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Delayed Inhibition of Agonist-Induced Granulocyte-Platelet Aggregation After Low-Dose Sevoflurane Inhalation in Humans

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BACKGROUND: Sevoflurane can be used as sedative-analgesic drug with endothelial protective properties. We tested whether low-dose sevoflurane inhalation provides sustained inhibition of detrimental granulocyte-platelet aggregation in humans.

METHODS: Ten healthy male volunteers were enrolled in this crossover study. Each subject inhaled sevoflurane for 1 h at 0.5–1 vol % end-tidal concentration in oxygen (50 vol %). Inhaling oxygen (50 vol %) alone served as control. Venous blood samples were collected at baseline before inhalation, immediately after inhalation, and 24 h thereafter, and were used for flow cytometry to determine platelet surface marker (CD41, CD42b, CD62P/P-selectin, and PAC-1) on platelets and granulocytes and for kaolin-induced clot formation, as assessed by thromboelastography. In flow cytometry experiments, platelets were stimulated with arachidonic acid (AA, 30 μM), adenosine diphosphate (ADP, 1 μM), and thrombin receptor agonist peptide-6 (TRAP-6, 6 μM).

RESULTS: AA, ADP, and TRAP-6 markedly increased the expression of CD62P on platelets, whereas CD42b (shedding) and PAC-1 (heterotypic conjugates) expression decreased. The amount of granulocyte-platelet aggregates increased upon agonist stimulation. Low-dose sevoflurane inhalation reduced ADP-induced CD62P expression on platelets 24 h after inhalation, and inhibited the formation of granulocyte-platelet aggregates under stimulation with AA and ADP after 1 and 24 h, and with TRAP-6 after 24 h compared with control. Inhibition of granulocyte-platelet aggregates was accompanied by reduced clot firmness 24 h after sevoflurane inhalation compared with control.

CONCLUSIONS: We demonstrated for the first time that inhaling low-dose sevoflurane (<1 vol % end-tidal) inhibits agonist-induced granulocyte-platelet interactions 24 h after administration and thus counteracts thromboinflammatory processes.

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An increasing body of evidence supports the potent anti-ischemic properties of halogenated ethers in patients undergoing coronary artery bypass graft surgery.[1–3] The protection is thought to be due to the preconditioning-mimicking effects by this class of drugs.[4,5] Our group previously demonstrated that inhaling low-dose sevoflurane protects the endothelium against reperfusion injury in a human forearm ischemia model.[3] We further showed in healthy volunteers that sevoflurane inhalation at subanesthetic concentrations modifies the human blood transcriptome and decreases the expression of proinflammatory L-selectin (CD62L) on granulocytes for up to 48 h,[6] consistent with the occurrence of a "second window of protection." Thus, sevoflurane, with its pleasant nonpungent odor, might serve to provide conscious sedation combined with organ protection in all clinical settings in which ischemia–reperfusion plays a role.

In contrast to these promising findings, which would suggest the use of low-dose sevoflurane inhalation as a valuable alternative to IV propofol sedation in cardiovascular at-risk patients, are reports on enhanced expression of platelet markers and increased formation of granulocyte-platelet aggregates by sevoflurane implying procoagulant effects.[7] Conversely, severe inhibition of platelet function[8,9] and prolonged bleeding times in patients undergoing sevoflurane anesthesia were also shown.[10] Another study found no inhibition of human

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platelet aggregation induced by thrombin.[11] In view of these conflicting findings obtained from studies with collected human blood exposed *in vitro* to the anesthetic gas, or from studies in patients undergoing surgery with sevoflurane anesthesia, we sought to determine the effects of sedative doses of sevoflurane (<1 vol % end-tidal) on platelet marker expression and function under well-controlled conditions using a crossover volunteer study.

Preconditioning was shown to enhance arterial patency in patients with preinfarction angina[4,12,13] and in sevoflurane-treated patients undergoing coronary artery bypass graft surgery.[14] In an *in vivo* dog model, intermittent coronary artery ligation before test ischemia was capable of enhancing arterial patency by selectively inhibiting procoagulant granulocyte-platelet aggregation.[15] Therefore, we hypothesized that inhaling sevoflurane would mimic ischemic preconditioning and attenuate platelet-mediated thrombosis by reducing the molecular indices of agonist-induced granulocyte-platelet aggregation. Because stimulation of several surface receptors is required for full platelet activation,[16] we studied inducible activation of several key platelet receptors using a variety of platelet agonists, including the P2Y12 receptor agonist adenosine diphosphate (ADP), the thromboxane A2 precursor arachidonic acid (AA) targeting the thromboxane A2 receptor, and the thrombin receptor agonist peptide-6 (TRAP-6) targeting the thrombin receptor. Finally, since sevoflurane continues to exert its actions long after its physical clearance from the human body,[6] and postoperative thromboembolic complications typically occur within the first 24 h after surgery,[17] we also evaluated the late effects of low-dose sevoflurane inhalation on platelets and heterotypic aggregation 24 h after administration.

METHODS

Subjects

The study was approved by the local ethics committee and all subjects gave written informed consent. The research was performed in accordance with the Declaration of Helsinki (2000). Clinicaltrials (www.clinicaltrials.gov) registration number is NCT00526695. Ten healthy male Caucasian volunteers participated in the study. All subjects were nonsmokers, and refrained from drinks and food containing caffeine and dark chocolate for 24 h. Participants fasted overnight and 20 mg of prophylactic esomeprazole was administered.

Study Protocols

In this study with crossover design, each participant underwent the same protocol with and without sevoflurane (Fig. 1A). Participants were randomly allocated to the protocols, which were at least 4 days apart (time between two inhalation periods). Experiments were performed in temperature-controlled (25°C) and quiet rooms. An IV line was placed into a large cubital or forearm vein, and 50 mL 0.9% NaCl solution was infused during inhalation. Prophylactic ondansetron against nausea and vomiting (0.5 mg) was given IV. In the protocol with sevoflurane inhalation, sevoflurane in 50 vol % oxygen was inhaled for 1 h by the spontaneously breathing volunteers using a tight facemask connected to the common gas outlet of an anesthesia machine (Siemens Servo 900D ventilator; Siemens Life Support Systems, Sona, Sweden) to achieve an end-tidal concentration of 1.0 vol %.[3,6] Control experiments consisted of 50 vol % oxygen inhalation for 1 h. Noninvasive arterial blood pressure, oxygen saturation, electrocardiogram, end-tidal carbon dioxide and sevoflurane concentrations (Draeger Infinity Delta XL, Draeger Medical Systems, Danvers, MA), and Bispectral Index (A2000 monitor ® with three adhesive electrodes to the forehead, single channel: Fp1-Fpz, version 3.3; Aspect Medical Systems, Inc., Norwood, MA) were recorded. Blood samples were taken from the IV line at indicated time points without the use of a tourniquet (Fig. 1A).

Determination of Platelet Surface Markers and Granulocyte-Platelet Aggregates Using Flow Cytometry

After collection, heparinized blood samples were immediately processed for flow cytometry. The expression of surface markers was determined at baseline, immediately after 1 h of sevoflurane or oxygen-control inhalation, and 24 h later in all 10 volunteers. Because stimulation of several surface receptors is required for full platelet activation,[16] we studied inducible activation of several key platelet receptors using a variety of platelet agonists. One milliliter aliquots of the collected whole blood were exposed to medium alone (unstimulated), AA (precursor of thromboxane A2 targeting the thromboxane A2 receptor, 30 μM, Sigma Chemicals, St. Louis, MO), ADP (targeting the P2Y12 receptor, 1 μM, Sigma Chemicals), or to TRAP-6 (targeting the thrombin receptor, 6 μM, Bachem, Heidelberg, Germany) *ex vivo* for 10 min. Preliminary experiments showed that these agonist concentrations elicited a strong and highly reproducible response of platelet markers, as previously reported.[9] Subsequently, 50 μL of stimulated blood was incubated in the dark with 3 μL of the primary fluorochrome-labeled antibody in endotoxin-free tubes for 10 min at room temperature. Erythrocytes were lysed by addition of lysis buffer (450 μL) (Becton Dickinson, San José, CA) for an additional 20 min at room temperature in the dark. The lysates were fixed for 30 min in 500 μL 0.2% paraformaldehyde solution at room temperature in the dark. The samples were centrifuged, and the cell pellets were resuspended in 0.5 mL of phosphate-buffered saline supplemented with 2% FCS (pH 7.2) and stored at 4°C in the dark until flow cytometric measurements. The FACS Calibur (Becton Dickinson) flow cytometer was used to measure R-phycoerythrin (PE)-fluorescence at 580 nm and FITC-fluorescence at 515 nm. Cells were distinguished from each other by typical physical characteristics, resulting in

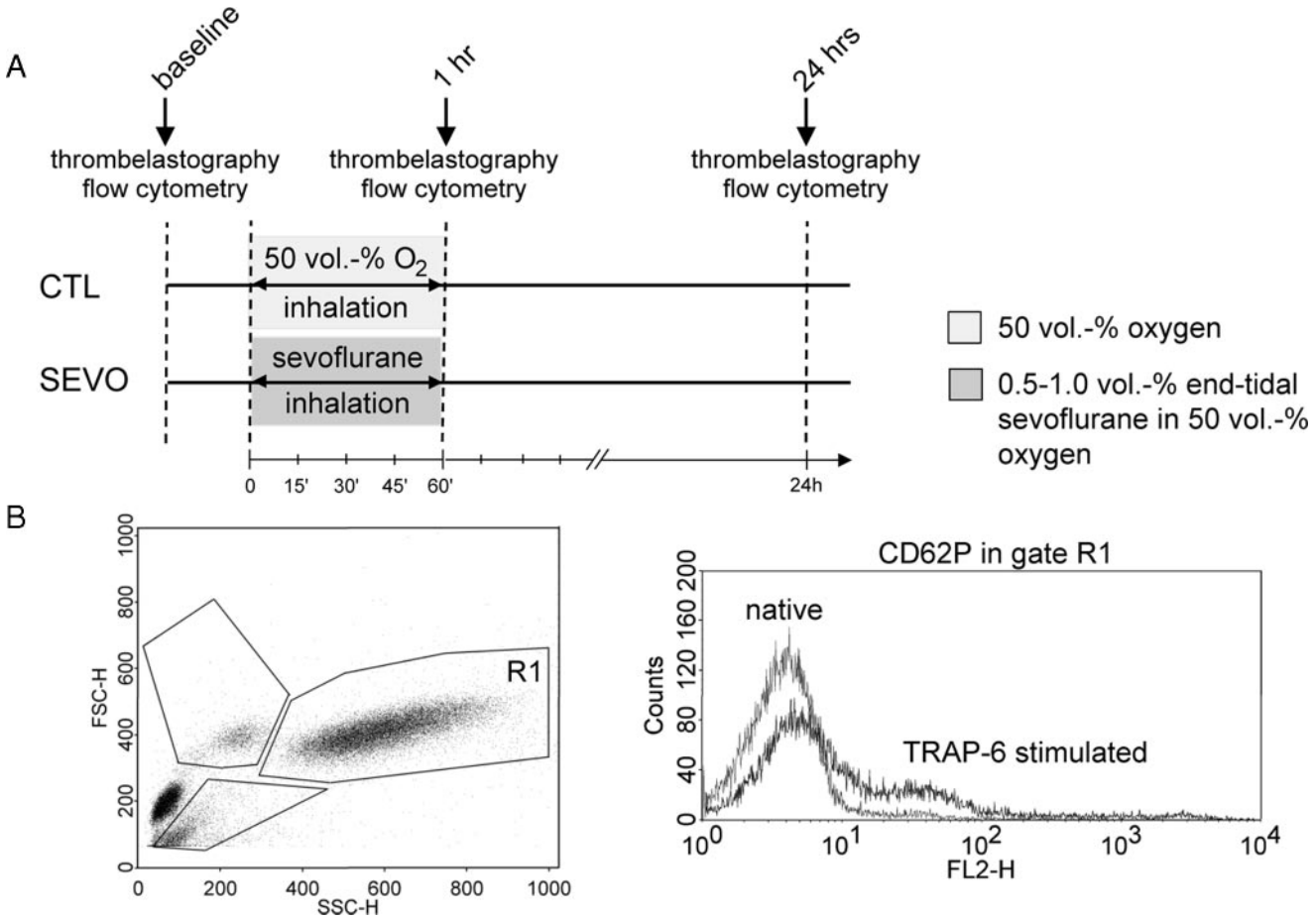

Figure 1. Panel A: Study protocols. Participants inhaled sevoflurane (SEVO) for 60 min at 0.5–1.0 vol % end-tidal concentration. Blood samples for flow cytometry and thromboelastography were collected before and immediately after sevoflurane inhalation, and 24 h thereafter. Inhalation of 50 vol % oxygen served as control (CTL). Panel B: Whole blood flow cytometric analysis of granulocyte-platelet aggregates in the absence (native) and presence of thrombin receptor agonist peptide-6 (TRAP-6) activation (6 μM). Granulocytes were identified by their characteristic light scatter properties (forward scatter FSC and side scatter SSC) and the binding of CD15-specific antibodies (not shown). Fluorescence intensity (FL2) of platelet-specific markers such as P-selectin (CD62P) measured in the granulocyte gate R1 was used as surrogate for granulocyte-platelet aggregate formation. Please note the shift in fluorescence intensity after TRAP-6 stimulation, indicating formation of granulocyte-platelet aggregates.

well-delineated cellular subpopulations that are easily identified on forward (cell size) and side-scatter (granularity) plots. Furthermore, granulocytes and platelets were identified by use of monoclonal antibodies against cell-specific antigens. To determine expression levels of surface markers on platelets and granulocyte-platelet aggregates, mean fluorescence intensities (MFI) of platelet markers were measured in the predefined gates containing the cellular subgroups of interest. The MFI of platelet markers in the granulocyte gate directly indicate the formation of granulocyte-platelet aggregates (Fig. 1B). The following surface markers were used: P-selectin (CD62P, PE-labeled, clone AK-4, Santa Cruz Biotechnology, CA), CD41a (GP IIb/IIIa, PE-labeled, clone HIP8, BD Biosciences, San José, CA), CD41b (GP αIIb of IIb/IIIa, PE-labeled, Santa Cruz Biotechnology, CA), CD42b (GPIb, clone AN51, FITC-labeled, DAKO, Glostrup Denmark), PAC-1 (epitope of the activated CD41a, FITC-labeled, Becton-Dickinson), and the marker for polymorphonuclear granulocytes CD15 (PE-labeled, clone 80H5, Immunotech, Marseille, France). The fluorescence signals from unstained samples and samples treated with isotype-matched antibodies (PE-labeled IgG, eBioscience, Wembley, UK, and FITC-labeled IgG, Becton Dickinson) were used for correction and subtracted from the signals obtained in samples exposed to true antibodies. A minimum of 20,000 events was counted on each sample, and recorded data were analyzed using CellQuest Software (Becton Dickinson). All data are shown as MFI or percent change of MFI from the respective baseline values.

Thromboelastography

Thromboelastographic (TEG®) measurements were also determined at baseline, 1 h after sevoflurane inhalation, and 24 h later in control and sevoflurane-treated blood samples. Quality control procedures were performed before each measurement, and procedures for TEG analysis were done according to the

Table 1. Volunteer Characteristics

Age (yr)	BMI (kg/m^2)	Hemoglobin (g/dL)	Leukocytes ($10^3/\mu L$)	Platelets ($10^3/\mu L$)	Fibrinogen (g/L)
32.3 ± 6.2	23.6 ± 1.5	14.6 ± 0.9	5.0 ± 1.3	223 ± 32	2.5 ± 0.4

Data are mean ± SD.

BMI = body mass index (kg/m^2).

manufacturer's descriptions (TEG Hemostasis Analyzer, Hemoscope Corporation, Niles, IL). Three milliliters of blood was gently collected through the IV cannula into a 5-mL syringe and discarded. Subsequently, 9 mL of blood was drawn into a 10-mL syringe from which 5 mL was immediately transferred to a citrate-(3.8%) containing tube. The tube was gently mixed, and TEG analysis was performed 1 h later.[18] One milliliter of citrated blood was transferred into a kaolin vial and mixed. Subsequently, 340 μL was transferred into prewarmed cups containing 20 μL of 0.2 M $CaCl_2$ and carefully mixed. From the recorded signals, four parameters of clot formation were measured including the reaction time (R-time corresponds to the distance measured from the beginning of the trace to the point where 2 mm of divergence is detected), K-time (time from start of tracing to 20 mm divergence), MA (maximum amplitude indicates maximal divergence of the tracing lines and clot firmness the components of which are fibrin and platelet interactions), and α angle (measured from the tracing slope over the time indicating rate of clot formation) were determined. *G*-modulus, which indicates normalized shear elastic modulus, was calculated as follows $G = 100 \times MA/(100 - MA)$. Please note that *G*-modulus increases exponentially in proportion to MA, and thus is more indicative of small changes in clot strength.

Statistical Analysis

Data are given as mean ± SD. The Kolmogorov–Smirnov test was used to test for normality of the underlying data distribution. Paired *t*-tests or Wilcoxon's signed rank test were used for comparisons between sevoflurane and control samples depending on the underlying distribution. $P < 0.05$ was considered significant. Analyses were performed using SigmaStat Version 2 (SPSS Science, Chicago, IL).

RESULTS

Table 1 shows the measured blood cell counts and fibrinogen levels, which were within normal limits in the 10 healthy volunteers. All subjects could be aroused on tactile stimulation, and tolerated the procedures well, except for one who experienced transient nausea. None of them experienced an excitement phase during sedation (Table 2).

Expression of Surface Markers on Platelets After Stimulation with AA, ADP, and TRAP-6

We first determined the effects of the three different platelet activators AA, ADP, and TRAP-6 on platelet surface markers including P-selectin (CD62P), GP IIb/IIIa (CD41a and GD41b representing different epitopes of the same protein), GPIb (CD42b), and PAC-1 (activated CD41) in blood samples of the untreated volunteers. Although CD41a exhibited increased expression, as measured by the MFI, upon AA and TRAP-6 but not ADP stimulation, CD41b, a different CD41 epitope, only showed increased expression after strong TRAP-6 stimulation (Supplementary Figures S1A–B, available at www.anesthesia-analgesia.org). In contrast, CD42b expression was reduced after stimulation, most probably due to shedding of this surface marker (Supplementary Figure S1C, available at www.anesthesia-analgesia.org), as previously reported.[19] P-selectin (CD62P) exhibited a graded increase in expression in response to AA, ADP, and TRAP-6 activation (Supplementary Figure S1D, available at www.anesthesia-analgesia.org), whereas PAC-1 was reduced after strong stimulation with ADP and TRAP-6, probably due to formation of platelet-derived microparticles or adhesion to granulocytes (see next paragraph), but unaffected after AA stimulation (Supplementary Figure S1E, available at www.anesthesia-analgesia.org).

Table 2. Recorded Vital Signs and BIS Values

	Baseline	30 min after initiation of inhalation
CTL		
SBP (mm Hg)	117 ± 8	112 ± 8
HR (bpm)	62 ± 9	57 ± 10
RR (min^{-1})	13 ± 2	14 ± 2
Spo_2 (%)	97 ± 2	99 ± 1
BIS	95 ± 5	93 ± 4
SEVO		
SBP (mm Hg)	117 ± 6	115 ± 8
HR (bpm)	67 ± 7	61 ± 7
RR (min^{-1})	14 ± 2	15 ± 3
Spo_2 (%)	98 ± 1	98 ± 1
ET(SEVO) (vol %)	—	0.8 ± 0.2
BIS	96 ± 2	72 ± 7*

Data are mean ± SD.

CTL = inhalation of 50 vol % oxygen; SEVO = sevoflurane 0.5–1 vol % in 50 vol % oxygen; ET(SEVO) = end-tidal sevoflurane concentration; SBP = systolic blood pressure; HR = heart rate (in beats per minute or bpm); RR = respiratory rate (breaths per minute), Spo_2 = oxygen saturation (finger tip); BIS = bispectral index.

* $P < 0.05$ vs baseline.

Formation of Granulocyte-Platelet Aggregates After Stimulation with AA, ADP, and TRAP-6

To determine the formation of heterotypic granulocyte-platelet aggregates, we measured the MFI of the various platelet markers in the predefined granulocytes (CD15-positive cells) containing gate (Fig. 1B). There was a significant increase in

CD41a and CD42b expression upon AA, ADP, and TRAP-6 stimulation on the granulocyte surface, whereas P-selectin (CD62P) and PAC-1 were only increased after strong stimulation with ADP and TRAP-6 (Supplementary Figure S2A–E, available at www.anesthesia-analgesia.org). Of note, although CD42b expression on platelets decreased after stimulation (shedding), a significant increase in CD42b expression on granulocytes was observed indicating a marked formation of granulocyte-platelet aggregates. Together, these experiments indicate significant granulocyte-platelet aggregation after stimulation in the blood of the untreated study subjects.

Delayed Beneficial Effects of Low-Dose Sevoflurane Inhalation on ADP-Induced P-Selectin (CD62P) Expression and Granulocyte-Platelet Aggregation

To reduce interindividual variability and to minimize possible confounding variables, the effects of sevoflurane on platelet marker expression and granulocyte-platelet aggregate formation were determined in a study with healthy volunteers using a crossover design. The effects of sevoflurane were evaluated at two time points: immediately after 1 h of low-dose sevoflurane inhalation and 24 h later. Differences in control and sevoflurane protocols were expressed as percent changes in MFI for the various markers from baseline. Although all platelet markers were unaffected after 1 h of inhalation (Figs. 2A–H), ADP-induced P-selectin expression on platelets was significantly reduced 24 h later ($P = 0.02$). Significant reduction in granulocyte-platelet aggregation was detected after sevoflurane treatment, occurring upon AA and ADP stimulation immediately after inhalation (Figs. 3A–D). This effect was even more pronounced 24 h later and also observed in native unstimulated as well as TRAP-6-activated blood samples (Figs. 3E–H). Together, these experiments show that sedative doses of sevoflurane administered for only 1 h effectively inhibit agonist-induced granulocyte-platelet aggregation 24 h after administration.

Low-Dose Sevoflurane Inhalation Maintains Normal Coagulation but Counteracts Procoagulant Actions 24 Hours Later

To test whether sevoflurane-induced changes in platelet surface markers would further affect clot formation, we used TEG. Measurements were performed at baseline, after 1 h of inhalation, and 24 h later. No changes in R-time, K-time, and α angle were observed in blood samples of sevoflurane-treated volunteers immediately after treatment compared to untreated controls (Fig. 4). In contrast, MA and even more *G*-modulus were increased in the control Group 24 h later, which was reversed by administration of sevoflurane. These results provide evidence that low-dose sevoflurane inhalation maintains normal hemostasis, but exerts late anticoagulant activity by counteracting hypercoagulation.

DISCUSSION

Using the well-controlled conditions of a crossover volunteer study, we were able to show that brief low-dose sevoflurane inhalation decreased ADP-induced P-selectin (CD62P) expression on platelets, effectively inhibited agonist-induced granulocyte-platelet aggregation, and further attenuated thrombus formation, as assessed by TEG 24 h after administration. Our findings are important because of the following reasons. First, previous human *in vivo* studies evaluating sevoflurane effects on platelet function and aggregation did not investigate the effects at lower sedative doses.[8,20] Second, no studies determined the late effects of sevoflurane on platelet function and coagulation, i.e., 1 day after the clearance of sevoflurane from the human body. A single study reported some residual effects of sevoflurane on platelets 1 hour after inhalation.[20] We now provide evidence that sevoflurane blunts the responsiveness of platelets, and exerts a strong delayed effect on granulocyte-platelet aggregation after stimulation with multiple physiological activators, which is consistent with the concept of an "anticoagulant preconditioning." Analogous to anesthetic preconditioning of the heart, in which the myocardium is rendered more resistant to potentially lethal ischemia,[5,21] granulocyte-platelet interaction was attenuated in response to thrombotic stimulation after sevoflurane pretreatment, representing different aspects of the same biological process termed "preconditioning." Since leukocyte-platelet interactions are of particular relevance in the pathogenesis of inflammation and thrombosis,[16] the observed delayed actions on platelet aggregation at subanesthetic concentrations may be beneficial in many clinical settings such as the postoperative period in which a hypercoagulable state is occurring.[17]

Using flow cytometry for the determination of the key platelet surface markers CD41a, its activated form PAC-1, CD42b, and P-selectin (CD62P) in the presence and absence of AA, ADP, and TRAP-6 activation, we were able to track the effects of sevoflurane on platelet markers and heterotypic aggregation.[22] The combined use of TEG, further allowed us to test whether the observed changes in markers affected clot formation. TEG, which is sensitive to all of the interacting cellular and plasma components, monitors shear elasticity of a whole blood sample, and was successfully used in the assessment of postoperative hypercoagulation.[23]

Platelet activation initiates arterial thrombosis, contributes to inflammation, and participates in host defense, cancer cell metastasis, and wound healing.[24] In our study, we were able to confirm previously described CD42b shedding upon platelet activation.[19] Reduced PAC-1 expression in the platelet gate after stimulation was also detected and may have been due to adherence of activated platelets to granulocytes, as

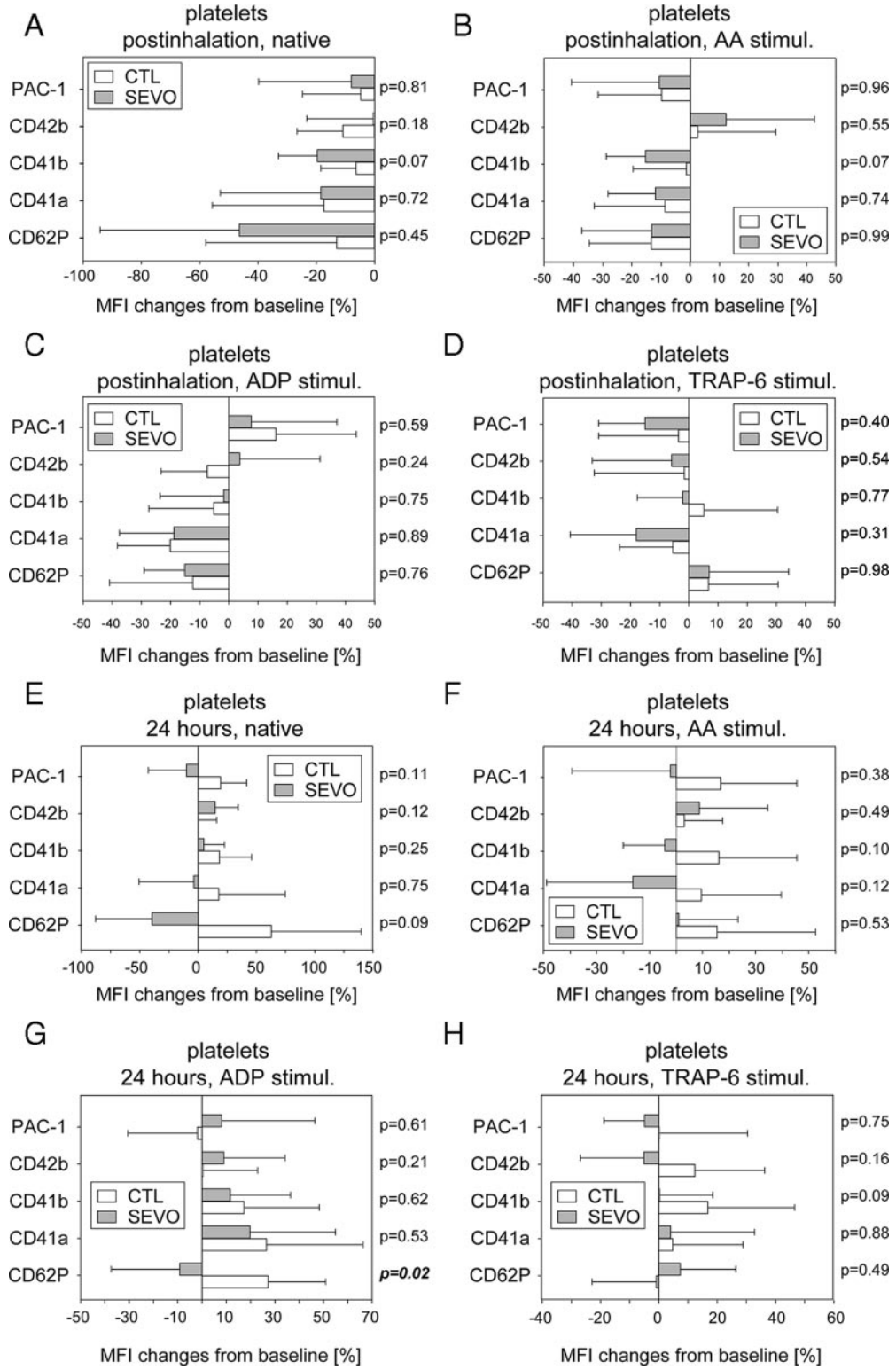

Figure 2. Platelet surface markers after sevoflurane administration. Differences in markers (CD41a, 41b, CD42b, CD62P, and PAC-1) between sevoflurane (SEVO) versus control treatment (CTL) are expressed in percent change in mean fluorescence intensity (MFI) from baseline. Panel A–D: after 1 h of sevoflurane inhalation (postinhalation). Panel E–H: 24 h after sevoflurane inhalation. *P* values are given on the right side of each graph and are bold if statistically significant ($P < 0.05$). Data are mean ± SD.

evidenced by the increased PAC-1 signal in the granulocyte gate. Alternatively, this might have been due to agonist-induced formation of platelet-derived microparticles.[25] Aggregation of platelets to leukocytes is predominantly P-selectin-dependent and mediated by binding to the counterligand PSGL-1 (CD162) on leukocytes.[26,27] Binding of CD11a/18 to ICAM-2 and CD11b/18 to CD42b (via fibrin) further enhance this interaction, but play a less important role. Platelets bound to leukocytes markedly enhance adhesion to

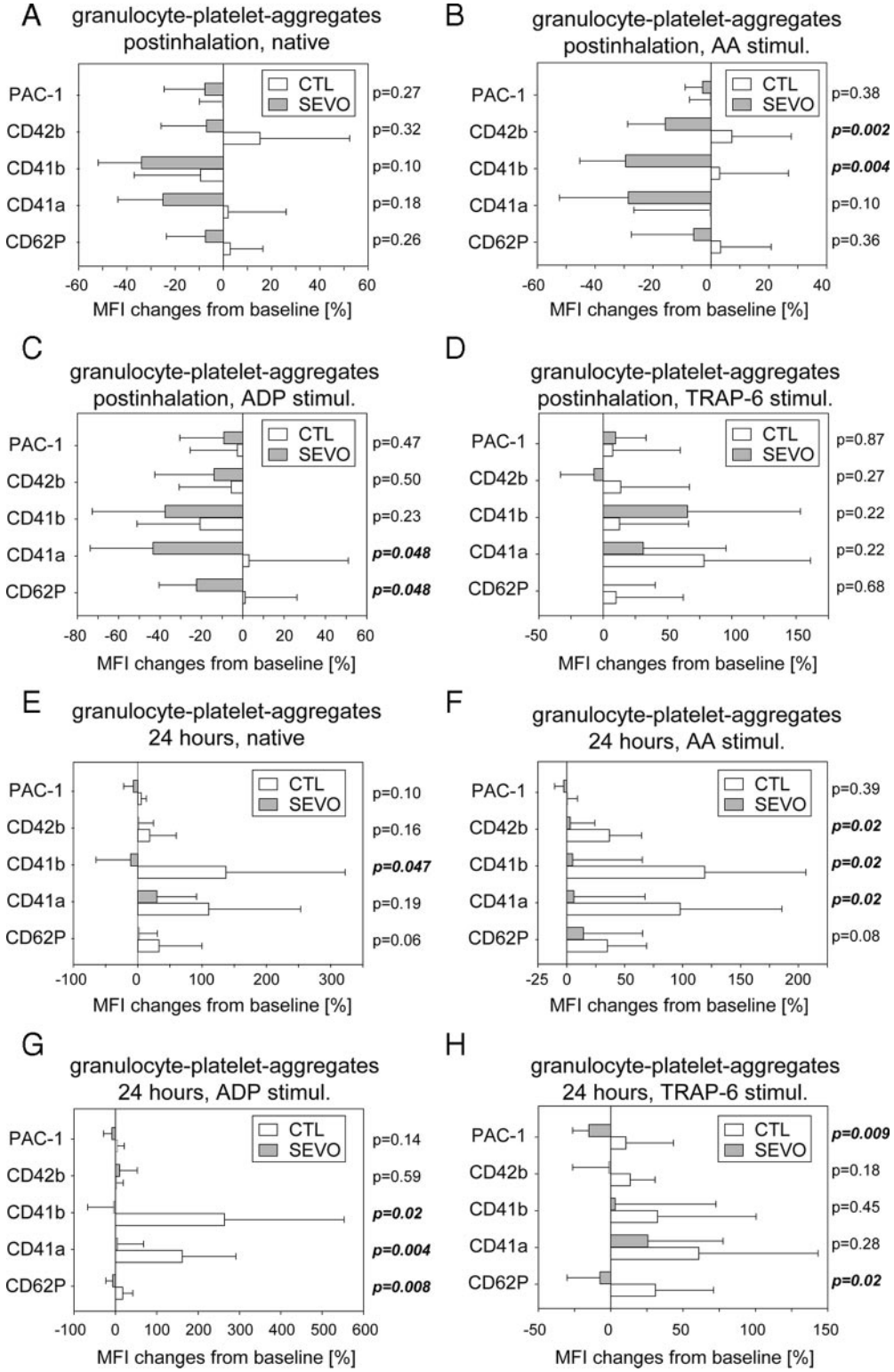

Figure 3. Granulocyte-platelet aggregation after sevoflurane administration. Please note that fluorescence intensities of platelet-specific markers were determined in the granulocyte gate (see Fig. 1B). Differences in platelet markers (CD41a, 41b, CD42b, CD62P, and PAC-1) between sevoflurane (SEVO) versus control treatment (CTL) are expressed in percent change in mean fluorescence intensity (MFI) from baseline. Panel A–D: after 1 h of sevoflurane inhalation (postinhalation). Panel E–H: 24 h after sevoflurane inhalation. *P* values are given on the right side of each graph and are bold if statistically significant ($P < 0.05$). Data are mean ± SD.

the endothelium by "secondary capture." Increased formation of leukocyte-platelet aggregates was reported in patients with stable and unstable angina, acute myocardial infarction, and in patients undergoing coronary artery interventions.[28–30] In myocardial infarction, leukocyte-platelet interaction at the site of

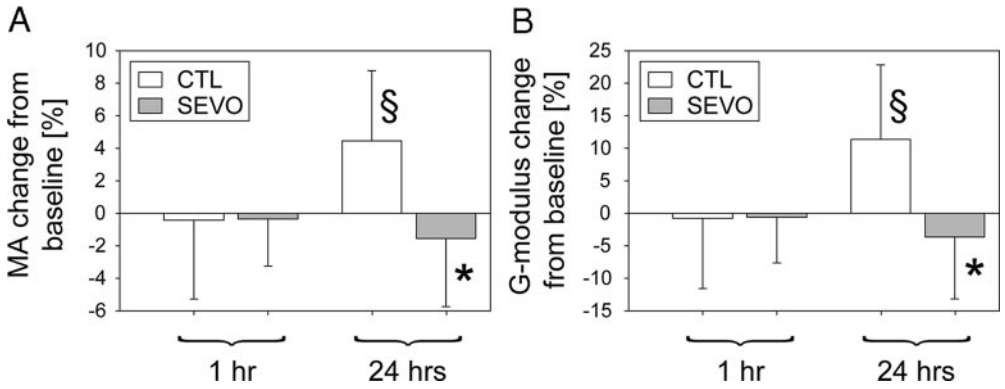

Figure 4. Clot strength, as determined by thromobelastography. Differences in maximum amplitude (MA, panel A) and *G*-modulus (panel B) between sevoflurane (SEVO) versus control treatment (CTL) are expressed in percent change from baseline. *$P < 0.05$ vs. CTL. §$P < 0.05$ vs respective baseline. Data are mean ± SD.

plaque rupture may represent an important mechanism for inadequate reperfusion ("no-reflow" phenomenon).[31] In the perioperative setting, vascular surgical patients with subsequent graft occlusion demonstrated greater postoperative increases in circulating granulocyte-platelet aggregates compared with patients with patent grafts.[32] Interestingly, this study also suggests that heterotypic aggregation may be a more sensitive marker of platelet activation than platelet surface markers themselves. Importantly, heterotypic aggregates do not only reflect, but also effect, cardiovascular disease.[33] Increased production of reactive oxygen species by platelet-activated leukocytes may destabilize atherosclerotic plaques and modify cardiovascular outcome.[34,35] In a pig model, administration of blocking recombinant PSGL-1-immunoglobulin, the P-selectin counterligand, was shown to decrease neointima formation after balloon injury and restenosis within the stent.[36] Hence, sustained reduction in heterotypic aggregation after low-dose sevoflurane inhalation, as observed in our study, suggests strong antithromboinflammatory actions.

Previous reports of sevoflurane's effects on platelet function have been highly conflicting. Obviously, *in vitro* conditions differ from *in vivo* conditions with intact endothelium, present plasma proteins, no artificial cellular activation, and the intact rheologic environment. *Ex vivo in vitro* studies found that even low sevoflurane doses (≤0.5 MAC) transformed platelets into more adhesive phenotypes.[9,7] Enhanced formation of leukocyte-platelet aggregates and increased expression of P-selectin,[7] especially in stimulated blood samples, combined with seemingly paradoxical impairment of clot formation,[9] were also reported. *In vivo*, however, Huang et al.[37] reported reduced P-selectin expression on platelets and reduced leukocyte-platelet aggregation in unstimulated and ADP-stimulated blood samples of sevoflurane-anesthetized patients (approximately 4 vol %). Sevoflurane also prolonged bleeding times in patients,[10] but this is not considered clinically relevant.[38] In our protocols with low-dose sevoflurane inhalation, we only detected a slight delayed reduction in clot strength compared with baseline. However, clot strength was increased 24 h after oxygen inhalation in control protocols, which was completely offset by sevoflurane administration. The reason for this hypercoagulable state is not clear, but may have been related to the reported procoagulant effects of oxygen on platelets.[39,40] Irrespectively, sevoflurane completely abolished this untoward effect and reestablished normal coagulation. Since maximal clot strength was reported to correlate with postoperative thrombotic complications, this finding may be of clinical significance.[23] Together, our results confirm previous findings but further extend these by showing strong late inhibitory effects of low-dose sevoflurane on heterotypic aggregation and thrombus formation.

What are the possible mechanisms underlying the inhibition of granulocyte-platelet aggregation by sevoflurane? Based on our results, the following explanations can be put forward. Suppression of thromboxane A_2 formation[41] and inhibition of calcium mobilization in platelets[42] were previously shown, and may be involved primarily in the immediate inhibitory effects of sevoflurane on heterotypic aggregation. However, delayed effects on aggregates may rather depend on transcriptional changes and changes in protein expression,[6] consistent with the concept of late preconditioning. Platelets, although anucleate cells, do contain functional mRNA,[43] and upon activation or inhibition, were shown to change their protein expression profile.[44] Thus, it may be speculated that an increase in adenosine elicited by anesthetic gases[21] might block the P2Y12 receptor for ADP on platelets and subsequently downregulate P-selectin expression ("clopidogrel-like" effect).[45] Indeed, a reduction in ADP-induced P-selectin expression by 40% as observed in our study (sevoflurane vs control) is equivalent to the effects on P-selectin after 7 days of clopidogrel therapy (300 mg loading and 75 mg daily dose). Additional mediators, such as nitric oxide and prostaglandins, might have further modulated platelet function. Another possibility would be that sevoflurane affects activation and adhesion of leukocytes rather than platelets. In support

of this view, we recently demonstrated that sevoflurane at subanesthetic concentrations induces transcriptional changes and downregulates L-selectin (CD62L) on granulocytes in humans 24–48 h later.[6] Sedative doses of sevoflurane further decreased CD11b expression after ischemia–reperfusion injury in the human forearm.[3] Hence, it can be postulated that counterligands for platelet markers on granulocytes such as PSGL-1 or ICAM-2 may be also decreased after sevoflurane administration, a hypothesis which needs to be confirmed in future studies.

STUDY LIMITATIONS AND SPECIFIC REMARKS

Our findings obtained in this well-controlled study with healthy volunteers should be confirmed in the clinical setting with patients suffering from multiple comorbidities. In the current study, platelet-specific markers measured in the granulocyte gate were used for identifying granulocyte-platelet aggregates. Although this is a well established method, which was previously used,[46] this approach does not allow a one-by-one identification of granulocyte-platelet aggregates, as would be possible in multicolor flow cytometry, and thus measured MFI must rather be considered surrogate markers for granulocyte-platelet aggregate formation. Stimulation with AA, ADP, and TRAP-6, as used in our study, only mimics part of the changes present in endothelial injury. Future studies should help to delineate all mechanisms underlying inhibition of granulocyte-platelet aggregation by sevoflurane and further determine whether higher doses of sevoflurane would affect platelet function and coagulation more profoundly. We used esomeprazole to protect the participants from the potential hazards of pulmonary aspiration, and ondansetron was used to prevent nausea and vomiting. Although no effects of these drugs on granulocyte-platelet aggregation or clot formation were previously reported, we cannot completely exclude any effects on blood cells. Finally, TEG with citrated blood, as opposed to native blood, was shown to have a trend toward hypercoagulation, but these measurements can be still used as reliable surrogates.[18]

In conclusion, in this human crossover study, we demonstrate that inhaling sevoflurane at low subanesthetic doses inhibits agonist-induced detrimental granulocyte-platelet aggregation 24 h after administration and attenuates thrombus formation. Since postoperative thromboembolic complications typically occur within the first 24 h after surgery, our findings are of clinical significance. Our results further suggest that conscious sedation with sevoflurane could benefit patients at high thrombotic risk undergoing interventional procedures in cardiology or endovascular procedures.

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