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Lidocaine Reduces Neutrophil Recruitment by Abolishing Chemokine-Induced Arrest and Transendothelial Migration in Septic Patients

Christian Berger,¹ Jan Rossaint,¹ Hugo Van Aken, Martin Westphal, Klaus Hahnenkamp, and Alexander Zarbock

The inappropriate activation, positioning, and recruitment of leukocytes are implicated in the pathogenesis of multiple organ failure in sepsis. Although the local anesthetic lidocaine modulates inflammatory processes, the effects of lidocaine in sepsis are still unknown. This double-blinded, prospective clinical trial was conducted to investigate the effect of lidocaine on leukocyte recruitment in septic patients. Fourteen septic patients were randomized to receive either a placebo (n = 7) or a lidocaine (n = 7) bolus (1.5 mg/kg), followed by continuous infusion (100 mg/h for patients >70 kg or 70 mg/h for patients <70 kg) over a period of 48 h. Selectin-mediated slow rolling, chemokine-induced arrest, and transmigration were investigated by using flow chamber and transmigration assays. Lidocaine treatment abrogated chemokine-induced neutrophil arrest and significantly impaired neutrophil transmigration through endothelial cells by inhibition of the protein kinase C-0 while not affecting the selectin-mediated slow neutrophil rolling. The observed results were not attributable to changes in surface expression of adhesion molecules or selectin-mediated capturing capacity, indicating a direct effect of lidocaine on signal transduction in neutrophils. These data suggest that lidocaine selectively inhibits chemokine-induced arrest and transmigration of neutrophils by inhibition of protein kinase C-0 while not affecting selectin-mediated slow rolling. These findings may implicate a possible therapeutic role for lidocaine in decreasing the inappropriate activation, positioning, and recruitment of leukocytes during sepsis. The Journal of Immunology, 2014, 192: 000–000.

Sepsis is a major healthcare problem, affecting millions of individuals around the world each year, and is associated with a high morbidity and mortality (1). The immune system combats microbial infections. In severe sepsis, however, circulating bacteria and LPS exposition may lead to an uncontrolled release of proinflammatory cytokines (e.g., TNF-α, IL-1, IL-6, and IL-8) from immune cells, including monocytes and macrophages. In response to this cytokine release and bacterial shedding of LPS during the initial phase of sepsis, leukocytes become inappropriately activated, get stuck in the microcirculation, and may subsequently be recruited into different organs, where they can cause tissue damage and organ failure (2). However, sepsis can also be caused by Gram-positive bacteria lacking LPS (3). Interestingly, although the inappropriate activation of leukocytes occurs in the early phase of sepsis, the later stage may be characterized by immune paralysis (4).

The mechanism by which inappropriately activated and recruited leukocytes induce injury is indicated by in vitro studies showing that endotoxin and other proinflammatory mediators directly and potently activate neutrophils to produce elastase (5) and superoxide ions (6). In vitro and in vivo data provide evidence that activated neutrophils may induce considerable endothelial injury via combined action of these agents, probably acting synergistically (7). Neutrophils incubated with endotoxin are capable of causing acute lung injury when reinjected into animals (8). Furthermore, endotoxin primes neutrophils to produce an enhanced respiratory burst in response to a second activating stimulus (9). This priming effect is characterized by the ability of trace amounts of endotoxin to act synergistically in producing lung injury in animals whose neutrophils have been exposed to small amounts of a chemotactic peptide (10). Injection of either agent alone does not cause lung injury in this model, demonstrating that sublethal doses of endotoxin can still provoke significant injury in the presence of other predisposing factors.

Neutrophil migration from the intravascular to the extravascular compartment predominantly occurs in the postcapillary venules, which proceeds in a cascade-like fashion (11–14). The first steps of this cascade are mediated by endothelial P- and E-selectin interacting with their counterreceptor P-selectin glycoprotein ligand (PSGL)-1 on leukocytes (14). Selectin engagement triggers the activation of an intracellular signaling pathway inducing the extended conformation of the β2 integrin LFA-1 (integrin αLβ2) on neutrophils (15). The extended conformation of LFA-1 enables the integrin to interact with ICAM-1 on endothelia, and this interaction leads to a reduction of the rolling velocity (14). During slow rolling on endothelial cells, neutrophils are exposed to different chemokines (14). Binding of chemokines to their receptors on neutrophils induces the high-affinity conformation of LFA-1, which mediates neutrophil adhesion to the endothelium. Following adherence, neutrophils require a chemokine gradient to complete the process of...
transmigration. The CXC-family chemokines, including IL-8 (CXCL-8) and growth-related oncogene, attract mainly neutrophils, whereas the C-C chemokines, such as MCP-1, are chemotactic for a variety of leukocytes. The excessive activation of neutrophils by chemokines contributes to uncontrolled leukocyte recruitment and organ damage in critically ill patients during sepsis (2).

The local anesthetic lidocaine has a variety of actions, including modulation of the inflammatory response, in addition to sodium channel blockade to relieve pain (16). In vivo, local anesthetics prevent or reduce inflammatory disorders such as reperfusion injury in heart, lung, and brain, as well as endotoxin- or hypoxia-induced pulmonary injury (16). In vitro, local anesthetics inhibit cellular functions of neutrophils that mediate early steps of the inflammatory response (16–21). However, the mechanisms behind these potentially beneficial effects of local anesthetics are largely unknown. It is clear that these actions do not result primarily from sodium channel blockade. The putative anti-inflammatory mechanisms of lidocaine and its exact effects on neutrophil recruitment in septic patients are completely unknown. The aim of this double-blinded, prospective, randomized clinical trial was to investigate the effects of lidocaine on neutrophil recruitment in septic patients.

Materials and Methods
Blinded, prospective, randomized clinical trial
To investigate the effects of lidocaine on the different steps of leukocyte recruitment, we conducted a prospective interventional study in septic patients. This study was approved by the local ethic committee of the University of Münster (Institutional Review Board). Patients were eligible for enrollment if they met all of the following inclusion criteria: 1) early sepsis due to suspected or proven infection and at least two of the following clinical signs of a systemic inflammatory response syndrome: hypothermia (≤36°C) or hyperthermia (≥38°C); tachycardia (≥90/min); tachypnoea (≥20/min) and/or PaCO₂ ≥33 mmHg and/or mechanical ventilation; leucocytosis (≥12 × 10³/μl) or leukopenia (≤4 × 10³/μl); diagnosis of sepsis within the past 24 h; and written informed consent.

Patients were excluded from the study if they met one of the following exclusion criteria: age <18 yr; pregnancy; known or suspected allergy to local anesthetics; severe sinus-atrial, atrial-ventricular, or intraventricular arrhythmias; terminal multiorgan failure; myocardial infarction during the past 3 months; cardiac ejection fraction <35%; cardiogenic shock; renal replacement therapy or chronic kidney disease; and liver failure with model for end-stage liver disease score ≥30 (22).

Eligible patients were randomly allocated to either the placebo or lidocaine group. Patients in the lidocaine group received a bolus infusion of 1.5 mg/kg lidocaine (equivalents 0.075 ml/kg 2% lidocaine solution), followed by a continuous infusion of 100 mg/h lidocaine (equivalents 5 ml/h) for patients with a body weight >70 kg or a continuous infusion of 70 mg/h lidocaine (equivalents 3.5 ml/h) for patients <70 kg over a period of 48 h. Lidocaine was obtained from Braun (Lidocard B. Braun 2%; B. Braun, Melsungen, Germany). This preparation contains lidocaine hydrochloride (20 mg/ml) and sodium chloride (94.9 mmol/ml) in aqueous solution. To avoid critically high plasma levels of lidocaine if patients suffered from acute kidney injury (glomerular filtration rate <50 ml/min) or received amiodarone, β-receptor antagonists, or norepinephrine at dosages >0.2 μg/kg/min, the continuous infusion of the study medication was decreased to 40 mg/h lidocaine (equivalents 2 ml/h) for patients with a body weight >70 kg or a continuous infusion of 20 mg/h lidocaine (equivalents 1 ml/h) for patients <70 kg. Control patients received a bolus of 0.075 ml/kg saline, followed by a continuous infusion of 5 ml/h saline over a period of 48 h; continuous infusion rates were reduced as specified for the study medication. Heparinized whole-blood samples and plasma samples were obtained from patients before administration of the study medication, 4 and 24 h after starting the study medication and 24 h after the cessation of the study medication.

Reagents
Unless otherwise stated, all reagents were obtained from Sigma-Aldrich (Taufkirchen, Germany).

Blood-perfused human microflow chamber
To investigate selectin-mediated slow neutrophil rolling, we used a whole-blood perfused human flow chamber system, as described previously (15, 23). Briefly, glass capillaries were coated with E-selectin (3.5 μg/ml; R&D Systems, Minneapolis, MN), P-selectin (20 μg/ml; R&D Systems), E-selectin/ICAM-1 (3.5/3.5 μg/ml; R&D Systems), or P-selectin/ICAM-1 (20/5 μg/ml; R&D Systems) for 2 h. Chambers were blocked with 1% casein (Fisher Scientific, Waltham, MA) for 1 h and afterwards perfused with heparinized whole-blood samples at a constant shear stress of 5–6 dyne/cm². It has been demonstrated that >90% of rolling cells in this system are neutrophils (15).

To investigate chemokine-induced arrest of neutrophils, flow chambers were coated with P-selectin (20 μg/ml), ICAM-1 (5 μg/ml), and IL-8 (50 μg/ml; PeproTech, Rocky Hill, NJ), or P-selectin and ICAM-1 as a control. The flow chambers were perfused with whole blood at a constant shear stress of 5–6 dyne/cm² for 2 min, and, subsequently, the number of rolling and adherent cells per field of view was determined and the ratio of adherent to rolling cells was calculated.

In vitro transmigration assay
The transepithelial migration of isolated human neutrophils through a monolayer of cultured HUVECs was performed, as described previously (30). Briefly, 6.5-mm Transwell filters (Corning Life Sciences, Corning, NY) were coated with fibronectin (0.01%) for 1 h. HUVECs were seeded into the wells and grown to confluence for 2 d. HUVECs were stimulated with TNF-α (5 nM; PeproTech) for 16 h. Human neutrophils were isolated from whole blood by Histopaque density centrifugation, resuspended in control plasma or plasma obtained from placebo- or lidocaine-treated septic patients at different time points, and incubated at 37°C for 30 min. The neutrophils were applied on top of each Transwell filter, and transmigration was allowed for 30 min, after which the number of transmigrated cells in the outer well was counted. To enforce transmigration, IL-8 (100 ng/ml; PeproTech) was added to the media in the outer well.

FACS analysis of neutrophils from human whole blood
The surface expression of neutrophil surface adhesion molecules on neutrophils was analyzed by flow cytometry. Human neutrophils were incubated with different Abs (anti-human PSGL-1 Ab, clone KPL-1, BD Biosciences, Franklin Lakes, NJ; anti-human LFA-1 Ab, clone MEM-25, ImmunoTools, Friesoythe, Germany; anti-human L-selectin Ab, clone LT-TD180, ImmunoTools) for 20 min. Samples were analyzed on a FACSCanto flow cytometer (BD Biosciences). FACS data were processed using FlowJo (version 7.5.3; Tree Star, Ashland, OR). Neutrophils were identified by their typical appearance in the forward light scatter/side light scatter plot. In separate experiments, isolated donor neutrophils were incubated with plasma obtained from healthy control volunteers or septic patients treated with placebo or lidocaine, and the conformational activation of β2 integrins was investigated using the reporter Ab mAb242 in 24 h flow cytometry analysis.

Western blotting
Western blot analysis from isolated human neutrophils was performed, as described previously (25). Following stimulation, cells were lysed using radioimmunoprecipitation assay buffer (25). Lysate was boiled with Laemmli sample buffer at 95°C for 10 min, run on a 10% SDS-PAGE gel, and immunoblotted with Abs against total PKC-θ and phospho–PKC-θ (pThr40, Cell Signaling Technology, Danvers, MA). Blots were developed using the Amersham ECL Prime detection system (GE Healthcare, Piscataway, NJ).

Rap1 activation assay
To investigate Rap1 activation, isolated human neutrophils were stimulated with LPS and immediately used with EDTA-free ice-cold lysis buffer (25). Detection of GTP-bound Rap1 (Rap1-GTP) in lysates was performed by Rap1 activation assay buffer (25). Lysate was boiled with Laemmli sample buffer at 95°C for 10 min, run on a 10% SDS-PAGE gel, and immunoblotted with Abs against total PKC-θ and phospho–PKC-θ (pThr40). Following stimulation, cells were lysed using radioimmunoprecipitation assay buffer (25).

Measurement of IL-8 plasma levels
IL-8 in patient plasma samples was analyzed by ELISA, according to the manufacturer’s instructions (R&D Systems).

Statistical analysis
The power analysis on the required patient group size was performed with G*Power software (G*Power 3.1.7) by Axel Buchner, University of
Based on preliminary in vitro experiments showing a completely abolished chemokine-induced arrest of neutrophils after lidocaine treatment, we expected a strong effect size. For the clinical trial, we used an effect size of 0.8 and an α-error probability of 0.05 and calculated a required group size of n = 7 patients with an actual power of 0.8029. The study population is described by absolute and relative frequencies, mean and SE, where appropriate. Statistical analysis was performed with SPSS 21 (IBM SPSS Statistics for Windows, version 21.0; IBM, Armonk, NY) using Student-Newman-Keuls test, post hoc correction, or t test, where appropriate. More than two groups were compared using one-way ANOVA. Continuous variables lacking normality were tested using Mann–Whitney U tests. All data are represented as means ± SEM. A p value < 0.05 was considered as statistically significant.

Results

Lidocaine does not affect selectin-mediated slow neutrophil rolling

Demographic variables, vital parameters, renal function, infectious pathogens, hepatic function, and vasopressor use did not differ significantly between the groups (Tables I, II). Details on the primary site of infection and isolated pathogens are provided in Table III. In patients of the lidocaine-treated group, the plasma levels of lidocaine averaged 1.7 ± 0.15 μg/ml and 2.2 ± 0.28 μg/ml at 4 and 24 h after initiation of lidocaine administration, respectively.

To investigate the effect of lidocaine on selectin-mediated slow neutrophil rolling, we used a previously published flow chamber system (15, 23). The flow chamber system has the advantage that neutrophil rolling behavior can be investigated in whole blood without isolating neutrophils. This is important, because it has been demonstrated that the isolation process might alter the activation status and rolling ability of neutrophils (26–28). Before administering the study medication, neutrophils from both patient groups showed the same rolling velocity on E-selectin or P-selectin alone and a reduction of the rolling velocity on E-selectin or P-selectin in the presence of the LFA-1 ligand ICAM-1 (Fig. 1A, 1B). Lidocaine did not alter the rolling velocity on P-selectin or E-selectin alone or in combination with ICAM-1 compared with the treatment with saline 4 and 24 h after starting the study medication and 24 h after the cessation of the study mediation (Fig. 1C–H), showing that lidocaine treatment does not affect slow neutrophil rolling in septic patients.

Lidocaine reduces chemokine-induced neutrophil arrest

Neutrophils interacting with inflamed vascular endothelial cells are exposed to chemokines presented by the vascular endothelium (29). Binding of chemokines to G protein–coupled receptors (GPCRs) on neutrophils induces an intracellular signaling cascade leading to the transition of LFA-1 to the high-affinity conformation that induces neutrophil adhesion (30). The chemokine IL-8, which is upregulated in the blood of septic patients, binds to the chemokine receptors CXCR1 and CXCR2 on human neutrophils. The mean IL-8 plasma levels were 914 ± 257 pg/ml in patients in the placebo group and 989 ± 339 pg/ml in patients in the lidocaine group (Supplemental Fig. 1) (p < 0.05). The mean IL-8 plasma levels decreased in both groups in the course of the diseases, with no significant differences between the groups (Supplemental Fig. 1). To investigate neutrophil arrest in whole-blood samples from septic patients, we used a separate set of flow chambers coated with P-selectin/ICAM-1/IL-8 or P-selectin/ICAM-1 as a control.
The ratio of adherent to rolling cells was similar in the placebo and lidocaine group prior to administration of study medication (Fig. 2A). Neutrophil arrest significantly increased during the acute phase of sepsis in placebo-treated patients 4 h after starting the study medication, with a decline at 24 h and a return to baseline values 24 h after stopping the study medication (Fig. 2A). Lidocaine strongly inhibited the increase of chemokine-induced neutrophil arrest at 4 and 24 h after starting the study medication (Fig. 2A).

To confirm that the observed reduction of chemokine-induced arrest is solely due to the effect of lidocaine on neutrophils, we conducted flow chamber experiments with healthy donor neutrophils incubated with various doses of lidocaine, which were similar to the levels measured in septic patients after treating with lidocaine. Untreated neutrophils showed the expected increase in the ratio of adherent to rolling cells in flow chambers coated with P-selectin/ICAM-1/IL-8 compared with flow chambers coated with P-selectin/ICAM-1 alone (Fig. 2B). Pretreatment of neutrophils with lidocaine caused a dose-dependent decrease of chemokine-induced arrest, with a nearly complete inhibition of chemokine-induced arrest at lidocaine concentration of 3 µg/ml (Fig. 2B).

To clarify whether lidocaine directly affects chemokine-induced arrest of neutrophils and to exclude potential confounding effects on plasma composition in patients from the lidocaine group, we incubated isolated neutrophils from healthy donors with plasma obtained from septic patients from the placebo group and added lidocaine at different concentrations in vitro. Lidocaine showed a dose-dependent inhibition of chemokine-induced arrest (Fig. 2C). Substitution of lidocaine at 2 µg/ml inhibited chemokine-induced arrest of neutrophils to the same degree as pretreatment with plasma obtained from septic patients 4 h after initiation of lidocaine administration. In accordance with this finding, the mean lidocaine levels in patients were 1.7 ± 0.15 µg/ml and 2.2 ± 0.28 µg/ml at 4 and 24 h after initiation of lidocaine administration, respectively. In the control group, lidocaine levels in the blood were not detectable.

FIGURE 1. Lidocaine does not affect selectin-mediated slow neutrophil rolling. The neutrophil rolling velocity in whole blood from patients treated with placebo (dashed line) or lidocaine (solid line) was analyzed before starting the study medication (A, B), 4 h (C, D), and 24 h (E, F) after starting the study medication, and 24 h after stopping the study medication (G, H) using microflow chambers coated with E-selectin or P-selectin alone and in combination with ICAM-1. The average rolling velocity of neutrophils is presented as mean ± SEM (n = 7 patients). The wall shear stress in all flow chamber experiments was 5–6 dynes/cm².
FIGURE 2. Lidocaine reduces chemokine-induced neutrophil arrest. (A) The chemokine-induced neutrophil arrest was investigated using the flow chamber assay. Flow chambers coated with P-selectin/ICAM-1 or P-selectin/ICAM-1/IL-8 were perfused with whole blood obtained from patients treated with placebo (black bars) or lidocaine (white bars) before starting the study medication, 4 and 24 h after starting the study medication, and 24 h after stopping the study medication. The ratio of adherent to rolling cells was calculated 2 min after starting the perfusion of the chamber with whole blood at a constant wall shear stress of 5–6 dynes/cm² (n = 7 patients). (B) Isolated donor neutrophils were pretreated with lidocaine, and chemokine-induced neutrophil arrest was investigated using the flow chamber assay (n = 4 experiments). (C) Isolated neutrophils from healthy volunteers were incubated with plasma from placebo-treated (P) or lidocaine-treated (L) patients and with different lidocaine concentrations. Chemokine-induced neutrophil arrest was investigated using the flow chamber assay (n = 3 experiments). (D and E) Flow chambers coated with E-selectin (D) or P-selectin (E) were perfused with whole blood obtained from patients treated with placebo (black bars) or lidocaine (white bars) before starting the study (Figure legend continues)
Rap1 fusion proteins (with wild-type (WT) or constitutively active (CA) Tat-blot of three experiments). Immunoblotted with Abs against total Rap1 (exemplary precipitated from lysates using Ral-GST beads and caine before stimulation with IL-8. GTP-bound Rap1 was healthy volunteers and preincubated with saline or lidocaine before stimulation with IL-8. Assays. Neutrophils were isolated from whole blood of healthy volunteers and preincubated with saline or lidocaine before stimulation with IL-8. GTP-bound Rap1 was precipitated from lysates using Ral-GST beads and immunoblotted with Abs against total Rap1 (exemplary blot of three experiments).

The activation of Rap1 was analyzed by GTP-pulldown assays. Neutrophils were isolated from whole blood of healthy volunteers and preincubated with saline or lidocaine before stimulation with IL-8. Neutrophils were isolated from whole blood of healthy volunteers and preincubated with saline or lidocaine before stimulation with IL-8. Neutrophils were isolated from whole blood of healthy volunteers and preincubated with saline or lidocaine before stimulation with IL-8.

**FIGURE 3.** Lidocaine inhibits phosphorylation of PKC-0. (A) The phosphorylation of PKC-0 was analyzed by Western blot. Neutrophils were isolated from whole blood of healthy volunteers and preincubated with saline or lidocaine before stimulation with IL-8. Lysates were prepared and immunoblotted with Abs against phosphorylated PKC-0 (phospho–PKC-0 (Thr538)) or total PKC-0 (exemplary blot of three experiments). (B) The activation of Rap1 was analyzed by GTP-pulldown assays. Neutrophils were isolated from whole blood of healthy volunteers and preincubated with saline or lidocaine before stimulation with IL-8. GTP-bound Rap1 was precipitated from lysates using Ral-GST beads and immunoblotted with Abs against total Rap1 (exemplary blot of three experiments). (C) Chemokine-induced neutrophil arrest was investigated using the flow chamber assay. Flow chambers coated with P-selectin/ICAM-1 or P-selectin/ICAM-1/IL-8 were perfused with isolated donor neutrophils that were pretreated with lidocaine and/or Ro-31-8220. In a separate set of experiments, lidocaine or Ro-31-8220–pretreated neutrophils were incubated with wild-type (WT) or constitutively active (CA) Tat-Rap1 fusion proteins (n = 4 experiments). *p < 0.05.

To exclude a possible effect of the total number of rolling cells on the observed effects, we determined the capturing capacity of neutrophils in whole-blood samples from both patient groups in flow chambers coated with E-selectin or P-selectin alone. There was no significant difference in the capturing capacity, measured as the total number of rolling neutrophils per field of view after 2 min between both study groups at the different time points (Fig. 2D, 2E).

To directly investigate the effect of lidocaine treatment on the conformational activation of LFA-1, we incubated healthy donor neutrophils with control plasma or plasma obtained from placebo- or lidocaine-treated patients before and 4 h after starting the study medication. The high-affinity conformational activation, which mediates chemokine-induced arrest, is selectively recognized by the reporter Ab mAb24 (32). Incubation of donor neutrophils with plasma obtained from lidocaine-treated patients 4 h after starting the study medication significantly decreased binding of the reporter Ab mAb24 both under baseline conditions and following stimulation with IL-8 (Fig. 2F). These data demonstrate that lidocaine inhibits the chemokine-induced conformational change of LFA-1 from the resting to the high-affinity state and thus inhibits neutrophil arrest.

**Lidocaine inhibits phosphorylation of protein kinase C-0**

Protein kinase C (PKC)-0 is involved in T cell activation via regulating the avidity of the β2 integrin LFA-1 in the immunological synapse (33). Western blot analysis showed that PKC-0 in neutrophils is phosphorylated after stimulation with IL-8 (Fig. 3A). Pretreatment with lidocaine abolished PKC-0 phosphorylation. The small GTPase Rap1 is a downstream effector of chemokine signaling in neutrophils. Rap1 is activated following IL-8 stimulation in neutrophils (Fig. 3B). Lidocaine pretreatment inhibited Rap1 activation. To investigate the functional consequence of reduced PKC-0 activation by lidocaine pretreatment on chemokine-induced arrest, we performed flow chamber assays. Pretreatment with lidocaine or the PKC inhibitor Ro-31-8220 significantly reduced chemokine-induced arrest (Fig. 3C). The combined pretreatment of neutrophils with lidocaine and Ro-8320 did not show an additive effect, indicating that lidocaine abolishes chemokine-induced arrest by inhibiting PKC-0 activation. Pretreatment of neutrophils with lidocaine or Ro-31-8220 and a constitutively active Tat-Rap1 fusion protein restored chemokine-induced arrest, whereas pretreatment with a wild-type Tat-Rap1 fusion protein as a control did not reverse the inhibition of chemokine-induced arrest (Fig. 3C).

The expression of neutrophil surface adhesion molecules is not altered by lidocaine

Alterations of neutrophil-rolling behavior and chemokine-induced arrest may be attributable to changes in the expression pattern of surface adhesion molecules on the surface of neutrophils (34–36). To investigate whether the observed effect of lidocaine treatment on chemokine-induced arrest may be due to changes in the expression of adhesion molecules on the surface of neutrophils, we analyzed the surface expression of different important adhesion molecules, including PSGL-1, LFA-1, and L-selectin. There was no significant difference in the surface expression of PSGL-1 (Fig. 4A), LFA-1 (Fig. 4B), and L-selectin (Fig. 4C) between the two groups before and after administration of study medication as well as 24 h after cessation of the study medication.

**Lidocaine abolishes transmigration of neutrophils**

After arresting on the inflamed vascular endothelium, neutrophils leave the vessel by transmigration into the surrounding tissue, medication, 4 and 24 h after starting the study medication, and 24 h after stopping the study medication. The number of rolling cells was analyzed 2 min after starting the perfusion of the chamber with whole blood (n = 7 patients). (F) To directly investigate the high affinity conformational of the integrin LFA-1, donor neutrophils were incubated with plasma obtained from placebo- or lidocaine-treated patients before and 4 h after starting the study medication. The binding of the reporter Ab mAb24 was analyzed by flow cytometry (n = 4 experiments). *p < 0.05.
where they participate in tissue damage and organ dysfunction during sepsis (14). To investigate the effect of lidocaine on transmigration, we used an in vitro transmigration assay with TNF-α-stimulated HUVECs (24, 31). Healthy donor neutrophils were preincubated with control plasma or plasma obtained from placebo- or lidocaine-treated septic patients. Neutrophils incubated with plasma from both patient groups obtained before the administration of the study medication showed a significantly higher transmigration rate compared with neutrophils incubated with plasma from healthy volunteers (Fig. 5A). However, the transmigration rate of neutrophils incubated with plasma from septic patients treated with lidocaine significantly decreased compared with the transmigration rate of neutrophils incubated with plasma from septic patients treated with saline (Fig. 5A). Astonishingly, incubating neutrophils with plasma from septic patients obtained 24 h after cessation of lidocaine infusion significantly reduced the neutrophil transmigration rate compared with the incubation of neutrophils with plasma from septic patients obtained 24 h after cessation of saline infusion (Fig. 5A).

Western blot analysis showed that PKC-\(\alpha\) is phosphorylated in neutrophils that transmigrated through a HUVEC monolayer in vitro (Fig. 5B). Pretreatment of neutrophils with lidocaine or Ro-31-8220 significantly reduced transmigration (Fig. 5C). The combined pretreatment of neutrophils with lidocaine and Ro-31-8220 did not show any additive effect (Fig. 5C). To investigate whether lidocaine directly affects transmigration of neutrophils and to exclude potential confounding effects due to an indirect action of lidocaine on plasma, we incubated isolated neutrophils from healthy donors with plasma from septic patients and added lidocaine (2 \(\mu\)g/ml) or saline as a control. The addition of lidocaine and plasma from septic patients to donor neutrophils caused a significant reduction in transendothelial migration compared with donor neutrophils incubated with plasma from septic patients and saline. These data show that lidocaine directly reduces neutrophil transendothelial migration (Fig. 5D).

**Discussion**

Sepsis is a major challenge in intensive care patients and largely contributes to the high morbidity and mortality in these patients (1). The uncontrolled systemic activation of immune cells, for example, due to circulating proinflammatory mediators, causes inappropriate activation and recruitment of leukocytes into peripheral tissues, followed by extensive tissue injury, a process that may ultimately lead to the organ dysfunction (37). Several laboratory and clinical strategies have been employed to diminish the uncontrolled systemic inflammation during sepsis. However, none have proven efficient to this date.

The aim of this study was to investigate the effects of systemic lidocaine application on the different steps of the leukocyte recruitment cascade, including selectin-mediated slow neutrophil rolling, chemokine-induced arrest, and transmigration in septic patients. Using flow chamber assays and flow cytometry analysis, we were able to demonstrate that lidocaine does not affect neutrophil slow rolling, but inhibits chemokine-induced arrest by inhibiting the affinity upregulation of the \(\beta_2\) integrin LFA-1. In addition, we provided evidence that lidocaine inhibits transendothelial migration. We identified the inhibition of PKC-\(\alpha\) activation as the molecular mechanism by which lidocaine inhibits chemokine-induced arrest and transmigration of neutrophils.

Several studies have indicated an anti-inflammatory effect of lidocaine (16). In this context, it has been shown that lidocaine suppresses superoxide release from stimulated neutrophils (38, 39). As superoxide production relies on integrin-mediated outside-in signaling and GPCR function (40, 41), these data go along with our results indicating an inhibitory role of lidocaine in chemokine-induced activation of the integrin LFA-1 and leukocyte arrest.

![FIGURE 4.](http://www.jimmunol.org/) The expression of neutrophil surface adhesion molecules is not altered by lidocaine. Surface adhesion molecules on human neutrophils obtained from patients treated with placebo (black bars) or lidocaine (white bars) were stained with fluorescently labeled Abs, and the surface expression was analyzed by flow cytometry. The mean fluorescence intensity (MFI) as a measure for surface expression of PSGL-1 (A), LFA-1 (B), and L-selectin (C) was quantified (\(n = 7\) patients).
addition, lidocaine has been reported to ameliorate several inflammatory conditions in vivo, including cardiac reperfusion injury and endotoxin- or hypoxia-induced pulmonary injury (16). However, these studies did not address the question how lidocaine suppresses leukocyte recruitment into the inflamed tissue.

Lidocaine at mean levels of 1.7 μg/ml in the blood of septic patients blocked neutrophil arrest and transmigration. The arrest was induced by coating the flow chamber with IL-8, a chemokine that is upregulated in the blood of septic patients (42). IL-8 binds to chemokine receptors CXCR1 and CXCR2 on human neutrophils and induces integrin activation, neutrophil arrest, and neutrophil transmigration (43, 44). Blocking these receptors prevents the activation of different signaling molecules, including PKC, the upregulation of integrin affinity, neutrophil arrest, and transmigration (43). Our in vitro experiments in which human neutrophils were incubated with plasma from septic patients treated with lidocaine showed that this plasma reduced neutrophil arrest and transmigration compared with plasma from septic patients treated with saline. It could be that lidocaine attenuates neutrophil recruitment by altering the chemokine homoeostasis and/or inhibiting intracellular signaling in neutrophils. However, our in vitro experiments in which the human neutrophils were preincubated with different concentrations of lidocaine suggest that lidocaine directly affects intracellular signaling following GPCR engagement. These data are in agreement with experiments showing that local anesthetics inhibit the activation of phospholipase D in the neutrophil-like human promyelocytic leukemia HL60 cell line and PKC in human neutrophils (45, 46). When reviewing these data together, it is most likely that lidocaine abolishes leukocyte arrest and transmigration by antagonizing GPCR signaling. By using Western blot analysis, flow chamber, and transmigration assays, we demonstrate that lidocaine affects GPCR signaling by inhibition of PKC-δ activation, leading to abolished adhesion and transmigration of neutrophils. Future studies have to address the question whether lidocaine reduces the release of proinflammatory mediators and/or increases the release of anti-inflammatory mediators, which are also released during inflammation (47). Recent studies showed that this mechanism is very important in limiting inflammation (48).

Although being of crucial importance, the chemokine-induced activation of neutrophil integrins is not the only prerequisite for leukocyte recruitment into peripheral tissues. The activation of
endothelial cells and the expression of adhesion molecules on endothelial cells may also influence leukocyte recruitment (14). Based on our experiments, we cannot exclude that lidocaine may also have an effect on vascular endothelial cells in septic patients. The available evidence for modulating the inflammatory response through the interaction of lidocaine with endothelial cells is limited. Lidocaine does not modulate the blood-aqueous permeability of the endothelium after intraocular injection in humans (49). In another study, lidocaine did not affect the mRNA transcription through the interaction of lidocaine with endothelial cells, the available data on this topic are not sufficient to completely exclude this possibility.

The lung is frequently one of the first organs affected during systemic inflammatory processes. Due to its unique microvasculature, the molecular mechanisms involved in neutrophil recruitment into the lung differ compared with other organs (51). Chemokine signaling plays a dominant role for neutrophil recruitment into the lung tissue, whereas selectins are only of minor importance in response to most inflammatory stimuli in the lung. However, as the mechanisms of neutrophil recruitment into the lung vary with different insults and may not necessarily require \( \beta_2 \) integrins, it is uncertain whether the inhibitory effects of lidocaine treatment would be more pronounced in this organ or not.

Our study has some limitations that we would acknowledge. If lidocaine inhibits chemokine-induced arrest and transendothelial migration, the systemic application during the acute phase of sepsis in patients may have an influence on the course of the disease. However, the number of intensive care patients included in this study was not sufficient to detect significance differences in clinical outcome parameters, for example, mortality and length of stay on the intensive care unit. Another limitation is that we could not investigate the effect of lidocaine on endothelial cells under septic conditions.

In conclusion, this blinded, prospective, randomized clinical trial demonstrates that lidocaine modulates inflammation in septic patients by decreasing chemokine-induced neutrophil arrest and transendothelial migration by inhibition of PKC-\( \theta \) activation. These findings may give rise to further research to investigate the effects of lidocaine treatment during the course of sepsis in critically ill patients.

Disclosures
The authors have no financial conflicts of interest.

References


