

# Volatile Anesthetics Differentially Affect Immunostimulated Expression of Inducible Nitric Oxide Synthase

## Role of Intracellular Calcium

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**Background:** Nitric oxide released by inducible nitric oxide synthase (iNOS) plays an important role in immune responses and systemic vasodilation in septic shock. Volatile anesthetics have been reported to interfere with signal transduction and gene expression. We studied the effect of volatile anesthetics on activity and expression of iNOS and potential mechanisms of action.

**Methods:** Nitrite release and iNOS expression were determined using the Griess reaction and Western and Northern blot techniques, respectively, in J774 murine macrophages stimulated with lipopolysaccharide and  $\gamma$ -interferon in the absence and presence of various concentrations (0.25–2.0 minimum alveolar concentration [MAC]) of volatile anesthetics (i.e., halothane, enflurane, isoflurane, desflurane). Furthermore, potential interference of volatile anesthetics with specific signal transduction pathways was investigated.

**Results:** All volatile anesthetics, studied in a time- and dose-dependent manner, suppressed nitrite production and iNOS expression in J774 macrophages stimulated by lipopolysaccharide or  $\gamma$ -interferon at clinically relevant concentrations. The inhibition was completely antagonized by ionomycin but unaffected by diacylglycerol, phorbol myristate acetate, and C2-ceramide. In contrast, in cells costimulated by lipopolysaccharide plus  $\gamma$ -interferon, volatile anesthetics significantly increased nitrite production and iNOS expression independent of ionomycin and other mediators studied.

**Conclusions:** Volatile anesthetics strongly reduced the mRNA

and protein levels of iNOS and NOS activity after a single stimulation with lipopolysaccharide or  $\gamma$ -interferon, most likely by attenuating intracellular calcium increase. Costimulation with lipopolysaccharide plus  $\gamma$ -interferon, however, results in maximum iNOS expression and activity, which are no longer inhibited but are potentiated by volatile anesthetics by unidentified mechanisms. (Key words: Anesthetics; calcium; gene expression; inducible nitric oxide synthase; transcription factors.)

NITRIC oxide (NO), a small radical gas, is described as an important messenger molecule with diverse functions throughout the body. NO is involved in the regulation of enzyme activities, vascular tone, platelet and leukocyte adhesion, neurotransmission, and mediation of excessive vasodilation and cytotoxic actions of macrophages against microbes and tumor cells.<sup>1,2</sup> There are two major classes of NO synthases (NOSs) distinguished on the basis of their expression and regulation: Two constitutively expressed isoforms in neurons and endothelial cells that require calcium and calmodulin for enzyme activity<sup>3,4</sup> and a calcium-independent isoform found in immunostimulated macrophages, vascular smooth muscle cells, fibroblasts, and hepatocytes, termed inducible NOS (iNOS).<sup>5</sup> In contrast to endothelial and brain NOS, iNOS activity is mainly controlled by gene expression<sup>6</sup> and is described to excessively produce NO responsible for immune responses and hemodynamic changes observed in septic shock.<sup>7</sup> Volatile anesthetics such as halothane, isoflurane, and sevoflurane have been shown to inhibit endothelial NOS and thereby to attenuate endothelium-dependent relaxation of vascular smooth muscle cells.<sup>8,9</sup> In addition, volatile anesthetics are reported to inhibit brain NOS activity in rat brain extracts<sup>10</sup> and neurotransmission by *N*-methyl-D-aspartate.<sup>11</sup> These findings could be explained, at least in part, by the inhibitory effect of volatile anesthetics on intracellular calcium mobilization<sup>12–15</sup> because constitutive NOSs in endothelial cells and neurons depend on elevated cytosolic calcium

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levels. Although iNOS activity is calcium-independent, the role of intracellular free  $\text{Ca}^{2+}$  for the induction of iNOS is controversially discussed.<sup>16-19</sup> Whether and whereby inhalational anesthetics affect gene expression and, in particular, iNOS induction has not yet been clearly shown. There are only a few reports demonstrating that halothane has selective effects on the expression of immediate-early genes *c-fos* and *jun-B*.<sup>20,21</sup> Recently, Zuo and Johns<sup>22</sup> described an up-regulation of constitutive NOS and iNOS by halothane and isoflurane in the murine macrophage cell line RAW 264.7 stimulated by lipopolysaccharide. However, dose-response and mechanism of action of volatile anesthetics were not evaluated. In addition, the question arises whether the stimulatory effect on iNOS expression holds true for different volatile anesthetics and various stimulatory conditions and cell types. Because of the importance of immunostimulated NO production in host defense and regulation of vascular tone, the current study was designed to investigate the effect of volatile anesthetics on expression and activity of iNOS in murine macrophages stimulated by lipopolysaccharide and  $\gamma$ -interferon ( $\gamma$ IFN) regarding dose- and time-dependency and the underlying mechanisms of action.

## Materials and Methods

### Reagents and Media

Halothane was obtained from Hoechst Marion Roussel (Bad Soden, Germany). Isoflurane and enflurane were purchased from Abbott (Wiesbaden, Germany). Desflurane was provided by Pharmacia & Upjohn (Erlangen, Germany).  $\gamma$ IFN and polyclonal IL-1 $\beta$ -neutralizing antibodies were obtained from R&D Systems (Wiesbaden, Germany). All other reagents, media and materials were obtained from Sigma Aldrich Chemical (Deisenhofen, Germany) unless otherwise indicated.

### Cell Culture

J774A.1 cells, a murine macrophage-like cell line, were obtained from American Type Culture Collection (Rockville, MD), and cultured in bottles (Becton Dickinson, Heidelberg, Germany) containing RPMI 1640 medium, 10% fetal bovine serum, penicillin (100 U/ml), and streptomycin (100  $\mu$ g/ml). For stimulation experiments, confluent cells were harvested and then resuspended and plated in two different 24-well culture plates (Costar, Cambridge, MA) at a concentration of  $1 \times 10^6$ /ml. Two hours after incubation at 37°C in 5%  $\text{CO}_2$ , supernatant was aspirated to remove nonadherent cells and replaced

by fresh culture medium. One cell culture plate was then incubated at 37°C in 5%  $\text{CO}_2$  for 18 h in a chamber preequilibrated with 0.25 minimum alveolar concentration (MAC), 0.5 MAC, 1.0 MAC, and 2.0 MAC volatile anesthetic. The MAC values used were isoflurane 1.15%, enflurane 1.75%, halothane 0.75%, and desflurane 7.2%. The other cell culture plate (anesthetic control) was treated in parallel and incubated at 37°C in 5%  $\text{CO}_2$  without volatile anesthetic. Equilibration with the volatile anesthetic was achieved and maintained by introducing an anesthetic/air mixture and controlling the concentration of the anesthetic and the  $\text{CO}_2$  by using a gas analyzer (PM 8050, Draeger Medizintechnik, Luebeck, Germany) that continuously monitored the gas concentration in the incubator in a closed-loop system. After 1 h of incubation in the presence or absence of volatile anesthetic, macrophages were stimulated with lipopolysaccharide (1  $\mu$ g/ml),  $\gamma$ IFN (200 U/ml), or lipopolysaccharide (1  $\mu$ g/ml) plus  $\gamma$ IFN (200 U/ml) and then further incubated at 37°C in 5%  $\text{CO}_2$  with and without volatile anesthetic. Nitrite, a stable metabolite and measure for NO production, was determined in the supernatant 1 h before and 18 h after stimulation. In addition, the expression of iNOS mRNA and iNOS protein was analyzed 4 h and 18 h after stimulation, respectively. To assess the time course of the effect of volatile anesthetics on immunostimulated NO production, cells were exposed to volatile anesthetics 1 h before and 2 h, 4 h, 6 h, and 12 h after stimulation. Nitrite concentration in the culture supernatant was measured 18 h after stimulation and compared with the nitrite level of immunostimulated cells not exposed to volatile anesthetics that were run in parallel. In additional experiments, calcium-ionophore ionomycin (1  $\mu$ M), diacylglycerol (Calbiochem, Bad Soden, Germany; 3  $\mu$ g/ml), phorbol myristate acetate (1  $\mu$ g/ml), C2-ceramide (12  $\mu$ M), or polyclonal interleukin-1 $\beta$  (IL-1 $\beta$ )-neutralizing antibodies (1 ng/ml) were added 30 min before immunostimulation in the presence or absence of 1.0 and 2.0 MAC of inhalational anesthetics, and nitrite concentrations in the culture supernatant and iNOS expression were determined.

### Nitrite Assay

Nitrite concentration in the culture supernatant medium was assessed as previously described.<sup>23</sup> In brief, Griess color reagent was freshly prepared by combining (1:1) reagent A (0.1% N-[1-naphthyl]ethylenediamine) and reagent B (1%, w/v, sulfanilamide in 5%  $\text{H}_3\text{PO}_4$ ). Samples (100  $\mu$ l) were transferred to a 96-well plate (Costar). Griess color reagent (100  $\mu$ l) was added, and 10 min

## ANESTHETIC EFFECTS ON NITRIC OXIDE SYNTHASE EXPRESSION

after incubation at 21°C, absorbance was read at 560 nm using a Multiskan MCC/340 plate reader (Titertek Instruments Inc., Huntsville, AL). Standard curves were constructed by using sodium nitrite (0–120  $\mu$ M) dissolved in supplemented culture medium.

#### *Preparation of Total Cell Extract*

At the end of stimulation (18 h), J774 mouse macrophages ( $10^6$ /well) were washed twice with phosphate-buffered saline (PBS) and lysed by treatment with 50  $\mu$ l boiling lysis solution (1% sodium dodecyl sulfate, 1.0 mM sodium ortho-vanadate, 10 mM Tris, pH 7.4). Lysed samples were transferred to a microcentrifuge tube and boiled for additional 5 min. To reduce viscosity, the samples were passed several times through a 27-gauge needle to shear DNA. Protein concentration of each sample was measured in a 10-fold-diluted aliquot using the bicinchoninic acid assay from Pierce (Rockford, IL). To all samples an equal volume of two times the concentrated electrophoresis sample buffer (125 mM Tris-HCl, pH 6.8, 4% sodium dodecyl sulfate, 10% glycerol, 0.006% bromophenol blue, and 2%  $\beta$ -mercaptoethanol) was added, and the samples were boiled for 5 min. Per lane, 15  $\mu$ g total cell extract protein, which was approximately 15  $\mu$ l, was used for iNOS Western blot analysis.

#### *Western Blot Analysis*

Cell extract proteins were separated by a standard sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE; 7.5 ExelGel SDS Homogenous, Pharmacia & Upjohn, Erlangen, Germany) and electrophoretically transferred to a nitrocellulose membrane (0.45  $\mu$ m, Schleicher & Schuell, Keene, NH) using a NovaBlot Electrophoretic Transfer Kit (Pharmacia & Upjohn). The membrane was then immersed for 1 h at 25°C in PBS containing 5% nonfat dry milk, washed twice with Tween-PBS (0.05% Tween 20), and incubated overnight at 4°C with anti-iNOS monoclonal antibody (Transduction Laboratories, Lexington, KY) diluted in PBS containing 1% nonfat dry milk. The membrane was then washed three times with Tween-PBS and incubated for 1 h at 25°C with sheep antimouse immunoglobulin G biotin conjugate (Boehringer Mannheim, Mannheim, Germany). After further washing steps, the membrane was incubated for 45 min at 25°C with avidin-peroxidase. For visualization of the protein, the membrane was washed again, incubated with ECL detection reagents (Amersham International, Braunschweig, Germany) and exposed to Kodak XR film (Kodak, Stuttgart, Germany). Lysate from immunostimulated RAW 264.7 cells were

used as a positive control for iNOS protein (Transduction Laboratories).

#### *RNA Preparation and Northern Blot Analysis*

Four hours after immunostimulation, total RNA was extracted from  $15 \times 10^6$  J774 cells using peqGOLD TriFas FL (peqlab Biotechnologie, Erlangen, Germany) and the method of Chomczynski.<sup>24</sup> Ten micrograms of total RNA per sample were denatured, run on formaldehyde/agarose gels, and transferred to Biotodyne B nylon membranes (Pall Filtron, Dreieich, Germany). These were then ultraviolet cross-linked, prehybridized, hybridized with a  $\alpha$ -[<sup>32</sup>P]dCTP random primer-labeled cDNA plasmid probe specific for iNOS, washed, and exposed to Kodak XAR films as previously described.<sup>25</sup> The iNOS cDNA clone was obtained from Dr. C. Nathan (Beatrice and Samuel A. Seaver Laboratory, Department of Medicine, Cornell University Medical College, New York, NY). The blots were also hybridized with a  $\gamma$ -[<sup>32</sup>P]ATP end-labeled oligoprobe specific for mouse 16S mitochondrial RNA to normalize the iNOS mRNA data for RNA loading and transfer. For quantitative comparison of mRNA levels, bands were analyzed using a BAS 3000 phosphor imager (Fuji Medical Systems, Stamford, CT), and the intensities were expressed as phosphor imager units. After correcting for background activity, the ratios of iNOS mRNA levels to mitochondrial RNA levels were calculated. These values were then expressed as percent of mRNA levels of cells immunostimulated in the absence of volatile anesthetics.

#### *Statistical Analysis*

Data are presented as mean  $\pm$  SD unless otherwise specified. For each volatile anesthetic studied, data for nitrite production at different anesthetic concentrations, different stimulatory conditions, and different time points after stimulation, respectively, were compared with control values using analysis of variance (ANOVA) followed by unpaired, two-tailed *t* tests with Bonferroni correction of the *t* statistic for multiple comparisons. Individual comparisons were carried out using the Mann-Whitney U test. Significance was accepted at *P* < 0.05.

### **Results**

We first studied the effect of volatile anesthetics on immunostimulated NO production. Table 1 shows that halothane and enflurane, and isoflurane and desflurane, significantly inhibit the nitrite release into the culture supernatant in response to lipopolysaccharide and  $\gamma$ IFN.

**Table 1. Effect of Volatile Anesthetics on Immunostimulated Nitrite Production**

	Control	Halothane	Control	Enflurane
Stimulus				
None	1.9 ± 0.5	1.2 ± 0.6	1.4 ± 0.8	0.6 ± 0.3
LPS	16.6 ± 2.1	6.0 ± 2.0*	14.3 ± 1.1	7.6 ± 1.7*
γIFN	12.5 ± 1.6	3.2 ± 1.0*	22.5 ± 2.0	10.0 ± 1.0*
LPS + γIFN	37.3 ± 2.2	43.7 ± 1.9*	41.2 ± 1.4	50.4 ± 2.0*
	Control	Isoflurane	Control	Desflurane
Stimulus				
None	0.6 ± 0.3	0.8 ± 0.3	1.8 ± 0.7	0.8 ± 0.3
LPS	12.8 ± 2.6	4.4 ± 1.2*	14.5 ± 1.3	5.9 ± 1.0*
γIFN	16.8 ± 3.0	6.1 ± 1.9*	14.8 ± 2.7	5.4 ± 1.7*
LPS + γIFN	50.2 ± 4.8	62.7 ± 5.9*	41.6 ± 2.8	50.8 ± 3.7*

Nitrite concentration ( $\mu\text{M}$ ) in the culture supernatant of J774 macrophages 18 h after stimulation in the absence (control) or presence of 1 MAC volatile anesthetic. Values are mean  $\pm$  SD of four separate experiments performed in duplicate.

LPS = lipopolysaccharide (1  $\mu\text{g}/\text{ml}$ );  $\gamma\text{IFN}$  = interferon gamma (200 U/ml); LPS +  $\gamma\text{IFN}$  = LPS (1  $\mu\text{g}/\text{ml}$ ) plus  $\gamma\text{IFN}$  (200 U/ml).

\* Significantly ( $P < 0.05$ ) different from control.

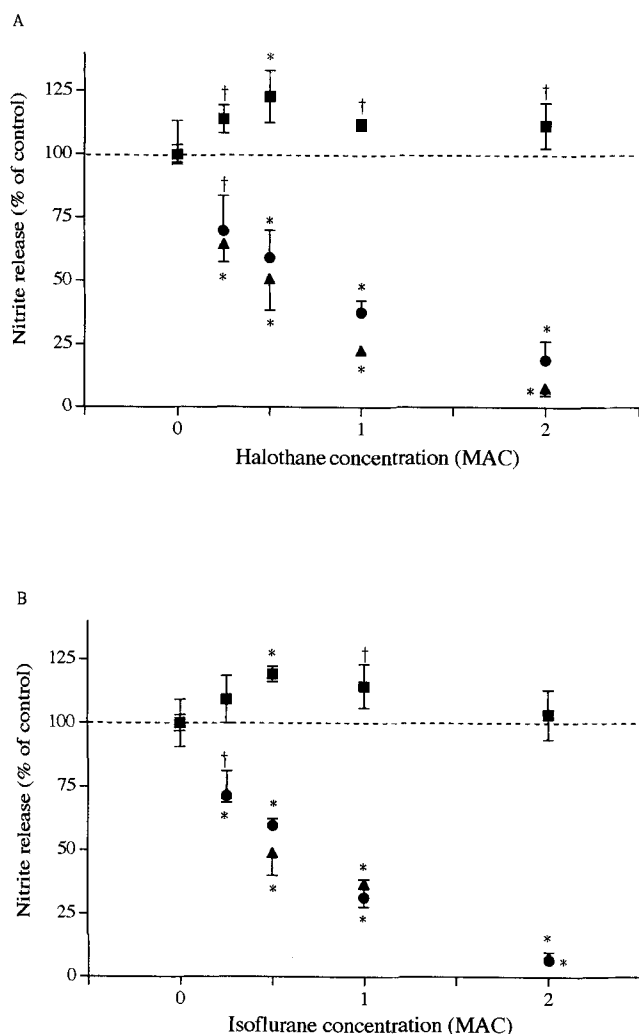
In contrast, all volatile anesthetics studied increased the nitrite release after costimulation with lipopolysaccharide plus  $\gamma\text{IFN}$ . The inhibitory effect of volatile anesthetics on the induced nitrite release in the culture supernatant after a single stimulation with lipopolysaccharide or  $\gamma\text{IFN}$  occurred dose-dependently with an 50% inhibitory concentration ( $\text{IC}_{50}$ ) of about 0.5 MAC, as shown in figures 1A and 1B for halothane and isoflurane, respectively. Similar data were obtained for enflurane and desflurane (not shown). At an anesthetic concentration of 0.25 MAC, we already found a significant inhibition of the induced NO production that continuously increased to almost complete inhibition at a concentration of 2 MAC. With costimulation with lipopolysaccharide and  $\gamma\text{IFN}$ , however, NO production was significantly increased (between 10% and 20%). With all anesthetics, maximum increase of costimulated nitrite release occurred at 0.5 MAC, showing a lower but still elevated nitrite level compared with controls, with anesthetic concentration of 1 MAC and 2 MAC.

To further investigate the mechanism of the modulatory effect of volatile anesthetics on nitrite production and whether there is a sensitive time period after lipopolysaccharide stimulation, the onset time of anesthetic exposure from 1 h before stimulation to 12 h after stimulation was varied. As shown in figure 2, a distinct time-dependence of the modulating effects on nitrite production could be detected (fig. 2). Regarding single stimuli, incubation with anesthetics 1 h before stimulation had the most profound inhibitory effect on nitrite release. The inhibitory effect of volatile anesthetics on lipopolysaccharide- and  $\gamma\text{IFN}$ -stimulated nitrite accumu-

lation was significantly less (about 50%) when anesthetics were applied 6 h after stimulation and absent when applied 12 h after stimulation. In contrast, nitrite production after costimulation with lipopolysaccharide plus  $\gamma\text{IFN}$  was maximally potentiated by halothane, and by other anesthetics studied (data not shown), when cells were exposed to the anesthetic 4 h after costimulation. Inhibitory and stimulatory effects of all volatile anesthetics studied were limited to the first 12 h after stimulation, indicating that they interfere with the induction of NOS expression and not with NOS activity. In fact, induced NOS activity in the homogenate of immunostimulated cells could not be inhibited by 2 MAC of any volatile anesthetic (data not shown).

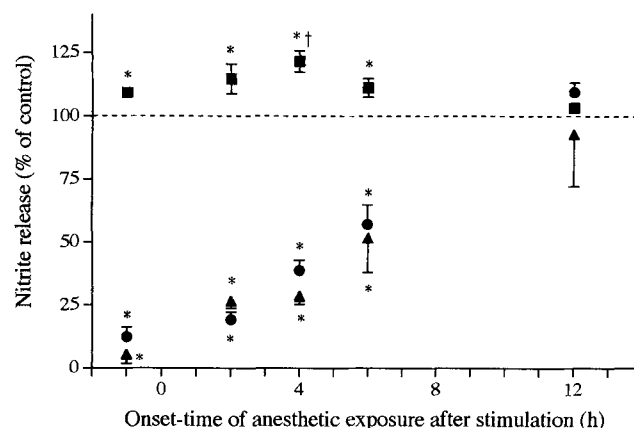
To determine whether volatile anesthetics modulate the iNOS protein expression of immunostimulated J774 macrophages, we analyzed total cell lysates by Western blot using murine iNOS-specific antibodies. As shown in figure 3A, iNOS protein, which was detected as a 130-kDa protein band only in stimulated cells, almost completely disappeared when cells were exposed to 2 MAC isoflurane during stimulation (lanes 3 and 6). The inhibitory effect of volatile anesthetics on nitrite production after single stimulation was caused by a specific reduction of the expression of inducible NOS and not by a reduction in the viability of the cells, which were studied by Trypan blue exclusion. In line with our findings on costimulated NOS activity, iNOS protein expression in costimulated cells was significantly increased by incubation with a volatile anesthetic (fig. 3B). This up-regulation of iNOS expression did not occur in unstimulated cells with volatile anesthetics alone (fig. 3B, lane 4).

## ANESTHETIC EFFECTS ON NITRIC OXIDE SYNTHASE EXPRESSION



**Fig. 1.** Dose-response curve for halothane (A) and isoflurane (B) on nitrite production in immunostimulated J774 macrophages. Cells ( $10^6/\text{ml}$ ) were stimulated with lipopolysaccharide ( $1 \mu\text{g}/\text{ml}$ ; circles),  $\gamma$ -interferon ( $\gamma\text{IFN}$ ;  $200 \text{ U}/\text{ml}$ ; triangles), and lipopolysaccharide plus  $\gamma\text{IFN}$  (squares). After 18 h of incubation in the presence of various concentrations of volatile anesthetic, nitrite was measured in the supernatant. All symbols represent mean  $\pm$  SD of at least six independent experiments in percentage of control (nitrite release of stimulated cells in the absence of volatile anesthetic = 100%).  $\dagger P < 0.05$  and  $*P < 0.01$  versus control.

Northern blot analysis revealed that the modulatory effect of inhalational anesthetics on induction of iNOS protein and enzyme activity was due to an interference with iNOS gene transcription (figs. 4A and 4B). Whereas single immunostimulation with lipopolysaccharide and  $\gamma\text{IFN}$  resulted in a distinct induction of iNOS mRNA in the absence of volatile anesthetic (fig. 4, lanes 6 and 7), iNOS transcription was almost completely suppressed

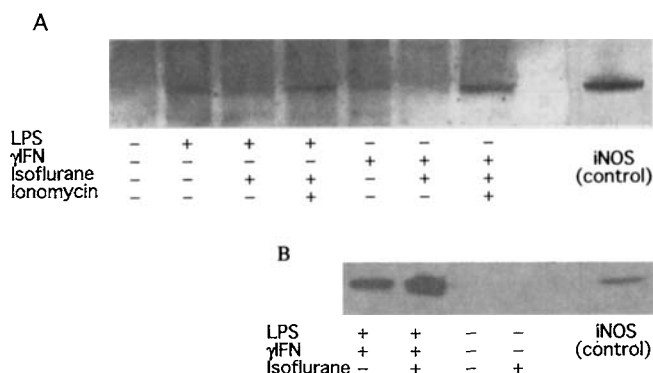


**Fig. 2.** Time-course of the effect of volatile anesthetics on the nitrite production of immunostimulated J774 macrophages. Nitrite release into the cell culture supernatant in the presence of 1 minimum alveolar concentration (MAC) halothane added 1 h before ( $-1 \text{ h}$ ) or at various time points after stimulation (2, 4, 6, and 12 h) with lipopolysaccharide (circles),  $\gamma$ -interferon ( $\gamma\text{IFN}$ ; triangles), and lipopolysaccharide plus  $\gamma\text{IFN}$  (squares), was determined after 18 h of incubation. All symbols represent mean  $\pm$  SD of six independent experiments in percentage of control (nitrite release of stimulated cells in the absence of volatile anesthetic = 100%).  $*P < 0.01$  versus control.  $\dagger P < 0.05$  versus  $-1$ , 6, and 12 h.

after 4 h of incubation with 2 MAC isoflurane (fig. 4, lanes 2 and 3). The ratios of the quantified iNOS mRNA and mRNA levels that were determined as a control for appropriate loading and transfer confirmed a specific modulatory effect of volatile anesthetics on iNOS gene transcription. In contrast to single immunostimulation, costimulation of the cells with lipopolysaccharide plus  $\gamma\text{IFN}$  induced a substantially higher iNOS mRNA level which was no longer inhibited by volatile anesthetics (fig. 4A, lane 1 vs. 5).

In three separate experiments, iNOS transcription induced by a single immunostimulation with lipopolysaccharide or  $\gamma\text{IFN}$  was significantly suppressed in the presence of 2 MAC isoflurane (by more than 80%) compared with iNOS mRNA levels in the absence of volatile anesthetics (fig. 4C). In contrast to single stimulation, mean mRNA level in costimulated cells was increased by 2 MAC isoflurane. Although not reaching statistical significance ( $P = 0.03$ ), the potentiating effect on costimulated iNOS transcription correlated well with the up-regulation in iNOS protein expression and induced enzyme activity that we observed under these conditions.

To investigate the underlying mechanism by which volatile anesthetics may modulate iNOS expression in immunostimulated macrophages, we assessed the role of intracellular calcium mobilization in iNOS expression



**Fig. 3.** Effect of isoflurane on inducible nitric oxide synthase (iNOS) protein expression of immunostimulated J774 macrophages in the presence and absence of ionomycin shown by representative Western blot results. (A) Cells were either stimulated for 18 h with lipopolysaccharide (1  $\mu$ g/ml; lane 2–4) or  $\gamma$ -interferon ( $\gamma$ IFN; 200 U/ml; lane 5–7) or left unstimulated (control; lane 1) in the presence or absence of 2 minimum alveolar concentration (MAC) isoflurane, as indicated. Color marker (29–205 kDa) and iNOS positive control were run in lanes 8 and 9, respectively. Ionomycin (1  $\mu$ M) was added 30 min before stimulation as indicated. (B) Cells were either costimulated with lipopolysaccharide plus  $\gamma$ IFN in the absence (lane 1) or presence (lane 2) of 2 MAC isoflurane or left unstimulated without (lane 3) and with (lane 4) 2 MAC isoflurane (lane 5, iNOS positive control). Total cell lysates were immunoblotted with anti-iNOS antibodies. Similar results were obtained in three separate experiments.

using the calcium ionophore ionomycin. As shown in figures 3A and 4B, addition of ionomycin 1  $\mu$ M 30 min before single stimulation of the cells with lipopolysaccharide or  $\gamma$ IFN completely restored the ability of lipopolysaccharide and  $\gamma$ IFN to induce iNOS protein and mRNA expression in cells exposed to a volatile anesthetic that, in the absence of ionomycin, were strongly inhibited to express iNOS protein and mRNA, respectively. The antagonistic effect of ionomycin on the isoflurane-induced inhibition of iNOS protein expression was also reflected by induced NOS activity determined by the nitrite accumulation in the culture supernatant (Tab. 2). Whereas ionomycin *per se* was unable to induce nitrite expression in unstimulated J774 cells, it significantly augmented the nitrite production of macrophages not exposed to anesthetics in response to a single stimulation with lipopolysaccharide or  $\gamma$ IFN. The presence of ionomycin significantly restored nitrite production that was profoundly reduced after exposure to 2 MAC isoflurane to levels measured in singly stimulated cells in the presence of ionomycin and absence of volatile anesthetic.

Costimulated cells, however, showed a much higher levels of nitrite production and iNOS mRNA, respectively, which was even further increased by exposure to

isoflurane but was not significantly affected by ionomycin, regardless of an incubation with the volatile anesthetic (tab. 2; figs. 4B and 4C).

Because halothane was described to increase IL-1 $\beta$  release by alveolar macrophages<sup>26</sup> and IL-1 $\beta$  is known to induce iNOS in macrophages,<sup>27,28</sup> we tested the hypothesis that the stimulatory effect of anesthetics on costimulated J774 cells is mediated by IL-1 $\beta$ . However, the addition of polyclonal IL-1 $\beta$ -neutralizing antibodies before costimulation with lipopolysaccharide plus  $\gamma$ IFN could not prevent the stimulatory effect of anesthetics on iNOS expression (data not shown). Similarly, the presence of cell-permeable diacylglycerol and phorbol myristate acetate (both activators of protein kinase C) during stimulation of the cells had no significant effect on the modulation of nitrite production seen under exposure to 1 MAC volatile anesthetic, as shown in figure 5 for enflurane and desflurane. Similar results were obtained when cells were stimulated in the presence of cell-permeable C2-ceramide, which is known as an activator of ceramide-activated protein kinase (data not shown). Thus, with exception of the calcium ionophore ionomycin, none of the protein kinase activators studied was able to prevent the changes in iNOS expression in immunostimulated macrophages mediated by volatile anesthetics.

## Discussion

NO generated by inducible NOS has been established as an important factor in the pathogenesis of sepsis.<sup>29–31</sup> Volatile anesthetics such as halothane and isoflurane have been reported to interfere with the activity of constitutive endothelial and brain NOS.<sup>8–10,15</sup>

In this study, we have shown that volatile anesthetics can have both a suppressive and a potentiating effect on immunostimulated NO production and iNOS gene expression in murine macrophages, depending on the stimulus used. Our findings suggest that volatile anesthetics do not directly interfere with already expressed iNOS activity, as assessed by the lack of effect on nitrite production in lysates of stimulated cells and activated cells 12 h after stimulation. Moreover, Northern blot analysis and immunoblotting of the cell extracts revealed that the modulatory effect of volatile anesthetics on stimulated NO production affected both the transcriptional and the translational level of iNOS gene expression within the first hours after immunostimulation.

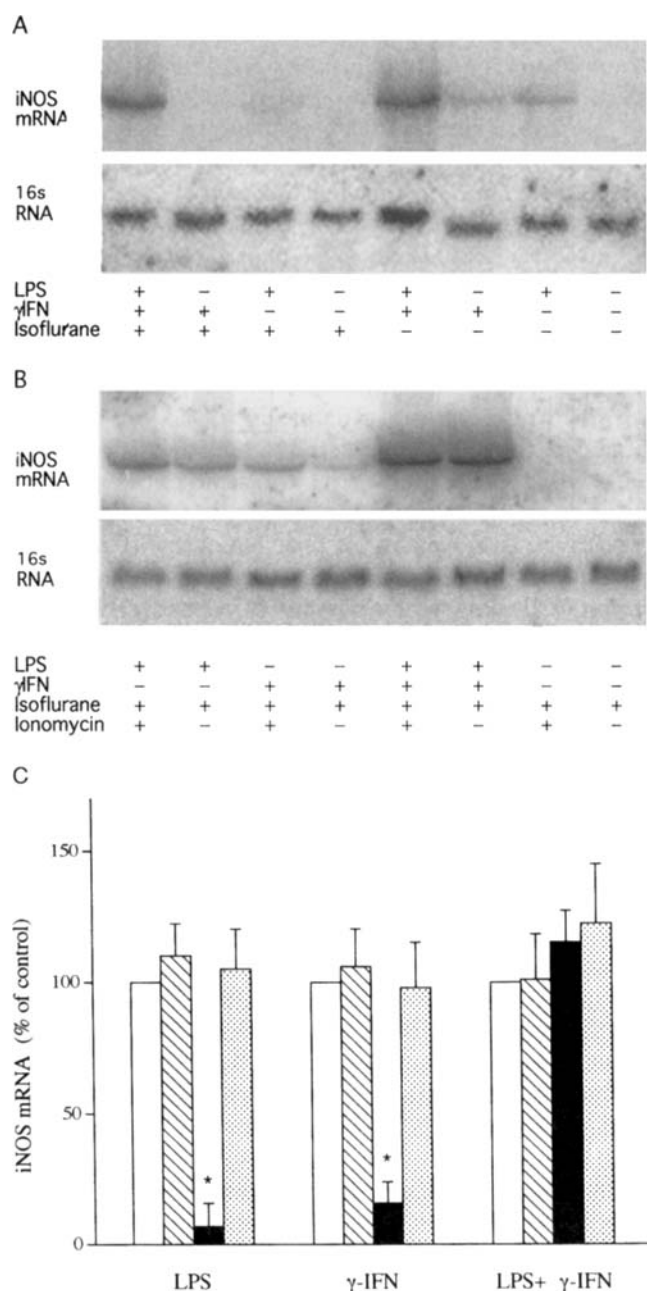
During costimulation with lipopolysaccharide plus  $\gamma$ IFN, which resulted in a strong induction of NOS ac-

## ANESTHETIC EFFECTS ON NITRIC OXIDE SYNTHASE EXPRESSION

tivity, iNOS protein, and mRNA, the presence of volatile anesthetics further increased NO production and iNOS expression in J774 macrophages. Time- and dose-response analysis of the stimulatory effect of volatile anesthetics revealed a nonlinear relation with a maximum at a concentration of 0.5 MAC after 4 h of costimulation. With increasing the concentrations above 0.5 MAC, the up-regulatory effect on costimulated nitrite production decreased. Similarly, exposure of the cells to inhalational

anesthetics 1 h before or 2 h and 6 h after costimulation of the cells resulted in significantly less stimulatory effect, indicating that an anesthetic-sensitive step is located around the hour 4 of stimulation.

We therefore assumed that volatile anesthetics could potentiate costimulated iNOS gene expression by stimulating a relatively late event, such as the release of a proinflammatory cytokine implicated in iNOS up-regulation. Because volatile anesthetics have been shown to increase IL-1 $\beta$  release in macrophages<sup>26</sup> and interleukin-1 $\beta$  is known to induce iNOS in macrophages,<sup>27,28</sup> we evaluated whether an increase in IL-1 $\beta$  by volatile anesthetics might account for the augmented iNOS expression in costimulated J774 cells. However, we found no evidence for this hypothesis because neutralizing IL-1 $\beta$  antibodies failed to prevent the up-regulation of iNOS expression induced by volatile anesthetics. Alternatively, additional activation of protein kinase C, which has been shown to occur by volatile anesthetics<sup>32</sup> and to play a role in lipopolysaccharide-stimulated iNOS induction,<sup>33</sup> must be considered. However, our data do not support this notion because protein kinase C activators had no effect on nitrite production by costimulated nor by singly stimulated cells. Another possible explanation for the finding of an up-regulated iNOS expression by volatile anesthetics could be that anesthetics increase the stabil-



**Fig. 4.** Effect of isoflurane on inducible nitric oxide synthase (iNOS) mRNA expression in immunostimulated murine J774 macrophages in the presence and absence of ionomycin shown by representative Northern blot results. (A) Cells were either stimulated for 4 h with lipopolysaccharide (1  $\mu$ g/ml; lanes 1, 3, 5, 7) or  $\gamma$ -interferon ( $\gamma$ IFN; 200 U/ml; lanes 1, 2, 5, 6) or left unstimulated (controls, lanes 1 and 8) in the presence (lane 1–4) or absence (lanes 5–8) of 2 minimum alveolar concentration (MAC) isoflurane, as indicated. Similar results were obtained in three separate experiments. (B) Antagonizing effect of ionomycin. Cells were stimulated for 4 h with lipopolysaccharide,  $\gamma$ IFN, or lipopolysaccharide plus  $\gamma$ IFN in the absence (lanes 2, 4, 6, 8) or presence (lanes 1, 3, 5, 7) of 1  $\mu$ M ionomycin and incubated with 2 MAC isoflurane (negative controls, lanes 7 and 8). Similar results were obtained in three separate experiments. (C) iNOS mRNA levels of immunostimulated cells from three separate experiments were quantified using a phosphor imager. Cells were stimulated and incubated with 2 MAC isoflurane either in the absence of ionomycin (filled bars) or in the presence of ionomycin (stippled bars). The effect of ionomycin on immunostimulated iNOS transcription in cells not incubated with isoflurane was also shown (hatched bars). Values are expressed as the percentage (mean  $\pm$  SEM) of the corresponding controls (100%) that were immunostimulated in the absence of isoflurane and ionomycin (open bars). \* $P$  < 0.05 compared with the corresponding controls by Mann-Whitney U test.

**Table 2. Effect of Isoflurane on Immunostimulated Nitrite Production in the Presence and Absence of Ionomycin**

Stimulus	Control	Control + Ionomycin	Isoflurane	Isoflurane + Ionomycin
None	1.2 ± 0.5	1.6 ± 0.6	1.4 ± 0.8	1.6 ± 0.4
LPS	5.4 ± 1.4	8.2 ± 1.8*	3.1 ± 1.1†	6.4 ± 1.3‡
γIFN	6.5 ± 1.6	8.7 ± 1.7*	2.3 ± 0.5†	9.6 ± 1.5‡
LPS + γIFN	33.7 ± 3.2	37.0 ± 4.3	41.2 ± 4.4†	42.4 ± 3.6

Nitrite concentration ( $\mu\text{M}$ ) in the culture supernatant of J774 macrophages 18 h after immunostimulation and incubation with and without 2 MAC isoflurane in the absence or presence of ionomycin ( $1 \mu\text{M}$ ). Data are presented as mean  $\pm$  SD of four separate experiments performed in duplicate.

LPS = lipopolysaccharide; γIFN = interferon gamma.

\* Significant ( $P < 0.05$ ) vs. control group without ionomycin.

† Significant vs. anesthetic control without ionomycin.

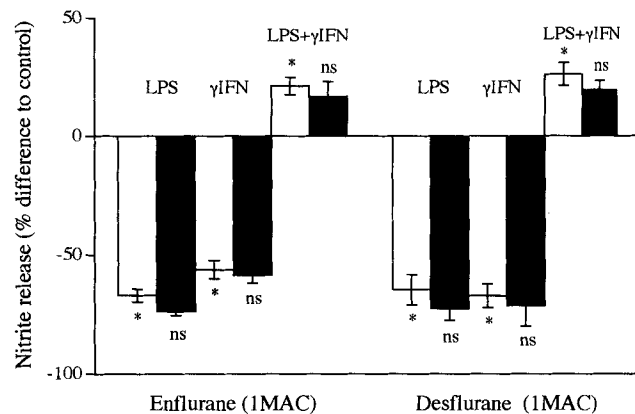
‡ Significant vs. 2 MAC isoflurane without ionomycin.

ity of iNOS mRNA transcripts by inhibition of ribonucleolytic action similar to a mechanism that has been reported for IL-10.<sup>34</sup>

In contrast to the potentiating effect on costimulated iNOS expression, after single stimulation with lipopolysaccharide or γIFN, which provides only a weak iNOS induction, volatile anesthetics were found to strongly inhibit nitrite production and iNOS gene expression at the transcriptional level. The assumption of a direct and additional inhibitory effect on the translational level (*i.e.*, iNOS protein synthesis) is not supported by our findings because in this case iNOS protein and nitrite production should also be reduced after costimulation with lipopolysaccharide plus γIFN. Likewise, the differential effect on iNOS expression according to the stimulatory condition does not advocate for the possibility of a generally reduced iNOS mRNA half-life, but instead indicates an inhibitory effect of the volatile anesthetics on the signal transduction upstream of iNOS gene transcription.

Furthermore, we demonstrate for the first time that the inhibitory effect of inhalational anesthetics can be completely antagonized by the calcium ionophore ionomycin. In addition, we found that the presence of ionomycin during single stimulation with lipopolysaccharide or γIFN enhanced the expression but not the activity of iNOS, whereas NO production of costimulated and unstimulated cells, respectively, was unaffected by ionomycin. In previous experiments, we also observed an inhibitory effect of verapamil ( $150 \mu\text{M}$ ) and EGTA ( $2 \text{ mM}$ ) on immunostimulated nitrite production by J774 macrophages (unpublished observations). These findings indicate that cytosolic-free  $\text{Ca}^{2+}$  potentiates those immune stimuli that moderately induce iNOS expression in J774 macrophages.

In several studies, volatile anesthetics have been shown to reversibly inhibit voltage-dependent calcium channels and affect intracellular calcium mobilization,



**Fig. 5. Effect of anesthetics on immunostimulated nitrite production in the presence and absence of cell-permeable diacylglycerol.** J774 macrophages ( $10^6/\text{ml}$ ) were stimulated for 18 h with lipopolysaccharide, γ-interferon (γIFN), and lipopolysaccharide plus γIFN in the presence of 1 minimum alveolar concentration (MAC) enflurane and desflurane. Diacylglycerol ( $3 \mu\text{g}/\text{ml}$ ; solid bars) or RPMI medium (open bars) was added 30 min before stimulation. All data represent mean  $\pm$  SD of the changes in nitrite release determined in at least three independent experiments in percentage of stimulated cells incubated without anesthetics (control). \* $P < 0.05$  versus control. ns = Not significantly different ( $P > 0.05$ ) from cells stimulated in the absence of diacylglycerol.

resulting in a decreased concentration of intracellular free  $\text{Ca}^{2+}$ .<sup>12-15,35</sup> Our data therefore support the conclusion that volatile anesthetics mediate the inhibitory effect on iNOS expression in singly stimulated cells by inhibiting mobilization of cytosolic free  $\text{Ca}^{2+}$ .

In line with our findings, there are several reports demonstrating a significant role of  $\text{Ca}^{2+}$  as a priming signal in the transcriptional regulation of iNOS in murine peritoneal macrophages and rat Kupffer cells after a single stimulation with lipopolysaccharide or γIFN.<sup>16-19</sup> In those studies, an increase in cytosolic free  $\text{Ca}^{2+}$  enhanced iNOS expression, whereas blocking of cytosolic



free  $\text{Ca}^{2+}$  significantly decreased iNOS expression and NO release. In contrast, in immunostimulated RAW 264.7 cells, an increase in intracellular free  $\text{Ca}^{2+}$  has been shown to inhibit iNOS transcription and NO production.<sup>19</sup> Moreover, RAW 264.7 cells respond to a single stimulation with lipopolysaccharide with a considerably higher production of iNOS activity than J774 cells (unpublished observations). This could explain the discrepancy between the effect of volatile anesthetics observed in RAW 264.7 cells<sup>22</sup> and J774 macrophages after single immunostimulation. Whereas Zuo and Johns<sup>22</sup> reported that halothane and isoflurane up-regulate constitutive and inducible NOS expression in RAW 264.7 cells after a single stimulation with lipopolysaccharide, we found that inhalational anesthetics potentiate iNOS expression in J774 macrophages only during costimulation with lipopolysaccharide plus  $\gamma\text{IFN}$ . Thus, the effect of volatile anesthetics and intracellular free  $\text{Ca}^{2+}$  on the transcriptional regulation of iNOS appears to partly depend on the stimulus used and the cell type studied.

There are several lines of evidence regarding the mechanism linking impaired up-regulation of cytosolic free calcium by volatile anesthetics to inhibition of further downstream activated transcription factors regulating iNOS gene expression. First, immediate early genes *jun-B* and *c-fos* have been shown to be up-regulated by intracellular calcium<sup>36</sup> and inhibited by halothane.<sup>20,21</sup> Second, *fos* and *jun* are known to form the AP-1 transcription factor that could in addition stimulate the promoter activity of the murine iNOS gene, together with other transcription factors such as nuclear factor  $\text{NF-}\kappa\text{B}$ .<sup>37,38</sup> In the light of these findings, we would like to speculate that volatile anesthetics might inhibit iNOS expression by interfering with calcium mobilization and subsequent activator protein-1 activation when stimuli are used that induce only submaximal promoter activity of iNOS gene. In contrast, when cells were costimulated with lipopolysaccharide plus  $\gamma\text{IFN}$  and the iNOS promoter was already maximally activated by nuclear factor  $\text{NF-}\kappa\text{B}$ <sup>39</sup> and IFN regulatory factor-1,<sup>40</sup> additional transcriptional activators such as AP-1 are redundant. Thus, costimulation of J774 macrophages with lipopolysaccharide plus  $\gamma\text{IFN}$  results in a strong iNOS induction, and volatile anesthetics can no longer impair iNOS expression by their interference with intracellular  $\text{Ca}^{2+}$  mobilization.

Furthermore, we addressed the question whether volatile anesthetics could exert their modulatory effect on iNOS expression by interference with the activation of protein kinase C and ceramide-activated protein kinase

that has been shown to be required for iNOS expression.<sup>33,41,42</sup> However, neither activation of protein kinase C by phorbol myristate acetate and diacylglycerol nor the activation of ceramide-activated protein kinase by cell-permeable C2-ceramide could overcome the modulatory effect of volatile anesthetics on iNOS expression.

We have shown that volatile anesthetics time- and dose-dependently inhibit nitrite production, iNOS protein, and mRNA expression at clinically relevant concentrations in J774 murine macrophages within the first hours after single stimulation with lipopolysaccharide or  $\gamma\text{IFN}$ . This inhibitory effect could be completely antagonized by ionomycin, suggesting an impaired up-regulation of intracellular free calcium as the pathophysiologic mechanism. By contrast, nitrite production and iNOS expression after costimulation with lipopolysaccharide plus  $\gamma\text{IFN}$  are moderately enhanced by volatile anesthetics, and this occurrence is not affected by ionomycin. The mechanisms for this potentiating effect are presently not known but may be related to an increased stability of iNOS mRNA. The results of the current study add further evidence that volatile anesthetics can have a specific and differential effect on signal transduction and gene expression at clinically relevant concentrations. Thus, our findings may have important implications for experimental studies investigating iNOS induction and also for clinical medicine in regard to anesthesia administration during major surgery and analgesedation in critically ill patients.

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