Adenosine Receptor Adora2b Plays a Mechanistic Role in the Protective Effect of the Volatile Anesthetic Sevoflurane during Liver Ischemia/Reperfusion

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ABSTRACT

Background: Liver ischemia/reperfusion (IR) injury is characterized by hepatic tissue damage and an inflammatory response. This is accompanied by the formation and vascular sequestration of platelet–neutrophil conjugates (PNCs). Signaling through Adora2b adenosine receptors can provide liver protection. Volatile anesthetics may interact with adenosine receptors. This study investigates potential antiinflammatory effects of the volatile anesthetic sevoflurane during liver IR.

Methods: Experiments were performed *ex vivo* with human blood and in a liver IR model with wild-type, Adora2a$^{-/-}$, and Adora2b$^{-/-}$ mice. The effect of sevoflurane on platelet activation, PNC formation and sequestration, cytokine release, and liver damage (alanine aminotransferase release) was analyzed using flow cytometry, luminometry, and immunofluorescence. Adenosine receptor expression in liver tissue was analyzed using immunohistochemistry and real-time polymerase chain reaction.

Results: *Ex vivo* experiments indicate that sevoflurane inhibits platelet and leukocyte activation (n = 5). During liver IR, sevoflurane (2 Vol%) decreased PNC formation 2.4-fold in wild-type ($P < 0.05$) but not in Adora2b$^{-/-}$ mice (n ≥ 5). Sevoflurane reduced PNC sequestration 1.9-fold ($P < 0.05$) and alanine aminotransferase release 3.5-fold ($P < 0.05$) in wild-type but not in Adora2b$^{-/-}$ mice (n = 5). In Adora2a$^{-/-}$ mice, sevoflurane also inhibited PNC formation and cytokine release. Sevoflurane diminished cytokine release (n ≥ 3) and increased Adora2b transcription and expression in liver tissue of wild-types (n = 4).

Conclusions: Our experiments highlight antiinflammatory and tissue-protective properties of sevoflurane during liver IR and reveal a mechanistic role of Adora2b in sevoflurane-associated effects. The targeted use of sevoflurane not only as an anesthetic but also to prevent IR damage is a promising approach in the treatment of critically ill patients. **(Anesthesiology 2016; 125:547-60)**

HEPATIC ischemia/reperfusion (IR) injury results from a prolonged ischemic insult followed by restoration of blood perfusion. During surgical procedures, liver IR injury is usually caused by prolonged portal triad clamping followed by reperfusion and is also associated with liver transplantation. Liver IR injury causes hepatic tissue damage and induces a systemic inflammatory response with potentially detrimental consequences for the patient.[1–4] During systemic inflammation, specific stimuli such as proinflammatory mediators induce the activation of platelets and leukocytes. In consequence, platelets act as proinflammatory effector cells, interact with granulocytes, and become bound in platelet–neutrophil conjugates (PNCs). On the receptor level, the platelet surface molecules P-selectin and glycoprotein IIb/IIIa and the leukocyte protein Mac-1 contribute to PNC formation. PNCs play a crucial role in the emergence and maintenance of systemic inflammation during

What We Already Know about This Topic

- Proinflammatory platelet–neutrophil conjugates formed after ischemia/reperfusion contribute to systemic inflammation and organ damage
- Adenosine can inhibit platelet and leukocyte activations and reduce ischemia/reperfusion injury of hepatocytes mediated by adenosine 2B receptors

What This Article Tells Us That Is New

- Sevoflurane inhibited platelet activation and platelet–neutrophil conjugate formation in human blood *ex vivo* and reduced systemic inflammation, platelet–neutrophil conjugate formation, and organ damage in a mouse model of liver ischemia/reperfusion injury
- The organ-protective and antiinflammatory effects of sevoflurane are reduced in genetically modified mice not expressing adenosine 2B receptors, indicating a critical role for this signaling pathway

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IR and can cause significant organ damage when they are sequestered into the microvasculature. Consequently, PNC formation is associated with devastating complications in the clinical setting, including the failure of vital organs.[5–13] Therefore, the successful treatment of systemic inflammation remains an ongoing challenge for clinicians. To this day, however, a specific therapy to treat IR-related platelet and leukocyte activation, proinflammatory mediator release, and inflammatory tissue damage is unavailable. A therapeutic approach that prevents the development of PNC formation would have very beneficial effects on many disease courses associated with systemic inflammation including IR.

Adenosine is a highly potent inhibitor of platelet and leukocyte activation, thereby exerting antithrombotic and antiinflammatory effects.[14,15] Of note, it has been reported previously that signaling through hepatocellular Adora2b adenosine receptors dampens IR injury of the liver.[16] In this regard, volatile anesthetics have been reported to interact with receptors of the antiinflammatory extracellular signaling molecule adenosine[17] and to exert antiinflammatory effects.[17–23] Therefore, the application of anesthetics with antiinflammatory properties may represent one possible therapeutic strategy to inhibit systemic inflammation.

The aim of our current study was to establish a therapeutic approach for the inhibition of PNC formation, the release of inflammatory mediators, and the hepatic tissue damage during liver IR. The underlying hypothesis of our analyses was that the volatile anesthetic sevoflurane may inhibit inflammatory events during liver IR *via* interaction with adenosine receptors. To investigate the translational potential of our therapeutic approach for the clinical setting, we evaluated the specific antiinflammatory effects of the volatile anesthetic sevoflurane in *ex vivo* experiments with human whole blood and also during liver IR in mice. Our experiments indicate that sevoflurane exerts significant antiinflammatory action and decreases IR-related organ damage. Furthermore, we show that sevoflurane augments Adora2b transcription and expression during liver IR and that sevoflurane's protective action is partially mediated *via* Adora2b. On the basis of these results, we propose a new sevoflurane-based antiinflammatory strategy for the inhibition of IR-associated complications.

Materials and Methods

Blood Sampling in Human Subjects

For *in vitro* experiments, human whole blood was collected by venipuncture with a 21-gauge butterfly needle from an antecubital vein of nonmedicated healthy subjects after signed informed consent. The adult human subjects volunteered for blood sampling and were recruited from the staff of the research laboratory of the Department of Anesthesiology and Intensive Care Medicine (Tübingen University Hospital, Tübingen, Germany). The following exclusion criteria for study participation and blood donation were applied: smokers, drug and medication intake (including medication that might affect the hemostatic systems and platelets) within 14 days before blood donation, and infectious diseases including viral hepatitis and human immunodeficiency virus. The human subjects consented that data obtained during the course of the study will be stored anonymously for 10 yr on password-secured computers of the research laboratory of the Department of Anesthesiology and Intensive Care Medicine (Tübingen University Hospital). Blood sampling procedures were approved by the ethics committee of the University of Tübingen, Tübingen, Germany. Blood samples were anticoagulated with citrate in commercially available sampling tubes (S-monovette; Sarstedt, Germany).

Mice

Mice deficient in Adora2a and Adora2b were generated, validated, and characterized as previously described.[24,25] Adora2a$^{-/-}$ were on a CD1 and Adora2b$^{-/-}$ on a C57BL/6 background. C57BL/6 and CD1 wild-type (WT) mice, obtained from Charles River Laboratories (Sulzfeld, Germany), served as respective controls. All mice were anesthetized with pentobarbital (80 mg/kg) as background anesthesia for performing surgical procedures. Sevoflurane (Baxter, USA) was administered in the clinically relevant concentration of 2 Vol% to spontaneously breathing mice using an inhalator device for rodents. Mice were randomly allocated to the experimental condition. Investigators were not blinded with regard to the experimental condition. We aimed to achieve equal anesthetic depths in all animals. The depth of anesthesia was monitored continuously by experienced personnel. Additional pentobarbital was administered when the level of anesthesia was not judged satisfactory according to pedal and eye blink reflexes. All protocols for animal experiments, as well as care and handling of the animals, were in accordance with the German guidelines for research on living animals (available on the webpage: https://www.gesetze-im-internet.de/tierschversv/BJNR312600013.html) and were approved by the Institutional Animal Care and Use Committee of the Tübingen University Hospital and the Regierungspräsidium Tübingen, Germany (regional council).

Administration of Liquid Sevoflurane to Whole Blood

In our *ex vivo* experiments, liquid sevoflurane was added to whole blood in 1.5-ml sampling tubes (Eppendorf, Germany). Because liquid sevoflurane does not dissolve well in blood, we evaluated different solvents including buffer and fat solutions to dissolve sevoflurane in preliminary experiments. However, a satisfactory solution of sevoflurane could not be achieved in any of the solvents. The solvent dimethyl sulfoxide was not used because it inhibits platelet aggregation,[26] which may disturb results. Therefore, we chose to add pure liquid sevoflurane to human whole blood using a gastight syringe (Gilson, USA). The lids of the sampling tubes were closed after sevoflurane addition. Using this approach, sevoflurane diffuses into the blood inside the sampling tube. To optimize the contact of the diffusing sevoflurane with blood components, the sampling tubes were carefully rotated.

In Vitro *Investigations Regarding the Effect of Sevoflurane on the Activation and Interaction of Platelets and Leukocytes*

The effect of sevoflurane on the activation and interaction of platelets and leukocytes was evaluated by flow cytometry, by measurement of adenosine triphosphate (ATP) levels, and by immunofluorescence under defined *in vitro* conditions. In these experiments, 100 μl of citrated human or murine whole blood was incubated with liquid sevoflurane (5% v/v) at 37°C. Incubation was performed in 1.5-ml test tubes (Eppendorf) with a lid to minimize sevoflurane evaporation.

Murine Liver Ischemia and Reperfusion

The murine liver IR experiments were performed with anesthetized mice as described previously.[27] Briefly, we used a hanging-weight system that induces ischemia in the lobus sinister and medianus of the liver. An ischemia time of 30 min was established followed by a reperfusion time of 3 h. Blood samples were taken from the right atrium and immediately anticoagulated with citrate 5 min or 3 h after start of reperfusion.

Incubation Protocols for Flow Cytometry

Human Whole Blood Samples: Detection of the Activation and the Interaction of Platelets and Leukocytes. Whole blood was incubated with sevoflurane, adenosine diphosphate (ADP), and prostaglandin E_1 (PGE_1) as indicated. Incubation steps were performed according to a previously described method.[28] Briefly, citrated whole blood samples were incubated with an antibody cocktail and ADP solution (Moelab, Germany) in a final concentration (fc) of 20 μM for 30 min at 37°C. The antibody cocktail consisted of the following: 2.5 μl fluorescein isothiocyanate (FITC)–labeled anti-CD41 (Beckman Coulter, USA) and 1.5 μl peridinin chlorophyll protein complex (PerCP)–labeled anti-CD45 (Beckman Coulter); 1.5 μl Brilliant Violet (BV)421–labeled anti-CD11b (Biolegend, USA) and 1.5 μl allophycocyanin conjugate (APC)–labeled anti-CD14 (Biolegend); 1.5 μl phycoerythrine-labeled anti-CD62P (Becton Dickinson [BD], Germany) and 2.5 μl phycoerythrine-Cy7–labeled anti-CD66b (eBioscience, Germany). After 30-min incubation at 37°C, samples were diluted in FACS tubes preloaded with 500 μl 1× Cell Fix (BD). Flow cytometry analyses were performed within 4 h after sample fixation.

For detecting PNCs, we used the following incubation protocol. Either 1 μM PGE_1 (Sigma, Germany) or sevoflurane (Baxter 5%; v/v) was added to 100 μl citrated whole blood and incubated for 10 min. Then ADP (fc: 2 μM) was added. Next, 2.5 μl FITC-labeled anti-CD41 (Beckman Coulter), 1.5 μl PerCP-labeled anti-CD45 (Beckman Coulter), and 2.5 μl phycoerythrine-Cy7–labeled anti-CD66b (eBioscience) were added, followed by 30-min incubation at 37°C. Stained samples were lysed with lysis buffer (BD), washed with phosphate buffered saline (PBS; Sigma), and resuspended using 500 μl 1× Cell Fix (BD).

Detection of Platelet Vasodilator-stimulated Phosphoprotein Phosphorylation. Platelet-rich plasma (PRP) was obtained as a supernatant after centrifuging whole blood at 160*g* for 10 min. PRP (100 μl) was diluted 1:50 in PBS buffer. PGE_1 (Sigma; fc = 1 μM) and/or ADP (Moelab; fc = 2 μM) were added to respective samples. Samples were resuspended in permeabilization buffer (0.1%; Sigma) and stained with 1.5 μl PerCP-labeled anti-CD45 (Beckman Coulter) and 1 μl FITC-labeled anti–vasodilator-stimulated phosphoprotein (VASP) phospho-Ser 157 antibody (FITC/VASP-5C6; Nanotools, Germany) for 30 min. Samples were washed with PBS, fixed with 100 μl 1× Cell Fix (BD), and stained for 30 min using 1 μl FITC-labeled monoclonal anti-VASP phospho-Ser 157 antibody (Nanotools). After staining, whole blood and PRP samples were washed and resuspended in 500 μl Cell Fix (BD). Events were acquired on a FACS CantoII flow cytometer (BD).

Murine Blood Samples. Citrated whole blood (16 μl) was incubated with an antibody cocktail and 3 μl ADP solution (Moelab, fc: 20 μM) for 30 min at 37°C. The antibody cocktail consisted of the following: 2.5 μl FITC-labeled anti-CD42b (emfret, Germany), 1.5 μl PerCP-labeled anti-CD45 (Biolegend), 2.0 μl BV421-labeled anti-CD11b (Biolegend), 1.5 μl Alexa fluor–labeled anti-CD62P (BD), 1.5 μl phycoerythrine-labeled JON-A (emfret), 2.0 μl phycoerythrine-Cy7–labeled anti-Ly6G/6C (Biolegend), and 2.0 μl BV510-labeled anti-F4/80 (Biolegend). Next, 3 μl of the stained samples was diluted in 500 μl 1× Cell Fix (BD). Flow cytometry was performed within 4 h after sample fixation.

Fluorescence-activated Cell Sorting Analysis

Samples were measured on a FACSCanto II flow cytometer (BD). The cytometer was calibrated routinely using the cytometer setup and tracking beads (BD) recommended by the manufacturer. BD FACSDiva software (version 6; BD) was employed to control the flow cytometer settings, to perform the calibration procedures, and to acquire data.

Platelets, leukocytes, and aggregates including PNCs were detected according to the principles of a previously described method[28] using their typical forward and sideward scatter properties and specific antibody fluorescence, which is given in arbitrary units (a.u.). The platelet-specific antibody anti–CD41-FITC was used as a human platelet detection marker, whereas CD42b-FITC was used as a murine platelet detection marker. The fluorescence of the antibodies targeted against specific epitopes and phosphorylated VASP (see Detection of Platelet Vasodilator-stimulated Phosphoprotein Phosphorylation) was used to evaluate the activation and interaction of platelets and leukocytes. Detailed data analysis was performed using FlowJo software (version 9.3.2; Ashland, USA).

Determination of ATP Concentrations in PRP

Lyophilized luciferin and d-luciferase (Chrono-Log; Havertown, USA) were dissolved according to the manufacturer's instructions. ATP release was measured in a luminometer

(Turner Designs; Sunnyvale, USA). Reconstituted reagent (50 or 100 μl) was incubated with 450 μl PRP, respectively.

Immunofluorescence Staining of PNCs

Whole blood was incubated with 2 μM ADP and liquid sevoflurane (5 Vol% v/v) for 10 min. Neutrophils were stained with a phycoerythrine-labeled anti-CD15 antibody (BD) and platelets with an anti-CD41 antibody (Beckman Coulter). After 30 min, erythrocytes were lysed, and the remaining cells were fixed, washed, and resuspended in permeabilization buffer (0.1% Triton X-100, 2.0% bovine serum albumin in PBS, pH = 7.4; Sigma) for 10 min. After another washing step, samples were resuspended in mounting medium with 4′,6-diamidino-2-phenylindole (DAPI; Carl Roth, Germany) as the counterstain. Images were acquired on a confocal microscope (Zeiss CLSM 510 series) using Zen 7.1 software (Zeiss, Germany).

Immunofluorescence Staining of Liver Tissue

Murine liver tissue was fixed in Tissue-Tek (Sakura, Japan), and 5-μm cryoslides were permeabilized in methanol/acetone 1:1 for 5 min at room temperature (RT), blocked in 5% bovine serum albumin (Sigma), stained with a goat anti-mouse E-cadherin primary antibody (Santa Cruz, USA) overnight at 4°C, and counterstained with 1:500 donkey anti-goat Alexa594 (Santa Cruz) for 1 h at RT. After washing, samples were stained further with 1:100 rabbit anti-mouse Adora2a or rabbit anti-mouse Adora2b antibodies (Santa Cruz) overnight at 4°C and counterstained with 1:500 goat anti-rabbit Alexa488 (Santa Cruz) for 1 h at RT. After a washing step, tissue cuts were incubated with mounting medium with DAPI (Carl Roth) as counterstain. Images were acquired on a confocal microscope (Zeiss CLSM 510 series) using Zen 7.1 software (Zeiss).

Cytometric Bead Arrays for the Analysis of Chemokine Release

Chemokine release was analyzed using antibody-coated beads that bind to interleukin-6 (BD CBA mouse interleukin-6 Flex Set; BD) and keratinocyte-derived chemokine (KC; BD). Plasma samples obtained from murine blood were processed according to the manufacturer's instructions before acquisition on the FACSCanto II (BD).

Serum Enzymatic Measurements of Alanine Aminotransferase

For the assessment of liver damage in mice, serum levels of the liver-specific enzyme alanine aminotransferase (ALT) were measured in blood serum using an enzymatic assay (TECO Diagnostics, USA). Tests were performed according to the manufacturer's instructions using a microtiter plate for quantitative colorimetric determination between 500 and 550 nm on a plate reader (Tecan Diagnostics, Austria).

Transcriptional Analysis

Relative expression levels of Adora2a and Adora2b were analyzed in liver tissue harvested from all animal groups after liver IR. Liver tissue was homogenized, RNA was extracted, and cDNA was synthesized (iScript; Bio-Rad Laboratories, Germany). The quantification of murine Adora receptor expression levels was performed using real-time polymerase chain reaction on an iCycler (CFX 96; Bio-Rad Laboratories) as described previously.[29] Data were normalized using the 18S ribosomal subunit as a house-keeping gene. The following primers were used—18S subunit: forward 5′-GTAACCCGTTGAACCCCATT-3′ and reverse 5′-CCATCCAATCGGTAGTAGCG-3′; Adora2a: forward 5′-TCAACAGCAACCTGCAGAAC-3′ and reverse 5′-GGCTGAAGATGGAACTCGC-3′; and Adora2b: forward 5′-GCATTACAGACCCCCACCAA-3′ and reverse 5′-TTTATACCTGAGCGGGACGC-3′. Amplification was achieved in 40 cycles of 95°C (3 min), 95°C (10 s), 65°C (15 s), and 72°C (45 s).

Immunohistochemistry of Platelets and Neutrophils in Murine Hepatic Tissue

Liver histologic sections were evaluated for PNC sequestration by immunohistochemistry staining as previously described.[7]

Statistical Analysis

Data are presented as mean ± SD or median and interquartile range (IQR) as indicated. Any analyses that were conducted with a sample size less than or equal to 4 are presented either in figures as a scatter plot together with mean and SD imposed on the scatter or in the text as mean ± SD and minimum/maximum values. For comparisons between two groups, we employed unpaired *t* tests or the Mann–Whitney test as indicated. The reported *P* values derived from the *t* tests and Mann–Whitney tests are two-tailed *P* values. For comparisons of three or more groups, we employed one-way ANOVA with Bonferroni *post hoc* test. *P* value of less than 0.05 was defined to indicate a statistically significant difference.

The sample size pursued in the reported experiments is based on previous experience. In the previous work of our research group, an inhibitory effect of sevoflurane on proinflammatory leukocyte activation during extracorporeal circulation could be assessed with a sample size of n = 5.[19] Therefore, we primarily aimed to achieve a sample size of n = 5 to evaluate the effect of sevoflurane on different parameters. However, in some experiments, sample sizes were adapted according to observed effect strengths. No adjustments to the *P* values were made for these interim analyses. All statistical analyses were performed using GraphPad Prism, version 5.0b, for Mac OS X (GraphPad Software, USA).

Results

Sevoflurane Inhibits the Activation and Interaction of Platelets and Neutrophils and Induces Platelet VASP Phosphorylation In Vitro

Our first aim was to evaluate the effect of sevoflurane on the expression of platelet and neutrophil activation markers and

on the binding of platelets and neutrophils under defined *in vitro* conditions. These analyses were performed using an *in vitro* flow cytometry assay. Data of these experiments are given as mean and SD and are depicted in figure 1. The results indicate that the amount of anti–CD41-FITC geometric mean fluorescence on neutrophils as a marker for PNC formation was decreased from 1.7 ± 0.2 a.u. in controls to 1.2 ± 0.2 a.u. in sevoflurane-treated samples ($P < 0.01$; n = 5), the expression of the platelet activation marker P-selectin on PNC-bound platelets decreased from 2.1 ± 0.5 a.u. in controls to 1.5 ± 0.5 a.u. in sevoflurane-treated samples ($P < 0.05$; n = 5), and the expression of the leukocyte activation marker Mac-1 on PNC-bound granulocytes decreased from 1.4 ± 0.3 a.u. in controls to 1.0 ± 0.3 a.u. in sevoflurane-treated samples ($P < 0.05$; n = 5).

Furthermore, the phosphorylation of VASP-157 in platelets was measured (fig. 2, A and B) to evaluate the potential effect of sevoflurane on intracellular signal transduction. Results of these experiments are given as scatter plots together with mean and SD imposed on the scatter in figure 2C and as mean and SD in figure 2D. These experiments reveal that VASP-157 phosphorylation in platelets increased from 1.0 ± 0.1 a.u. in controls to 1.7 ± 0.1 a.u. in sevoflurane-treated samples ($P < 0.001$; n = 3). As another indicator of platelet activation, ATP release was investigated. In these experiments, ADP-induced ATP release in PRP was decreased from 53 ± 9.5 to 20 ± 4.3 a.u. in sevoflurane-treated samples ($P < 0.001$; n = 5).

To further visualize the effect of sevoflurane on PNC formation, we performed immunofluorescence staining of platelets and neutrophils in human whole blood after treatment with ADP and sevoflurane. Results are given as mean and SD and are depicted in figure 3. These analyses confirmed that sevoflurane inhibits ADP-induced PNC formation and decreases PNC size from 0.17 ± 0.05 mm^2 in controls to 0.07 ± 0.007 mm^2 in sevoflurane-treated samples ($P < 0.001$; n = 5).

Sevoflurane Dampens Platelet Activation and PNC Formation after Liver Ischemia: A Protective Effect Mainly Mediated by Adora2b

Further, we sought to evaluate the protective effect of sevoflurane and a potential mechanistic role of Adora2b in a previously described[27] murine liver IR model. In these experiments, C57BL/6 WT mice and Adora2b$^{-/-}$ mice were

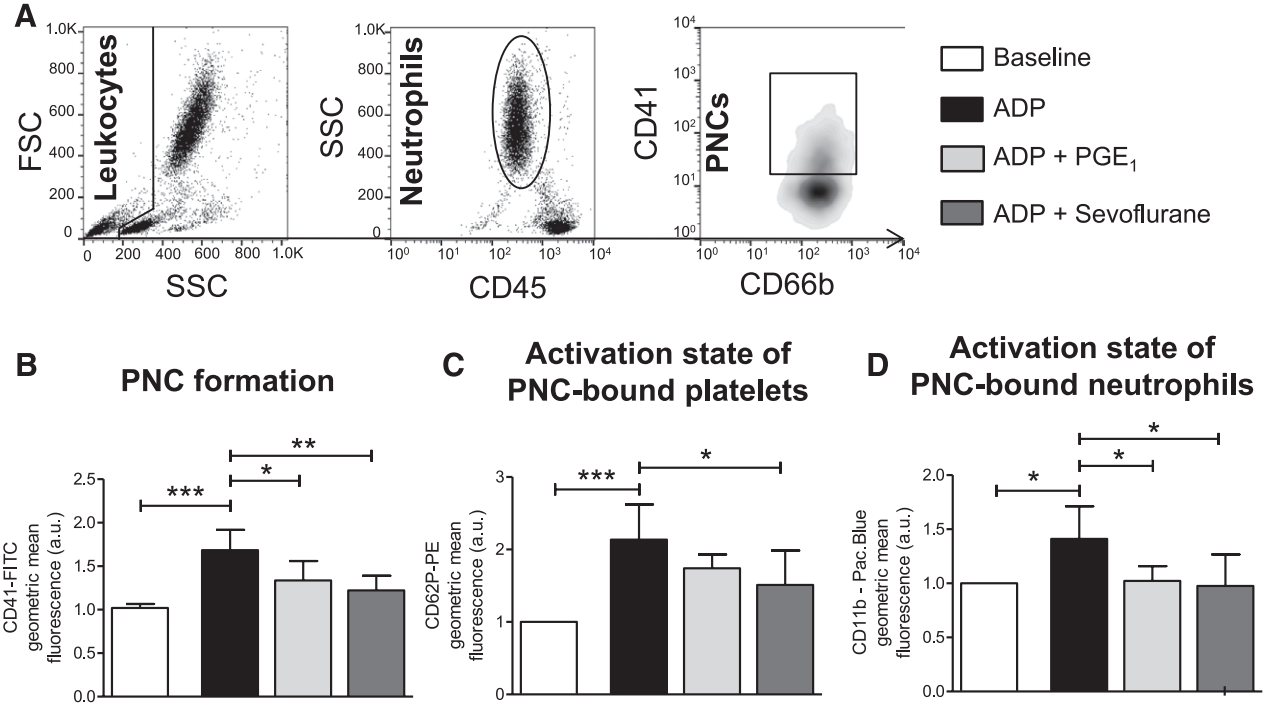

Fig. 1. Sevoflurane dampens the activation and binding of platelets and neutrophils in human whole blood *in vitro*. Whole blood was incubated with sevoflurane (5% v/v), adenosine diphosphate (ADP; 20 μM), and the platelet inhibitor prostaglandin E_1 (PGE_1; 1 μM) as indicated and then further processed for flow cytometry. (*A*) Flow cytometric strategy to investigate platelet–neutrophil conjugate (PNC) formation: PNCs were identified in whole blood according to their forward scatter/sideward scatter (FSC/SSC) properties and positive staining for CD45, CD66b, and CD41. (*B*) Sevoflurane inhibits PNC formation in whole blood. (*C*) Sevoflurane lessens expression of the platelet activation marker P-selectin (CD62P) of PNC-bound platelets. (*D*) Sevoflurane dampens expression of the leukocyte activation marker Mac-1 (CD11b) of PNC-bound neutrophils. Antibody fluorescence of the fluorophores fluorescein isothiocyanate (FITC), phycoerythrin (PE), and pacific blue (Pac. Blue) is given in arbitrary units (a.u.). Data are shown as mean ± SD; n = 5 per group. *P* values derived from one-way ANOVA and Bonferroni *post hoc* test: *$P < 0.05$, **$P < 0.01$, and ***$P < 0.001$.

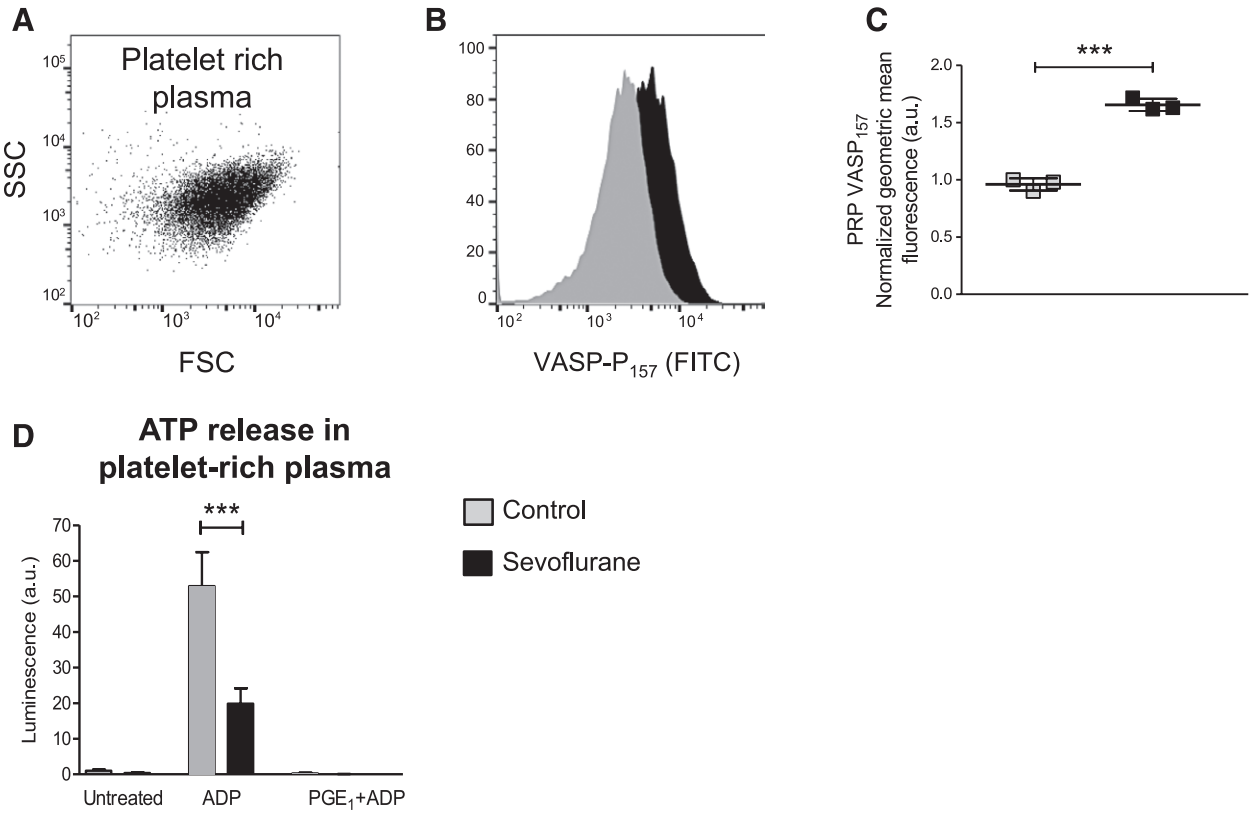

Fig. 2. Sevoflurane induces vasodilator-stimulated phosphoprotein (VASP) phosphorylation in platelets and dampens platelet adenosine triphosphate (ATP) release. Platelet-rich plasma (PRP) was incubated with sevoflurane (5% v/v) and then processed for analysis. (*A*) Platelets were identified in PRP using flow cytometry according to their typical forward scatter/sideward scatter (FSC/SSC) properties. (*B*) VASP phosphorylation in platelets was measured using an anti–$VASP_{157}$-fluorescein isothiocyanate (FITC)–labeled antibody. (*C*) Sevoflurane induces platelet $VASP\text{-}P_{157}$ (n = 3). (*D*) Sevoflurane decreases platelet ATP release in PRP (n = 5). Antibody fluorescence is given in arbitrary units (a.u.). Data are shown as scatter plots together with mean and SD imposed on the scatter in (*C*) and as mean and SD in (*D*). *P* values are derived from unpaired *t* tests; ***$P < 0.001$. ADP = adenosine diphosphate; PGE = prostaglandin E_1.

investigated. All mice were anesthetized with pentobarbital throughout the entire experiment. In addition, mice were either treated with sevoflurane in a clinically relevant concentration (2 Vol%) by inhalation or left to breathe room air as controls. Liver ischemia was induced over a period of 30 min. Blood was sampled in all mice after 5-min reperfusion. In WT mice, we aimed to achieve a sample size of eight animals per group to confirm the effect of sevoflurane. However, because blood clotting occurred in the sampling tube of one sevoflurane-treated animal, flow cytometry analysis could not be performed. Therefore, in WT mice, n = 8 untreated control mice are compared with n = 7 sevoflurane-treated mice. In $Adora2b^{-/-}$ mice, n = 5 control animals are compared with n = 5 sevoflurane-treated animals.

The activation and interaction of platelets and leukocytes were analyzed in blood samples using flow cytometry (fig. 4, A and B). Results of these experiments are given as median (IQR). In WT mice (C57BL/6), the expression of the platelet activation marker P-selectin was decreased from 8.8 (7.1 to 13) a.u. in controls to 7.6 (5.5 to 8.5) a.u. in sevoflurane-treated samples ($P < 0.05$), the activation of glycoprotein IIb/IIIa was decreased from 88% (86 to 91%) in controls to 84% (80 to 90%) in sevoflurane-treated samples ($P < 0.05$), and consecutive PNC formation was decreased 2.4-fold from 110 (48 to 200) a.u. in controls to 44 (30 to 92) a.u. in sevoflurane-treated samples ($P < 0.05$). Of note, in $Adora2b^{-/-}$ mice, the sevoflurane-associated reduction of platelet activation and PNC formation was not observed (fig. 4, C to E).

In further experiments with $Adora2a^{-/-}$ mice and CD1 WT controls (n = 4), the effect of sevoflurane (2 Vol%) on PNC formation was also investigated in the liver IR model.[27] Results of these experiments are given as mean ± SD and minimum/maximum values. In these experiments, PNC formation in WT mice (n = 4 per group) was decreased from 480 ± 130 (minimum/maximum: 360/660) a.u. in controls to 350 ± 36 (minimum/maximum: 310/380) a.u. in sevoflurane-treated samples ($P < 0.05$). In $Adora2a^{-/-}$ mice (n = 4 per group), PNC formation was decreased from 340 ± 23 a.u. (minimum/maximum: 310/360) in controls to 240 ± 90 (minimum/maximum: 130/310) a.u. in sevoflurane-treated samples ($P < 0.01$).

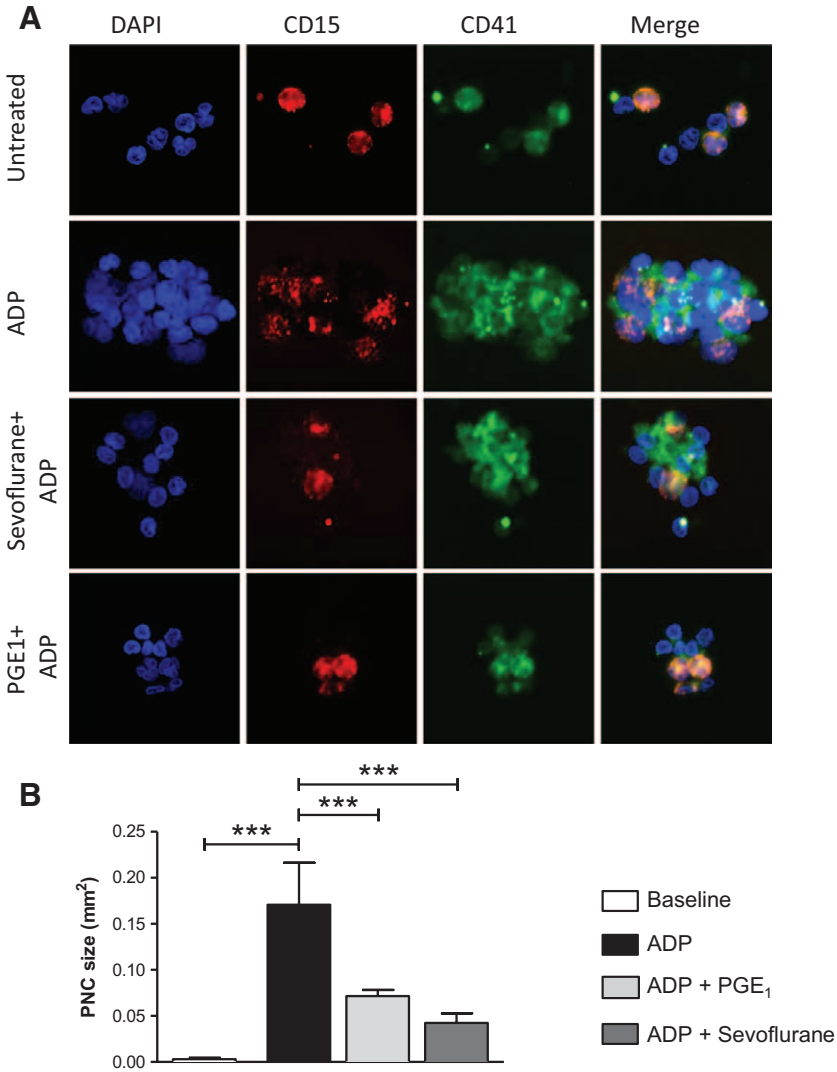

Fig. 3. Sevoflurane decreases platelet–neutrophil conjugate (PNC) formation in human whole blood *in vitro*. PNC formation was induced in whole blood *in vitro* by adding 20 μM adenosine diphosphate (ADP). Next, sevoflurane (5% v/v) or the platelet inhibitor prostaglandin E_1 (PGE_1; 1 μM) was added as indicated. (*A*) Samples were processed for immunofluorescence staining and investigated in confocal microscopy. Representative images are shown: platelets are labeled green with anti–CD41-fluorescein isothiocyanate (*green*), neutrophils stained red with anti–CD15-phycoerythrin (*red*), and cellular nuclei counterstained with 4′,6-diamidino-2-phenylindole (DAPI, *blue*). (*B*) Calculation of PNC size was performed according to the fluorescence areas derived from PNCs. Data are shown as mean ± SD; n = 5 per group. *P* values are derived from one-way ANOVA and Bonferroni *post hoc* test: ***$P < 0.001$.

Sevoflurane Decreases IR-induced Proinflammatory Cytokine Release, PNC Sequestration, and Liver Tissue Damage: A Protective Effect Mainly Mediated by Adora2b

To quantify the extent of systemic inflammation during liver IR in mice, proinflammatory cytokines (interleukin-6 and KC) were measured using cytometric bead arrays (fig. 5, A to D) in plasma, which was sampled after 3 h of reperfusion. Data are given as scatter plots together with mean and SD imposed on the scatter in figure 5, C and D and as median (IQR) in figure 5, F and G.

In WT mice (C57BL/6; n = 3 per group), interleukin-6 release was decreased from 320 ± 100 pg/ml in controls to 230 ± 59 pg/ml in sevoflurane-treated mice ($P < 0.05$). This antiinflammatory effect of sevoflurane on interleukin-6 release is completely abolished in $Adora2b^{-/-}$ mice (n = 4 per group). In WT mice (C57BL/6; n = 3 per group), KC release was decreased from 720 ± 95 pg/ml in controls to 530 ± 170 pg/ml in sevoflurane-treated samples ($P < 0.05$). In $Adora2b^{-/-}$ mice (n = 4 per group), KC release was decreased from 280 ± 94 pg/ml in controls to 66 ± 21 pg/ml in sevoflurane-treated samples ($P < 0.001$).

The effect of sevoflurane on interleukin-6 and KC release was also investigated in $Adora2a^{-/-}$ mice and respective CD1 WT mice (n = 4 per group). In these experiments, the inhibitory effect of sevoflurane was confirmed in CD1 WT and also in $Adora2a^{-/-}$ mice. Results of these experiments

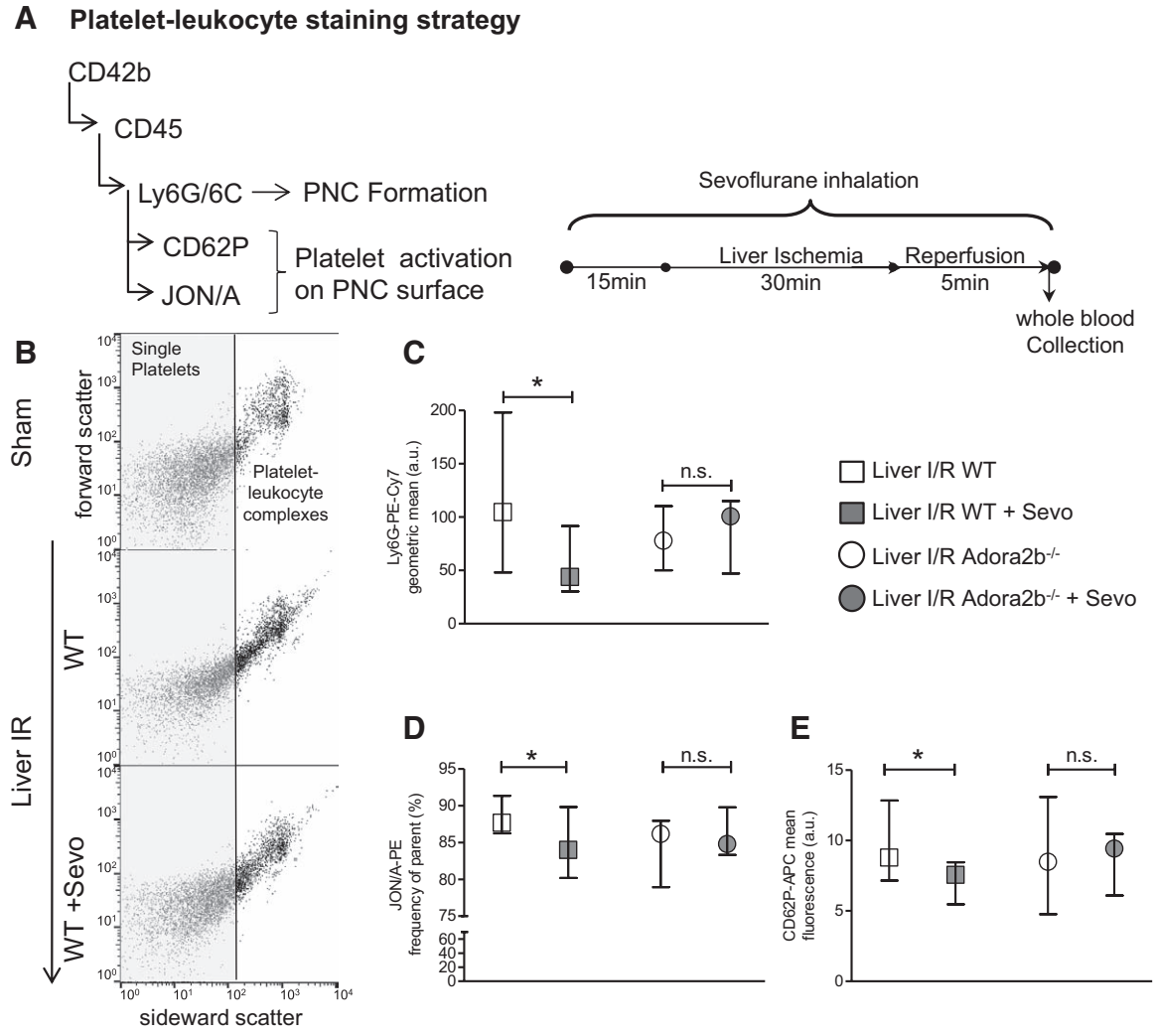

Fig. 4. Sevoflurane (Sevo) dampens the activation and interaction of platelets and neutrophils during ischemia/reperfusion (I/R) of the liver in mice. A 30-min period of liver ischemia was induced in C57BL/6 wild-type (WT) and Adora2b$^{-/-}$ mice. All mice were anesthetized with pentobarbital (as basis anesthesia). Mice were then either left as controls (without Sevo—referred to as "liver I/R WT") or treated with Sevo (2 Vol%—referred to as "liver I/R WT + Sevo") per inhalation. Blood was sampled after 5 min of reperfusion. The activation and interaction of platelets and neutrophils was investigated by flow cytometry. (*A*) Staining strategy for evaluating platelet–neutrophil conjugate (PNC) formation and the platelet activation state in flow cytometry. PNCs were detected according to positive fluorescence for CD42b, CD45, and Ly6G/6C epitopes. Antibodies against P-selectin (CD62P) and activated glycoprotein IIb/IIIa (antibody: JON/A) were used to detect platelet activation. (*B*) Representative dot plots showing the detection of platelet–leukocyte complexes in flow cytometry in animals that underwent liver I/R with and without Sevo treatment. Sevo significantly decreases I/R-induced PNC formation (*C*) and platelet activation as measured using the glycoprotein IIb/IIIa activation–specific JON/A ab (*D*) and an anti–P-selectin antibody (*E*). The inhibitory effect of Sevo is not observed in Adora2b$^{-/-}$ mice ("liver I/R Adora2b$^{-/-}$" compared to "liver I/R Adora2b$^{-/-}$ + Sevo"). (*C* to *E*) Data are shown as median ± interquartile range; n = 8 for WT mice not treated with Sevo and n = 7 for WT mice treated with Sevo; n = 5 for both Adora2b$^{-/-}$ groups (with and without Sevo treatment); antibody fluorescences are given in arbitrary units (a.u.) or percentages as indicated. *P* values are derived from the Mann–Whitney test: *$P < 0.05$; n.s. = not significant.

are given in the following as mean ± SD and minimum/maximum values. Interleukin-6 release in WT mice was decreased from 890 ± 210 (minimum/maximum: 680/1200) pg/ml in controls to 480 ± 280 (minimum/maximum: 250/840) pg/ml in sevoflurane-treated samples ($P < 0.01$). In Adora2a$^{-/-}$ mice, interleukin-6 release was decreased from 560 ± 120 (minimum/maximum: 430/730) pg/ml in controls to 390 ± 100 (minimum/maximum: 250/470) pg/ml in sevoflurane-treated samples ($P < 0.01$). KC release in WT mice was decreased from 680 ± 420 (minimum/maximum: 320/1300) pg/ml in controls to 360 ± 140 (minimum/maximum: 220/550) pg/ml in sevoflurane-treated samples ($P < 0.05$). In Adora2a$^{-/-}$ mice, KC release was decreased from 260 ± 110 (minimum/maximum: 190/430) pg/ml in controls to 160 ± 17 (minimum/maximum: 140/180) pg/ml in sevoflurane-treated samples ($P < 0.05$).

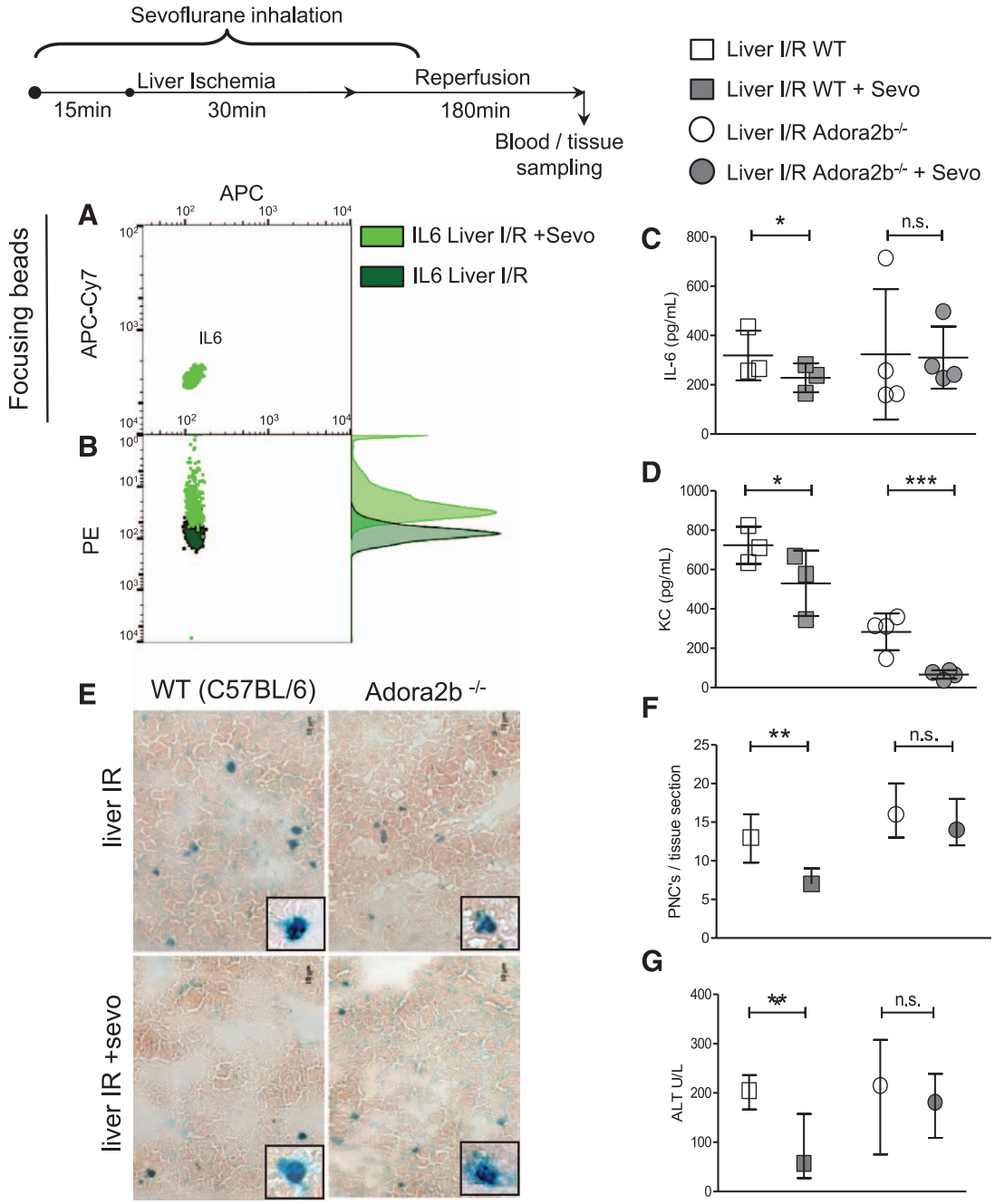

Fig. 5. Sevoflurane (Sevo) decreases proinflammatory cytokine release, platelet–neutrophil conjugate (PNC) sequestration, and liver damage during ischemia/reperfusion (I/R) of the liver in mice. A 30-min period of liver ischemia was induced in C57BL/6 wild-type (WT) and Adora2b$^{-/-}$ mice. All mice were anesthetized with pentobarbital (as background anesthesia). Mice were either left as controls (without Sevo—referred to as "liver I/R WT") or treated with Sevo (2 Vol%—referred to as "liver I/R WT + Sevo") per inhalation. Blood and liver tissue were collected for further analysis after 3-h reperfusion. (*A* and *B*) The proinflammatory cytokines interleukin-6 (IL-6) and keratinocyte-derived chemokine (KC) were measured using cytometric bead arrays using specific allophycocyanin conjugate-Cy7 (APC-Cy7)/phycoerythrin (PE) dot plots. (*C*) Sevo treatment decreases the release of IL-6 after liver I/R in C57BL/6 WT mice. This effect is completely abolished in Adora2b$^{-/-}$ mice (n = 3 per group for WT and n = 4 for Adora2b$^{-/-}$). Data are presented as scatter plots together with mean and SD imposed on the scatter. (*D*) Sevo decreases the release of KC after liver I/R in C57BL/6 WT and Adora2b$^{-/-}$ mice (n = 3 per group for WT and n = 4 for Adora2b$^{-/-}$). Data are presented as scatter plots together with mean and SD imposed on the scatter. (*E* and *F*) Sevo decreases sequestration of PNCs in liver tissue after I/R in WT mice. This protective effect of Sevo was not observed in Adora2b$^{-/-}$ mice (n = 5 per group). (*G*) Sevo decreases alanine aminotransferase (ALT) release after liver I/R in WT mice. A decrease of ALT release after Sevo treatment was not observed in Adora2b$^{-/-}$ mice (n = 5 per group). (*F* and *G*) Data are shown as median and interquartile range. All *P* values are derived from the Mann–Whitney test: *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$. n.s. = not significant.

Histologic analyses of liver tissue were performed in WT and Adora2b$^{-/-}$ mice after 3-h reperfusion to quantify the amount of sequestered PNCs. Data are shown in figure 5, E and F and are given as median and IQR. In WT mice (C57BL/6; n = 5 per group), proinflammatory PNC sequestration in the liver was significantly ($P < 0.05$) decreased by 1.9-fold from 13 (9.8 to 16) PNC/tissue section in controls to 7 (7 to 9) PNC/tissue section in sevoflurane-treated mice ($P < 0.05$). This antiinflammatory effect of sevoflurane is completely abolished in Adora2b$^{-/-}$ mice (n = 5 per group).

Furthermore, the release of the liver-specific enzyme ALT in WT mice (n = 5 per group) after 3-h reperfusion was significantly ($P < 0.05$) decreased by 3.5-fold from 210 (170 to 240) U/l in controls to 57 (27 to 160) U/l in sevoflurane-treated mice. In Adora2b$^{-/-}$ mice (n = 5 per group), sevoflurane does not inhibit IR-related ALT release (fig. 5G).

Sevoflurane Increases Transcription and Expression of Adora2b. To further elucidate the observed mechanistic role of Adora2b during sevoflurane-mediated protective effects, we analyzed whether the transcription and expression of Adora2b are influenced by sevoflurane treatment (fig. 6). Results of these experiments are given as scatter plots together with mean and SD imposed on the scatter. Polymerase chain reaction analyses of liver tissue reveal that sevoflurane inhalation significantly increases Adora2b transcript levels in sham-treated controls (2.2-fold increase; $P < 0.01$; n = 4 per group) and in the setting of liver IR (3.4-fold increase; $P < 0.001$; n = 4 per group). Immunofluorescence stainings of liver tissue reveal that the observed sevoflurane-associated increase of intracellular Adora2b transcription leads to increased Adora2b expression in liver tissue. In further experiments, no effect of sevoflurane inhalation on Adora2a transcription and expression was observed (data not shown).

Discussion

The data presented in this study provide new insights into protective properties of the volatile anesthetic sevoflurane during liver IR. Our *in vitro* experiments indicate that sevoflurane lessens proinflammatory activation and the formation of platelet and neutrophil complexes and induces platelet VASP phosphorylation. Our *in vivo* experiments confirm the antiinflammatory properties of sevoflurane. During IR damage of the liver in mice, sevoflurane dampens platelet activation, PNC formation and sequestration, and the release of proinflammatory cytokines. These effects are directly associated with decreased liver organ damage. To further understand the underlying mechanism, we analyzed the role of Adora2a and Adora2b receptors in the protective effects caused by sevoflurane. These experiments reveal that Adora2b plays a key role in driving sevoflurane's antiinflammatory effects. Also, sevoflurane treatment increases Adora2b expression in liver tissue. Altogether, these findings demonstrate that Adora2b acts as an important mediator of the sevoflurane-associated tissue-protective effects during liver IR.

A modulation of inflammatory events and IR injury by volatile anesthetics has been described previously for the heart, lung, liver, and kidney, and during extracorporeal circulation.[19,30–35] Our current *in vivo* data confirm findings from previous reports, which indicate that sevoflurane reduces leukocyte and platelet adhesion after IR, inhibits cytokine release under *in vivo* inflammatory conditions, and has tissue-protective properties.[36–38] Nevertheless, the underlying mechanism is not entirely understood as of yet. A better understanding of the mechanism of how sevoflurane decreases inflammatory events and potentially improves survival during IR and inflammation could further promote a targeted administration of this agent during respective disease states. During ischemia, ATP is released from ischemic and necrotic cells. Next, ATP is metabolized by the enzymes CD39 and CD73, which finally results in adenosine generation.[39] Adenosine acts through four receptors, namely, Adora1, Adora2a, Adora2b, and Adora3, to transmit antiinflammatory and organ-protective effects during ischemia.[14,15,17,40] Adenosine inhibits platelet aggregation,[14] decreases leukocyte binding to the microvasculature,[41] and is involved in platelet VASP phosphorylation.[14] VASP is expressed in many cell types, particularly in high levels in platelets, where it can negatively modulate platelet activation.[42] Phosphorylation of VASP at Ser157 correlates well with glycoprotein IIb/IIIa inhibition.[43] We have shown previously that VASP is crucially involved in PNC formation in the setting of liver and myocardial IR injury and have identified VASP as a potential target for organ-protective strategies.[7,8] Our current finding that sevoflurane induces platelet VASP phosphorylation underlines sevoflurane's antiinflammatory and antithrombotic potential.

It has been reported previously that hepatocellular-specific Adora2b signaling plays a unique role in liver protection during IR injury. In human liver biopsies, a selective induction of Adora2b transcript and protein after IR was detected. Furthermore, during liver IR, Adora1$^{-/-}$, Adora2a$^{-/-}$, and Adora3$^{-/-}$ animals had similar degrees of hepatic tissue injury as corresponding littermate controls. In contrast, Adora2b$^{-/-}$ mice exhibited significantly higher levels of tissue injury.[16] Apart from its expression on hepatocytes, the adenosine receptor Adora2b is expressed on a wide variety of cell types. Signaling *via* Adora2b has been shown to play a crucial antiinflammatory and tissue-protective role in many disease models.[39,41] Not only adenosine itself but also volatile anesthetics mediate through Adora receptors protective effects against IR damage.[17] Our results clearly reveal that Adora2b plays a major role in sevoflurane's antiinflammatory properties during liver IR. Moreover, our results provide clear evidence that sevoflurane has several effects on Adora2b function and expression. Notably, sevoflurane increases Adora2b transcription and expression. An important regulatory factor for Adora2b transcription is hypoxia-inducible factor (HIF). During cardioprotection against ischemia, Adora2b induction and signaling is dependent on HIF-1.

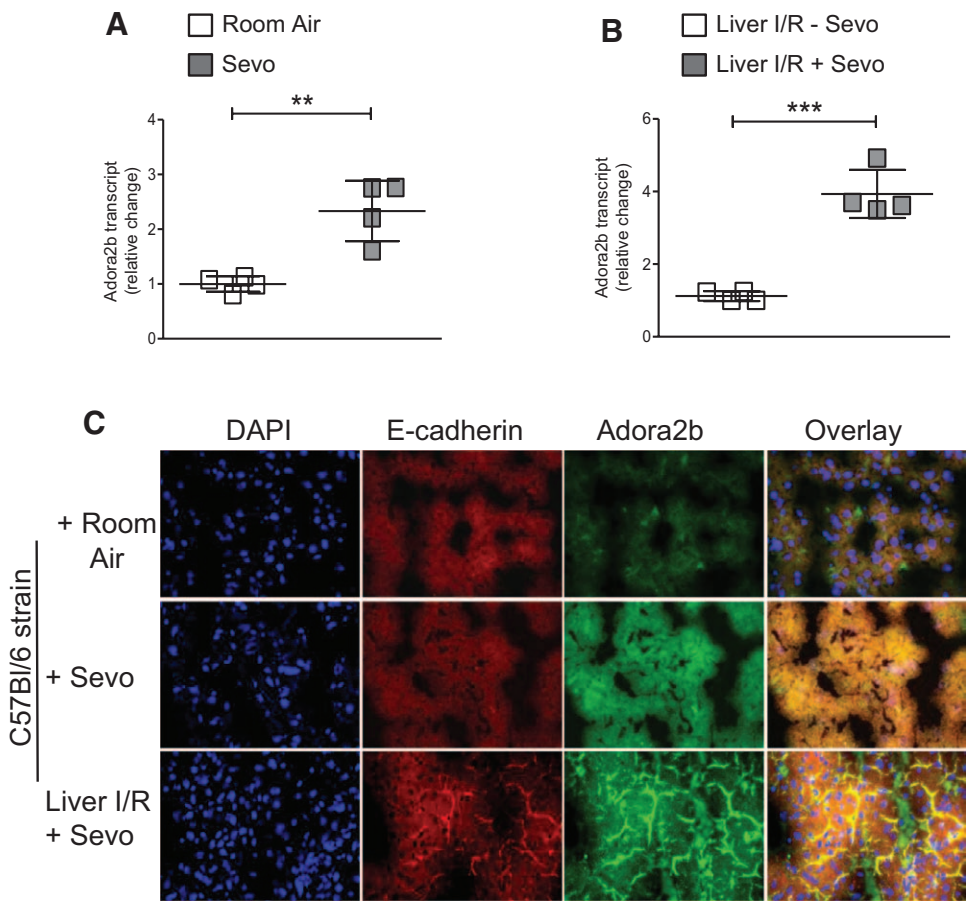

Fig. 6. Sevoflurane (Sevo) induces the transcription and expression of Adora2b. The effect of Sevo on the transcription and expression of Adora2b in liver tissue was analyzed using polymerase chain reaction (PCR) and immunofluorescence staining. All mice were anesthetized with pentobarbital (background anesthesia). (*A*) Mice were then either left to breathe room air (as controls without Sevo) or treated with Sevo (2 Vol%) inhalation for 60 min. Then, the liver was harvested and investigated for the transcription of the Adora2b receptor. (*B*) Mice underwent liver ischemia/reperfusion (IR) for 30 min. After 3-h reperfusion, the liver was harvested and investigated for the transcription and expression of the Adora2b receptor. (*C*) Representative images are shown: Murine liver tissue is stained with anti–E-cadherin Alexa Fluor 594 (*red*) and anti-Adora2b Alexa Fluor 488 (*green*) and counterstained with 4′,6-diamidino-2-phenylindole (DAPI; *blue*). PCR and immunofluorescence analyses indicate a significant upregulation of Adora2b transcription and expression in liver tissue of Sevo-treated mice. Data are presented as scatter plots together with mean and SD imposed on the scatter; n = 4 per group. *P* values are derived from an unpaired *t* test: $^{**}P < 0.01$ and $^{***}P < 0.0001$.

Further studies reveal a functional binding site for HIF-1 as part of the Adora2b promoter.[44–46] Therefore, HIF might participate in the observed effect of sevoflurane on Adora2b transcription.

In our *in vivo* experiments, the effects of sevoflurane on Adora2b directly translate into decreased activation, interaction, and tissue sequestration of platelets and leukocytes, as well as decreased proinflammatory cytokine release. As a result, sevoflurane decreases liver cell necrosis and liver enzyme release. Sevoflurane thereby exerts a significant organ-protective effect. These findings identify Adora2b as an important target for pharmacologic protection during liver IR, *e.g.*, through the administration of sevoflurane. Another strategy to achieve liver protection would be the application of an Adora2b agonist, which has been shown to attenuate hepatic injury in an animal model.[16] Furthermore, application of prolyl hydroxylase domain (PHD) inhibitors, which function as HIF activators, should be taken into account as a means for liver protection during IR. However, while studies with Adora agonists and HIF activators are promising, the translation of these compounds into the clinical setting has not yet been performed. In particular, pharmacologic approaches to using these agents have yet to be established, and their safety profile and potential side effects need to be evaluated.[16,47–50]

The choice of whether sevoflurane or an Adora2b agonist or a PHD inhibitor would be used for liver protection in the clinical setting should depend on the specific situation. In settings where anesthesia is required, such as during liver surgery, sevoflurane could be employed to provide liver protection. However, when liver protection needs to be established in patients who do not require anesthesia, an

Adora2b agonist or HIF activator could be administered systemically. Also a pharmacologic approach of administering an Adora2b agonist directly into the vasculature of the liver or the kidney before or during IR seems to be feasible. Such an approach would be of interest especially in settings of surgery and transplantation. This approach should produce a beneficial effect similar to that of inhaled Adora2b agonist treatment described for the treatment of endotoxin-induced lung injury.[49,51]

PNCs are formed through the binding of platelets and leukocytes after stimulation by proinflammatory agonists. PNCs are mechanistically involved in systemic inflammatory events, including extracorporeal circulation–associated inflammation and inflammatory vasculopathy during sepsis.[6,13] In particular, PNCs also participate in the pathomechanism of IR damage of the heart and liver and contribute to the development of acute lung injury.[7–9] Various effects of sevoflurane on platelet and leukocyte function have been described previously. In detail, it has been reported that sevoflurane impairs platelet glycoprotein IIb/IIIa activation,[20] attenuates platelet P-selectin expression,[22] decreases platelet–leukocyte conjugate formation,[21,22] and dampens extracorporeal circulation–induced neutrophil activation including Mac-1 expression.[19] All of these sevoflurane-associated properties are confirmed by our current results. Moreover, it has been reported that sevoflurane binds to lymphocyte function–associated antigen-1 on leukocytes and blocks ligand-binding functions of lymphocyte function–associated antigen-1.[18] Sevoflurane also inhibits the nuclear translocation of the proinflammatory transcription factors nuclear factor-κB and activator protein 1 and can reduce necrosis and inflammation in the kidney.[23] In combination, these findings suggest that the antiinflammatory action of sevoflurane is not transmitted by a single pathway. Furthermore, the antiinflammatory effects of sevoflurane act multifactorial and may differ between cell types and tissues. In our current experiments, an inhibitory effect of sevoflurane on KC release was detectable in WT and also in Adora2b$^{-/-}$ animals. This finding confirms that aside from Adora2b, other factors also contribute to the antiinflammatory properties of sevoflurane. However, our data clearly reveal that Adora2b plays an important role in the antiinflammatory effects of sevoflurane. The identification of Adora2b as a key mediator of sevoflurane's antiinflammatory properties significantly expands the understanding of the mechanism of sevoflurane's tissue-protective properties.

Limitations

During our liver IR experiments, the depth of anesthesia was monitored continuously to adjust equal anesthetic depths in all animals. However, we cannot definitely exclude that the anesthetic depth may have differed in animals that received pentobarbital alone in comparison to animals that received pentobarbital plus sevoflurane. Differing anesthetic depths may have influenced the degree of inflammation.

A potential mechanistic role of Adora1, Adora3, and HIF in sevoflurane-mediated effects was not investigated in our current study. Effects transmitted through these factors may, however, also contribute to the protective effect of sevoflurane.

In conclusion, our data indicate that the volatile anesthetic sevoflurane exerts antiinflammatory action and tissue-protective effects. Under the conditions of liver IR, the sevoflurane-associated antiinflammatory effects are mainly mediated by Adora2b receptors. In many clinical scenarios, IR damage and associated inflammatory complications significantly contribute to postoperative morbidity and mortality. Therefore, our finding that sevoflurane exerts antiinflammatory and organ-protective action may have an important meaning for the clinical situation. Our current results are valid for the situation of liver IR and the related noninfectious inflammatory response. Future studies should evaluate whether the Adora2b-mediated antiinflammatory properties of sevoflurane are also valid during sepsis. A specific use of sevoflurane not only as anesthetic but also as antiinflammatory agent may be a promising therapeutic approach for critically ill patients. We believe that our findings certainly warrant further investigations for application in the clinical setting. The use of sevoflurane, specific Adora2b agonists, and PHD inhibitors for tissue protection in IR settings should be investigated further in carefully planned clinical studies in the future.`

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Competing Interests

The authors declare no competing interests.

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