmacol 29:1891–1896
10. Vulliemoz Y, Verosky M, Alpert M, Triner L (1983) Effects of enflurane on cerebellar cGMP and on motor activity in the mouse. Br J Anaesth 55:79–84
11. Gonzales JM, Loeb AL, Reichard PS, Irvine S (1995) Ketamine inhibits glutamate *N*-methyl-D-aspartate and quisqualate-stimulated cGMP production in cultured cerebral neurons. Anesthesiology 82:205–213
12. Johns RA, Moscicki JC, DiFazio CA (1992) Nitric oxide synthase inhibitor dose-dependently and reversibly reduces the threshold for halothane anesthesia. Anesthesiology 77:779–784
13. Ichinose F, Huang PL, Zapol WM (1995) Effects of targeted neuronal nitric oxide synthase gene disruption and nitro^{G}-L-arginine methylester on the threshold for isoflurane anesthesia. Anesthesiology 83:101–108
14. Pajewski TN, DiFazio CA, Moscicki JC, Johns RA (1996) Nitric oxide synthase inhibitors 7-nitro indazole and nitro^{G}-L-arginine methyl ester, dose dependently reduced the threshold for isoflurane anesthesia. Anesthesiology 85:1111–1119
15. Kawabata A, Umeda N, Takagi H (1993) L-Arginine exerts a dual role in niciceptive processing in the brain: involvement of the kyotorphin-Met-enkephalin pathway and NO-cyclic GMP pathway. Br J Pharmacol 109:73–79
16. Ahr HJ, King LJ, Nastainczyk W, Ullrich V (1982) The mechanism of reductive dehalogenation of halothane by liver cytochrome P-450. Biochem Pharmacol 41:383–390
17. Baker MT, Nelson RM, Van Dyke RA (1983) The release of inorganic fluoride form halothane and halothane metabolites by cytochrome P-450, hemin and hemoglobin. Drug Metab Dispos 11:308–311

18. Masaki E, Van Dyke RA (1992) Effect of nitric oxide on the anaerobic metabolism of halothane by rat hepatic cytochrome P-450. FASEB J 6:A1568 (abstract)
19. Hart JL, Jing M, Bina S, Freas W, Van Dyke RA, Muldoon SM (1993) Effects of halothane on EDRF/cGMP-mediated vascular smooth muscle relaxation. Anesthesiology 79:323–331
20. Jing M, Hart JL, Masaki E, Van Dyke RA, Bina S, Muldoon SM (1995) Vascular effects of halothane and isoflurane: cGMP dependent and independent actions. Life Sci 56:19–29
21. Zuo Z, Johns RA (1995) Halothane, Enflurane, and isoflurane do not affect the basal or agonist-stimulated activity of partially isolated soluble and particulate guanylyl cyclases of rat brain. Anesthesiology 83:395–404
22. Bradford MM (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein binding. Anal Biochem 72:248–254
23. Moy JA, Bates JN, Fisher RA (1991) Effects of nitric oxide on platelet-activation factor and α-adrenergic-stimulated vasoconstriction and glycogenolysis in perfused rat liver. J Biol Chem 266:8092–8096
24. Myers PR, Minor RJ Jr, Bates JN, Harrison DG (1990) Vasorelaxant properties of the endothelium-derived relaxing factor more closely resembles-nitrosocysteine than nitric oxide. Nature (Lond) 345:161–163
25. Ignarro LJ, Wood KS, Wolin MS (1982) Activation of purified soluble guanylate cyclase by protoporphyrin IX. Proc Natl Acad Sci USA 79:2870–2873
26. Brume B, Ullrich V (1987) Inhibition of platelet aggregation by carbon monoxide is mediated by activation of guanylate cyclase. Mol Pharmacol 32:497–504
27. Marks GS, Brien JF, Nakatsu K, McLaughlin BE (1991) Does carbon monoxide have a physiological function? Trends Pharmacol Sci 12:185–188
28. Palmer RMJ, Ferrige AG, Moncada S (1987) Nitric oxide release accounts for the biological activity of endothelium-derived relaxing factor. Nature (Lond) 327:524–526
29. Ignarro LJ, Adams JB, Horwitz PM, Wood KS (1986) Activation of soluble guanylate cyclase by NO-hemeproteins involves NO-heme change. Comparison of heme-containing and heme-deficient enzyme forms. J Biol Chem 261:4997–5002
30. Baker MT, Van Dyke RA (1992) Biochemical and toxicological aspects of the volatile anesthetics. In: Barash PG, Cullen BF, Stoelting RK (eds) Clinical anesthesia, 2nd edn. JB Lippincott, Philadelphia, pp 467–480
31. Van Dyke RA, Wineman CG (1971) Enzymatic dechlorination: dechlorination of chloroethanes and propanes in vitro. Biochem Pharmacol 20:463–470
32. Jing M, Bina S, Verma A, Hart JL, Muldoon SM (1996) Effects of halothane and isoflurane on carbon monoxide-induced relaxations in the rat aorta. Anesthesiology 85:347–354
33. Blaise G, To Q, Parent M, Lagarde B, Asenjo F, Sauve R (1994) Does halothane interfere with the release, action or stability of endothelium-derived relaxing factor/nitric oxide? Anesthesiology 80:417–426
34. Yoshida K, Okabe E (1992) Selective impairment of endothelium-dependent relaxation by sevoflurane: oxygen free radicals participation. Anesthesiology 76:440–447
35. Terasako K, Nakamura K, Miyawaki I, Toda H, Karuyama M, Mori K (1994) Inhibitory effects of anesthetics on cyclic guanosine monophosphate (cGMP) accumulation in rat cerebellar slices. Anesth Analg 79:91–96