Sevoflurane Inhibits Phorbol–Myristate–Acetate-induced Activator Protein-1 Activation in Human T Lymphocytes in Vitro: Potential Role of the p38-Stress Kinase Pathway

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Background: Modulation of immune defense mechanisms by volatile anesthetics during general anesthesia may compromise postoperative immune competence and healing reactions and affect the infection rate and the rate of tumor metastases disseminated during surgery. Several mechanisms have been suggested to account for these effects. The current study was undertaken to examine the molecular mechanisms underlying these observations.

Methods: Effects of sevoflurane, isoflurane, and desflurane were studied in vitro primary human CD3⁺ T-lymphocytes. DNA-binding activity of the transcription factor activator protein-1 (AP-1) was assessed using an electrophoretic mobility shift assay. Phorbol-myristate-acetate-dependent effects of sevoflurane on the phosphorylation of the mitogen-activated protein kinases were studied using Western blots, the trans-activating potency of AP-1 was determined using reporter gene assays, and the cytokine release was measured using enzyme-linked immunosorbent assays.

Results: Sevoflurane inhibited activation of the transcription factor AP-1. This effect was specific, as the activity of nuclear factor κB, nuclear factor of activated T cells, and specific protein-1 was not altered and several other volatile anesthetics studied did not affect AP-1 activation. Sevoflurane-mediated suppression of AP-1 could be observed in primary CD3⁺ lymphocytes from healthy volunteers, was time-dependent and concentration-dependent, and occurred at concentrations that are clinically achieved. It resulted in an inhibition of AP-1-driven reporter gene activity and of the expression of the AP-1 target gene interleukin-3. Suppression of AP-1 was associated with altered phosphorylation of p38 mitogen-activated protein kinases.

Conclusion: The data demonstrate that sevoflurane is a specific inhibitor of AP-1 and may thus provide a molecular mechanism for the antiinflammatory effects associated with sevoflurane administration.

GENERAL anesthesia can modulate immune defense mechanisms within the postoperative period. In particular, inhaled anesthetics contribute to postoperative immunosuppression, especially when applied in higher concentrations or doses or for longer periods of time. For example, two studies have demonstrated peripheral lymphocytopenia after inhalation anesthesia with halothane and nitrous oxide in patients undergoing elective hysterectomy. Giraud et al. recently reported that halothane >1 Vol.%, delivered by mechanical ventilation, abated the early lipopolysaccharide-induced lung inflammation in the rat. Similarly, isoflurane pretreatment prevented the increase in tumor necrosis factor α associated with lipopolysaccharide-induced inflammation in rats. Previous studies have demonstrated that the total number of circulating neutrophils decreased during anesthetic exposure, whereas the number of lymphocytes increased.

In contrast to the well documented clinical effects of inhaled anesthetics, the precise molecular mechanism of their immunosuppressive action remains to be identified. The following in vitro studies have provided evidence that volatile anesthetics may alter the innate immune response. Halothane, enflurane, and sevoflurane inhibited the respiratory burst. Likewise, sevoflurane, isoflurane, and enfurane inhibit interleukin-1β and tumor necrosis factor α release as well as chemotaxis in human mononuclear cells and neutrophil adhesion to endothelial cells. How ever, exposure of interleukin-1β-stimulated alveolar type II cells to halothane, enfurane, and isoflurane reversibly decreased interleukin-6, macrophage inflammatory protein-2, and monocyte chemoattractant protein-1 concentrations in cell culture supernatants.

Much less is known about the effect of inhalation anesthetics on the function of lymphocytes. The CD3⁺ cell population encompasses all known T cell populations, including CD4⁺, CD8⁺, and Th1 or Th2. We chose T cells in our model because they play a crucial role in the defense against nosocomial infections. Previous studies have demonstrated that halothane inhibits phytohemmagglutinin-induced RNA and protein synthesis in human lymphocytes. This anesthetic also induces apoptosis, which may be mediated through the suppression of interleukin-2 receptor expression.

The latter finding suggests that anesthetics could exert their immunosuppressive effects in part by inhibiting the transcription factor function of immune cells. This hypothesis is supported by the observation that thiopental suppresses induction of the nuclear factor κB (NF-κB) in human T lymphocytes. We therefore investigated whether volatile anesthetics likewise interfere with the
activation of the transcription factor activator protein-1 (AP-1), a central transcriptional regulator of the immune and stress response in activated T cells.

AP-1 is an immediate early transcription factor that is involved in the regulation of cellular proliferation, transformation, differentiation, and the death of immune cells. AP-1 activity is induced by a wide range of physiologic stimuli and environmental insults and is composed of homodimers of the Jun family (c-Jun, JunD, and JunB) or of Jun heterodimers with any of the Fos family (c-Fos, FosB, Fra1, and Fra2) (fig. 1). AP-1 proteins are targets of the mitogen-activated protein (MAP) kinase cascade. The MAP kinases are a family of serine-threonine protein kinases, of which four distinct subgroups have been described: 1) extracellular signal-regulated kinases (ERKs), 2) c-jun N-terminal or stress activated protein kinases (JNK/SAPK), 3) ERK5/big MAP kinase 1 (BMK1), and 4) the p38 group of protein kinases (fig. 1). Five isoforms of p38 kinases have been identified in mammalian cells. These proteins, termed p38α, p38β, p38γ/SAPK3, p38δ/SAPK4, and p38 –2, are similar in size, show about 60–75% sequence homology, and are activated by tumor necrosis factor α, interleukin-1, ultraviolet irradiation, and hyperosmolar medium. The α- and β-isofoms of p38 kinases are inhibited by a novel class of antiinflammatory drugs that block the access of adenosine triphosphate to the catalytic site of the kinase. In contrast, the γ and δ isoforms of p38 are insensitive to these pyridinyl-imidazole inhibitors.

It was the aim of this study to examine whether sevoflurane, desflurane, or isoflurane interfere with the
activation of the transcription factor AP-1 in primary human T lymphocytes and, if so, whether they target the upstream signal transduction pathway.

Materials and Methods

Reagents
The following anesthetics and substances were used: sevoflurane, isoflurane (Abbott, Wiesbaden, Germany), and desflurane (Baxter, Unterschleißheim, Germany). All other reagents were purchased from Sigma (Deisenhofen, Germany) unless otherwise specified.

Isolation of CD3⁺ T lymphocytes
Peripheral blood mononuclear cells were isolated from buffy coats that had been obtained from healthy donors using density centrifugation on Ficoll-Hypaque® (Amer sham-Pharmacia, Freiburg, Germany) according to manufacturer recommendations. The cells were microscopically analyzed and counted in a Neubauer chamber. For the isolation of CD3⁺ T lymphocytes, peripheral blood mononuclear cells (3–4 × 10⁶) were incubated for 15 min on ice with anti CD3-antibodies conjugated to magnetic beads (Miltenyi Biotech, Bergisch-Gladbach, Germany). Separation of CD3⁺ cells was performed using an L/S column (Miltenyi Biotech, Bergisch-Gladbach, Germany) and confirmed by fluorescence associated cell sorting (>92% purity). For electrophoretic mobility shift assays (EMSA) > 2 × 10⁶ T lymphocytes were analyzed per sample.

Cell Culture
Jurkat T cells (ACC 282, DSMZ, Braunschweig, Germany) and primary human T lymphocytes isolated as described above, were maintained in Roswell Park Memorial Institute medium 1640 supplemented with 10% fetal calf serum, 1% glutamine, and 50 μg/ml penicillin and streptomycin (all from Gibco-BRL, Karlsruhe, Germany). Cells were incubated for 15 min at 37°C on ice with anti CD3-antibodies conjugated to magnetic beads (Miltenyi Biotech, Bergisch-Gladbach, Germany) and confirmed by fluorescence associated cell sorting (>92% purity). For electrophoretic mobility shift assays (EMSA) > 2 × 10⁶ T lymphocytes were analyzed per sample.

Exposure to Volatile Anesthetics and Experimental Protocol
Jurkat T cells or primary CD3⁺ T lymphocytes were exposed to either air or volatile anesthetic/air mixture using a 12 l airtight glass chamber. The chamber atmosphere was kept continuously saturated with water at 37°C. Gas was prepared using a gas mixing unit by directing a 95% air/5% CO₂ mixture at 6 l/min through calibrated vaporizers (Draeger, Lübeck, Germany) that were placed at the entrance of the chamber. In preliminary experiments, we observed that over a 48 h period the different culture conditions used in our experiments did not influence AP-1 activation in cells; the DNA binding activity was similar in a standard incubator and in the scaled chamber flushed with a 95% air/5% CO₂ mixture. Volatile anesthetic concentrations were monitored at the chamber exit port using a halogen monitor (PM 8050, Dräger, Lübeck, Germany). Concentrations of the inhalation anesthetics dissolved in the cell culture medium were measured by a fully automated solid-phase microextraction procedure followed by gas chromatography and mass spectrometry (CTC Combi PAL autosampler, Agilent model 6890 Series plus Gas Chromatograph with Agilent 5973 N Mass Selective Detector; Chromtech, Idstein, Germany). Using an external standard method, calibration curves were achieved for sevoflurane, desflurane, and isoflurane. Based on this method the following concentrations of volatile anesthetics were measured in the culture medium after 24 h of incubation with volatile anesthetics: sevoflurane (8 Vol.%) 982 ± 4 μmol/l, isoflurane (5 Vol.%) 755 ± 6 μmol/l, desflurane (18 Vol.%) 1799 ± 12 μmol/l.

For each exposure condition, a control sample was obtained simultaneously from T cells cultured in a standard 95% air/5% CO₂ incubator without anesthesia exposure. At the end of each experiment, both supernatants and T cells were frozen immediately in liquid nitrogen and stored at −80°C until protein extraction.

To analyze the effects of volatile anesthetics on AP-1, NF-κB, nuclear factor of activated T cells (NFAT), and specific protein-1 (SP-1) activation, cells were treated with sevoflurane, isoflurane, or desflurane. Thirty minutes before harvesting, the cells were stimulated with phorbol-myristate-acetate (PMA; 15 ng/ml) and ionomycin (700 ng/ml), after which nuclear cell protein extracts were prepared and analyzed for the DNA binding activity by electrophoretic mobility shift assays (EMSA).

Electrophoretic Mobility Shift Assays
Cells were harvested by centrifugation and washed once in ice-cold phosphate-buffered saline, and nuclear cell extracts were prepared according to the procedure described.³¹ Briefly, cells were incubated for 15 min at 4°C in 400 μl extraction buffer (10 mM HEPES (pH 7.9), 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, and 2.5 μl Nonidet P40). Pellets were solubilized in suspension buffer (20 mM HEPES (pH 7.9), 0.4 mM NaCl, 1 mM EDTA, 1 mM EGTA, and 1 mM dithiothreitol). Supernatants were used for EMSA. Inhibitors of proteinases and phosphatases were added at the following concentrations to the extraction and suspension buffer: 10 μg/ml aprotinin, 25 μM leupeptin, 2 μM phenylmethyl-sulfonyl-fluoride, 2 μM iodoacetamide, 10 μM sodium fluoride, and 10 μM sodium pyrophosphate. EMSAs were performed using a 3²²P-labeled AP-1, NF-κB, or SP-1 oligonucleotide (25 ng/μl, Promega, Mannheim, Germany). EMSAs for NFAT were performed using the NFAT motif of the human granulocyte-monocyte colony stimulating factor enhancer region (5'-TTTCTCATGAAAAATGACAT A-3') as a probe. The kinase reaction consisted of 37 μl purified water, 1 μl AP-1 (or

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NF-κB, or SP-1) oligonucleotides, 5 μl kinase buffer, 5 μl γ-
32P-d-adenosine triphosphate (Amersham International,
Braunschweig, Germany), and 1.5 μl T4 kinase (PNK buffer
and PNK T4 kinase; New England Biolabs, Schwalbach,
Germany) and was incubated for 30 min at 37°C. The
protein content of the cell lysates was determined using a
Bradford-Phaston system (Bio-Rad Laboratories, München,
Germany), and equal amounts of protein (30 μg) were
added to a 20 μl EMSA reaction mixture containing 20 μg
bovine serum albumin, 2 μg poly(dII-dC) (Roche, Mann-
heim, Germany), 2 μl buffer D+ (20 mM HEPES, pH 7.9,
20% glycerol, 100 mM KCl, 0.5 mM EDTA, 0.25% Nonidet
P-40, 2 μm dithiothreitol, 0.1% phenyl-methyl-sulfonyl-fluo-
ride), 4 μl 5× Ficoll buffer (20% Ficoll 400, 100 mM HEPES,
300 mM KCl, 10 mM dithiothreitol, 0.1% phenyl-methyl-
sulfonylfluoride), 1 μl 50 mM MgCl₂, 3 μl ddH₂O, and 1 μl
AP-1 (or NFAT, NF-κB, or SP-1) 32P-labeled oligonucleotide.
These samples were incubated at room temperature for 30
min and then loaded on an acrylamide gel containing 60 μl
ddH₂O, 10 ml 30% acrylamide, 3.8 ml 10× Tris-borate-
EDTA buffer, gels were vacuum dried and then loaded on an acrylamide gel containing 60 ml
ddH₂O, 1 ml ammonium persulfate, and 40 μl tetramethylethene diamine. After running the gel in 0.5×
Tris-borate-EDTA running buffer (900 m, TRIS-HCl, 900 μM boric acid, 20 mM
EDTA [pH 8.0]), 400 μl ammonium persulfate, and 40 μl
tetramethylethylene diamine. After running the gel in 0.5×
Tris-borate-EDTA running buffer, gels were vacuum dried
(Gel dryer 543, Bio-Rad, Hercules, CA) for 30 min on a
3 mm chromatography filter (Whatman, Maidstone, En-
gland) and exposed to radiographic film (Kodak, Stuttgart,
Germany). For the supershift assays, 2.5 μl of antibody were
added to the reaction simultaneously with the protein
and incubated as described. Anti-jun, anti-jun (AP-1), anti-
JunB, anti-JunD, anti-fos, anti-Fra1 and -Fra2, anti-FosB and
and anti-RelB antibodies were purchased from Santa Cruz Bio-
technology (Santa Cruz, CA).

Transfections and Luciferase Assays
Jurkat cells were plated 12–16 h before transfection at
a density of 3 × 10⁵ cells per well in a six-well plate.
Cells were transiently transfected with a luciferase re-
porter gene construct (pAP1(PMA)TA-Luc vector, Clon-
tech, Palo Alto, CA). Transfections were performed us-
ing the superfect reagent (Qiagen, Hilden, Germany)
according to manufacturer descriptions. To exclude the
possibility of differences in transfection efficiency, all
cells were pooled at 6 h after transfection, gently mixed,
and then equally distributed into individual wells for all
further determinations. The experiment was repeated
six times. Cells were pretreated with sevoflurane or
desflurane for 1 h before stimulation with PMA and
ionomycin (15/700 ng/ml) and harvested in situ after
23 h using a commercial lysis buffer and luciferase assay
system (Promega, Mannheim, Germany). Luciferase ac-
activity was determined using a microplate luminometer
(Eg & G-Berthold, Bad Wilsbach, Germany) measuring
light emission over an interval of 30 s. The results are
expressed in arbitrary light units as percentage of the
respective positive control.

Cytokine Analysis
Cell culture supernatants were analyzed for interleu-
kin-3 (IL-3) 23 h after stimulation with PMA and ionomy-
cin (15/700 ng/ml). Cells were pretreated with sevoflu-
rane (8 Vol.%) for 1 h before stimulation. Measurements
were performed using enzyme-linked immunosorbent
assay kits purchased from R&D Systems (Minneapolis,
MN) according to manufacturer instructions.

Western Blotting for p38, JNK1/2 and ERK1/2
Phosphorylation of p38, JNK1/2, and ERK1/2 was anal-
yzed by Western blotting. To determine whether sevoflurane may interfere with the phosphorylation of
these three MAP-kinases, CD3⁺ T cells were pretreated
with sevoflurane (8 Vol.%) for 24 h and subsequently
stimulated with PMA and ionomycin (15/700 ng/ml) for
15 min. Total cell extracts of CD3⁺ T cells (30 μg) were
boiled in Laemmli sample buffer and subjected to 10%
sodium dodecyl sulfate–polyacrylamide gel. Before trans-
gens, gels were equilibrated for 15 min in cathode buffer,
(25 mM Tris, 40 mM glycine, 10% methanol). Using a semi-
dry blotting apparatus (Bio-Rad Laboratories, München,
Germany), proteins were transferred at 0.8 mA/cm² for 1 h
onto Immobilon P membranes (Millipore Corp., Eschborn,
Germany), pre-equilibrated in methanol (15 s), ddH₂O
(2 min each side), and anode buffer II (25 mM Tris/10%
methanol). Equal loading and transfer were monitored by
amido-black staining of the membranes. Nonspecific bind-
ing sites were blocked by immersing the membrane into
blocking solution (10 mM Tris-HCl, pH 8.0, 150 mM NaCl,
0.1% Tween-20 [v/v]) containing 5% milk powder (Fluka,
Buchs, Switzerland) for 1 h at room temperature. Mem-
branes were washed in blocking solution and incubated
in a 1:1000 dilution of anti-p38 (Cat. No. 9212), anti-JNK1/2
(Cat. No. 9252), anti-ERK1/2 (Cat. No. 9102) antibody, or
antiphosphorylated-p38, JNK1/2, ERK1/2 (Cat. No. 9910;
all Cell Signaling Technology Inc., Beverly, MA) in blocking
solution plus 5% bovine serum albumin overnight at 4°C,
followed by extensive washing with blocking solution.
Bound antibody was decorated with goat-antirabbit/horse-
radish peroxidase conjugate (Amersham Pharmacia,
Freiburg, Germany), diluted 1:2000 in blocking solution,
for 30 min at room temperature. After washing four times
(5 min each), the immunocomplexes were detected using
ECL Western blotting reagents (Amersham-Pharmacia,
Freiburg, Germany) according to manufacturer instruc-
tions. Exposure to Kodak XAR-5 films (Stuttgart,
Germany) was performed for 15 s to 1 min.

Quantitative and Statistical Analysis
Differences in measured variables between the exper-
imental conditions were assessed using one-way analysis
of variance on ranks followed by a nonparametric Stu-
dent-Newman-Keuls test for multiple comparisons. Re-
sults were considered statistically significant if P < 0.05.
The tests were performed using the SigmaStat software package (Jandel Scientific, San Rafael, CA).

Results

Effects of Sevoflurane on AP-1 Activation

Treatment of CD3⁺ T lymphocytes with PMA (15 ng/ml) and ionomycin (700 ng/ml) induced AP-1 DNA binding activity compared with untreated cells (fig. 2, lanes 1 and 2). Induced AP-1 activation was inhibited by pretreatment of cells with sevoflurane (8 Vol.%) (fig. 2, lane 3). In contrast, incubation of T cells with sevoflurane alone had no effect on the activation of AP-1 (fig. 2, lane 4).

Concentration-dependent and Time-dependent Effects of Sevoflurane and AP-1 Activation

We evaluated whether the concentration and the exposure time to sevoflurane may affect the activation of AP-1 DNA binding. CD3⁺ T cells were incubated with different concentrations of sevoflurane (2, 4, 6, and 8 Vol.%) and stimulated with PMA and ionomycin. As shown in figure 3, activation of AP-1 was inhibited after exposure to 2, 4, 6, and 8 Vol.% sevoflurane (fig. 3, lanes 2, 5, 8, and 11 compared with lanes 3, 6, 9, and 12 respectively). AP-1 DNA binding activity was suppressed beginning at 12 h of sevoflurane exposure (fig. 4, lanes 9 and 12).

Effects of Sevoflurane on SP-1 Activation

In order to determine whether sevoflurane specifically inhibits AP-1 activation or whether DNA binding of an immunologically nonrelevant transcription factor is also perturbed, we performed additional EMSAs using a DNA probe containing the consensus motif for the transcription factor SP-1. Treatment of CD3⁺ T cells with sevoflurane (8 Vol.%) with subsequent administration of PMA (15 ng/ml) and ionomycin (700 ng/ml) did not detectably affect DNA binding of SP-1 to its DNA probe (fig. 5, lanes 2 and 3).}

Effects of Sevoflurane on NF-κB and NFAT Activation

To determine whether sevoflurane specifically inhibits AP-1 activation or whether DNA binding of other immunologically relevant transcription factors is also perturbed, we performed additional EMSAs using a DNA probe containing the consensus motif for the transcription factor NF-κB and NFAT.
tion factors NF-κB and NFAT. Treatment of CD3⁺ T cells with sevoflurane (8 Vol.%) with or without subsequent administration of PMA and ionomycin did not affect DNA binding of NF-κB and NFAT to their respective DNA elements (fig. 6 A and B, lanes 1 to 4).

**Effects of Desflurane and Isoflurane on AP-1 Activation**

The results described above raised the question whether the suppression of AP-1 activation by sevoflurane is specific for this drug or a common biologic effect of other volatile anesthetic agents. Therefore, we tested whether desflurane or isoflurane, when applied at maximal concentrations, also affect the activation of AP-1. However, in contrast to the results obtained with sevoflurane, desflurane, and isoflurane did not inhibit AP-1 activation in CD3⁺ T lymphocytes (fig. 7 A and B, lanes 1 to 4).

**Sevoflurane and AP-1-Driven Reporter Gene Activity**

Sevoflurane potently inhibits AP-1 DNA binding in EM-SAs (figs. 2–4), raising the question whether this results in a comparable inhibition of AP-1-dependent gene expression. Therefore, Jurkat T cells were transiently transfected with a luciferase reporter gene driven by AP-1 binding sites preceding a minimal promoter (pAP1(PMA)TA-

**Fig. 4.** The effect of the time of incubation with sevoflurane on the phorbol-myristate-acetate (PMA)-mediated and ionomycin-mediated activation of Activator Protein-1 (AP-1). CD3⁺ T cells were pretreated for 2, 5, 11, and 23 h with sevoflurane 8 Vol.%, and subsequently stimulated with 15 ng/ml PMA and 700 ng/ml ionomycin for 1 h (lanes 2 and 3, 5 and 6, 8 and 9, 11 and 12) or the respective volumes of ppH₂O (lanes 1, 4, 7, and 10) as vehicle control. Equal amounts of protein from cell extracts were analyzed for AP-1 activity by electrophoretic mobility shift assay. A section of a fluorogram is shown. △ = position of AP-1 DNA complexes; ○ = a nonspecific activity binding to the probe; ▼ = unbound oligonucleotide. The results are representative of six independent experiments.

**Fig. 5.** The effect of sevoflurane on SP-1 DNA binding after phorbol-myristate-acetate (PMA) and ionomycin stimulation. CD3⁺ T lymphocytes were treated for 23 h with sevoflurane 8 Vol.% (lane 3) and subsequently stimulated with 15 ng/ml PMA and 700 ng/ml ionomycin for 1 h (lanes 2 and 3) or the respective volume of ppH₂O (lane 1) as vehicle control. Equal amounts of protein from cell extracts were analyzed for SP-1 activity by electrophoretic mobility shift assay. A section of a fluorogram is shown. △ = position of SP-1 DNA complexes; ○ = a nonspecific activity binding to the probe; ▼ = unbound oligonucleotide. The data shown are representative of six independent experiments.

Luc). PMA/ionomycin stimulation resulted in a profound increase in reporter gene activity (fig. 8). Desflurane and sevoflurane alone had no effect on the activation of the AP-1-driven promoter; likewise, the PMA/ionomycin-induced activation was unaffected by desflurane. In contrast, AP-1-dependent reporter gene activity was suppressed by sevoflurane (fig. 8).

**Effects of Sevoflurane on Expression of Interleukin-3**

To determine whether inhibition of AP-1 transcriptional activity by sevoflurane in CD3⁺ T lymphocytes is associated with altered production of a target gene of AP-1, the effect of sevoflurane on IL-3 release was studied. In CD3⁺ T cells the release of IL-3 increased upon PMA and ionomycin stimulation (fig. 9). Sevoflurane attenuated this increase in the production of IL-3 (fig. 9).

**Effects of Sevoflurane on MAP-Kinase Phosphorylation**

The effect of sevoflurane on MAP-kinase phosphorylation was studied by using phospho-specific antibodies...

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Pre-treatment of CD3 and ERK1/2 (700 ng/ml) induced the phosphorylation of p38, JNK1/2, and p38α MAP-kinases (lanes 2 and 3) or the respective volumes of pH2O (lanes 1 and 4) as vehicle control. Equal amounts of protein from cell extracts were analyzed for NF-κB (A) and NFAT (B) activity by electrophoretic mobility shift assay. A section of a fluorogram is shown. = position of NF-κB (A) and NFAT (B) DNA complexes; = a nonspecific activity binding to the probe; = unbound oligonucleotide. The data shown are representative of six independent experiments.

Fig. 6. The effect of sevoflurane on NF-κB (A) and NFAT (B) DNA binding after phorbol-myristate-acetate (PMA) and ionomycin stimulation. CD3+ T lymphocytes were treated for 23 h with sevoflurane 8 Vol.% (lanes 3 and 4) and subsequently stimulated with 15 ng/ml PMA and 700 ng/ml ionomycin for 1 h (lanes 2 and 3, A and B) or the respective volumes of pH2O (lanes 1 and 4) as vehicle control. Equal amounts of protein from cell extracts were analyzed for NF-κB (A) and NFAT (B) activity by electrophoretic mobility shift assay. A section of a fluorogram is shown. = position of NF-κB (A) and NFAT (B) DNA complexes; = a nonspecific activity binding to the probe; = unbound oligonucleotide. The data shown are representative of six independent experiments.

against p38, JNK1/2, and ERK1/2. Treatment of CD3+ T lymphocytes with PMA (15 ng/ml) and ionomycin (700 ng/ml) induced the phosphorylation of p38, JNK1/2, and ERK1/2 (fig. 10, A, B, C, upper panel, lanes 1 and 2). Pretreatment of CD3+ T cells with sevoflurane (8 Vol.%) with subsequent administration of PMA and ionomycin did not affect the phosphorylation of JNK1/2 and ERK1/2 MAP-kinases (fig. 10, B, C, upper panel, lane 3). However, after sevoflurane exposure, the PMA/ionomycin-induced phosphorylation of p38 was more pronounced than after PMA/ionomycin stimulation alone (fig. 10, A, upper panel, lane 2 and 3). Furthermore, even in the absence of PMA/ionomycin exposure to sevoflurane (8 Vol.%) alone significantly increased the phosphorylation of p38, whereas JNK1/2 and ERK1/2 were not affected (fig. 10, A - C, upper panel, lane 4). The total amount of p38, JNK1/2, and ERK1/2 was constant throughout each experiment (fig. 10, A-C, lower panel, lanes 1 - 4).

Effects of p38-Inhibitors on Sevoflurane-Suppressed AP-1 Activation

The transcription factor AP-1 can be activated by the different isoforms of the p38 MAP-kinases in a selective and opposite manner. Therefore, we wanted to assess AP-1 DNA binding activity after pretreatment with specific inhibitors of the p38α and p38β isoforms (SD203580 and SD202190 respectively, No. 19–134 and 19–135; Upstate Biotechnologies, Lake Placid, NY). As demonstrated previously, treatment of CD3+ T lymphocytes with PMA and ionomycin induced AP-1 DNA binding activity (fig. 11, lanes 1 and 2), which was inhibited by pretreatment of cells with sevoflurane (8 Vol.%) (fig. 11, lane 3). Preincubation of CD3+ T cells with either SD203580 or SD202190 before sevoflurane exposure reduced AP-1 induction on PMA and ionomycin stimulation more potently than sevoflurane alone (fig. 11, lanes 4 and 5).

Protein Binding to the AP-1 Site Induced by PMA and Ionomycin

To determine which protein species binds to the AP-1 site induced by PMA and ionomycin, we performed supershift analysis using antibodies against different AP-1 subunits. These experiments indicated that the AP-1 complex induced by PMA/ionomycin is composed mainly of c-jun, JunB, JunD, c-Fos and FosB (fig. 12, lanes 3 to 10).

Effect of Sevoflurane on Protein Binding to the AP-1 Site after Induction by PMA and Ionomycin

To determine which protein binding to the AP-1 site is inhibited by sevoflurane, we performed supershift anal-
ysis on extracts of cells pretreated with sevoflurane using the same antibodies described above. These experiments indicated that the treatment of cells with sevoflurane appeared to influence mainly JunD and Fra2 DNA binding most and affect c-jun, JunB and FosB to a lesser extent (fig. 13, lanes 4 to 11).

**Effect of Sevoflurane on Various Stimulators of AP-1 DNA Binding Activity**

To consider the effect of other inducing agents that may stimulate AP-1 activity, we have exposed CD3⁺ T cells to CD3/CD28-beads, tumor necrosis factor α, and serum. AP-1 DNA binding activity was induced upon all stimuli (fig. 14, lanes 2, 4, 6, and 8). Sevoflurane inhibited CD3/CD28-dependent (fig. 14, lane 5) and tumor necrosis factor-induced (fig. 14, lane 7; slight) AP-1 activity. In contrast, serum-induced AP-1 activation was unaffected by sevoflurane (fig. 14, lane 9).

**Discussion**

It has been recently recognized that general anesthetics may modulate and impair immune defense mechanisms.²²⁻³⁷ This is of particular importance because anesthetics are exclusively administered to patients undergoing surgical procedures who are therefore at
particular risk of developing infections. Compromised immunity could affect the postoperative infection rate, healing reactions, and the rate and size of tumor metastases disseminated during surgery.\(^1\),\(^3\),\(^13\),\(^38\) However, evidence regarding immunomodulatory effects of volatile anesthetics is conflicting. This may result from differences in study designs as well as the inherent limitations of in vitro models. Thus, it was the aim of the current study to identify the molecular mechanism by which sevoflurane, isoflurane, or desflurane may modulate the activation of various transcription factors in human T lymphocytes.

Our data demonstrate that the volatile anesthetic sevoflurane inhibits activation of the transcription factor AP-1 in isolated CD3\(^{+}\) T lymphocytes. The effect of sevoflurane had the following characteristics:

1) it appeared to be a specific pharmacological characteristic of sevoflurane, as it occurred in a dose-dependent and time-dependent manner and the other volatile anesthetics tested (isoflurane and desflurane) did not exert any inhibitory effect on AP-1 activation. In addition, DNA-binding activity of NF-\(\kappa\)B, NFAT, and SP-1 in T cells was not affected by sevoflurane treatment;

2) it was associated with an inhibition of AP-1-driven reporter gene activity and of expression of the AP-1 target gene IL-3;

3) sevoflurane-mediated AP-1 inhibition is associated with the activation of p38;

4) in contrast, ERK and JNK activation is not involved; and

5) it may involve with the \(\delta\) or \(\gamma\) isoform of p38 because sevoflurane-mediated inhibition of AP-1 activation was even more pronounced after treatment with inhibitors of the p38\(\alpha\) and \(\beta\) isoforms. We thus propose that sevoflurane may exert its antiinflammatory and antiproliferative effects through inhibition of the activation of AP-1.

Several previously reported effects of volatile anesthetics on the regulation of immune cells could be explained by their inhibitory effect on AP-1. AP-1 acts as an envi-

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Fig. 11. The effect of sevoflurane and specific p38 inhibitors on Activator Protein-1 (AP-1) DNA binding after phorbol-myristate-acetate (PMA) and ionomycin stimulation. CD3\(^{+}\) T lymphocytes were pretreated for 1 h with either the specific p38\(\alpha\)/p38\(\beta\) inhibitors SD202190 or SD203580 at the concentrations indicated, incubated with sevoflurane (8 Vol\%) for 23 h, and subsequently stimulated with 15 ng/ml phorbol-myristate-acetate (PMA) and 700 ng/ml ionomycin for 1 h (lanes 2 through 6) or the respective volumes of ppH\(\kappa\)O (lane 1) as vehicle control. Equal amounts of protein from cell extracts were analyzed for AP-1 activity by electrophoretic mobility shift assay. A section of a fluorogram is shown. \(\bullet\) = position of AP-1 DNA complexes; \(\circ\) = a nonspecific activity binding to the probe; \(\triangle\) = unbound oligonucleotide. The data shown are representative of six independent experiments.

Fig. 12. Supershift analysis to evaluate protein binding to the Activator Protein-1 (AP-1) site induced by phorbol-myristate-acetate (PMA) and ionomycin. These experiments indicated that the AP-1 complex induced by PMA/ionomycin is composed of mainly of c-jun, JunB, JunD, c-Fos, and FosB (lane 3 to 10). Equal amounts of protein from cell extracts were analyzed for AP-1 activity by electrophoretic mobility shift assay. \(\bullet\) = position of AP-1 DNA complexes; \(\circ\) = a nonspecific activity binding to the probe; \(\triangle\) = unbound oligonucleotide. The data shown are representative of three independent experiments.
Environmental biosensor to various external stimuli. AP-1 proteins are key regulatory molecules that convert MAP kinase cascade signals into expression of specific target genes. In unstimulated T cells, AP-1 expression is low or undetectable. However, there is a rapid induction of AP-1 activity after T cell stimulation, which is mediated through the T cell/CD3 receptor complex. Thus, it would be tempting to speculate that the inhibitory effect of volatile anesthetics alters T cell stimulation. Indeed, previous studies demonstrated that halothane inhibited PMA-induced transcription, RNA and protein synthesis in cultured human lymphocytes. Furthermore, in an inflammatory pain model, noxious stimulation-induced c-Fos protein expression in the spinal cord of the rat is suppressed after halothane-based or isoflurane-based general anesthesia. These results argue that volatile anesthetics can attenuate T cell stimulation. Indeed, previous studies demonstrated that halothane inhibited PMA-induced transcription, RNA and protein synthesis in cultured human lymphocytes. Furthermore, in an inflammatory pain model, noxious stimulation-induced c-Fos protein expression in the spinal cord of the rat is suppressed after halothane-based or isoflurane-based general anesthesia. These results argue that volatile anesthetics can attenuate T cell stimulation. Indeed, previous studies demonstrated that halothane inhibited PMA-induced transcription, RNA and protein synthesis in cultured human lymphocytes. Furthermore, in an inflammatory pain model, noxious stimulation-induced c-Fos protein expression in the spinal cord of the rat is suppressed after halothane-based or isoflurane-based general anesthesia.

Fig. 13. Effect of sevoflurane on protein binding to the Activator Protein-1 (AP-1) site after induction by phorbol-myristate-acetate (PMA) and ionomycin. These supershift analyses indicated that the AP-1 complex induced by PMA/ionomycin after sevoflurane exposure is composed mainly by JunD and Fra2 DNA binding and affects c-Jun, JunB, and FosB to a lesser extent (lanes 4 to 11). Equal amounts of protein from cell extracts were analyzed for AP-1 activity by electrophoretic mobility shift assay. ▲ = position of AP-1 DNA complexes; ○ = a nonspecific activity binding to the probe; ◄ = unbound oligonucleotide. The data shown are representative of three independent experiments.

Fig. 14. The effect of other inducing agents that stimulate Activator Protein-1 (AP-1) DNA binding activity. Exposure of CD3+ T cells to CD3/CD28-beads, tumor necrosis factor α, and serum induced AP-1 DNA binding activity (lanes 2, 4, 6, and 8). Sevoflurane inhibited CD3/CD28-induced (lane 5) and slightly tumor necrosis factor-induced (lane 7) AP-1 activity. In contrast, serum-induced AP-1 activation could not be inhibited by sevoflurane (lane 9). Equal amounts of protein from cell extracts were analyzed for AP-1 activity by electrophoretic mobility shift assay. ▲ = position of AP-1 DNA complexes; ○ = a nonspecific activity binding to the probe; ◄ = unbound oligonucleotide. The data shown are representative of three independent experiments.
ulation of the DNA-binding activity of AP-1 in T lymphocytes. Therefore, it is of particular interest that based on the medium/gas partition coefficients, concentrations obtained in the culture medium of our model were calculated to be $\approx 1.0$ ms sevoflurane, which would be higher than the clinical dose.\(^{34,43}\) Finally, incubation of cells with sevoflurane caused a profound attenuation of IL-3 production, which plays a functional role in T cell growth, differentiation, and leukocyte adhesion.\(^{46-48}\) Likewise, beside IL-3, genes such as interleukin-2 and granulocyte-monocyte colony stimulating factor are under AP-1 control through the putative cisacting AP-1 sites in their promoter and enhancer regions.\(^{49}\) Therefore, volatile anesthetic-induced apoptosis in lymphocytes could be explained in the same manner because withdrawal of interleukin-2, IL-3, or granulocyte-monocyte colony stimulating factor promotes cell death.\(^{23,50,51}\)

Our observation raises the question by which underlying mechanism sevoflurane may inhibit the activation of AP-1. AP-1 activity is induced by a variety of environmental stress factors.\(^{26}\) These stimuli activate MAP kinase cascades that enhance AP-1 activity through converting phosphorylation of distinct substrates (fig. 1). The MAP kinases consist of three main protein kinase families: the extracellular signal-regulated kinases (ERKs), the c-Jun N-terminal kinases (JNKs), and the p38 family of kinases (fig. 1). Furthermore, the protein kinase C, cyclic-adenosine-monophosphate, and calcium pathways regulate c-fos expression, whereas protein kinase C and calcium are the main upstream modulators of c-jun, the most important transcriptional activator of the AP-1 menagerie of proteins.\(^{26}\) Protein kinase C is also an important initiator of the MAP kinase pathway.\(^{52}\) Previous studies indicated that volatile anesthetics activate protein kinase C and Ca\(^{2+}\)-calmodulin-dependent protein kinase II in various tissue preparations.\(^{53}\) In addition, it has been shown that isoflurane increased phosphorylated ERK1/2 in cultured smooth muscle cells.\(^{53}\) In this study, a dose-dependent increase in phosphorylation of ERK1/2 was initiated upstream by activation of Ca\(^{2+}\)-calmodulin-dependent protein kinase II and Ca\(^{2+}\)-independent protein kinase C. This raises the question whether volatile anesthetics may interfere with the signal transduction cascade leading to the activation of other MAP kinases. Indeed, ERK and JNK did not seem to be involved in the inhibition of AP-1 activity; sevoflurane exposure did not alter the phosphorylation of these MAP kinases. In contrast, appearance of immunoreactive phospho-p38 could be observed after sevoflurane exposure alone, even without stimulation by PMA. In addition, we observed an additive phosphorylation of p38 after sevoflurane incubation and subsequent stimulation with PMA and ionomycin. Enzymes in the p38 MAP kinase module are subject to phosphorylation and are generally activated by environmental stresses. The five p38 isoforms defined to date (p38\(\alpha\), p38\(\beta\), p38\(\gamma\), p38\(\delta\), and p38\(\varepsilon\)) vary in functional relevance based on their substrate specificity and in their affinity for the pyridylinhibitors SB203580 and SB202190. These drugs are nearly equipotent against p38\(\alpha\) and \(\beta\) but do not inhibit p38\(\gamma\) or p38\(\delta\).\(^{30,54}\) The latter p38 kinase isoforms have nearly 60% identity to p38\(\alpha\), but less is known about their physiologic function.\(^{29}\) Previous data indicate that the different p38 isoforms have opposite effects on AP-1-dependent transcription through differential regulation of c-Jun.\(^{55}\) Whereas the p38\(\beta\) isoform increases the activation of AP-1 transcriptional activity, p38\(\gamma\)/p38\(\delta\) inhibits it by regulating the c-jun transcription.\(^{55}\) In our study, we were not able to definitively identify which p38 isoform was induced by sevoflurane. However, selective blockade of p38\(\alpha\)/p38\(\beta\) by the inhibitors SB202190 and SB203580, which are inactive against p38\(\gamma\)/p38\(\delta\), increased the sevoflurane-mediated AP-1 inhibition. This was not reflected by the slight increase of pMA/ionomycin-induced p38 phosphorylation after sevoflurane exposure compared with stimulated T cells alone, which may have contributed to an increase in the phosphorylation of the p38\(\gamma\)/p38\(\delta\) isoforms. Therefore, our data would be consistent with the assumption that sevoflurane may interfere with the p38\(\delta\) isoform, which, in contrast to p38\(\gamma\), exists in T cells and is not inhibited by these compounds.\(^{56}\)

In conclusion, our data suggest that sevoflurane is a specific inhibitor of AP-1, which may exert its effect by interfering with the p38 MAP kinase cascade. Our data may provide a potential molecular mechanism for the immunomodulating effects associated with sevoflurane administration.

References


