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Inhalational Anesthetics Up-Regulate Constitutive and Lipopolysaccharide-Induced Inducible Nitric Oxide Synthase Expression and Activity

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SUMMARY

Nitric oxide (NO) is an important biological messenger involved in the regulation of blood vessel tone, neurotransmission, inflammatory responses, and host defenses. Inhalational anesthetics have been shown to inhibit the function of the NO signaling pathway in a variety of tissues. In addition, acute inhibition of the NO signaling pathway significantly reduced the required alveolar concentration of halothane or isoflurane for anesthesia, which suggests a role for the NO signaling pathway in mechanisms of anesthesia and consciousness. We now report that inhalational anesthetics affect gene expression of nitric oxide synthases (NOS) (EC 1.14.13.39), the enzymes that synthesize NO from L-arginine. Both halothane and isoflurane, at clinically relevant concentrations, significantly up-regulate the mRNA, protein, and activity level of NOS in lipopolysaccharide-treated macrophages (inducible NOS; type II NOS), and bovine pulmonary endothelial cells (endothelial constitutive NOS; type III NOS). This is a novel interaction between inhalational anesthetics and the NO signaling pathway and has wide-ranging implications for both clinical medicine and experimental biology.

NO is a novel cell-signaling molecule that has been demonstrated to be involved in a variety of physiological and pathophysiological processes (1–6). Two types of enzymes, constitutive and inducible NOS (EC 1 14.13.39), are found to be responsible for the production of NO *in vivo*. Two constitutive NOS isoforms are known, one of which was found originally in endothelial cells and the other was found originally in neurons. Both isoforms require calcium, calmodulin, β-NADPH, FAD, flavin mononucleotide, and tetrahydrobiopterin as cofactors for activation (7). iNOS (type II NOS) is inducible in many cell types, such as macrophages and smooth muscle cells in response to LPS and cytokines, and also requires β-NADPH, FAD, flavin mononucleotide, and tetrahydrobiopterin, but not calcium and calmodulin as cofactors for activation (7). Therefore, iNOS will produce NO continuously once it is induced, whereas constitutive NOS activity is regulated by intracellular Ca^{2+} level. It is believed that iNOS has a significant role in immune responses, where it has been implicated in killing tumor cells and microbes, and the development of inflammatory responses and septic shock (8). eNOS (type III NOS) is primarily involved in the regulation of blood vessel tone and is implicated in mechanisms of a wide range of cardiovascular diseases (8).

Halothane and isoflurane, two widely used inhalational anesthetics, have been demonstrated to inhibit the function of the NO signaling pathway in blood vessels and brain (9, 10). We have shown that the inhibition of NOS dose-dependently and significantly decreased the required alveolar concentration of halothane or isoflurane for anesthesia, demonstrating a possible role for NO in consciousness (6). Therefore, the inhibitory effect of inhalational anesthetics on the function of the NO signaling pathway may be a mechanism of anesthesia and of the side-effects of these inhalational anesthetics. However, one potential target of anesthetic action, gene expression within cells, has been largely neglected. Indeed, both pentobarbital and halothane have been demonstrated to have selective effects on the gene expression of c-*fos*, and *jun*-B, two protooncogene members, in rat brain (11). Our current study, using cultured mouse macrophages and BPAEC, addresses the hypothesis that inhalational anesthetics may alter NOS expression.

Materials and Methods

All reagents and materials were obtained from Sigma Chemical (St. Louis, MO) unless specified in the text.

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ABBREVIATIONS: NO, nitric oxide; NOS, nitric oxide synthase; BPAEC, bovine pulmonary artery endothelial cells; eNOS, endothelial constitutive nitric oxide synthase; iNOS, inducible nitric oxide synthase; L-NAME, nitro^{G}-L-arginine-methyl-ester; LPS, lipopolysaccharide; PKC, protein kinase C; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid.

Cell culture. Mouse macrophages and BPAEC were cultured in glass roller bottles (850 cm^2; Wheaton, Millville, NJ) to provide a model to study the expression of iNOS and eNOS. Glass bottles with Teflon caps were used to avoid the possible absorption or interaction of anesthetics by plastic bottles.

Mouse RAW 264.7 macrophages were obtained from the American Type Culture Collection (Rockville, MD), and cultured in RPMI 1640 (Gibco/BRL, Grand Island, NY) containing 10% fetal bovine serum. BPAEC were isolated, characterized, and cultured as we described previously (12). In summary, endothelial cells were sorted out by fluorescence-activated cell sorting using acetylated low-density lipoprotein as the fluorescent marker. They were then further identified by: 1) typical cobblestone morphology; 2) antibody staining for factor VIII antigen; and 3) demonstrating a single mRNA for endothelial cell β-actin by Northern blot and the absence of immunostaining for α-actin by immunocytochemistry. The cells were cultured in medium 199 containing 10% fetal bovine serum, 2.4 μg/ml thymidine, 100 units/ml penicillin, and 100 μg/ml streptomycin.

Incubation. Only confluent mouse RAW 264.7 macrophages (passage 20 to 36) or BPAEC (passage 9 to 12) were used in the experiments. Macrophages were incubated with LPS (100 ng/ml) in the presence or absence of 2% isoflurane (Ohmeda Caribe, Liberty Corner, NJ) for 0.5, 1, 3, 7, 15, and 24 hr at 37° in RPMI 1640 containing 1% fetal bovine serum. Endothelial cells were incubated with or without 2% isoflurane for 0.5, 1, 3, 7, 15, and 24 hr at 37° in the endothelial cell culture medium. In another set of experiments, BPAEC or LPS-activated macrophages were incubated with 2% halothane (Halocarbon Laboratories, Hackensack, NJ) for 7 hr at 37° in the respective incubation medium as used in isoflurane experiments.

Northern analysis. Total RNA from both macrophages, and endothelial cells was purified using TRIREAGENT (MRC, Cincinnati, OH), and the method of Chomczynski (13). Twenty micrograms of total RNA per sample was analyzed using standard Northern blot and hybridization techniques with cDNA probes (14). The eNOS cDNA clone was from Dr. Sessa (Department of Pharmacology, Boyer Center for Molecular Medicine, Yale University School of Medicine, New Haven, CT) (15). The iNOS cDNA clone was from Drs. Xie and Nathan (Beatrice and Samuel A. Seaver Laboratory, Department of Medicine, Cornell University Medical College, New York, NY) (16). cDNA probes were labeled with ^{32}P using a random primed labeling kit (Boehringer Mannheim, Indianapolis, IN) (17). The blot was also hybridized with an 18S rRNA oligonucleotide probe labeled with ^{32}P using a terminal transferase system (Boehringer Mannheim) (18) to normalize the iNOS and eNOS mRNA data for RNA loading and transfer. Bands were quantified using a PhosphorImager and IMAGEQUANT software (Molecular Dynamics, Sunnyvale, CA).

Western analysis. Western blot of cell homogenates was performed as detailed by North *et al.* (19). Cell homogenates were prepared by homogenizing the cells in 25 mM Tris·HCl, pH 7.4, containing 1 mM EDTA, 1 mM EGTA, 0.1% (v/v) 2-mercaptoethanol, 1 mM phenylmethylsulfonyl fluoride, 2 μM leupeptin, and 1 μM pepstatin A, and then centrifuging the crude homogenates at 1000 $\times$ g for 10 min at 4°. Fifteen or thirty micrograms of total protein per lane was used for iNOS or eNOS studies, respectively. Mouse anti-iNOS IgG2a monoclonal antibody and mouse anti-eNOS IgG1 monoclonal antibody were from Transduction Laboratories (Lexington, KY). The intensity of the autoradiographic bands was quantitated by densitometry (Personal Densitometer/IMAGEQUANT; Molecular Dynamics).

Nitrite analysis. The culture media were collected from the glass roller bottles of the cultured cells used to produce the Northern and Western blots of Figs. 1 and 4. The nitrite level in the medium was measured by a fluorometric assay detailed by Misko *et al.* (20). The samples were read with excitation at 365 nm and emission at 450 nm. To confirm that the accumulation of nitrite in the roller bottles reflected the NOS activity of the cells, LPS-activated macrophages or BPAEC were incubated with various combinations of agents including 2% isoflurane, L-NAME, 1.0 mM L-arginine, and 1.0 mM D-arginine for 7 hr at 37°. The culture media were then collected for measurements of nitrite accumulation.

Statistical analysis. Results are presented as mean $\pm$ standard error in the bar graphs. Statistical analysis was performed by Student t test. $p < 0.05$ was accepted as significant (21).

Results

The level of mRNA and protein for NOS in the LPS-activated macrophages and BPAEC were detected by Northern and Western blot analysis, respectively. Northern analysis detected a single iNOS band at 4.2 kilobase pairs in activated macrophages, and a single eNOS band at 4.5 kilobase pairs in BPAEC. Western blot analysis found a 130-kDa iNOS protein in activated macrophages and a 140-kDa eNOS protein in BPAEC. No eNOS mRNA or protein band was detected in activated macrophages, and no iNOS mRNA or protein band was detected in BPAEC. Isoflurane caused a significant increase in the level of mRNA and protein of both iNOS and eNOS. Activated macrophages incubated with 2% isoflurane for longer than 3 hr expressed significantly more iNOS mRNA than the corresponding control cells (Fig. 1A). However, if macrophages are not activated, inhalational anesthetics alone did not induce iNOS expression (data not shown). The BPAEC incubated with 2% isoflurane for longer than 7 hr expressed significantly more eNOS mRNA than their respective control cells (Fig. 1C). This up-regulation of mRNA was the same regardless of whether 18S rRNA (as presented here) or β-actin or glyceraldehyde-3-phosphate dehydrogenase (data not shown) was used as the loading control. Consistent with this up-regulation of mRNA, 2% isoflurane greatly increased the level of iNOS protein in activated macrophages after 3 hr of incubation and slightly but significantly increased the level of eNOS protein in BPAEC after 7 hr of incubation (Fig. 1, B and D).

To confirm that this up-regulation of NOS mRNA and protein led to functional NO production, NO synthesis by iNOS or eNOS in activated macrophages or BPAEC was quantified by measuring the accumulation of nitrite (the NO oxidation product) in the conditioned culture medium (22). Consistent with the temporal pattern of the up-regulation of NOS mRNA, and protein, macrophages, and BPAEC incubated with 2% isoflurane for longer than 7 hr produced significantly more nitrite than the corresponding control cells (Fig. 2). This increased nitrite production is caused by NOS because 0.1 mM L-NAME, a specific NOS inhibitor, abolished this increase and 1 mM L-arginine but not D-arginine reversed this inhibition by L-NAME (Fig. 3). These results suggest that the up-regulation of NOS mRNA and protein caused by isoflurane leads to enhanced NO production.

The effect of halothane on NOS expression has also been investigated. Similar to the effect of isoflurane, 2% halothane, after incubating with the cells for 7 hr, significantly increased the level of mRNA and protein for iNOS in activated macrophages and for eNOS in BPAEC (Fig. 4). This up-regulation of mRNA and protein level by halothane was also functionally expressed by the increase of nitrite accumulation in the conditioned culture medium of both activated macrophages and endothelial cells (Fig. 5).

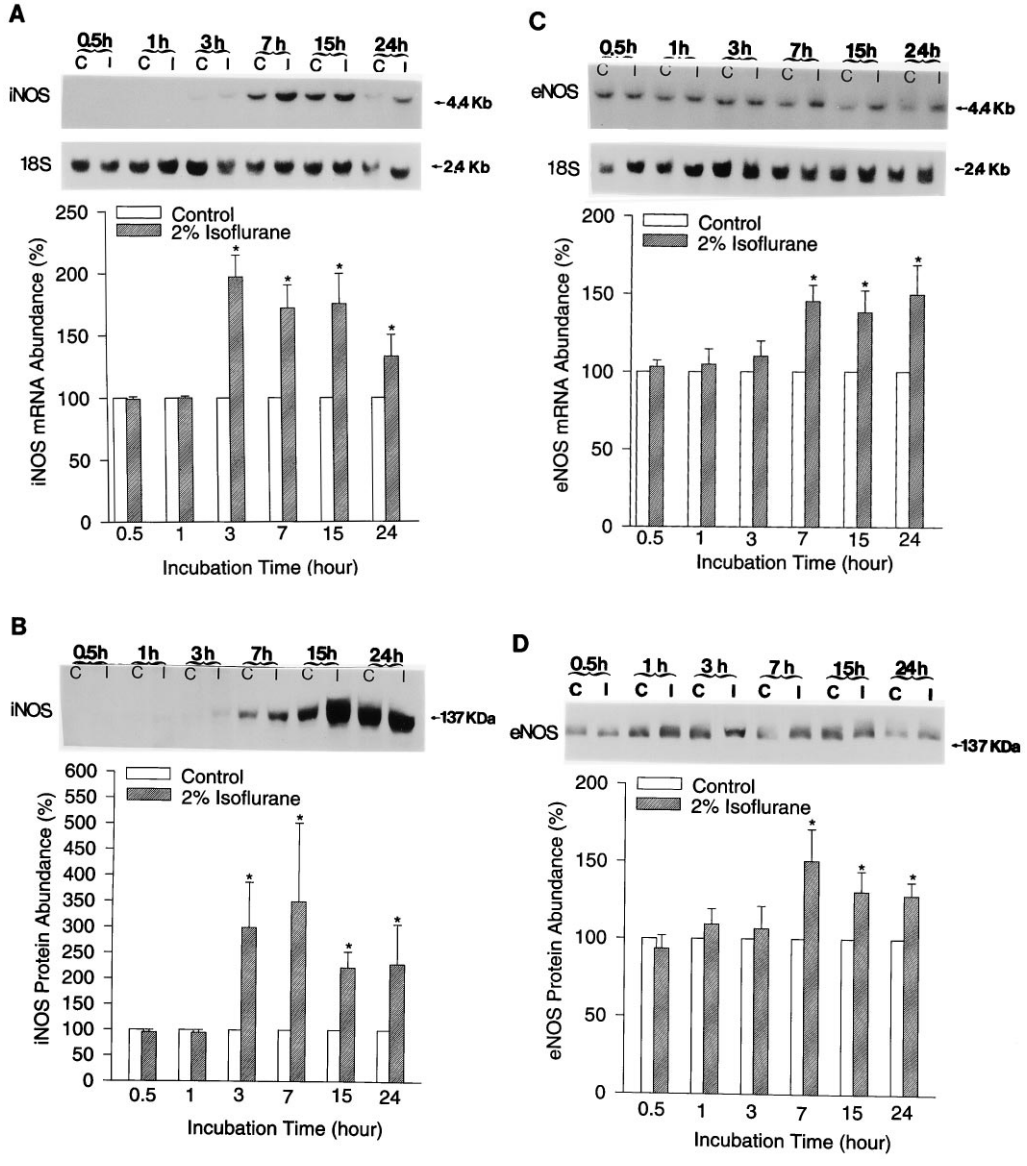

Fig. 1. Time-course of 2% isoflurane-induced up-regulation of the mRNA and protein of iNOS and eNOS in LPS-activated mouse RAW 264.7 macrophages, and bovine pulmonary artery endothelial cells. *Left,* demonstration of up-regulation of iNOS mRNA (A) or iNOS protein (B) in activated macrophages by a representative Northern or Western blot (*top*) and the graphic presentation of iNOS mRNA or protein abundance quantified by integrating the volume of autoradiograms from 4 separate experiments in duplicate (*bottom*). *Right*, demonstration of up-regulation of eNOS mRNA (C) or eNOS protein (D) in BPAEC by a representative Northern or Western blot (*top*) and the graphic presentation of eNOS mRNA or protein abundance quantified by integrating the volume of autoradiograms from four or five separate experiments in duplicate (*bottom*). Values in graphs are expressed as the percentage of corresponding time controls (100%) incubated without isoflurane. *Bars*, mean ± standard error of four or five experiments. *, $p < 0.05$ compared with the corresponding controls by *t* test. *h*, hour; *C*, control; *I*, 2% isoflurane.

Discussion

NO has been implicated in the function of macrophages to kill tumor cells and microbes (8). We now show that inhalational anesthetics increased iNOS expression and NO production in activated macrophages. These results suggest that inhalational anesthetics may have a significant effect on the NO-related functions of the immune system. Consistently, both *in vivo* and *in vitro* studies have demonstrated that inhalational anesthetics significantly affect humoral and cell-mediated immunity (23, 24). The fact that anesthetics potentiate the induction of iNOS by LPS is of great clinical significance because inhalational anesthetics are commonly administered to patients with sepsis and patients with acute inflammatory lesions (e.g., adult respiratory distress syndrome, pneumonia, and inflammatory bowel disease) when iNOS is likely to be up-regulated.

Inhalational anesthetics have been demonstrated to significantly inhibit the function of the eNOS signaling pathway (9, 25), although there is some controversy; under some circumstances, inhalational anesthetics may enhance endothelium-dependent vasodilation (26). Our current study showed a novel interaction between inhalational anesthetics and the eNOS signaling pathway. This may be one of the mechanisms by which inhalational anesthetics cause vessel relaxation in the clinical setting.

Apart from their roles in the immune and cardiovascular systems, iNOS and eNOS have been suggested to have other functions. For example, iNOS can be induced in vascular smooth muscle cells, fibroblasts, and myocytes of the cardiovascular system and astrocytes and other glial cells in the central nervous system (7, 27). iNOS has also been implicated in mechanisms of hyperalgesia in the spinal cord (28). eNOS has been implicated in the pathogenesis of several disease states such as diabetes, ischemic heart disease, hypertension, and myocardial ischemia-reperfusion injury (27, 29). Therefore, our novel observations may have multiple important physiological and pathophysiological implications. Furthermore, as many studies of the NO signaling pathway are performed in animals anesthetized with inhalational anesthetics, this novel action of anesthetics may significantly alter the outcome of those studies.

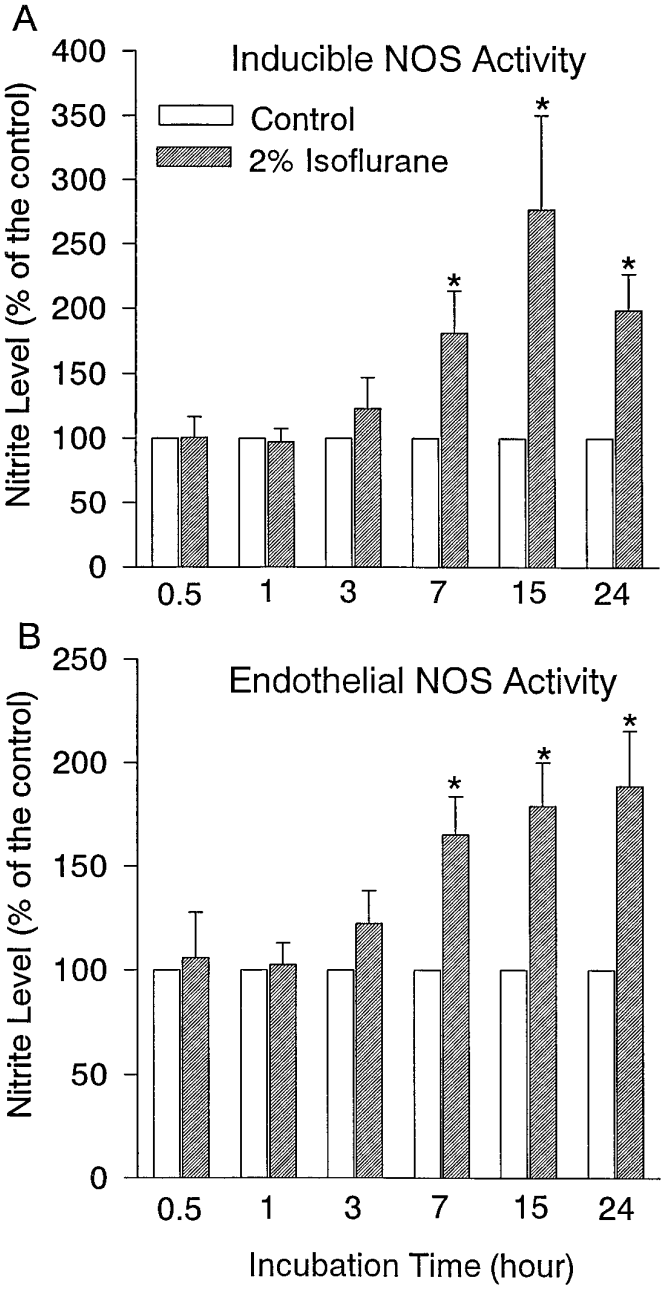

Fig. 2. Time-course of 2% isoflurane-induced increase of nitrite level in the conditioned culture medium of LPS-activated mouse RAW 264.7 macrophages (A) or bovine pulmonary artery endothelial cells (B). Values in bar graphs are expressed as the percentage of corresponding time controls (100%) incubated without isoflurane. *Bars*, mean ± standard error of four separate experiments performed in duplicate. *, $p < 0.05$ compared with the corresponding controls by *t* test.

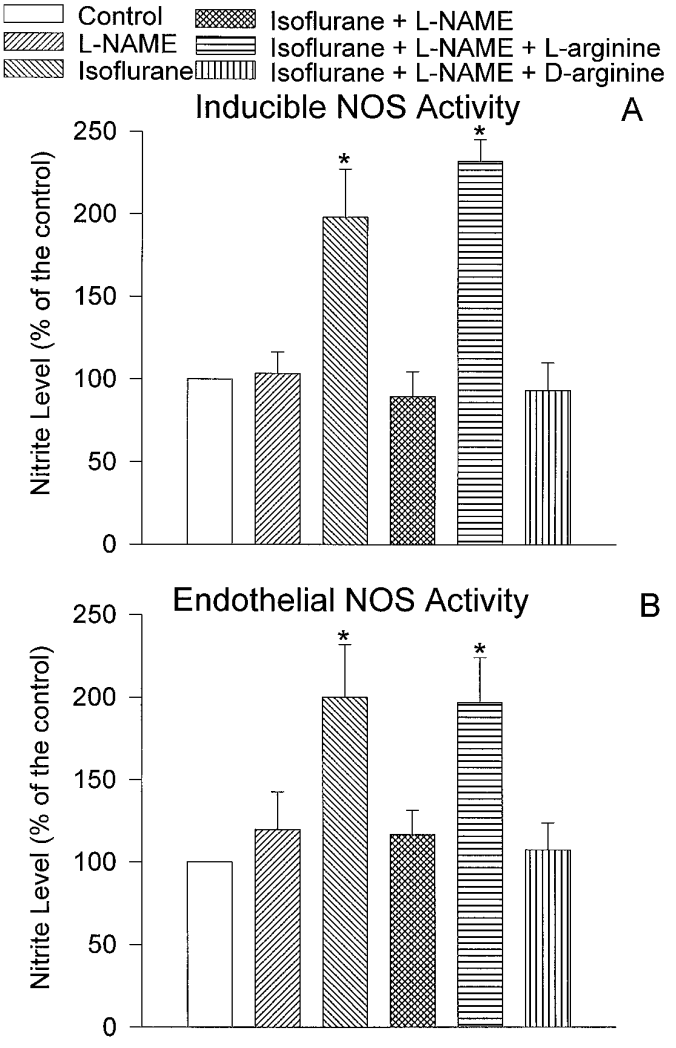

Fig. 3. The reversibility by 1.0 mM L-arginine and not by 1.0 mM D-arginine of the inhibitory effect of 0.1 mM L-NAME on the increased nitrite accumulation by 2% isoflurane in the conditioned culture medium of LPS-activated mouse RAW 264.7 macrophages (A) or bovine pulmonary artery endothelial cells (B). Values are expressed as the percentage of corresponding time control (100%) incubated without isoflurane. *Bars,* mean ± standard error of three separate experiments performed in duplicate. *, $p < 0.05$ compared with the corresponding controls by *t* test.

The mechanisms for the up-regulation of NOS expression by inhalational anesthetics are not known; they may be mediated through alterations in the rate of transcription and/or stability of the mRNA. The cloning and analysis of the promoter regions for iNOS and eNOS have revealed the potential for a complex array of transcription factor interactions with these promoters (30–33). Both iNOS and eNOS promoters have AP-1 and p53 sites. Other PKC responsive *cis*-elements, including the serum response element of the c-*fos* gene, the nuclear factor-κB binding site, and the AP-2 binding site are present in the promoters of NOS isoforms (30–33). Because inhalational anesthetics have been shown to activate PKC, and PKC up-regulates NOS (34, 35), this would be one potential mechanism of NOS up-regulation by anesthetics. Inhalational anesthetics have also been shown to increase the level of interleukin-1β in the culture medium of human lung macrophages (36). Interleukin-1β induces iNOS expression in macrophages (37). Thus, this may be a mechanism by which inhalational anesthetics up-regulate iNOS expression. In addition, NO has been demonstrated to down-regulate NOS gene expression (38). Because inhalational anesthetics clearly inhibit NO production and the NO signaling pathway (9), this may therefore prevent the inhibitory effect of NO on NOS gene expression, accounting for the up-regulation of NOS expression induced by anesthetics. The mechanism of NOS gene regulation by inhalational anesthetics, however, awaits further investigation, and detailed anal-

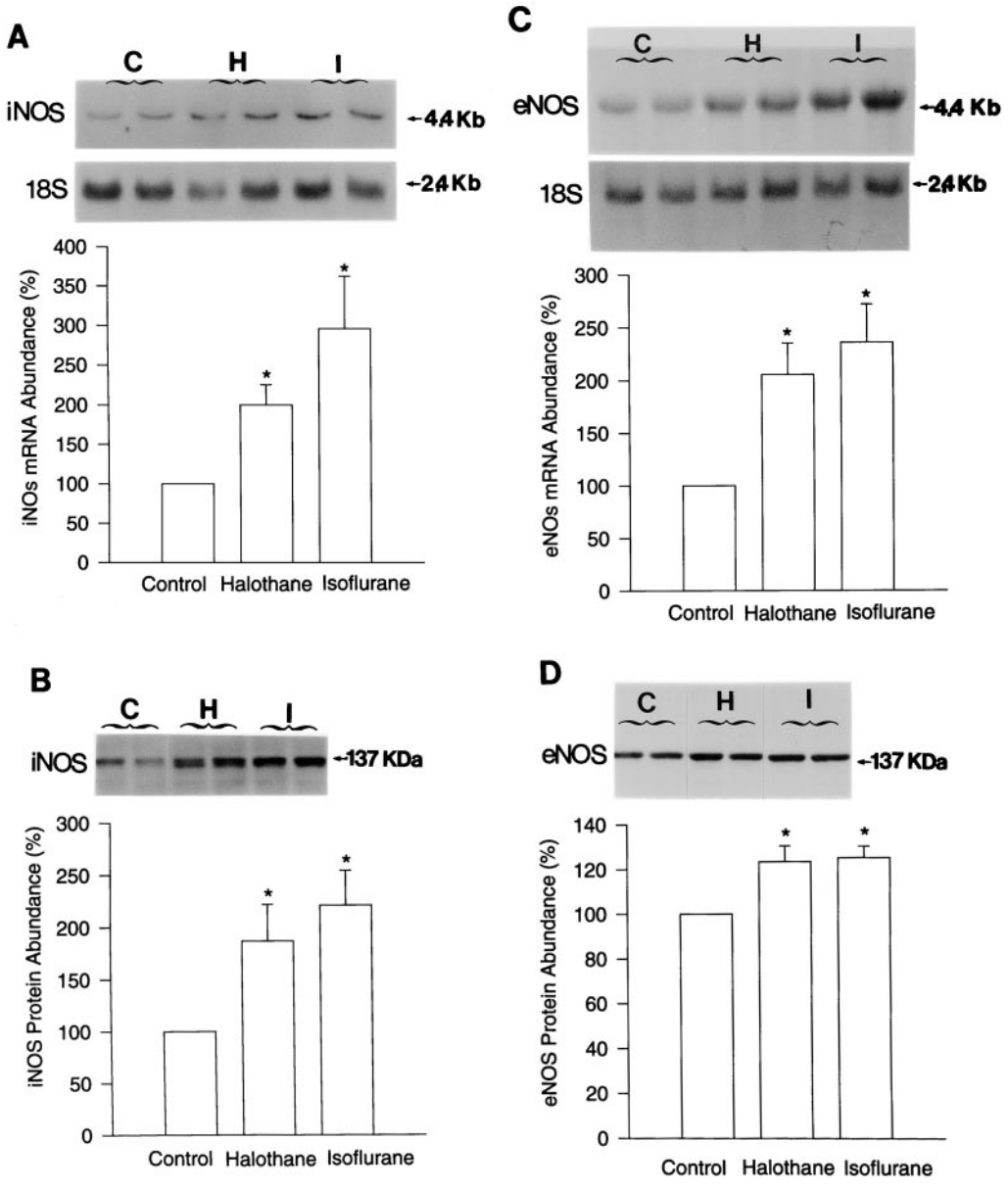

Fig. 4. The up-regulation of mRNA and protein level of iNOS and eNOS in the LPS-activated mouse RAW 264.7 macrophages and bovine pulmonary artery endothelial cells by 2% halothane or 2% isoflurane. The cells were incubated with 2% halothane or 2% isoflurane for 7 hr at 37°. *Left*, Up-regulation of iNOS mRNA (A) or iNOS protein (B) in activated macrophages by a representative Northern or Western blot (*top*), and the graphic presentation of iNOS mRNA or protein abundance quantified by integrating the volume of autoradiograms from three separate experiments in duplicate (*bottom*). *Right*, up-regulation of eNOS mRNA (C) or eNOS protein (D) in BPAEC by a representative Northern or Western blot (*top*), and the graphic presentation of eNOS mRNA or protein abundance quantified by integrating volume of autoradiograms from three separate experiments in duplicate (*bottom*). Values in bar graphs are expressed as the percentage of corresponding time controls (100%) incubated without anesthetics. *Bars*, mean ± standard error of the three experiments. *, $p < 0.05$ compared with the corresponding controls by *t* test. *C*, control; *H*, 2% halothane; *I,* 2% isoflurane.

ysis of anesthetic interactions with the 5′-flanking regions of the NOS isoforms.

Intravenous anesthetics, such as pentobarbital, have been demonstrated to alter the expression of many genes (39). There has been lack of systematic investigation, however, of the effects of inhalational anesthetics on gene expression. Perhaps it has been overshadowed by the concept that changes in gene expression occurred slowly and, therefore, could not be important for a highly dynamic phenomenon such as neuronal information processing or cardiovascular hemodynamics and the changes anesthesia produces in them. However, evidence suggests that anesthetics can regulate gene expression in rats, which, after inhaling halothane for 2 hr, express significantly lower levels of *jun*-B mRNA in their cerebral tissue compared with the controls (11). It is notable from our study that the effect of up-regulation of eNOS, and LPS-induced inducible NOS is apparent after three or more hours of exposure to isoflurane. This is consistent with the typical time course of changes in gene expression. Three hours may be a long exposure time for many surgeries. However, anesthetics are very frequently administered in excess of 3 hr for numerous surgical procedures including major vascular surgery, hepatic surgery, major brain surgery, organ transplantation, etc. Knowledge of the multiple physiologic actions of NO suggests that NOS up-regulation could have many highly significant effects on the postoperative state including wound healing, inflammatory responses, metabolism, vascular responsiveness, renal function, and reperfusion following vascular clamp release, and organ transplantation.

In summary, we demonstrated that halothane and isoflurane, two inhalational anesthetics very commonly used in clinical medicine and in laboratory animal experimentation, increased the mRNA, protein, and activity level of iNOS and eNOS in cultured cells at clinically relevant concentrations. This represents a novel site of interaction between inhalational anesthetics, and the highly important NO signaling pathway and may contribute significantly to the physiologi-

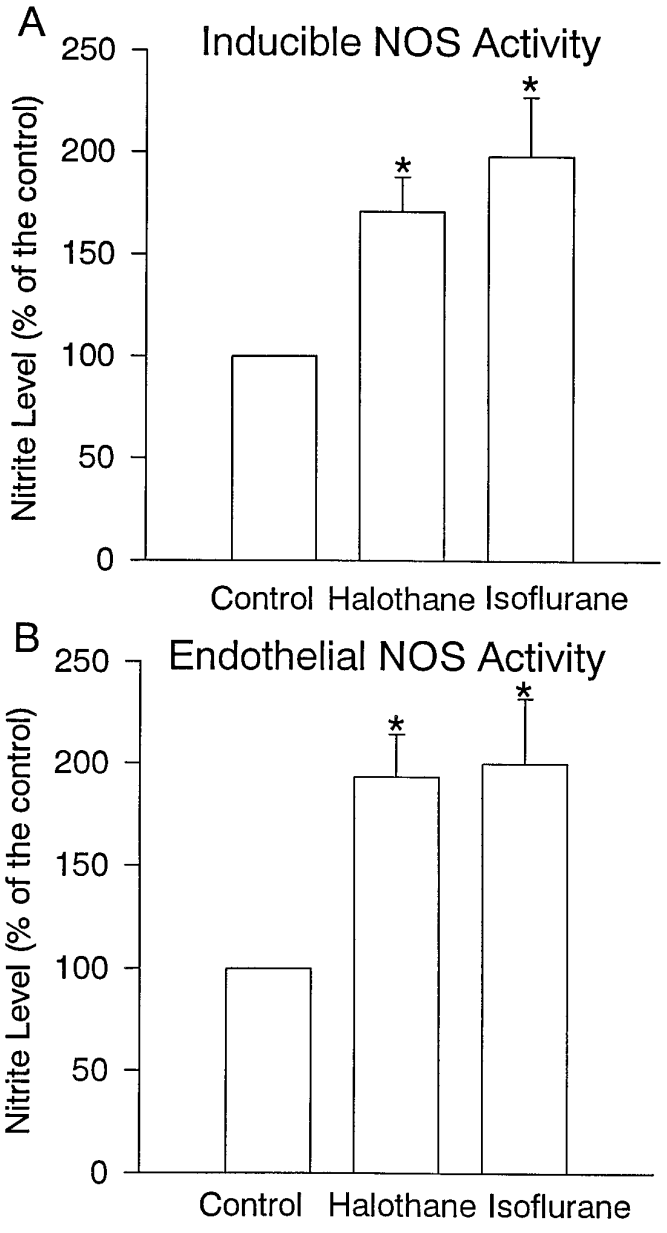

Fig. 5. The increase in nitrite level in the conditioned culture medium of LPS-activated mouse RAW 264.7 macrophages (A) or bovine pulmonary artery endothelial cells (B) after incubation with 2% halothane or 2% isoflurane for 7 hr at 37°. Values are expressed as the percentage of corresponding time controls (100%) incubated without isoflurane. *Bars*, mean ± standard error of three separate experiments. *, $p < 0.05$ compared with the corresponding controls by *t* test.

cal and pathophysiological changes after exposure to inhalational anesthetics.

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