Sevoflurane Promotes Bactericidal Properties of Macrophages through Enhanced Inducible Nitric Oxide Synthase Expression in Male Mice

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ABSTRACT

Background: Sevoflurane with its antiinflammatory properties has shown to decrease mortality in animal models of sepsis. However, the underlying mechanism of its beneficial effect in this inflammatory scenario remains poorly understood. Macrophages play an important role in the early stage of sepsis as they are tasked with eliminating invading microbes and also attracting other immune cells by the release of proinflammatory cytokines such as interleukin-1B, interleukin-6, and tumor necrosis factor-α. Thus, the authors hypothesized that sevoflurane mitigates the proinflammatory response of macrophages, while maintaining their bactericidal properties.

Methods: Murine bone marrow–derived macrophages were stimulated in vitro with lipopolysaccharide in the presence and absence of 2% sevoflurane. Expression of cytokines and inducible NO synthase (iNOS) were measured. The in vivo endotoxemia model consisted of an intraperitoneal lipopolysaccharide injection after anesthesia with either ketamine and xylazine or 4% sevoflurane. Male mice (n = 6 per group) were observed for a total of 20 h. During the last 30 min fluorescently labeled E. coli were intraperitoneally injected. Peritoneal cells were extracted by peritoneal lavage and inducible NO synthase expression as well as E. coli uptake by peritoneal macrophages was determined using flow cytometry.

Results: In vitro, sevoflurane enhanced lipopolysaccharide-induced inducible NO synthesis expression after 6 h by 466% and increased macrophage uptake of fluorescently labeled E. coli by 70% compared with vehicle-treated controls. Inhibiting inducible NO synthase expression pharmacologically abolished this increase in bacteria uptake. In vivo, inducible NO synthase expression was increased by 669% and phagocytosis of E. coli by 49% compared with the control group.

Conclusions: Sevoflurane enhances phagocytosis of bacteria by lipopolysaccharide-challenged macrophages in vitro and in vivo via an inducible NO synthase–dependent mechanism. Thus, sevoflurane potentiates bactericidal and antiinflammatory host-defense mechanisms in endotoxemia.

Nuclear factor–κB, a well-known nuclear transcription factor, is seen as one of the key regulators for initiating an immune response toward inflammation. Inhalation of sevoflurane also appears to be beneficial during acute lung injury. Less is known about the effects of sevoflurane on infectious inflammation. Animal studies suggest favorable influences as sevoflurane improved survival in bacterial sepsis. This finding is particularly interesting because propofol, another commonly used anesthetic agent, showed adverse effects in infectious conditions, as exposure to...
propofol increased bacterial burden of infected animals and decreased survival in sepsis.16

Sevoflurane interacts with immune cells like neutrophils and decreases their adhesion to the endothelium as well as transmigration, and reduces apoptosis. Only a few studies have investigated the effects of sevoflurane on other immune cells. Macrophages, as part of the innate immune system, are among the first to interact with microbial invaders and defend the host against pathogens. They are recruited to the site of infection and attract other immune cells by releasing proinflammatory cytokines such as interleukin-1β, interleukin-6, and tumor necrosis factor-α.9,32

Invading microorganisms are eradicated when macrophages engulf them via phagocytosis, whereas bactericidal proteins such as inducible NO synthase are upregulated, leading to increased expression of NO.22,23 Antigens from eradicated pathogens are then presented to the adaptive immune system and a specific immune response can be initiated.24,25 Thus, macrophages play a pivotal role in restoring tissue homeostasis and overcoming inflammation.23

We hypothesized that sevoflurane prevents macrophages from eliciting an exaggerated immune response by attenuating the expression of nuclear factor-κB-dependent gene products, thereby contributing to sevoflurane’s overall beneficial effect in severe inflammation. The first aim was thus to assess the inflammatory response of murine macrophages on stimulation with bacterial lipopolysaccharide in the presence and absence of sevoflurane. The second aim was to evaluate macrophage function under the influence of sevoflurane in vitro as well as in vivo in a lipopolysaccharide-induced endotoxemia model in mice.

Materials and Methods

Cell Culture

All cell culture procedures were conducted under sterile conditions in a laminar flow cabinet, with reagents warmed to 37°C before use, if not stated otherwise. Abelson murine leukemia virus-transformed macrophages from BALB/c mice (RAW) 264.7 cells (ATCC TIB-71), were cultured under standard cell culture conditions (37°C, 80% relative humidity) and 5% carbon dioxide [CO2] in Dulbecco’s Modified Eagle’s Medium with 10% heat-inactivated fetal bovine serum (Gemini Bio-Products, West Sacramento, USA), 1% (volume/volume) Antibiotic-Antimycotic, 1 mM sodium pyruvate (both from Thermo Fisher Scientific, USA), and 10 to 15% L929 conditioned cell culture medium. For experimental procedures in six-well plates, macrophages were differentiated in the same plate. For experiments in other plates, bone marrow–derived macrophages were removed after 5–7 days with 5 mM EDTA (Calbiochem, USA) in Dulbecco’s phosphate buffered saline and reseeded into the appropriate cell culture dish.

Experimental Exposure In Vitro

Cells were incubated with bacterial lipopolysaccharide from *Escherichia coli* (E. coli) serotype 055:B5 (Sigma-Aldrich, USA) at a concentration of 100 ng/ml for up to 24 h. Sevoflurane (Baxter, USA) was vaporized with a Penlon (USA) Sigma Elite vaporizer, and the cells were incubated in an airtight Oxoid chamber (Pratteln, Switzerland) for the entire time of lipopolysaccharide exposure. This time varied according to the endpoint (inflammatory mediators interleukin-1β, interleukin-6, and tumor necrosis factor-α; mRNA 0–24 h, protein: 4–24 h; inducible NO synthase mRNA 0–24 h; inducible NO synthase protein 4 and 8 h; nitrite 24 h; nuclear factor-κB inhibitor-α 0–90 min; extracellular signal-regulated kinase 8 h; bone marrow–derived macrophage *E. coli* uptake: in vitro 8 h in vivo experiments 20 h). A schematic illustration of the experimental setting is shown in figure 1.

For inducible NO synthase inhibition, the selective inducible NO synthase inhibitor 1400W (Calbiochem, USA) was used at a final concentration of 1 μM. Sevoflurane...
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concentration in the outflowing air from the airtight chamber was measured with an E-CAiO gas analyzer and displayed on a CARESCAPE Monitor B650, both from GE Healthcare (USA). Sevoflurane was vaporized in synthetic air with 5% CO₂ (21% O₂, 74% N₂, and 5% CO₂).

**Quantitative Real-time Polymerase Chain Reaction Analysis**

Total RNA was isolated from bone marrow-derived macrophages with TRIzol reagent (Invitrogen, USA) following the manufacturer’s protocol. After RNA purification, 1 μg RNA was used to produce cDNA using the high-capacity cDNA reverse transcription kit (Applied Biosystems, USA). Quantitative real-time polymerase chain reaction was performed with Fast SYBR green master mix (Applied Biosystems, USA) on a ViiA 7 real-time polymerase chain reaction system (Applied Biosystems). The following primers were used: interleukin-1β forward primer 5’-TTC CCA TTA GAC AAC TGC ACT AC-3’ and reverse primer 5’-GTC GTG TCC TTG AC-3’; interleukin-6 forward primer 5’-CAC AAG TCC GGA GAG GAG AC-3’ and reverse primer 5’-TTC TGC AAG TGC ATG CTG-3’; glyceraldehyde 3-phosphate dehydrogenase forward primer 5’-GGG TGG AAG CTA AAT-3’ and reverse primer 5’-GTC ATG ACC CCT TCC ACA AT-3’; inducible NO synthase forward primer 5’-CAG CTG GGC TGT ACA AAG CTT-3’ and reverse primer 5’-CAT TGG AAG TGA AGC GTT TGC-3’; tumor necrosis factor-α forward primer 5’-AGT TCT ATG GCC CAG ACC CT-3’ and reverse primer 5’-CAC TGG GTT TGC TGC TAC GA-3’. Cycle threshold

Fig. 1. Schematic illustration of the experimental setting. (A) Stimulation of bone marrow–derived macrophages (BMDM) with lipopolysaccharide (LPS) in the presence and absence of 2% sevoflurane. (B) Experimental setup for degradation of nuclear factor-κB inhibitor-α (IκBα). (C) In vitro update of fluorescein isothiocyanate (FITC)-labeled *Escherichia coli* (*E. coli*). (D) In vivo inducible NO synthase (iNOS) expression and uptake of FITC-labeled *E. coli* by murine peritoneal macrophages.
Glyceraldehyde 3-phosphate dehydrogenase or β-actin were used as loading controls. The following primary antibodies were used; β-actin from BD Bioscience (USA); total and phosphorylated (pThr202/pTyr204) extracellular signal-regulated kinases from Cell Signaling (USA); glyceraldehyde 3-phosphate dehydrogenase and nuclear factor-κB inhibitor-α from Santa Cruz Biotechnology (USA); inducible NO synthase from EMD Millipore (USA).

Measurement of Uptake of Fluorescently Labeled E. coli

Macrophage-mediated uptake of heat inactivated E. coli was performed as previously described.28 Bone marrow-derived macrophages (10⁵ per well) were seeded in a black, clear-bottom 96-well plate (Corning, USA) and pretreated for 8 h with or without 100 ng/ml lipopolysaccharide in the presence or absence of 2% sevoflurane. Thereafter, the medium was removed and replaced with medium containing approximately 10⁸ fluorescein isothiocyanate-labeled heat-inactivated E. coli and incubated for 30 min. The fluorescent signal of extracellular E. coli was quenched with Trypan blue (Sigma-Aldrich, USA), as previously described,28–30 and the cells were then fixed in 4% paraformaldehyde (Electron Microscopy Science, USA) and stained with 4',6-diamidino-2-phenylindole (Molecular Probes, USA). The fluorescent signal from fluorescein isothiocyanate (λExcitation 494 nm/λEmission 518 nm) and 4',6-diamidino-2-phenylindole (λExcitation 364 nm/λEmission 454 nm) were obtained using a Synergy H4 fluorescent microplate reader (BioTek, USA). Uptake of heat-inactivated fluorescein isothiocyanate-labeled E. coli was calculated as: Uptake = (fluorescein isothiocyanate signal-background)/(4',6-diamidino-2-phenylindole signal). Uptake was normalized to bone marrow-derived macrophages that were exposed to medium without additives.

Lipopolysaccharide-induced Endotoxemia Model

Animals were randomly assigned to the following groups: (1) Sham, (2) Sevoflurane/Sham, (3) lipopolysaccharide, and (4) Sevoflurane/lipopolysaccharide. Endotoxemia was induced by intraperitoneal injection of 5 μg lipopolysaccharide, Serotype O111:B4 (Sigma-Aldrich, USA) in 200 μl of sterile prewarmed saline.31 Experiments were always initiated at the same time of day (start at 2 pm). To ensure solubilization the suspension was sonicated for 10 min prior to injection. Mice were anesthetized either with ketamine/xylazine (Sham and lipopolysaccharide) or with sevoflurane (Sevoflurane/Sham and Sevoflurane/lipopolysaccharide). The goal was to reach a group size of six, resulting in a total of 24 animals based on previous experiments, respecting replacement, refinement, or reduction of animals in research (3R) without sample size calculation. Animals were randomly assigned to one of the groups without blinding. Sevoflurane-treated animals were exposed to 4% sevoflurane for 2 h after injection of lipopolysaccharide. Sevoflurane concentration was used as previously described.

Western Blot

Cells were rinsed one time with ice-cold Dulbecco’s phosphate buffered saline (Cellgro, USA) and then lysed with RIPA buffer (Boston Bioproducts, USA) containing phosphatase and protease inhibitors (both from Sigma-Aldrich, USA). Thereafter, the lysate was scraped from the cell culture plate and left on ice for 30 min, followed by centrifugation at 4°C and 12,000 g for 20 min. Total protein was measured using a detergent compatible protein assay (Bio-Rad, USA) using albumin as the protein standard (Thermo Fisher, USA). The supernatant was stored at −80°C until further analysis, at which time equal amounts of protein were diluted using Laemmli buffer (Bio-Rad) plus beta-mercaptoethanol (Bio-Rad) and boiled for 5 min at 95°C. Proteins were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis and transferred to a nitrocellulose membrane. After blocking for 1 h with either 5% milk or 5% bovine serum albumin in tris-buffered saline (4’,6-diamidino-2-phenylindole signal). Uptake was normalized to the T ecan (Switzerland) GENios Pro microplate reader.

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described. For the last 30 min (at 19.5 h), 10⁷ fluorescein isothiocyanate-labeled heat-inactivated E. coli in 500 μl pre-warmed phosphate-buffered saline were injected intraperitoneally. After a total of 20 h, the anesthetized mice were euthanized by cervical dislocation. Subsequently, peritoneal lavage with 6 ml ice-cold phosphate-buffered saline was performed. The lavage was centrifuged for 5 min at 300g and the cell pellet resuspended in 1 ml ice-cold FACS buffer (phosphate-buffered saline and 0.5% bovine serum albumin). Before initiation of the experiments outcomes were defined. The primary outcome was uptake of E. coli by peritoneal macrophages; the secondary outcome was inducible NO synthase expression by peritoneal macrophages.

Flow Cytometry

Before staining, the cells were treated with anti-CD16/CD32 monoclonal antibody (BD Bioscience, USA) according to the manufacturer's recommendation to block FcγRII/III receptors. If not stated otherwise, all stainings were conducted on ice and protected from light. First, surface staining with the following antibodies was performed for 30 min: PerCP Cy5-5 labeled Ly6C, Pacific Blue labeled Ly6G, Apc Cy7 labeled CD11b, Brillian Violet 510 labeled CD11c and PE labeled F4/80 (all from Biolegend, USA). Thereafter, the cell suspension was washed in phosphate-buffered saline and stained for dead cells with fixable viability dye eFluor 660 (eBioscience, USA) for 15 min. To prepare the cells for the intracellular inducible NO synthase staining, they were fixed and permeabilized with a kit (eBioscience) according to the manufacturer's protocol. This step was followed by intracellular staining with PE Cy7 labeled inducible NO synthase antibody, which was performed for 20 min at room temperature. For compensation, Ultracomp eBeads (Invitrogen, USA) were stained with 0.5 μl of each dye for 20 min. The cells and beads were analyzed with a BD LSR Fortessa flow cytometer. For analysis, FlowJo v10 (FlowJo, LCC, USA) software was used; fluorescence minus one controls were used to set the gates.

Statistical Analysis

Values are shown as means ± SD. Data analysis and graphical presentations were performed using GraphPad Prism version 7.0 and 8.0 (GraphPad, USA). Normal distribution was visually analyzed using Q-Q plots. Comparisons between two groups were performed by an unpaired two-tailed t test, and for three or more groups using one-way ANOVA with Bonferroni's post hoc comparison. In experiments with one single time point analysis, comparison of one-way ANOVA was used. To assess the effect of the treatment and time an ordinary two-way ANOVA (no repeated measures) with...
Bonferroni’s post hoc test was used with time and treatment as factors to be evaluated. No statistical power analysis was conducted prior to the study. The sample size was based on previous experience with the experimental design(s). There was no exclusion of outliers. A P value of less than 0.05 was considered statistically significant. All experimental procedures were performed at least three times.

**Sevoflurane Suppresses Lipopolysaccharide-induced Expression and Production of Proinflammatory Cytokines in Bone Marrow-derived Macrophages**

Expression of the proinflammatory cytokines interleukin-1β, interleukin-6, and tumor necrosis factor-α in bone marrow-derived macrophages after lipopolysaccharide stimulation was determined using quantitative real-time polymerase chain reaction. As expected, we observed a time-dependent increase in the expression of proinflammatory cytokines after stimulation with lipopolysaccharide as compared with control (t₀ min; fig. 2, A–C). Lipopolysaccharide-induced interleukin-1β expression in bone marrow-derived macrophages was reduced by sevoflurane at 1 h (P = 0.0006; fig. 2A), whereas no difference was observed in the expression of tumor necrosis factor-α at any of the observed time points (fig. 2C).

To evaluate whether reduced interleukin-1β and interleukin-6 mRNA expression was associated with a decrease in protein level, the concentration of these cytokines was measured in the supernatant from lipopolysaccharide-treated bone marrow-derived macrophages using enzyme-linked immunosorbent assay. Stimulation with lipopolysaccharide increased the release of interleukin-1β on lipopolysaccharide stimulation at 24 h (546 ± 346 ng/ml [mean ± SD]) was reduced in the presence of 2% sevoflurane to a value of 145 ± 155 ng/ml (P < 0.0001; fig. 3A). Similarly, after lipopolysaccharide stimulation there was a time-dependent increase in interleukin-6 protein concentration over time to a value of (9,006 ± 5,817 ng/ml) at 24 h, while sevoflurane reduced lipopolysaccharide-induced interleukin-6 release to 4,183 ± 2,797 ng/ml; P < 0.0001; fig. 3B).

**Sevoflurane Differentially Modulates Inducible NO Synthase Expression on Lipopolysaccharide Stimulation**

We also assessed the effect of sevoflurane on another classic nuclear factor-κB-dependent inflammatory mediator, namely inducible NO synthase. Lipopolysaccharide stimulation promoted a time-dependent increase in inducible NO synthase mRNA expression, which peaked at 4 h (fig. 4A). The effect of sevoflurane on lipopolysaccharide-stimulated inducible NO synthase expression level, however, differed over the 24-hr time course as compared with other proinflammatory cytokines. Sevoflurane increased inducible NO synthase gene expression at 4 h (P < 0.0001). At 8 h, inducible NO synthase expression did not differ (P = 0.099) in sevoflurane-treated cells compared with cells exposed to air only (fig. 4A). Effects of sevoflurane on inducible NO synthase protein levels mirrored the changes observed by quantitative real-time polymerase chain reaction. At 4 h of lipopolysaccharide stimulation, sevoflurane reduced inducible NO synthase expression by 41% compared with bone marrow-derived macrophages treated with lipopolysaccharide alone (P = 0.0005; fig. 4B) and sevoflurane increased inducible NO synthase expression by 466% at 8 h compared with lipopolysaccharide alone (P = 0.0012; fig. 4C).

**Sevoflurane Enhances Nitrite Production during Lipopolysaccharide Stimulation**

To assess whether the increase in inducible NO synthase protein resulted correspondingly in enhanced NO production, we measured nitrite concentration in the culture media. As expected, lipopolysaccharide stimulation led to an increase in nitrite production by macrophages compared with...
Fig. 4. Inducible NO synthase (iNOS) gene expression and NO production in bone marrow–derived macrophages. (A) Cells were exposed to lipopolysaccharide (LPS) in the presence and absence of 2% sevoflurane (Sevo) for 0, 1, 2, 4, 8, and 24 h. RNA was extracted and quantitative real-time PCR was performed to determine iNOS mRNA. Values represent means ± SD; n = 4, two-way ANOVA (group and time interaction). ***P < 0.0001. (B and C) Protein levels of iNOS were determined after 4 h or 8 h of LPS stimulation in the presence or absence of 2% sevoflurane. Whole-cell lysates were used for Western blot analysis. Representative Western blots are shown. Values represent means ± SD; n = 3; unpaired two-tailed t test; **P = 0.0012, ***P = 0.0005. (D) NO production in bone marrow–derived macrophages after 24-h stimulation with LPS in the presence or absence of 2% sevoflurane was measured indirectly by determination of nitrite production using Griess reagent. To inhibit iNOS production 1400W was added for the entire incubation. Values represent means ± SD; n = 15 samples without 1400W, n = 6 samples with 1400W; one-way ANOVA; LPS versus Sevo/LPS **P = 0.009, Sevo/LPS versus Sevo/LPS/1400W ***P = 0.0005.
unstimulated controls ($P < 0.0001$; fig. 4D). The nitrite production after 24 h of lipopolysaccharide stimulation (5.5 ± 2.9 μM [mean ± SD]) was enhanced when cells were coexposed to sevoflurane (8.8 ± 4.3 μM; $P = 0.009$). Furthermore, lipopolysaccharide-induced increase in nitrite concentration was completely abolished in cells treated with the inducible NO synthase specific inhibitor 1400W (1μM; lipopolysaccharide vs. lipopolysaccharide 1400W, $P = 0.0007$; Sevoflurane/lipopolysaccharide vs. Sevoflurane/lipopolysaccharide/1400W, $P = 0.0005$), confirming increased nitrate level is promoted by inducible NO synthase activation.

Sevoflurane Decreases Lipopolysaccharide-induced Extracellular Signal-regulated Kinase Phosphorylation Whereas Nuclear Factor-κB Inhibitor–α Degradation Is Not Altered

As proinflammatory genes in macrophages were observed to be differentially modulated by sevoflurane, we next assessed potential molecular mechanisms by interrogating signaling proteins associated with the various inflammatory pathways. As the data indicated sevoflurane promotes a decrease in the expression of nuclear factor-κB–dependent genes, such as proinflammatory cytokines interleukin-1β and interleukin-6, we next assessed whether degradation of nuclear factor-κB inhibitor–α, which suppresses nuclear factor-κB activation, would play a role in the reduced expression of interleukin-1β and interleukin-6. Because this step is found at the very beginning of the inflammatory cascade, early time points (0, 5, 20, 30, 60, and 90 min) were chosen.34 Lipopolysaccharide led to a time-dependent degradation of nuclear factor-κB inhibitor–α, which peaked at 20 min ($P = 0.004$ compared with medium), which was still reduced at 30 min ($P = 0.002$ compared with medium), and finally returned to baseline at 60 min ($P > 0.999$ compared with controls). In the presence of sevoflurane, lipopolysaccharide-induced degradation of nuclear factor-κB inhibitor–α was not affected ($P > 0.999$; fig. 5, A and B).

![Fig. 5. Degradation of the nuclear factor-κB inhibitor IκBα in bone marrow–derived macrophages. (A) Cells were exposed to lipopolysaccharide (LPS) in the presence and absence of 2% sevoflurane (Sevo) for 0, 5, 20, 30, 60, and 90 min, followed by Western blot analysis. Scatter plot with actual values and the connecting line representing the mean; n = 3, two-way ANOVA (group and time interaction). (B) Representative Western blots.](http://pubs.asahq.org/anesthesiology/article-pdf/131/6/1301/461374/20191200_0-00020.pdf)
Next, mitogen-activated protein kinase pathways were explored. Extracellular signal-regulated kinase, which is part of the mitogen-activated protein kinase pathway, is activated by phosphorylation at Thr202/Tyr204 by MEK1. Phosphorylated extracellular signal-regulated kinase then influences the activity of several transcription factors. After 8 h of lipopolysaccharide stimulation, extracellular signal-regulated kinase phosphorylation increased (control vs. lipopolysaccharide, \( P = 0.0001 \); control vs. sevoflurane/lipopolysaccharide \( P = 0.0004 \)) and sevoflurane decreased lipopolysaccharide-induced extracellular signal-regulated kinase phosphorylation (\( P = 0.036 \)) compared with lipopolysaccharide alone (fig. 6).

**Sevoflurane Increases Uptake of Fluorescently Labeled Heat-inactivated E. coli by Macrophages In Vitro and In Vivo**

To test whether enhanced inducible NO synthase expression affects phagocytosis by macrophages, following exposure to medium alone, lipopolysaccharide or lipopolysaccharide and sevoflurane for 8 h, bone marrow–derived macrophages were incubated with fluorescein isothiocyanate-labeled heat-inactivated *E. coli* for 30 min. All samples were normalized to bone marrow–derived macrophages pretreated with medium without additional additives. There was no difference between bone marrow–derived macrophages pretreated with medium (100% ± 32.4% [mean ± SD]) and lipopolysaccharide (99.8% ± 29.6%; \( P > 0.999 \)).

![Fig. 6. Expression of extracellular signal-regulated kinase (ERK) phosphorylation (p-ERK) in bone marrow–derived macrophages normalized by total ERK (t-ERK). Cells were stimulated for 8 h with lipopolysaccharide (LPS) in the presence or absence of 2% sevoflurane (Sevo), followed by Western blot analysis. Representative Western blots are shown below. Values represent means ± SD; \( n = 7 \), one-way ANOVA). *\( P = 0.036 \).](image)

However, in lipopolysaccharide/sevoflurane treated bone marrow–derived macrophages, we observed a statistically significant increase in the uptake of fluorescein isothiocyanate-labeled *E. coli* (169.9% ± 79.2%, \( P = 0.004 \)). In addition, in the presence of the inducible NO synthase inhibitor 1400W, the increase in *E. coli* uptake induced by sevoflurane was abolished (fig. 7A), indicating *E. coli* uptake in the presence of sevoflurane was mediated by inducible NO synthase.

Finally, we assessed whether results obtained *in vitro* can be observed *in vivo*. Six animals per group were evaluated of a total number of 31 animals included. In each group, cell count or assessment was not possible in two animals (in the sham group only one animal) because of severe blood contamination of the lavage or technical difficulties when performing cell harvest and analysis. The average weight was 27.4 g. Using the lipopolysaccharide model of endotoxemia, macrophage inducible NO synthase expression and *E. coli* uptake in the peritoneal cavity was determined. Macrophages were identified as CD11c− CD11b+ F4/80+ cells. Data showed that inducible NO synthase expression was very low in all three control groups (Sham mean fluorescence intensity equals 1,813, lipopolysaccharide mean fluorescence intensity equals 6,736, Sevoflurane/Sham mean fluorescence intensity equals 6,757). In the Sevoflurane/lipopolysaccharide group, inducible NO synthase expression was markedly increased by 669% (mean fluorescence intensity equals 51,768) compared with the lipopolysaccharide group (\( P = 0.0003 \); fig. 7B). To quantify the number of bacteria internalized by macrophages, we analyzed the mean fluorescence intensity of fluorescein isothiocyanate-labeled *E. coli*. Consistent with results of our *in vitro* study, we detected an increase in *E. coli* uptake by 49% in the Sevoflurane/lipopolysaccharide treated group compared with lipopolysaccharide alone (lipopolysaccharide mean fluorescence intensity equals 6,756, Sevoflurane/lipopolysaccharide mean fluorescence intensity equals 10,068; \( P = 0.006 \); fig. 7C), demonstrating that the increased uptake of *E. coli* by macrophages after exposure to sevoflurane is also occurring *in vivo*.

**Discussion**

The present findings demonstrate that sevoflurane differentially modulates proinflammatory genes in murine macrophages. Whereas sevoflurane suppressed the expression of proinflammatory cytokines, it enhanced the expression of inducible NO synthase. Furthermore, sevoflurane increased the uptake of *E. coli* in an inducible NO synthase–dependent manner *in vitro* and *in vivo*.

This study demonstrates that endotoxin-induced release of proinflammatory cytokines in murine macrophage can be reduced by sevoflurane, as shown previously in other cell types. Because nuclear factor–κB is the primary mechanism associated with the regulation of proinflammatory genes, we assumed that sevoflurane would reduce the activation of nuclear factor–κB in bone marrow–derived...
As we did not observe a difference in the degradation of nuclear factor-κB inhibitor–α in presence of sevoflurane, we concluded that sevoflurane might be interacting directly with nuclear factor-κB. Boost et al. postulated that sevoflurane decreases nuclear factor-κB inhibitor–α degradation on tumor necrosis factor–α stimulation, but because lipopolysaccharide stimulates Toll-like receptor 4 we concluded that it affects the Toll-like receptor 4 pathway differently than the tumor necrosis factor–α pathway, the latter with involvement of tumor necrosis factor receptors, tumor necrosis factor receptor type 1–associated death domain and tumor necrosis factor receptor–associated factor 2.

Interestingly, we observed that sevoflurane affected lipopolysaccharide-induced expression of nuclear factor-κB–dependent genes differentially. Sevoflurane promoted the expression of inducible NO synthase, which is in agreement with previous findings. Because sevoflurane differentially modulates proinflammatory genes in murine macrophages, we concluded that different inflammatory pathways may be involved. Our data showed that sevoflurane affected the mitogen-activated protein kinase pathway by decreasing extracellular signal-regulated kinase phosphorylation. This may be a potential mechanism by which sevoflurane promotes inducible NO synthase expression in murine macrophages. Further, other groups reported decreased levels of extracellular signal-regulated kinase phosphorylation in the presence of sevoflurane and that inhibition of extracellular signal-regulated kinase phosphorylation was associated with enhanced inducible NO synthase expression. Because reduced extracellular signal-regulated phosphorylation was observed at later time points, and decreased expression of nuclear factor-κB–dependent genes was found as early as 1 h of stimulation, we postulate that sevoflurane-induced downregulation of inducible NO synthase at the early time point may be attributable to decreased activation of nuclear factor-κB during the initial phase. A question remains: Which transcription factor is involved in the increase in inducible NO synthase expression? A possible explanation is that reduced extracellular signal-regulated kinase phosphorylation leads to increased nuclear factor-κB activation. Bhatt et al. showed reduced extracellular signal-regulated kinase phosphorylation on stimulation of the Toll-like receptor 2 pathway enhances nuclear factor-κB activation. However, it remains unclear whether the same mechanism occurs with the Toll-like receptor 4 pathway or whether a different transcription factor is involved.

This is the first study to investigate the effect of sevoflurane on uptake of heat-inactivated E. coli by murine macrophages. We observed increased uptake of E. coli after exposure to sevoflurane, and the mechanism appears to be linked to the activity of inducible NO synthase as enhanced uptake was abolished when inducible NO synthase was pharmacologically inhibited. It has been shown that NO donors increase phagocytosis and that inhibiting NO release with L-N(G)-Nitroarginine methyl ester reduces bacterial uptake and bacterial killing. However, the role of inducible NO synthase in bacterial clearance remains controversial, as there is a report showing higher

Fig. 7. (A) Bone marrow–derived macrophages: uptake of fluorescein isothiocyanate (FITC)-labeled heat-inactivated Escherichia coli (E. coli). Cells were exposed to lipopolysaccharide (LPS) in the presence or absence of 2% sevoflurane (Sevo) for 8 h. Thereafter, the cells were exposed to E. coli for an additional 30 min. Fluorescence was determined using a fluorescence microplate reader. The same experiments were performed in the presence or absence of 1400W, an inhibitor of the inducible NO synthase (iNOS), which was added for the entire incubation. Values represent means ± SD; n = 15 samples without 1400W and n = 9 samples with 1400W; one-way ANOVA; LPS versus Sevo/LPS **P = 0.004, Sevo/LPS versus Sevo/LPS/1400W ***P = 0.009. (B) Mice were exposed to intraperitoneally applied LPS for a total of 20 h. Mice in the sevoflurane group received 2-hr anesthesia with 4% sevoflurane, whereas control animals were anesthetized with ketamine/xylazine. Thirty minutes before collection of peritoneal macrophages for analysis, FITC-labeled E. coli were injected intraperitoneally. Mean fluorescence intensity (MFI) of iNOS, after staining with fluorescently-labeled antibody, was measured using flow cytometry. (C) MFI of FITC-labeled E. coli using flow cytometry. Exposition as described. For B, and C, values represent means ± SD; n = 6, one-way ANOVA; >**P = 0.006 LPS versus Sevo/LPS, ***P = 0.0003 LPS versus Sevo/LPS.
bacterial clearance and resistance to bacterial meningitis in inducible NO synthase–deficient newborn mice. These findings are in contrast with the current data. Furthermore, various groups have observed that inducible NO synthase knockout animals exhibit an increase in mortality induced by different sepsis models, consistent with the idea that reactive nitrogen species play a significant role in host defense against microbes.

Inducible NO synthase is an important mediator in the inflammatory response to lipopolysaccharide stimulation. Although inducible NO synthase protein levels are decreased in the Sevoflurane/lipopolysaccharide group compared with lipopolysaccharide alone shortly after initiating endotoxin stimulation, inducible NO synthase expression in the Sevoflurane/lipopolysaccharide group exceeds the lipopolysaccharide values at later time points. With regard to inducible NO synthase expression, sevoflurane appears to be most likely interacting with at least two different inflammatory pathways, with opposed effects on inducible NO synthase expression. This is consistent with the findings that exposure to sevoflurane leads to decreased proinflammatory cytokine expression while enhancing bactericidal properties.

Obviously, the effect of sevoflurane upregulating inducible NO synthase can be evoked by using varying exposure times to sevoflurane. This becomes evident when comparing in vitro with in vivo results (in vitro: 8h, in vivo: 2h). However, in vitro scenarios are for sure not comparable with in vivo experimental setups, the latter being more complex with many different cell types over time involved. Moreover, it is known from previous studies that sevoflurane preconditioning increases nitric oxide release, and that conditioning with a volatile anesthetic for a short time provides long-term protection.

We observed markedly increased inducible NO synthase expression on lipopolysaccharide stimulation when animals were exposed to sevoflurane. Additionally, we demonstrated that exposure to sevoflurane increased the uptake of E. coli by peritoneal macrophages in vitro in mice. However, because the impact of sex on inducible NO synthase expression and uptake of E. coli was not evaluated, conclusions have to be drawn in a careful way.

Erol et al. reported that the phagocytic function of human polymorphonuclear leukocytes was not altered by sevoflurane when compared with desflurane and propofol, whereas another study showed reduced phagocytic function of human granulocytes after exposure to sevoflurane. Interestingly, this reduction was observed for granulocytes but not in monocytes, implying that the effects of sevoflurane on phagocytic function may be different between granulocytes and cells of monocyte-macrophage lineage.

Considering these data, we provide a possible mechanism by which sevoflurane increases bacterial uptake and survival in animal models of endotoxemia/sepsis. Stimulation of the Toll-like receptor 4 pathway with lipopolysaccharide leads to a well-understood inflammatory response. After stimulation of Toll-like receptor 4 in the plasma membrane, a signal transduction cascade is initiated which culminates in the phosphorylation of nuclear factor-κB inhibitor–α. Once nuclear factor-κB inhibitor–α is phosphorylated, it is degraded and nuclear factor-κB is no longer inhibited, enabling it to translocate to the nucleus to increase transcription of dependent genes. Because sevoflurane did not affect lipopolysaccharide–induced nuclear factor-κB inhibitor–α degradation, we assume that sevoflurane may directly interact with the translocation of nuclear factor-κB into the nucleus or reduce transcription of nuclear factor-κB–dependent genes as highlighted in figure 8. At later time points, sevoflurane reduced extracellular signal-regulated kinase phosphorylation, and this effect may be associated with an increase in inducible NO synthase expression through a mechanism that is not yet understood.

This study demonstrates that sevoflurane differentially modulates proinflammatory genes in murine bone marrow–derived macrophages. Whereas sevoflurane reduced lipopolysaccharide–induced release of proinflammatory cytokines, it enhanced the production of anti-microbial inducible NO synthase. Sevoflurane also increased macrophage uptake of heat-inactivated E. coli by a mechanism that seems to be linked to the activity of inducible NO synthase. These observations made in vitro were corroborated by in vivo studies that demonstrated sevoflurane promotes inducible NO synthase expression and bacterial uptake by peritoneal macrophages in the murine model of endotoxemia. These results provide an explanation, at least in part, for increased survival of septic rodents exposed to sevoflurane. It remains unclear whether results observed in rodents will hold true in higher mammals such as humans.

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Competing Interests

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References


Fig. 8. Schematic illustration of proposed antiinflammatory mechanism of action of sevoflurane. Exposure to lipopolysaccharide (LPS) leads to activation of Toll-like receptor 4 (TLR4) and initiates proinflammation cascade. Degradation of nuclear factor-κB inhibitor-α (IκBα) was not altered by sevoflurane and thus sevoflurane may directly interact with nuclear factor-κB (NF-κB; highlighted with "?"). Sevoflurane reduces phosphorylation of extracellular signal-regulated kinases (ERK) that may cause enhanced expression of inducible NO synthase (iNOS) and increased macrophage-mediated bacterial clearance.
against renal ischemia-reperfusion injury \textit{in vivo}. \textit{Anesthesiology} 2004; 101:1313–24


19. Möbert J, Zahler S, Becker BF, Conzen PF: Inhibition of neutrophil activation by volatile anesthetics decreases adhesion to cultured human endothelial cells. \textit{Anesthesiology} 1999; 90:1372–81


27. Kelm M, Feelisch M, Deussen A, Schrader J, Graue WE: The role of nitric-oxide in the control of coronary vascular tone in relation to partial oxygen-pressure,
34. Cruz MT, Duarte CB, Gonçalo M, Carvalho AP, Lopes MC: LPS induction of I kappa B-alpha degradation and iNOS expression in a skin dendritic cell line is prevented by the janus kinase 2 inhibitor, Typhostatin b42. Nitric Oxide 2001; 5:53–61


