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Title Page

Inhibition of COX-1 attenuates the formation of thromboxane A_2 and ameliorates the acute decrease in glomerular filtration rate in endotoxemic mice

Katharina Mederle[1], Manuel Meurer[2], Hayo Castrop[1], and Klaus Höcherl[2]

[1]Institute of Physiology, University of Regensburg, D-93040 Regensburg, Germany

[2]Institute of Experimental and Clinical Pharmacology and Toxicology, Friedrich-Alexander-Universität Erlangen-Nürnberg (FAU), D-91054 Erlangen, Germany

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Corresponding author:

Prof. Dr. Klaus Höcherl

Institut für Experimentelle und Klinische Pharmakologie und Toxikologie

Friedrich-Alexander-Universität Erlangen-Nürnberg (FAU)

Fahrstr. 17, D-91054 Erlangen, Germany

Tel.: x49-(0)9131-8522883, Fax.: x49-(0)9131-8522774, E-Mail: klaus.hoecherl@fau.de

Abstract

Thromboxane (Tx) A_2 has been suggested to be involved in the development of sepsis-induced acute kidney injury (AKI). Therefore, we investigated the impact of cyclooxygenase (COX)-1 and COX-2 activity on lipopolysaccharide (LPS)-induced renal TxA_2 formation, and on endotoxemia-induced AKI in mice.

Injection of LPS (3 mg/kg; i.p.) decreased glomerular filtration rate (GFR) and the amount of thrombocytes to about 50% of basal values after four hours. Plasma and renocortical tissue levels of TxB_2 were increased about 10- and 1.7-fold in response to LPS, respectively. The COX-1 inhibitor SC-560 attenuated the LPS-induced fall in GFR and in platelet count to about 75% of basal levels. Further, SC-560 abolished the increase in plasma and renocortical tissue levels of TxB_2 in response to LPS. The COX-2 inhibitor SC-236 further enhanced the LPS-induced decrease in GFR to about 40% of basal values. SC-236 did not alter thrombocyte levels nor the LPS-induced increase in plasma and renocortical tissue levels of TxB_2. Pretreatment with clopidogrel inhibited the LPS-induced drop in thrombocyte count, but did not attenuate the LPS-induced decrease in GFR and the increase in plasma TxB_2 levels.

This study demonstrates that endotoxemia-induced thromboxane A_2 formation depends on the activity of COX-1. Our study further indicates that the COX-1 inhibitor SC-560 has a protective effect on the decrease in renal function in response to endotoxin. Therefore, our data support a role for thromboxane A_2 in the development of AKI in response to LPS.

Keywords

lipopolysaccharide, cyclooxygenase, acute kidney injury, thromboxane, inflammation

Introduction

Sepsis still remains a major cause of death in intensive care units (50). The development of acute kidney injury (AKI) is a common, serious complication of sepsis. AKI occurs in about 41% of septic patients, which results in higher morbidity and mortality. With regard to intensive care units, the mortality rate of septic patients with AKI is about 67%, as compared with 43% in patients without sepsis (52). Thus, drugs for the treatment of sepsis-induced AKI are urgently required. An increase in serum creatinine with or without a concomitant decrease in urine output is used as criteria for the detection and classification of AKI (64). Since both parameters represent markers for a decrease in glomerular filtration rate (GFR), a hallmark of AKI in general and also of septic AKI is the loss of GFR. Although in experimental models of sepsis and in patients changes in renal vascular resistance (RVR) as well as in renal blood flow (RBF) vary widely, a decrease in GFR is commonly observed (8, 61). This decrease is largely independent of changes in blood pressure (14, 72). There is experimental evidence that early in sepsis-related AKI, the predominant pathogenetic factor is renal vasoconstriction (37). Micropuncture studies in rodents suggest that GFR and glomerular plasma flow are reduced in response to endotoxemia, likely due to an increase in afferent arteriolar resistance (46). Moreover, findings obtained from in vitro blood-perfused juxta-medullary nephron preparations suggest that endotoxin elicits vasoconstriction probably due to direct, endothelium-mediated effects of LPS on vascular smooth muscle cells (70). It has been assumed that an imbalance between vasodilatory and vasoconstrictory substances is responsible in the pathogenesis of septic AKI. In this regard, elevated renal and/or circulatory levels of vasoconstrictors, such as angiotensin II, catecholamines, endothelin, and thromboxane, for example, may play an important role in septic AKI (5, 18, 40, 72).

Thromboxane (Tx) A_2 is a pro-inflammatory, vasoconstrictive lipid mediator. The generation of TxA_2 involves three steps: (I) the release of arachidonic acid from membrane

66 phospholipids by phospholipase A2; (II) the formation of prostaglandin (PG) H_2 from
67 arachidonic acid by cyclooxygenase (COX); and (III) the conversion of PGH_2 into TxA_2 by
68 thromboxane synthase (TxS). TxA_2 mediates its effects through a G protein–coupled receptor,
69 named TP receptor, which has been localized to the renal vasculature, glomerular mesangial
70 cells, podocytes and various nephron segments (49). TxA_2 is a likely candidate to play a role
71 in sepsis-induced AKI. The TxA_2 agonist U-46619 mimics the fall in GFR and the increase in
72 RVR commonly seen in response to injection of LPS (7). In addition, U-46619 preferentially
73 constricts the afferent arteriole (28). Further, genetic deletion of the thromboxane TP receptor,
74 TP receptor antagonism and TxS inhibition have been shown to improve renal function in
75 animal models of sepsis (5, 12, 68). During physiological conditions, TxA_2 mainly derives
76 from platelets, where the formation depends on the activity of cyclooxygenase (COX)-1 (57).
77 During inflammation, PGH_2 production is thought to depend mainly on the inducible form of
78 cyclooxygenase, COX-2. Therefore, it has been suggested that COX-2 is responsible for the
79 formation of TxA_2 during septic AKI (12). However, inhibition of COX-2 does not attenuate
80 the fall in GFR in response to LPS (41). Further, endotoxemia induces an immediate release
81 of TxA_2, at a time point when COX-2 synthesis just begins (5, 35). Therefore, it is still
82 unclear, which isoform of COX is responsible for the formation of TxA_2 during severe
83 inflammation.
84 We investigated therefore the impact of COX-1 and COX-2 activity for the increased
85 formation of TxA_2 in response to LPS, which is an experimental approach commonly used in
86 examining the pathogenesis of septic AKI. We used a low, nonlethal dose, which has been
87 found to induce AKI in the absence of severe hypotension (39). Because glomerular filtration
88 rate decreases shortly after the injection of endotoxin (12), we examined the effect of LPS on
89 COX-1 and COX-2 activity at an early time-point, to gain insight into the development of
90 septic AKI. Since we found that TxA_2 formation depends on the activity of COX-1 at this
91 time point, we further investigated the effect of COX-1 inhibition on glomerular filtration

rate. Because antiplatelet drugs such as the $P2Y_{12}$ receptor antagonist clopidogrel have been reported to attenuate the drop in platelet count and to improve end organe damage in animal models of sepsis (26, 62), we further investigated the effect of clopidogrel on glomerular filtration rate.

MATERIALS AND METHODS

Animal experiments

All animal experiments were performed according to the National Institutes of Health Guide for the Care and Use of Laboratory Animals and were approved by the local ethics committee. Male C57BL/6J mice (8 weeks old) were obtained from Charles River (Sulzfeld, Germany). Mice were acclimated for at least one week in the animal facility with free access to food and water. The animals were housed with a 12 hour:12 hour day:night cycle. Controls received i.p. injections of 500 µl of 0.9% saline. To induce acute kidney injury, mice were injected intraperitoneally with 3 mg/kg Lipopolysaccharide (LPS) (i.p. injection of 500 µl of LPS from Escherichia coli serotype 0111:B4; Sigma Aldrich Chemical, Germany, dissolved in 0.9% of saline), a low, nonlethal dose, which has been found to worsen renal function in the absence of severe hypotension (39). Further, mice received a single dose of SC-560 (20 mg/kg, ip; Cayman Chemical, Ann Arbor, MI) 30 min before or 15 min after the injection of LPS. Since there was no difference in our determinations between pre- and posttreatment with SC-560, we combined these two groups. SC-236 (10 mg/kg, i.p.; Cayman Chemical, Ann Arbor, MI) was given as a single dose 30 min before the injection of LPS. Clopidogrel sulfate (20 mg/kg; Sigma Aldrich Chemical, Germany) was given orally 48, 24 and 2h prior to injection of LPS. The animals (n=6-12 per group) were killed 4, 8 or 16h after injection of LPS or saline during isoflurane anesthesia. Blood was collected into tubes containing indomethacin (final concentration 10 µM) and EDTA. The kidneys were quickly removed, dissected, frozen in liquid nitrogen, and stored at –80°C until extraction of total RNA. The doses of clopidogrel

and SC-236 were selected from the literature (23, 74). The dose of SC-560 was selected from previous studies demonstrating inhibition of constitutive prostaglandin synthesis (16, 25, 45). The selectivity for SC-560 (COX-1 inhibitor) and SC-236 (COX-2 inhibitor) has been previously established (55, 65, 71). Therefore, it seems unlikely that SC560 is inhibiting both COX isoforms at the dose used in our study. However, since it has been reported that SC-560 may act at least in some cell types as an unselective COX inhibitor (13), we cannot exclude a small effect of SC-560 on COX-2 activity.

Measurement of Renal and Blood Parameters

GFR was calculated from FITC-sinistrin plasma clearance (60). In brief, three hours after the injection of LPS or isotonic saline 3.7µl/g BW of FITC-sinistrin (1.5% wt/wt dissolved in 0.9% NaCl) was injected into the retroorbital plexus during brief isoflurane anesthesia from which the animals recovered within ~20 s. At 3, 7, 10, 15, 35, 55, and 75 min after the injection, mice were placed in a restrainer, and 2 µl of blood were drawn from the tail vein using a 30-g atraumatic needle. Samples were centrifuged and 500 nl of plasma were transferred into a microcapillary and diluted 1:10 in 500 mmol HEPES (pH 7.4). To generate a standard curve, 1 µl of 1.5%-FITC-sinistrin was diluted 1:100, 1:500, and 1:1000 and 1:2000 in 500 mmol HEPES (pH 7.4). Fluorescence was determined in 1.7 µl of each sample in a NanoDrop 3300 Fluorospectrometer (Peqlab Biotechnologie GmbH, Erlangen, Germany) at an excitation wavelength of 470 nm and an emission wavelength of 520 nm. Quantification was achieved by using FITC-sinistrin standard curves. GFR was calculated using a two-compartment model of two-phase exponential decay. Pretreatment GFR values were obtained from each mouse one week prior to the experiments. Platelet counts were done by phase microscopy using Thrombo Plus test tubes (Sarstedt, Nürnbrecht, Germany). Plasma levels of urea were determined by commercially available kits (BioAssay Systems, Hayward, CA). Plasma and tissue levels of thromboxane B_2 were determined as described previously by a

commercially available immunoassay kit (Cayman Chemical, Ann Arbor, MI) (31, 32). Renocortical levels of arachidonic acid were determined by a commercially available assay kit (ShangHai BlueGene Biotech CO. Ltd, Shanghai, PR China).

mRNA extraction and real-time PCR analysis

Total tissue RNA was extracted from homogenized tissue with TRIzol Reagent (Invitrogen) according to the manufacturer´s instruction. Total RNA was reverse transcribed into cDNA according to standard protocols as described previously (47). Real-time PCR for IL-1β, TNFα and β-actin was performed in a LightCycler®480 (Roche, Mannheim, Germany). All PCR experiments were performed using the LightCycler DNA Master SYBR Green I kit provided by Roche Molecular Biochemicals (Mannheim, Germany) as described previously (47). The following primers were used: mouse IL-1β (NM_008361) sense: tctcgcagcagcacatca, antisense: cacacaccagcaggttat; mouse TNFα (NM_013693) sense: ctcagcctcttctcattc, antisense: ggtggtttgctacgacgt; mouse β-actin (NM_007393) sense: ccgccctaggcaccagggtg, antisense: ggctggggtgttgaaggtctcaaa; and mouse TP receptor (NM_009325) sense: gccttgttctcaccgacttcc, antisense: cagcccgaagaacaccatag.

Protein preparation and immunoblotting

Protein preparation and immunoblotting were performed as described previously (32). In brief, protein samples (20μg) were electrophoretically separated on 10% polyacrylamide gels and transferred to nitrocellulose membranes, which were blocked overnight in 5% nonfat dry milk diluted in Tris-buffered saline with 0.1% Tween-20, and then incubated for 1 h at room temperature with antibodies against COX-1 (Cayman Chemical; 1:1000), COX-2 (Cayman Chemical; 1:2000) or TxS (ProteinTech Group; 1:2000). After being washed, the membrane was incubated for 2 h with the secondary antibody (Santa Cruz Biotechnology; 1:2000) and subjected to a chemiluminescence detection system. Quantitative assessment of band densities was performed densitometrically using ImageJ Software.

Immunohistochemistry for TxS, COX-1 and COX-2

Kidneys from vehicle- and LPS-treated mice were fixed in 4% paraformaldehyde solution by retrograde perfusion through the abdominal aorta. Immunolabeling was performed on 5-µm paraffin sections as described previously (32). In brief, after being boiled in a microwave oven followed by cooling, kidney sections were incubated with an anti-TxS antibody (Proteintech; batch 11321-1-AP; Chicago, IL; 1:100), anti-COX-1 antibody (Cayman Chemical; batch 160109; Ann Arbor, MI; 1:1000), or an anti-COX-2 antibody (Cayman Chemical; batch 160126; Ann Arbor, MI; 1:200) overnight at 4°C, followed by incubation with a secondary antibody. As a negative control, we used the secondary antibody without incubation with the primary antibody.

Statistical Analyses

Data are expressed as the means ± SEM. Statistical significance was assessed with Student´s t-test or with 1- or 2-way ANOVA followed by Tukey´s post hoc test using GraphPad Prism version 6.05 (GraphPad Software, San Diego, CA, USA). Significance was taken at $P<0.05$.

Results

Endotoxemia increases thromboxane A_2 formation and renocortical COX-2 expression

Already one hour after the injection of LPS, endotoxemic mice were inactive and displayed piloerection. First, we investigated the time-dependent effect of a single dose of LPS (3 mg/kg; i.p.) on platelet count, the major physiological source of TxA_2. We found that endotoxemia decreased the amount of platelets to about 53, 41 and 35% of control values at 4, 8 and 16 hours after the injection of LPS, respectively ($n = 6$, $P < 0.05$; Fig. 1A). Next, we determined plasma and renocortical tissue concentrations of TxB_2, a stable metabolite of TxA_2. Plasma levels of TxB_2 were elevated about 7.4-, 2.7- and 2.0-fold at 4, 8 and 16 hours after the injection of LPS, respectively ($n = 6$, $P < 0.05$; Fig. 1B). We further found that

endotoxemia increased renocortical tissue levels of TxB_2 about 2.3- and 1.7- fold at 4 and 8 hours after the injection of LPS ($n = 6$, $P < 0.05$; Fig. 1B). Further, we investigated the effect of LPS on enzymes involved in renocortical TxA_2 biosynthesis. We found, that endotoxemia for 4h did not alter renocortical COX-1 and TxS protein expression, but strongly increased renocortical COX-2 protein abundance about 10-fold ($n = 6$, $P < 0.05$; Fig. 1C). COX-2 protein expression was still increased 5.6- and 4.0-fold at 8 and 16 hours after the injection of LPS, respectively ($n = 6$, $P < 0.05$; Fig. 1C). In contrast, the expression of COX-1 and TxS was decreased to about 70% of control levels at 8 and 16 hours after the injection of LPS ($n = 6$, $P < 0.05$; Fig. 1C). Endotoxemia time-dependently decreased thromboxane TP receptor mRNA abundance to 60, 24 and 21% of control values at 4, 8 and 16h after the injection of LPS, respectively ($n = 6$, $P < 0.05$). In addition, we determined renocortical tissue concentration of arachidonic acid (AA). Renocortical AA tissue concentration was increased 1.7-, 1.4- and 1.5-fold at 4, 8 and 16 hours after the injection of LPS ($n = 6$, $P < 0.05$; Fig. 1D).

Immunolocalization of TxS, COX-1 and COX-2

To determine the impact of endotoxemia on the renocortical localization of TxS, COX-1 and COX-2 we investigated the immunolocalization of these enzymes. TxS immunoreactivity was mainly detected in cortical arterioles of vehicle-treated mice. Only a mildly positive TxS-immunoreactive staining was observed in glomeruli. The distribution pattern was not altered in mice treated with LPS (3 mg/kg) at 4h. Renocortical COX-1 immunoreactivity was detected in the glomerulus, extraglomerular mesangium, tubular segments (most likely distal convoluted tubule, connecting tubule and the collecting duct), cortical interstitial fibroblasts and in some endothelial cells of vehicle-treated mice. The distribution pattern was not altered in mice treated with LPS (3 mg/kg) at 4h. Renocortical COX-2 immunoreactivity was detected in some macula densa cells of vehicle-treated mice (not shown). Endotoxemia increased COX-2 immunoreactivity mainly in cortical interstitial cells. Vascular and arteriolar

endothelial cells only showed a mildly positive COX-2-immunoreactive staining in response to LPS at 4h (Fig. 2).

COX-1 inhibition attenuates the LPS-induced increase in plasma and renocortical tissue TxB_2 concentration and the decrease in platelet count

Because thromboxane formation was highest at 4 hours after LPS injection, subsequent studies were performed at this time point. To determine the impact of COX-1 and COX-2 on endotoxemia-induced formation of TxA_2 we used the COX-1 inhibitor SC-560 (20 mg/kg) and the COX-2 inhibitor SC-236 (10 mg/kg). Inhibition of COX-1 decreased basal tissue and plasma levels of TxB_2 and abolished the LPS-induced increase in renocortical tissue and plasma concentration of TxB_2 at 4 hours ($P < 0.05$; Fig. 3A and B). Inhibition of COX-2 did not alter basal levels of TxB_2 or the LPS-induced increase in renocortical tissue and plasma levels of TxB_2 (Fig. 3A and B). Neither SC-560 nor SC-236 altered the amount of thrombocytes in vehicle-treated mice. However, inhibition of COX-1, but not of COX-2, attenuated the LPS-induced fall in platelet count ($P < 0.05$; Fig. 3C).

COX-1 inhibition ameliorates endotoxemia-induced AKI

Four hours after injection of LPS, GFR decreased to about 52% of pretreatment values ($P < 0.05$; Fig. 4A). Neither the COX-1 inhibitor SC-560 nor the COX-2 inhibitor SC-236 influenced basal GFR at 4h after injection. However, inhibition of COX-1 attenuated the LPS-induced decrease of GFR to about 77% of pretreatment values at 4 hours after injection of endotoxin ($P < 0.05$). In contrast, inhibition of COX-2 enhanced the LPS-induced decrease of GFR to about 41% of pretreatment values at 4 hours after injection of endotoxin ($P < 0.05$; Fig. 4A).

In addition, we investigated plasma urea levels. Neither the COX-1 inhibitor SC-560 nor the COX-2 inhibitor SC-236 altered basal plasma urea levels (Fig. 4C). Four hours after injection

of LPS plasma urea concentration increased from 24 ±2 to 36 ±2 mg/dl. Inhibition of COX-1 attenuated the LPS-induced increase in plasma urea and COX-2 inhibition enhanced the LPS-induced rise in plasma urea concentration at 4 hours after LPS-injection ($P < 0.05$; Fig. 4B).

Effect of clopidogrel pretreatment on LPS-induced decrease in platelet count and GFR

We further investigated the effect of clopidogrel, which has been shown to attenuate the fall in platelet count and to improve end organ damage in animal models of sepsis (26, 62). Therefore, we pretreated mice with the $P2Y_{12}$ receptor antagonist clopidogrel. Clopidogrel inhibited the LPS-induced drop in platelet count ($n = 6$, $P < 0.05$) but did not attenuate the LPS-induced increase in plasma TxB_2 levels or the LPS-induced decrease in GFR (Fig. 5).

Effect of SC-560 on LPS-induced renal inflammation

To investigate the effect of SC-560 on renal inflammation, we determined the renal mRNA abundance of IL-1β and TNFα. We found, that renal IL-1β mRNA abundance was increased about 4-fold 4 hours after the injection of LPS ($n = 6$, $P < 0.05$). SC-560 did not alter basal and LPS-induced levels of IL-1β (Fig. 6A). Further, renal TNFα mRNA abundance was increased about 7-fold 4 hours after the injection of LPS ($n = 6$, $P < 0.05$). SC-560 did not alter basal or LPS-induced levels of TNFα (Fig. 6B).

Discussion

In the present study, we demonstrate that SC-560, an experimental COX-1 inhibitor, inhibited the formation of thromboxane A_2 and attenuated the early decrease in GFR in an animal model of endotoxemia-induced AKI without affecting the LPS-induced proinflammatory response.

In line with previous observations, we found that renocortical COX-2 expression was strongly increased and that renocortical COX-1 expression moderately decreased after the injection of

LPS (30, 32, 34, 51). Further, renocortical TxS expression was not changed at four hours after injection of LPS, confirming a previous report where no alteration in TxS mRNA abundance was observed in IL-1/LPS-stimulated rat glomeruli (38). However, we now found that TxS expression decreased at later time-points. Furthermore, we observed a time-dependent downregulation of the thromboxane TP receptor mRNA in response to LPS, which may be due to an activation of the NF-κB pathway (76).

Injection of LPS caused an increase in renocortical tissue TxB_2 concentration as described before (5, 22). Therefore, one may conclude that LPS-induced renocortical TxA_2 formation mainly depends on COX-2 activity. Such an implication was previously assumed by others with regard to renal and cardiac TxA_2 formation in response to LPS (12, 24). To verify this assumption, we performed experiments with the selective COX-1 inhibitor SC-560 and with the selective COX-2 inhibitor SC-236 (55, 65). We now found that the COX-1 inhibitor SC-560, but not the COX-2 inhibitor SC-236, inhibited the LPS-induced increase in renocortical TxB_2 concentration. Therefore, our data suggest that COX-1, but not COX-2, is responsible for the endotoxemia-induced increase in TxB_2 formation. Since the expression of COX-1 and of TxS were unaltered in the early response to LPS, one may conclude that an increased release of arachidonic acid could be responsible for an increased formation of PGH_2 and subsequently for thromboxane (33). In line with this, we found that renocortical arachidonic acid concentration was increased in response to LPS. To outline the role of COX-1 for the formation of TxA_2, we determined the amount of platelets and measured plasma levels of TxB_2. Confirming previous findings we observed a strong increase in plasma levels of TxB_2, which was accompanied by a drop in platelet count after injection of LPS (35, 63). In line with the concept of a COX-1-dependent formation of platelet-derived TxA_2, we found that inhibition of COX-1, but not of COX-2, attenuated the drop in platelet count and inhibited the LPS-induced rise in plasma TxB_2 levels. Since SC-560 did not completely inhibit LPS-

induced thrombocytopenia, additional factors and/or a direct effect of LPS have to be involved in platelet activation in response to endotoxemia (75).

There is growing evidence that LPS exerts a direct effect on platelets. It has been reported that platelets express the toll-like receptor (TLR) 4, the receptor for the bacterial endotoxin LPS, and that TLR4 expression is responsible for LPS-induced thrombocytopenia (1, 4). It has been found that TLR4 polymorphism decreases thromboxane biosynthesis (53), suggesting that a direct activation of platelets by LPS is responsible for the marked increase in systemic TxA_2 formation. In addition to the direct effect of LPS on platelet activation, an inflammation-induced thrombin formation can activate platelets via protease-activated receptors. Further, endothelial cell damage exposes subendothelial collagen and induces the expression of the von Willebrand factor and of tissue factor on endothelial cells, which in turn activates platelets and further stimulates thrombin formation (19). Activated platelets further stimulate platelet activation via the release of TxA_2 and ADP (43), suggesting that platelets are the main source of thromboxane synthesis in response to endotoxemia. However, because COX-1 and TxS are expressed not only in platelets but also in vascular cells, for example (21), also vascular-derived thromboxane may contribute in part to the increase in endotoxin-induced thromboxane formation (17). Since our study does not specifically address this issue, further studies are necessary to investigate the contribution of vascular-derived TxA_2 for the overall formation of TxA_2 in response to LPS.

Endothelial dysfunction is one of the hallmarks of sepsis and a role for platelets in endothelial dysfunction has been supposed (59). It is now well accepted that platelets are major effector cells in both haemostasis and inflammation. Platelets are involved in the development of hyperinflammation, disseminated intravascular coagulation and microthrombosis (19). In experimental endotoxemia, inhibition of platelet function was found to reduce endothelial dysfunction and to ameliorate multiple organ failure including acute lung injury (26, 56, 62). Recently, a role for platelets in the development of renal failure has been proposed for septic

patients (69). However, it is still unclear if antiplatelet drugs such as clopidogrel improve renal function in response to endotoxemia. Therefore, we used the $P2Y_{12}$ receptor antagonist clopidogrel, which has been shown to inhibit LPS-induced thrombocytopenia and to improve acute lung injury in mice (74). Indeed, clopidogrel inhibited the drop in platelet count in response to endotoxemia in our study. Although there is evidence that inhibition of platelet $P2Y_{12}$ receptors decreases basal platelet TxA_2 formation and inhibits platelet activation and aggregation mediated by TxA_2 pathways, by reducing platelet formation of TxA_2 and by inhibiting responses after TP receptor activation (2, 3, 10, 42, 54), clopidogrel did not affect the increase in plasma TxB_2 levels after injection of LPS in our present study. A similar finding was previously observed for the $P2Y_{12}$ receptor antagonist ticlopidine (35). We further found that clopidogrel did not improve the fall in GFR in response to LPS. Thus, our data indicate that inhibition of $P2Y_{12}$ does not have a protective effect on endotoxemia-induced AKI. In line with this, it has been reported that LPS-induced systemic inflammation is more severe in P2Y12 null mice (44).

Our data indicate that COX-1 is responsible for the increased formation of TxA_2 in response to LPS. Therefore, we investigated the effect of SC-560 on endotoxemia-induced acute kidney injury. Neither COX-1 nor COX-2 inhibition affected GFR in control mice as described before (15). We now found that inhibition of COX-1 attenuated the early decrease in GFR in response to LPS, without affecting the inflammatory response to LPS. It has been reported that inhibition of thromboxane synthase, pharmacological blockade of TP receptors or genetic disruption of TP receptors ameliorates the LPS-induced decrease in GFR (5, 12). Similarly to these reports, the endotoxemia-induced decrease in GFR in our present study was not completely prevented by SC-560 (5, 12). Since high concentrations of all 5 primary prostanoids are reported to be low-affinity agonists at the TP receptor (29), one may argue that also COX-2 derived prostanoids could contribute to the decrease in GFR in response to endotoxemia. Further, an increased formation of TP receptor activating isoprostanes could be

involved in the decline in GFR (6, 73). However, this assumption seems to be unlikely, because genetic disruption of TP receptors is also not sufficient to fully reverse the decline in GFR (12). Interestingly, a very similar effect was recently reported after ischemic acute kidney injury in rats (11). In this study, SC-560 at a dose of 1 mg/kg, but not the COX-2 inhibitor SC-58125, attenuated the decrease of GFR 24h after renal ischemia. Taken together, it seems likely that COX-1-derived prostanoids negatively influence renal function in animal models of AKI. However, the precise mechanism for the protective effect of COX-1 inhibition has to be determined in future studies. In addition, other vasoconstrictive factors, like angiotensin II, endothelin-1 or the renal nervous system could also be involved in the LPS-induced decrease in GFR (46, 58, 72).

A limitation of our study is that neither blood pressure nor renal blood flow were measured. In normal conditions, autoregulatory mechanisms attempt to maintain renal blood flow constant during hypotension by decreasing renal vascular resistance. With regard to endotoxemia, the altered renal function has been attributed predominantly to renal hypoperfusion, and it has been suggested that the reduction of GFR and perfusion pressure are secondary to the decline in renal blood flow after induction of sepsis or endotoxemia (36). Thus, one may argue that a possible strong hypotensive effect of LPS in our study negatively influences renal function. Indeed, it has been found, that an intravenous dose of 2 mg/kg LPS reduces blood pressure from 106 mmHg to about 75 mmHg 6h after the injection of LPS in conscious freely moving mice (66). Recently, we determined blood pressure in endotoxemic mice by radio-telemetry. In this study, we found that one hour after intraperitoneal injection of LPS at a dose of 3 mg/kg MAP dropped significantly from 104 to 93 mmHg. However, during the following two to three hours, MAP recovered in the wildtype mice (48). The different route of administration may explain the discrepancies obtained in conscious mice. Further, one may argue that the two different COX inhibitors have different effects on blood pressure in response to LPS. However, it has recently been found, that the early decrease in blood pressure was attenuated

by pretreatment with either SC-560 or SC-236 in rats treated with LPS at a dose of 1 mg/kg i.v. (67). Although we did not measure renal blood flow, one may speculate from our findings that COX-1 inhibition, like TxS inhibition and TP receptor antagonism, may also improve renal blood flow. In line with this assumption, it has been found that SC-560 improved renal blood flow after renal ischemia and reperfusion (11). In contrast to COX-1 inhibition, inhibition of COX-2 enhanced the effect of LPS on GFR, suggesting that COX-2 produces mainly vasodilatory prostanoids, which are of importance to maintain GFR by opposing vasoconstrictive influences. The role of COX-2-derived prostanoids for the LPS-induced decrease in GFR is somewhat confusing. We recently found that the COX-2 inhibitor parecoxib ameliorated the LPS-induced decrease in creatinine clearance in rats, probably because of the attenuation of LPS-induced hypotension (30, 32), whereas others did not observe an effect of COX-2 inhibition on plasma urea and plasma creatinine levels (41). The discrepancies may be due to the higher doses of LPS used in previous studies, differences in fluid infusion, the time point of the determination, and the determination in conscious or anaesthetized animals, for example. Moreover, it has recently been demonstrated that a reduced production of creatinine limits its use as marker of kidney injury in sepsis (20). Since nonselective cyclooxygenase inhibition is expected to decrease both vasodilatory and vasoconstrictive derivatives of arachidonic acid metabolism, our data may also explain why nonselective cyclooxygenase inhibition fails to improve renal function in human sepsis (9, 27). Together with previous studies, our data support therefore a role for TxA_2 in the development of AKI in response to LPS. However, it should be noted that this study was not designed to specifically address this issue.

In summary, we found that inhibition of COX-1 during basal conditions has a minor role on glomerular filtration rate. However, inhibition of COX-1 ameliorates the reduction in GFR in response to endotoxemia. This effect may result from a decrease in the formation of TxA_2 via

COX-1. Thus inhibition of COX-1, but not of $P2Y_{12}$ receptors, may be a potential preventive intervention for sepsis-induced AKI.

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Disclosures

No conflicts of interest, financial or otherwise, are declared by the author(s).

Authors´ contributions

KM, HC and KH designed the study. KM, MM and KH performed the animal experiments and analyzed the data. KH finalized the manuscript. KM, HC and KH drafted the manuscript. All authors approved the final version of the manuscript.

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Figure legends

Fig. 1. Time-dependent effects of endotoxemia.

A) Thrombocyte count and B) plasma thromboxane (Tx) B_2 levels and renocortical TxB_2 concentration at 4, 8 and 16 hours after the injection of lipopolysaccharide (LPS; 3 mg/kg; i.p.). C) The expression of cyclooxygenase (COX)-1, COX-2 and thromboxane synthase (TxS) was determined 4, 8 and 16 hours after the injection of LPS (Insets show representative immunoblots for cyclooxygenase (COX)-1, COX-2 and thromboxane synthase (TxS). The COX-2 blot is composed of two pieces: basal COX-2 protein expression was determined with 100μg of total protein per sample and LPS-induced COX-2 protein expression was determined with 20μg of total protein per sample.) D) Renocortical arachidonic acid (AA) concentration at 4, 8 and 16 hours after the injection of LPS. Values are mean±s.e.m. for six animals. ★$P<0.05$ vs LPS.

Fig. 2. Distribution of TxS, COX-1 and COX-2 in mouse renal cortex.

(A) TxS immunoreactivity in renal cortex of LPS-treated mice. Arrows indicate labeling of renal vasculature. (B) COX-1 immunoreactivity in renal cortex of LPS-treated mice. #, labeling of renal tubule; *, labeling of cortical interstitial cells; arrows indicate labeling of renal arteries (RA) or renal veins (RV). (C) COX-2 immunoreactivity in renal cortex of LPS-treated mice. Immunoreactivity from endotoxemic mice was determined 4h after the injection of lipopolysaccharide (LPS; 3 mg/kg; i.p.). Arrows indicate labeling of renal veins (RV); *, labeling of cortical interstitial cells. Approximate magnification: x100 (A) or x400 (B, C)

Fig. 3. Effect of COX inhibition on thromboxane formation and on platelet count.

A) Plasma thromboxane (Tx) B_2 concentration, B) renocortical TxB_2 levels and C) platelet count were determined 4h after the injection of lipopolysaccharide (LPS; 3 mg/kg; i.p.) in

mice treated with or without the COX-1 inhibitor SC-560 (20 mg/kg; i.p.) or the COX-2 inhibitor SC236 (10 mg/kg; i.p.). Values are mean±s.e.m. for 6-12 animals per group. ★P<0.05 vs pre-treatment or vehicle-treated controls. #P<0.05 vs LPS.

Fig. 4. Effect of COX-1 and COX-2 inhibition on glomerular filtration rate and plasma urea concentration.

Glomerular filtration rate (GFR) and plasma urea concentration were determined four hours after injection of lipopolysaccharide (LPS; 3 mg/kg; i.p.) in mice treated with or without the COX-1 inhibitor SC-560 (20 mg/kg; i.p.) or the COX-2 inhibitor SC-236 (10 mg/kg; i.p.). A) GFR related to body weight, and B) effect of SC-560 and SC-236 on plasma urea concentration. Values are mean±s.e.m. for 6-12 animals per group. ★P<0.05 vs pre-treatment or vehicle-treated controls. #P<0.05 vs LPS.

Fig. 5. Effect of clopidogrel pretreatment on platelet count, plasma thromboxane B_2 levels and on GFR.

Clopidogrel (10 mg/kg x d; orally) was administered 48, 24 and 2 hours before injection of lipopolysaccharide (LPS; 3 mg/kg; i.p.). A) Thrombocyte count, B) plasma thromboxane (Tx) B_2 levels and C) glomerular filtration rate (GFR) 4 hours after injection of LPS. Values are mean±s.e.m. for six animals. ★P<0.05 vs LPS.

Fig. 6. Effect of SC-560 on renal IL-1β and TNFα mRNA expression.

The renal expression of IL-1β and TNFα mRNA was determined four hours after injection of lipopolysaccharide (LPS; 3 mg/kg; i.p.). Effect of the COX-1 inhibitor SC-560 (20 mg/kg; i.p.) on A) renal IL-1β mRNA expression related to β-actin mRNA expression and B) on renal TNFα mRNA expression related to β-actin mRNA expression. Values are mean±s.e.m. for six animals. ★P<0.05 vs vehicle-treated controls.

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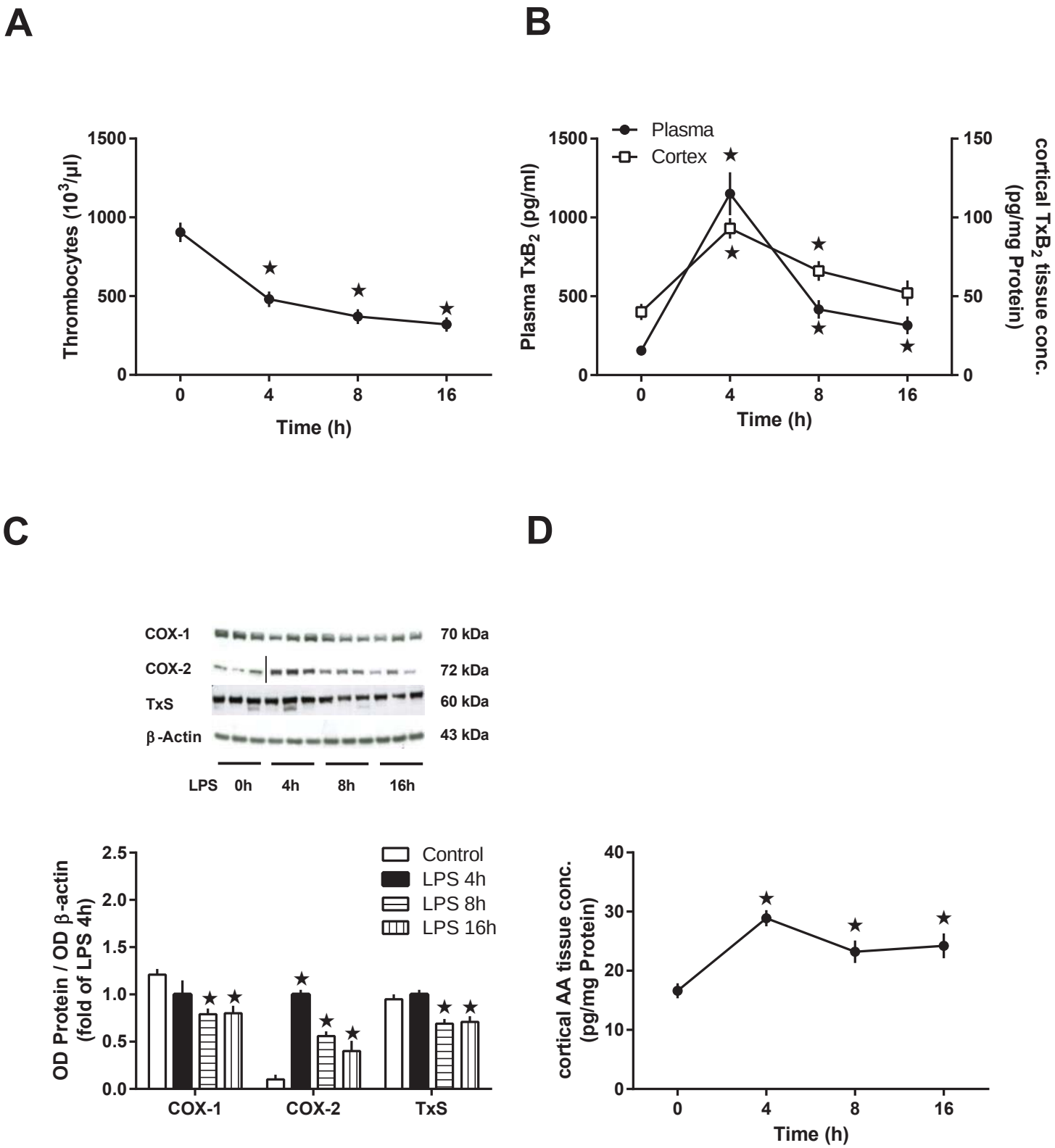
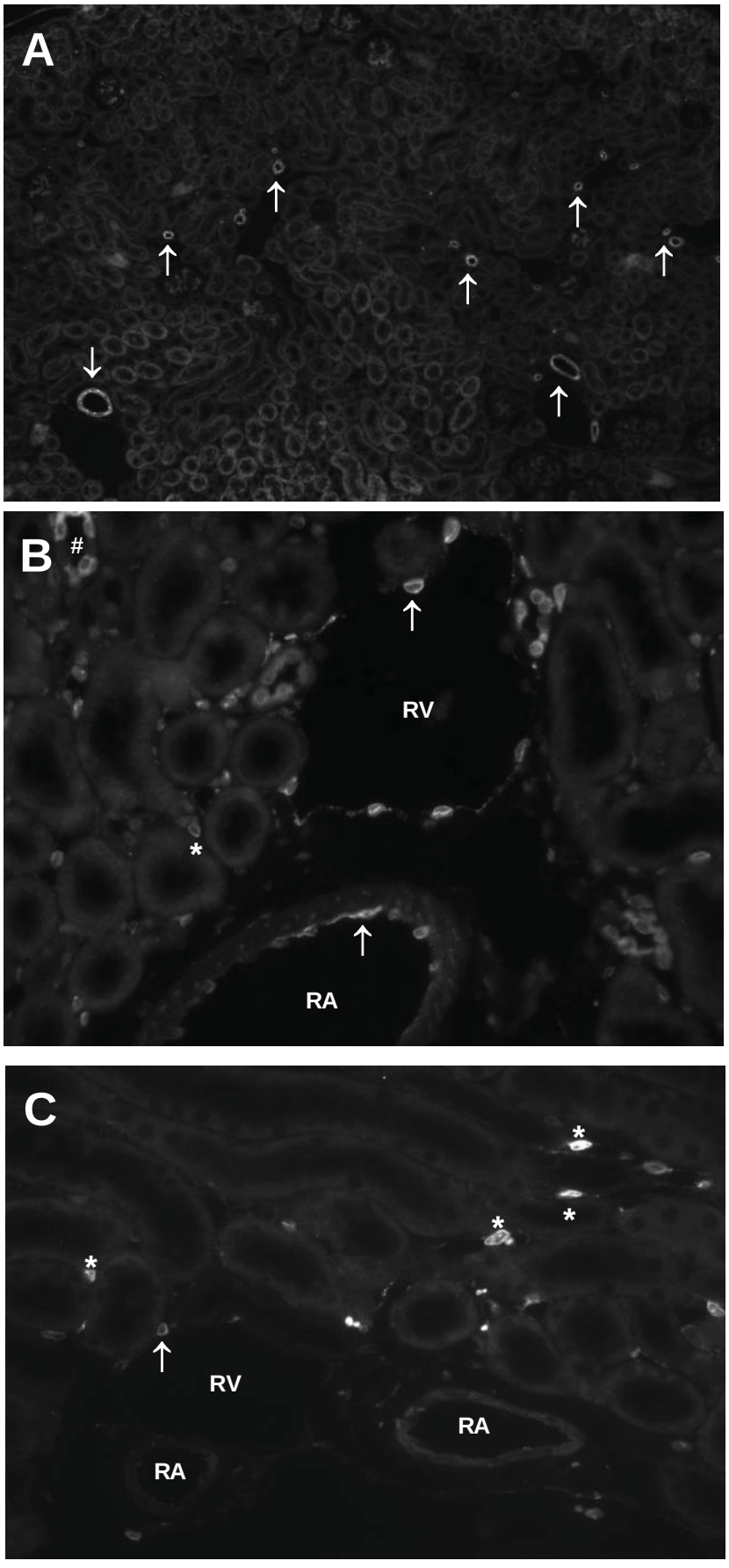

A
Thrombocytes (10^3/µl)
1500
1000
500
0
0
4
8
16
Time (h)
B
Plasma
Cortex
Plasma TxB2 (pg/ml)
cortical TxB2 tissue conc. (pg/mg Protein)
1500
1000
500
0
150
100
50
0
0
4
8
16
Time (h)
C
COX-1
COX-2
TxS
β-Actin
70 kDa
72 kDa
60 kDa
43 kDa
LPS 0h 4h 8h 16h
OD Protein / OD β-actin (fold of LPS 4h)
2.5
2.0
1.5
1.0
0.5
0.0
Control
LPS 4h
LPS 8h
LPS 16h
COX-1
COX-2
TxS
D
cortical AA tissue conc. (pg/mg Protein)
40
30
20
10
0
0
4
8
16
Time (h)


A
B
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RV
*
RA
C
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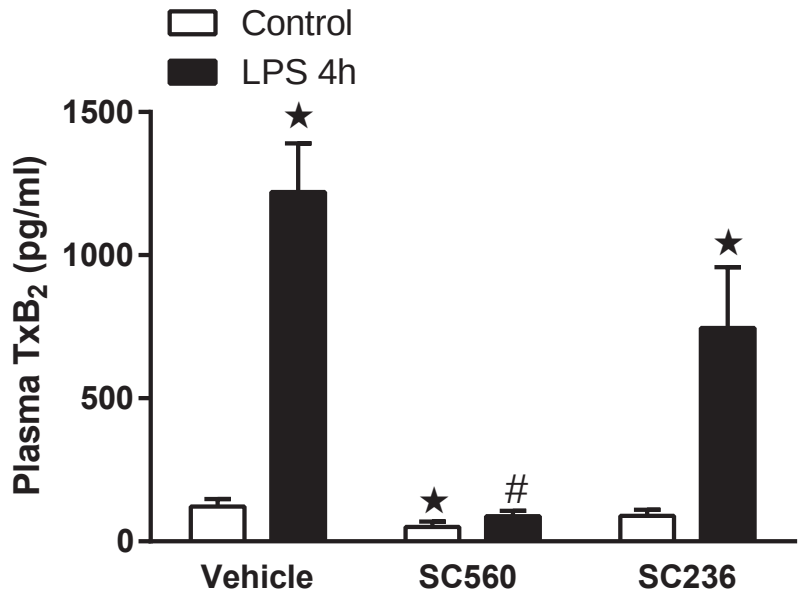
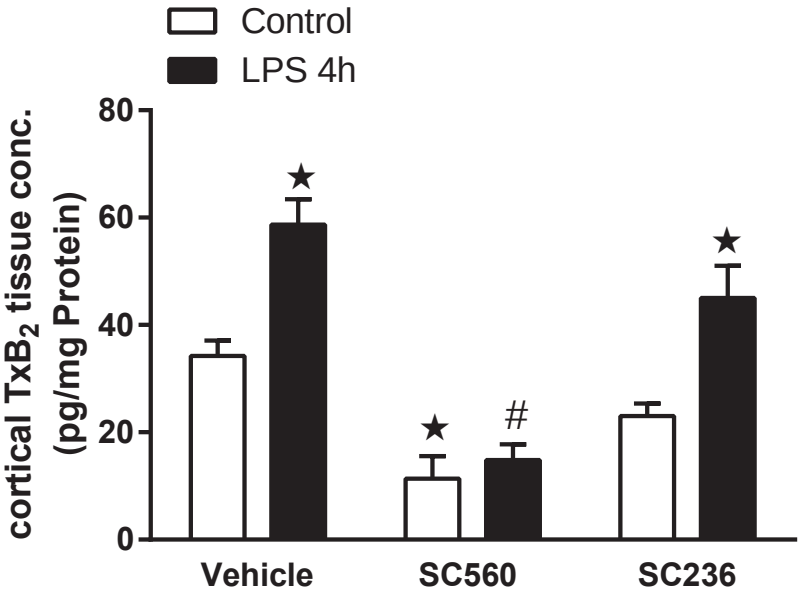
*
*
RV
RA
RA

A


Control
LPS 4h
Plasma TxB2 (pg/ml)
1500
1000
500
0
Vehicle
SC560
SC236

B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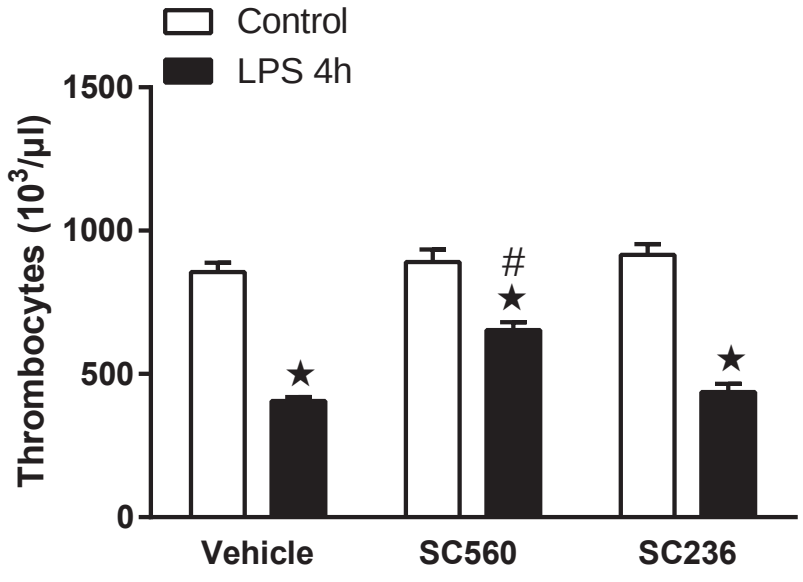
cortical TxB2 tissue conc. (pg/mg Protein)
80
60
40
20
0
Vehicle
SC560
SC236

C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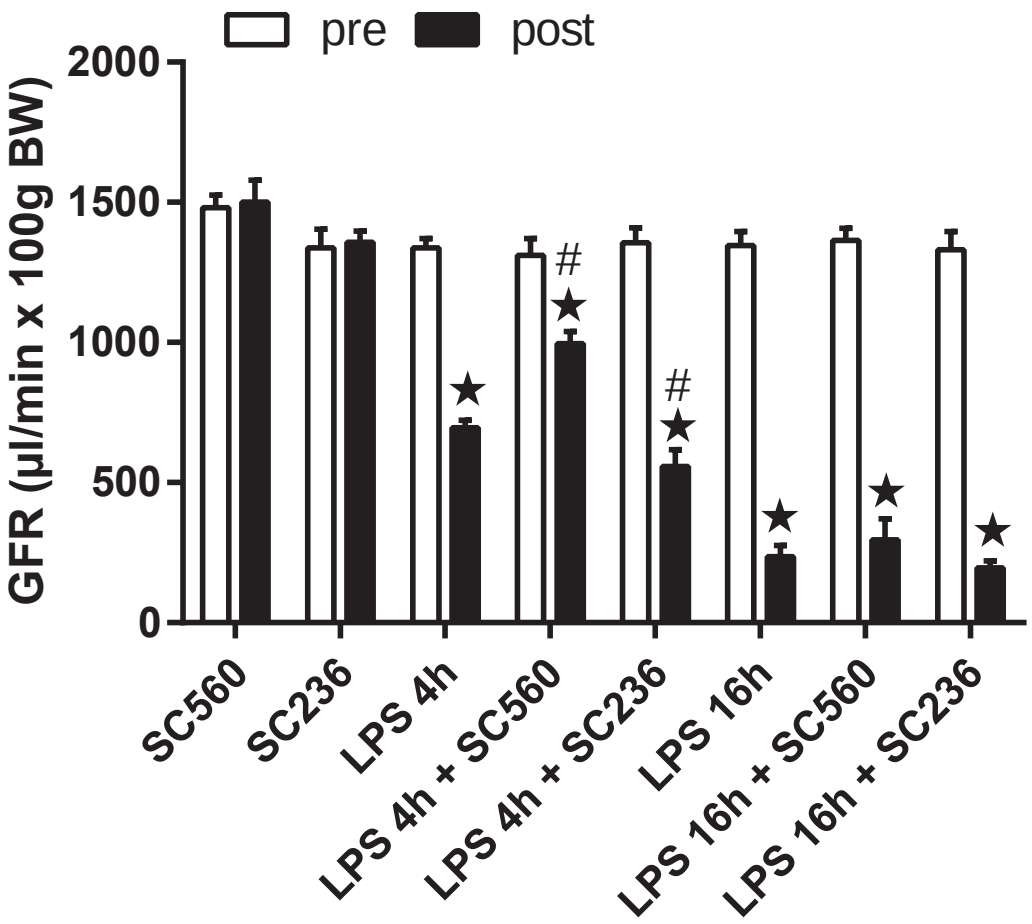
LPS 4h
Thrombocytes (10^3/µl)
1500
1000
500
0
Vehicle
SC560
SC236

A


pre
post
GFR (µl/min x 100g BW)
2000
1500
1000
500
0
SC560
SC236
LPS 4h
LPS 4h + SC560
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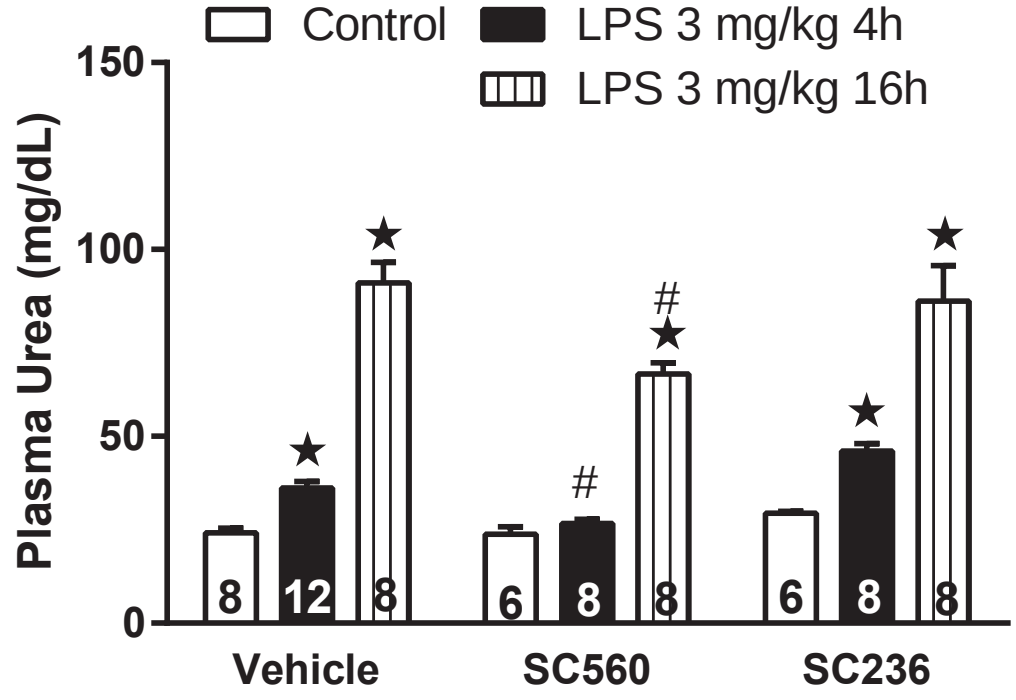
LPS 16h + SC560
LPS 16h + SC236

B


Control
LPS 3 mg/kg 4h
LPS 3 mg/kg 16h
Plasma Urea (mg/dL)
150
100
50
0
8
12
8
6
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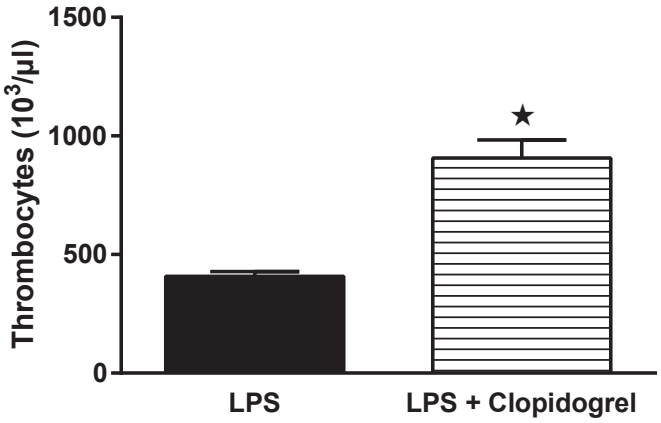
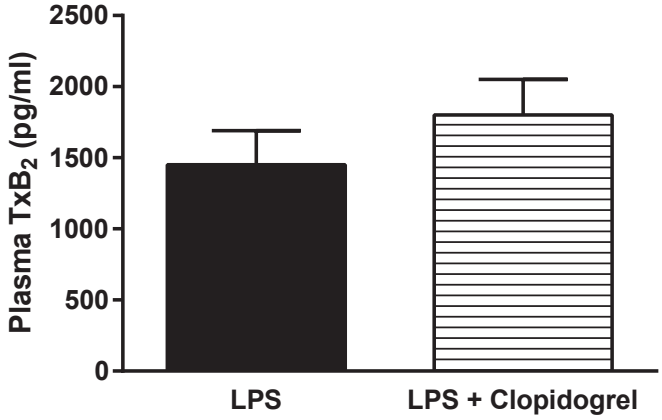
8
6
8
8
Vehicle
SC560
SC236

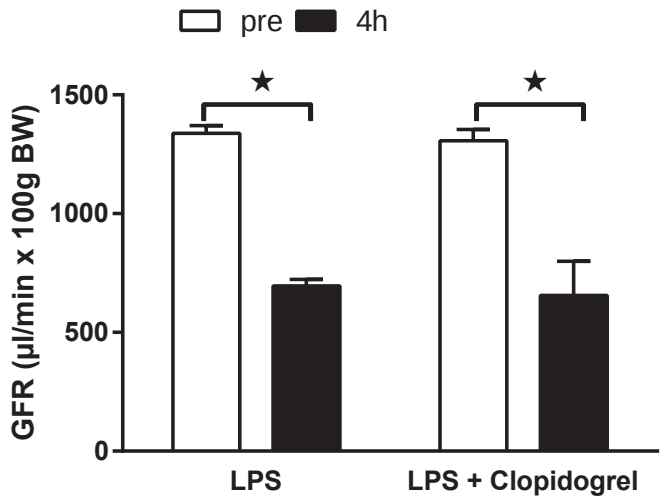
A

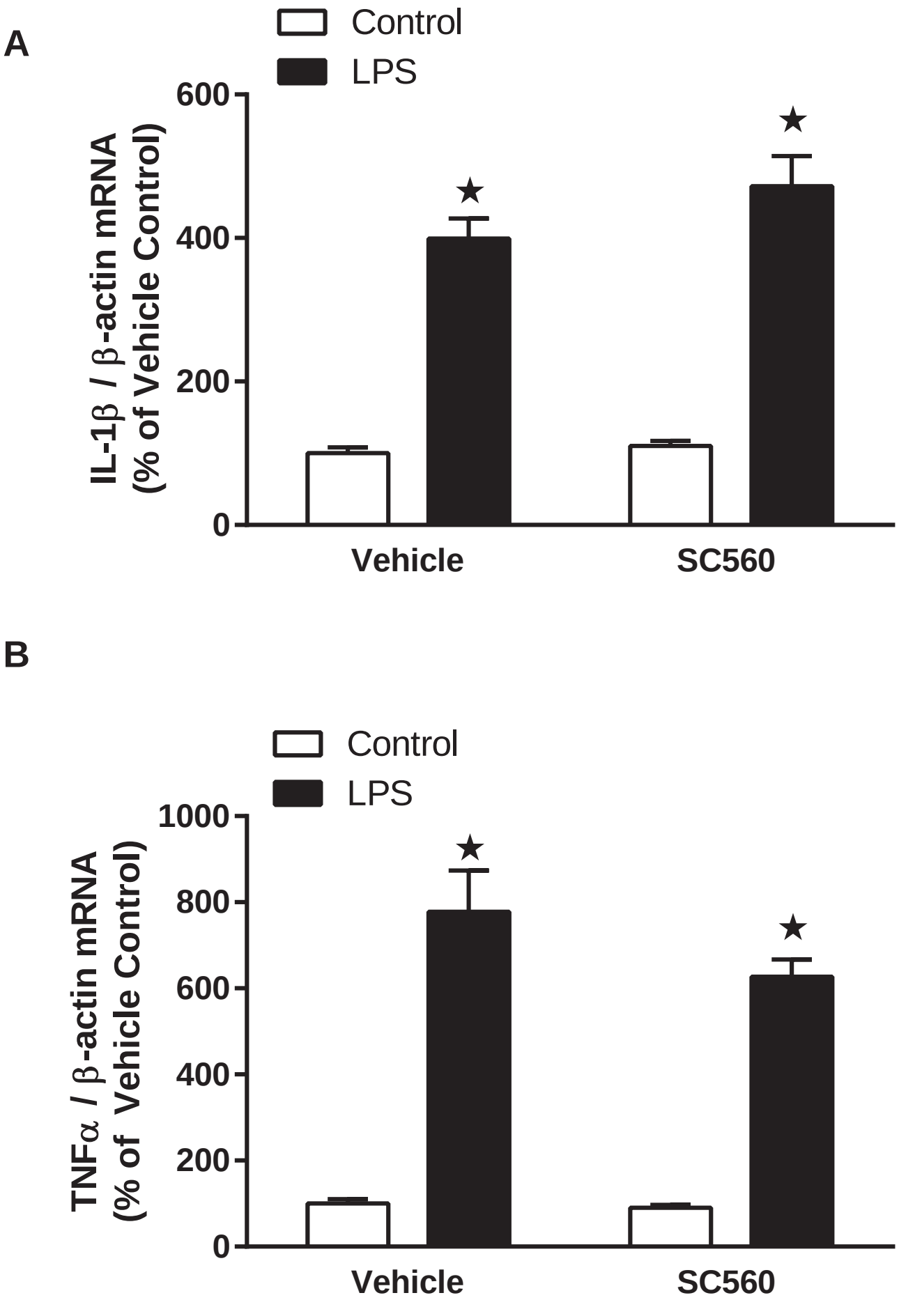

1500
1000
500
0
Thrombocytes (10³/µl)
★
LPS
LPS + Clopidogrel

B


2500
2000
1500
1000
500
0
Plasma TxB2 (pg/ml)
LPS
LPS + Clopidogrel

C


pre
4h
★
★
1500
1000
500
0
GFR (µl/min x 100g BW)
LPS
LPS + Clopidogrel


A
Control
LPS
IL-1β / β-actin mRNA
(% of Vehicle Control)
600
400
200
0
Vehicle
SC560
B
Control
LPS
TNFα / β-actin mRNA
(% of Vehicle Control)
1000
800
600
400
200
0
Vehicle
SC560