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Effects of a human recombinant alkaline phosphatase on renal hemodynamics, oxygenation and inflammation in two models of acute kidney injury

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A B S T R A C T

Two small clinical trials indicated that administration of bovine intestinal alkaline phosphatase (AP) improves renal function in critically ill patients with sepsis-associated acute kidney injury (AKI), for which the mechanism of action is not completely understood. Here, we investigated the effects of a newly developed human recombinant AP (recAP) on renal oxygenation and hemodynamics and prevention of kidney damage and inflammation in two in vivo AKI models. To induce AKI, male Wistar rats ($n = 18$) were subjected to renal ischemia (30 min) and reperfusion (I/R), or sham-operated. In a second model, rats ($n = 18$) received a 30 min infusion of lipopolysaccharide (LPS; 2.5 mg/kg), or saline, and fluid resuscitation. In both models, recAP (1000 U/kg) was administered intravenously (15 min before reperfusion, or 90 min after LPS). Following recAP treatment, I/R-induced changes in renal blood flow, renal vascular resistance and oxygen delivery at early, and cortical microvascular oxygen tension at late reperfusion were no longer significantly affected. RecAP did not influence I/R-induced effects on mean arterial pressure. During endotoxemia, recAP treatment did not modulate the LPS-induced changes in systemic hemodynamics and renal oxygenation. In both models, recAP did exert a clear renal protective anti-inflammatory effect, demonstrated by attenuated immunostaining of inflammatory, tubular injury and pro-apoptosis markers. Whether this renal protective effect is sufficient to improve outcome of patients suffering from sepsis-associated AKI is being investigated in a large clinical trial.

1. Introduction

Sepsis-associated acute kidney injury (AKI) is a serious complication in critically ill patients and is accompanied by high morbidity and mortality rates (Bagshaw et al., 2008). Treatment is limited to supportive care, as up to now pharmacological interventions are not available. The current lack of treatment options appears primarily due to the multifactorial pathogenesis of AKI, in which inflammation, hemodynamic instability, renal microcirculatory dysfunction and unbalanced renal bioenergetics play pivotal roles (Peters et al., 2014). Sepsis is accompanied by the systemic release of pro-inflammatory mediators, activation of complement and coagulation pathways, and endothelial leukocyte adhesion and extravasation, thereby promoting inflammation (Gustot, 2011). Within the kidney, the vascular endothelium is directly exposed to pathogens and danger-associated molecular patterns that induce a local inflammatory response. The release of harmful mediators like cytokines, reactive oxygen (ROS) and nitrogen species (RNS) characterize this response which, together with endothelial swelling and arteriolar vasoconstriction, leads to a compromised renal microcirculation and renal hypoxia (Ince, 2005). However, the role of renal vasoconstriction and a reduced renal blood flow (RBF) is still under debate since animal and human data suggest that during sepsis, AKI can develop in the setting of sustained or even increased renal blood flow (RBF) (Langenberg et al., 2005; Langenberg et al., 2006). This is in contrast to renal ischemia/reperfusion (I/R) injury, during which hemodynamic instability,

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hypoperfusion and a dysbalance of renal oxygen delivery and oxygen consumption may play key roles (Thurman, 2007; Legrand et al., 2008).

Considering its complex pathogenesis, a new treatment option for AKI should be able to modulate the various processes involved in disease development. One of a limited number of candidate drugs that might facilitate such a multimodal approach is the enzyme alkaline phosphatase (AP). According to two patient studies, treatment with bovine intestinal AP increased endogenous creatinine clearance, reduced the need for and duration of renal replacement therapy and decreased the urinary excretion of renal injury markers during sepsis-associated AKI (Heemskerk et al., 2009; Pickkers et al., 2012). Following these promising results, a human recombinant AP (recAP) was developed, which is both highly stable and biologically active (Kiffer-Moreira et al., 2014).

The possible mechanism of action that accounts for the renal protective effects of AP is still not completely understood and requires further investigation. Recently, using human renal proximal tubule cells it was demonstrated that recAP exerts protective effects through dephosphorylation of endotoxin (LPS, lipopolysaccharide), part of the outer membrane of gram-negative bacteria involved in sepsis pathogenesis, as well as by dephosphorylation of detrimental purines adenosine triphosphate (ATP) and adenosine diphosphate (ADP), released during inflammation (Peters et al., 2015). These results offer a plausible explanation of the anti-inflammatory effects of recAP locally in the kidney. Considering the various processes involved in the development of sepsis-associated AKI, we now investigated whether recAP can modulate renal microcirculatory dysfunction and hemodynamic changes following I/R- or endotoxin-induced AKI.

2. Material and methods

2.1. Animals

All experiments in this study were approved and reviewed by the Animal Research Committee of the Academic Medical Center at the University of Amsterdam (DFL 102669). Care and handling of the animals were in accordance with the guidelines for Institutional and Animal Care and Use Committees (NIH Publication 85-23, 1985). Experiments were performed with 36 male Wistar rats (Harlan, Horst, The Netherlands) with a mean ± SD body weight of 402 ± 74 g.

2.2. Anesthesia and surgical preparation

Rats were anesthetized and instrumented as described previously (Ergin et al., 2015a; Aksu et al., 2015). Rats received an intraperitoneal injection of 90 mg/kg b.w. ketamine (Nimatek®; Eurovet, Bladel, The Netherlands), 0.5 mg/kg b.w. medetomidine (Domitor®; Pfizer, New York, NY) and 0.05 mg/kg b.w. atropine-sulfate (Centrafarm, Etten-Leur, The Netherlands). After tracheotomy, rats were mechanically ventilated with a fraction of inspired O_2 (FiO_2) of 0.4. Body temperature was maintained at 37 ± 0.5 °C during the entire experiment using a heating pad. The ventilator settings were adjusted to maintain an end-tidal partial pressure of carbon dioxide (PCO_2) between 30 and 35 mmHg and an arterial PCO_2 between 35 and 40 mmHg.

Blood vessels were cannulated with polyethylene catheters (outer diameter 0.9 mm; Braun, Melsungen, Germany). A catheter in the right carotid artery was connected to a pressure transducer to monitor mean arterial pressure (MAP). The right jugular vein was cannulated for continuous infusion of Ringer's lactate (Baxter, Utrecht, The Netherlands) at a rate of 15 mL/kg/h and maintenance anesthesia (50 mg/kg ketamine dissolved in Ringer's lactate, 5 mL/kg/h). The right femoral artery was cannulated for blood sampling, and the right femoral vein was cannulated to enable fluid resuscitation, oxyphor G2, recAP and LPS administration.

The left kidney was exposed, decapsulated, and immobilized in a Lucite kidney cup (K. Effenberger, Pfeffingen, Germany) via an ~4 cm incision of the left flank. Renal vessels were carefully separated under preservation of nerves and adrenal gland. A perivascular ultrasonic transient time flow probe (type 0.7 RB; Transonic Systems Inc., Ithaca, NY) was placed around the left renal artery and connected to a flow meter (T206; Transonic Systems Inc.) to allow continuous measurement of RBF.

After the surgical protocol (approximately 60 min) one optical fiber was placed 1 mm above the decapsulated kidney and another optical fiber 1 mm above the renal vein to measure oxygenation using the phosphorescence decay time technique (as described below). A small piece of aluminum foil was placed on the dorsal site of the renal vein to prevent contribution of underlying tissue to the phosphorescence signal in the venous partial pressure of oxygen (PO_2) measurements. The operation field was covered with a humidified gauze compress throughout the entire experiment to prevent desiccation of the tissue exposed. At the end of the experiments, animals were sacrificed by systemic administration of 1 mL/kg 20% pentobarbital sodium (Euthasol; AST Farma, Oudewater, The Netherlands) through the femoral vein. Kidneys were harvested, weighed, and sectioned into two parts. One part was fixed in 10% buffered formalin for immunohistochemical analysis and the other part of the kidney was snap frozen in liquid nitrogen and stored at −80 °C before homogenization.

2.3. Ischemia/reperfusion protocol

Instrumented rats (369 ± 72 g) were randomized and divided into two groups: I/R (n = 12) or sham control (n = 6). Rats were subjected to renal ischemia for 30 min by supra-renal aortic occlusion with a custom-made vascular occluder, followed by 2.5 h reperfusion as described previously (Ergin et al., 2015a). The vascular occluder was placed on the aorta above the renal arteries, and beneath the mesenteric artery. The sham control group was instrumented and surgically prepared without subsequent cross-clamping of the aorta. Subsequently, ischemic rats were randomized to receive an intravenous bolus injection in the femoral vein with 1000 U/kg b.w. recAP (n = 6) or saline (n = 6) 15 min after initiation of ischemia (Supplemental Fig. 1A). Previous dose-response experiments in different animal models indicated this dose to be optimal to modulate AKI (not shown). The sham control group received saline. Arterial blood samples (0.5 mL) were taken at 1) before aortic occlusion (T0), 2) 15 min after reperfusion (T15), and 3) 150 min after reperfusion (T150). Blood samples were replaced by the same volume of 6% HES130/0.4 (Voluven®; Fresenius Kabi Nederland B.V., Schelle, Belgium).

2.4. Endotoxemia protocol

Endotoxemia was induced on fully instrumented rats (430 ± 65 g) as described previously (Legrand et al., 2011). Rats were randomized at baseline (T0) to receive an intravenous 30 min infusion of 2.5 mg/kg b.w. LPS (*E. coli* 0127:B8; Sigma-Aldrich, Paris, France; n = 12), or placebo (saline; n = 6). Fluid resuscitation was performed with 20 mL/kg/h 6% HES130/0.4 and continued after cessation of the LPS infusion for a period of 1 h. After this period, LPS-treated groups were randomized to receive either an intravenous infusion of recAP (1000 U/kg b.w.) or saline (n = 6 per group) over 30 min. Control groups received saline. Arterial blood (0.5 mL) was sampled at 1) baseline (T0), 2) 90 min after start of LPS administration (T1.5), 3) 120 min post LPS (T2; 30 min after start of recAP treatment), and 4) 4.5 h post-LPS (T4.5; 3 h after recAP infusion) (Supplemental Fig. 1B). Each blood sample drawn was replaced by the same volume of 6% HES130/0.4.

2.5. Phosphorimetric measurement of renal microvascular and venous PO_2

The oxygen-quenched phosphorescent decay time of a systemically infused albumin-targeted phosphorescent dye (Oxyphor G2; Oxygen Enterprises, Ltd. Philadelphia, PA) was used for measuring the microvascular oxygen tension in the renal cortex (Cortical μPO_2), outer medulla (Medullar μPO_2) and renal venous oxygen tension ($P_{rv}O_2$). Oxyphor G2 was infused (6 mg/kg b.w.) intravenously for 15 min followed by 30 min stabilization time. Oxyphor G2 has two excitation peaks (440 and 632 nm) and one emission peak (800 nm), which allowed simultaneous decay time measurements in the kidney cortex and the outer medulla due to different optical penetration depths of the light excitated by dual-wavelength phosphorimetry (Johannes et al., 2006). For the measurement of $P_{rv}O_2$, a mono-wavelength frequency-domain phosphorimeter was used (Mik et al., 2008). Phosphorescence was quenched by energy transfer to oxygen, resulting in shortening of the phosphorescence decay time. PO_2 was calculated by determining the correlation between reciprocal phosphorescence decay time and oxygen tension (Stern-Volmer relation) (Bezemer et al., 2010). Decay curves of phosphorescent intensity were analyzed using software programmed in Labview 6.1 (National Instruments, Austin, TX).

2.6. Renal vascular resistance and derivative oxygen parameters

Blood samples were used to determine blood gas values (ABL505 blood gas analyzer; Radiometer, Copenhagen, Denmark) as well as hemoglobin concentration and hemoglobin oxygen saturation. Renal vascular resistance (RVR) was estimated by the formula MAP / RBF × 100. Renal oxygen delivery (DO_2) was determined by the formula RBF × arterial oxygen content (C_aO_2 = hemoglobin × 1.31 × S_aO_2 + 0.003 × P_aO_2), where S_aO_2 is the arterial oxygen saturation and P_aO_2 the arterial partial oxygen pressure. Renal oxygen consumption (VO_2) was determined by the formula RBF × ($C_aO_2 - C_vO_2$), where renal venous oxygen content (C_vO_2) was calculated as hemoglobin × 1.31 × $S_{rv}O_2$ + 0.003 × $P_{rv}O_2$. The $S_{rv}O_2$ (renal venous O_2 saturation) was estimated with the Hill equation, with P50 = 37 Torr (4.9 kPa) and Hill coefficient = 2.7. The renal oxygen extraction ratio (ER) was estimated as VO_2 / DO_2 × 100.

2.7. NO metabolism

Kidney tissue samples were homogenized in ice-cold 5 mM sodium phosphate buffer using the Heidolph homogenizer DIAX 900 (Sigma-Aldrich, St. Louis, MO). Homogenates were centrifuged at 12,000*g* for 15 min at 4 °C, and supernatant was used for NO determination. The index of total NO production is the sum of NO and its reaction products nitrite, nitrate and nitrosothiols accumulated in tissue samples. The reducing agent used for total NO analysis was a saturated solution of vanadium (III) chloride (VCL_3) in 1 M HCL. At a temperature of 90 °C, VCL_3 reagent quantitatively converts all reaction products into NO in a glass vial vessel. NO is flushed out of the reaction vessel by flow of helium gas, which is then measured by the NO chemiluminescense signal analyzer (Sievers 280i, GE Analytical Instruments, Manchester, United Kingdom) as the amount of light from the ozone-NO reaction in the NO measurement chamber. NO tissue protein content was expressed as NO release per g protein, determined by the Bradford assay (ThermoFisher Scientific, Chicago, IL).

2.8. Oxidative stress, inflammatory cytokines and AP

Supernatant of kidney homogenates were obtained as described above. Supernatant was used to measure tissue malondialdehyde (MDA) levels to quantify oxidative stress by assessing lipid peroxidation by tandem mass spectrometry as described previously (Ergin et al., 2015a). Lipid peroxide content was expressed as MDA content per g protein, determined by the Bradford assay. TNF-α and IL-6 were determined by ELISA (DY510, DY506; R&D Systems, Minneapolis, MN).

2.9. Immunohistochemical analysis

After fixation in 10% formalin, tissue was processed and embedded in paraffin. Kidney sections (5 μm) were deparaffinized, antigen retrieval was accomplished using citrate buffer (pH 6.0) and endogenous peroxidase activity was blocked using 3% H_2O_2. Sections were incubated o/n at 4 °C with antibody rabbit anti-rat inducible nitric oxide synthase (iNOS) (RB-1605-P; NeoMarkers Fremont, CA) or rabbit-anti-human IL-6 (ab6672; Cambridge, UK; cross-reactive to rat), or incubated for 1 h at RT with antibody rabbit anti-rat myeloperoxidase (MPO) (RB-373-A; NeoMarkers), rabbit anti-human Lipocalin-2 (NGAL, neutrophil gelatinase-associated lipocalin) (ab41105; Abcam; cross-reactive to rat), rabbit-anti-rat liver-type fatty acid binding protein (L-FABP) (HP8010; Hycult Biotect, Uden, The Netherlands), or rabbit-anti-rat Bcl-2-associated X protein (Bax) (4 μm; sc-493; Santa Cruz, Heidelberg, Germany). Antibodies were used at a 1:100 dilution in UltraAb Diluent (TA-125-UD; LabVision, Thermo Scientific, Chicago, Illinois), or 1:50 in case of Bax. Subsequently, sections were stained with secondary biotinylated goat-anti-rabbit antibody (1:100; TR-125-BN; LabVision) for 30 min at RT. Immunostaining was visualized with AEC substrate (TA-007-HAC; LabVision), followed by hematoxylin counterstain. Both intensity and distribution were scored in a semiquantitative fashion. Positively stained cells were counted and graded according to staining intensity: 0 (no staining), 1+ (weak but detectable above control, 2+ (distinct), 3+ (intensive). A histological score (HSCORE) value was derived by counting cumulative percentages of cells that stained at each intensity multiplied by the weighted intensity of the staining (HSCORE = $\sum$ Pi (i + 1), where i is the intensity score and Pi is the corresponding percentage of the cells) (Senturk et al., 1999). MPO was scored in 240 selected glomeruli and peritubular areas in each group using a binary scoring system; 1 if leukocytes were observed, 0 if not. Scoring was performed by investigators blinded to the treatment protocol.

2.10. Statistical analysis

Normal distribution of the data could not be assumed due to the small sample size. Therefore, data is expressed as median [25th percentile, 75th percentile]. Significant differences within groups were estimated using Friedman test with Dunn's post-test, differences between groups were estimated using two-way repeated measures ANOVA with Bonferonni's post-test on log-transformed data. Chi-square test was used to analyze binary outcomes. A two-sided p-value <0.05 was considered statistically significant. All tests were performed with Graphpad Prism 5.00 for Windows (Graphpad Software Inc., La Jolla, CA).

3. Results

3.1. RecAP prevents I/R-induced alterations of renal hemodynamics immediately following reperfusion

The renal protective effect of recAP was investigated during I/R-induced AKI in rats, performed by 30 min suprarenal aortic clamping followed by a 2.5 h reperfusion period (Supplemental Fig. 1A). MAP was reduced at the end of reperfusion period in all groups compared to baseline ($p < 0.05$), which was more pronounced in the I/R group, and not affected by recAP treatment (Fig. 1A), administered 15 min preceding reperfusion. Immediately following reperfusion (T15), recAP treatment attenuated the detrimental effect of I/R on RVR, and I/R-induced changes in RBF were no longer significantly affected. At 2.5 h post-reperfusion (T150), RBF was impaired in all three groups compared to baseline (T0) (Fig. 1A) and to a greater extent in the I/R groups. Regarding renal oxygenation (Table 1), both renal oxygen delivery (DO_2) and oxygen consumption (VO_2) were significantly reduced post-reperfusion compared to baseline. Following recAP treatment,

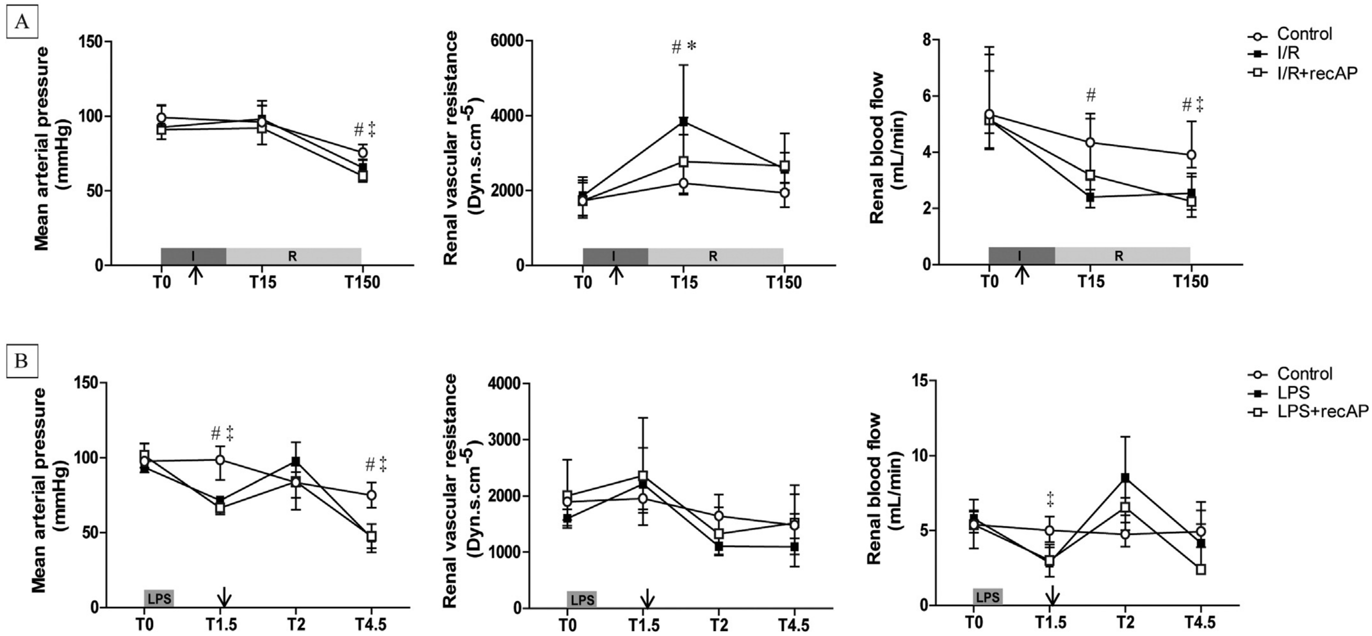

Fig. 1. Hemodynamic parameters during I/R-induced AKI (A) or LPS-induced AKI (B). A) Parameters were determined at baseline (T0) and after 15 min (T15) and 150 min (T150) reperfusion. RecAP was administered 15 min preceding reperfusion. B) Parameters were determined at baseline (T0) and 1.5 (T1.5), 2 (T2) and 4.5 h (T4.5) after start of LPS infusion, which was followed by fluid resuscitation until T1.5. RecAP was administered directly after T1.5. Data is expressed as median [25th percentile, 75th percentile]. Significant differences were estimated using two-way repeated measures ANOVA with Bonferonni's post-test on log-transformed data ($n = 6$ per group). #$p < 0.05$ control vs. I/R or control vs. LPS, *$p < 0.05$ I/R vs. I/R + recAP, ‡$p < 0.05$ control vs. I/R + recAP or control vs. LPS + recAP. I/R, ischemia/reperfusion; LPS, lipopolysaccharide, recAP, human recombinant alkaline phosphatase.

this was not observed immediately following reperfusion (T15). The oxygen extraction ratio (ER%) increased in all three groups at the end of reperfusion, with no differences between groups. While cortical microvascular oxygen tension (μPO_2) was impaired in both I/R groups at the end of reperfusion, recAP treatment significantly improved this parameter compared to the I/R group. Medullar μPO_2 was decreased in all groups after the 2.5 h reperfusion period compared to baseline, and remained unaffected by recAP administration.

3.2. RecAP treatment prevents I/R injury-induced renal inflammation

Renal MDA and NO levels, markers of oxidative and nitrosative stress respectively, were not affected by I/R itself or by I/R + recAP treatment (Table 2). However, immunohistochemistry demonstrated that recAP treatment attenuated iNOS staining, representing nitrosative stress, which was not significantly increased by I/R injury alone (Fig. 2). The renal content of pro-inflammatory mediators TNF-α and IL-6 was significantly increased following I/R injury, which was not observed following recAP treatment (Table 2). Immunohistochemistry data showed that IL-6 tended to be increased by I/R and was significantly reduced in recAP treated kidneys (Fig. 2). As biomarkers of renal injury, staining of L-FABP and proapoptotic protein Bax was not affected by I/R compared to control animals, but was significantly reduced in the recAP-treated kidneys (Fig. 2). NGAL expression was not increased by I/R injury, nor influenced by recAP treatment (data not shown).

Table 1
Renal oxygenation during I/R-induced AKI.

		T0	T15	T150
Cortical μPO_2 (mmHg)	Control	79 [62–89]	76 [69–87]	68 [64–77]
	I/R	80 [75–87]	70 [64–75]	54 [46–60]$^{\ddagger,\#}$
	I/R + recAP	85 [80–86]	79 [67–89]	67 [56–79]‡,*
Medullar μPO_2 (mmHg)	Control	60 [55–66]	56 [50–62]	49 [46–53]‡
	I/R	63 [58–64]	53 [51–58]	44 [39–48]‡
	I/R + recAP	65 [62–68]	54 [51–57]	44 [39–53]‡
DO_2 (mL $O_2 \cdot min^{-1}$)	Control	4.2 [3.9–6.4]	3.4 [2.6–4.5]	3.0 [2.5–3.7]‡
	I/R	4.4 [3.9–5.4]	2.1 [1.7–2.5]$^{\ddagger,\#}$	1.9 [1.6–2.4]$^{\ddagger,\#}$
	I/R + recAP	4.3 [3.5–6.1]	2.7 [2.1–4.0]	1.6 [1.2–2.2]$^{\ddagger,\#}$
VO_2 (mL $O_2 \cdot min^{-1}$)	Control	2.3 [2.0–3.4]	1.9 [1.5–2.1]	1.8 [1.5–2.3]
	I/R	2.4 [2.3–2.7]	1.2 [1.0–1.6]‡	1.2 [1.2–1.7]‡
	I/R + recAP	2.4 [1.7–3.2]	1.6 [1.0–2.2]	0.9 [0.7–1.4]$^{\ddagger,\#}$
ER (%O_2)	Control	52 [46–57]	56 [50–60]	61 [55–63]‡
	I/R	53 [50–62]	60 [56–68]	67 [62–76]‡
	I/R + recAP	52 [49–58]	54 [50–58]	65 [51–71]‡

Parameters were determined at baseline (T0) and after 15 min (T15) and 150 min (T150) reperfusion, which followed a 30 min period of ischemia. RecAP was administered 15 min preceding reperfusion. Data is expressed as median [25th percentile, 75th percentile]. Significant differences within groups were estimated using Friedman test with Dunn's post-test, differences between groups were estimated using two-way repeated measures ANOVA with Bonferonni's post-test on log-transformed data ($n = 6$ per group).

DO_2, oxygen delivery; ER, oxygen extraction ratio; I/R, ischemia-reperfusion; recAP, human recombinant alkaline phosphatase; μPO_2, microvascular oxygen tension; VO_2, oxygen consumption.

‡ $p < 0.05$ compared to baseline within a group.
\# $p < 0.05$ compared to control.
* $p < 0.05$ compared to I/R.

Table 2
Renal tissue levels of inflammatory and oxidative stress during AKI.

	I/R protocol			Endotoxemia protocol		
	Control	I/R	I/R + recAP	Control	LPS	LPS + recAP
MDA (μmol/g)	4.3 [3.4–4.6]	5.5 [4.6–6.0]	4.7 [4.4–5.0]	1.5 [1.1–1.7]	3.0 [2.9–3.7][#]	2.9 [2.1–3.6][#]
NO (μmol/g)	2.4 [1.4–2.9]	3.8 [2.9–4.5]	3.3 [1.9–4.9]	0.9 [0.6–1.1]	2.5 [1.8–3.7][#]	2.3 [1.3–2.5][#]
TNF-α (pg/μg)	1.5 [1.3–2.2]	6.9 [6.4–7.9][#]	5.8 [2.9–6.6]	0.9 [0.8–1.2]	0.8 [0.6–0.8]	0.8 [0.6–0.9]
IL-6 (pg/μg)	5.3 [4.2–6.8]	22.8 [16.9–26.2][#]	18.6 [10.1–21.1]	7.6 [5.7–9.4]	5.7 [4.8–6.9]	5.7 [4.7–6.8]

Data is expressed as median [25th percentile, 75th percentile]. Significant differences were estimated using Kruskal-Wallis test with Dunn's post-test (n = 6 per group).
IL-6, interleukin-6; I/R, ischemia/reperfusion; MDA, malondialdehyde; NO, nitric oxide; recAP, human recombinant alkaline phosphatase; TNF-α, tumor necrosis factor-α.
[#] $p < 0.05$ compared to control.

As another marker of acute inflammation, MPO-stained leukocyte influx (Ysebaert et al., 2000) was assessed in the glomerulus and in the peritubular areas. MPO staining was similar in the glomeruli of the control group compared to the I/R group (positive areas: 80 vs. 75%; p = NS), while it was decreased in the I/R + recAP group compared to control (68%; p = 0.01). In the peritubular areas, I/R injury decreased leukocyte influx compared to control (83 vs. 65%; $p < 0.0001$), which was further attenuated by recAP treatment (52%; $p < 0.0001$ compared to control; $p < 0.05$ compared to I/R).

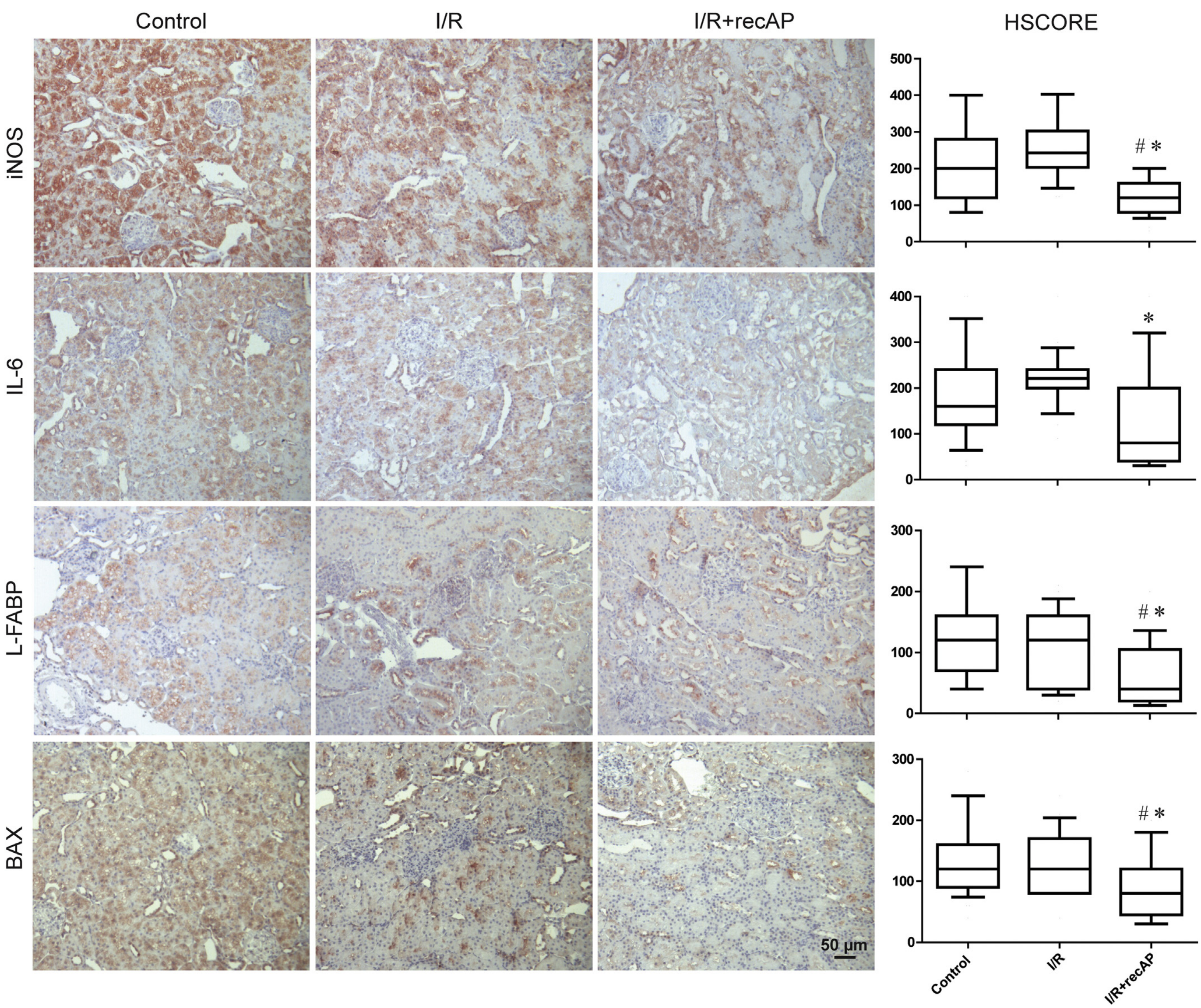

Fig. 2. Immunohistochemical results during I/R-induced AKI. Kidneys were harvested after the 2.5 h reperfusion period. A histological score (HSCORE) value was derived counting cumulative percentages of cells that stained at each intensity multiplied by the weighted intensity of the staining. Data is expressed as box and whiskers plots with 10–90 percentile. Significant differences were estimated using Kruskal-Wallis test with Dunn's post-test (n = 6 per group). #$p < 0.05$ control vs. I/R + recAP, *$p < 0.05$ I/R vs. I/R + recAP. Bax, bcl-2-associated X protein; IL-6, interleukin-6; iNOS, inducible nitric oxide synthase; I/R, ischemia/reperfusion; L-FABP, liver-type fatty acid binding protein; recAP, human recombinant alkaline phosphatase.

3.3. LPS-induced systemic hemodynamic instability and impaired renal oxygenation is not influenced by recAP

In a second rat AKI model, the renal protective effect of recAP was assessed following shock induced by LPS infusion in rats (Supplemental Fig. 1B). LPS administration decreased MAP ($p < 0.01$) compared to placebo, and this decrease in MAP was not affected by recAP administration (Fig. 1B). RVR was not different between the groups (Fig. 1B), but was reduced in the LPS + recAP group 2 h post-LPS (T2, 30 min after recAP administration) compared to baseline ($p < 0.05$). RBF remained stable over time in all groups compared to baseline.

Endotoxemia did not affect DO_2 or VO_2 significantly, but DO_2 was significantly lower in the LPS + recAP group at T1.5 and T4.5 compared to control (Table 3). The oxygen extraction ratio was increased in all groups at T4.5 compared to baseline, and was higher in the LPS and the LPS + recAP group compared to control (Table 3). Similar results were observed for cortical and medullar μPO_2, which were impaired during endotoxemia at T4.5, with no effect of recAP administration (Table 3).

3.4. RecAP inhibits markers of renal inflammation and damage during endotoxin-induced shock

Tissue NO and MDA levels were significantly increased by LPS treatment, irrespective of recAP co-administration (Table 1). However, recAP treatment attenuated LPS-induced iNOS reactivity (Fig. 3). While LPS had no effect on tissue levels of TNF-α and IL-6, immunohistochemistry data demonstrated a significant increase in expression of IL-6, L-FABP and Bax, which was ameliorated by recAP treatment (Fig. 3). Similar results were shown for NGAL immunoreactivity (HSCORE control: 90 [60–200], LPS: 200 [135–310], LPS + recAP: 100 [60–160]; $p < 0.0001$), and for leukocyte infiltration. LPS administration induced leukocyte influx in both the glomerulus (positive areas: 93 vs. 75% in the control group; $p < 0.0001$) and the peritubular areas (77 vs. 65%; $p < 0.01$). RecAP treatment did not reduce the influx in the glomerulus (87%; p = NS compared to the LPS group), but did prevent the peritubular influx (66%, $p < 0.05$).

4. Discussion

Sepsis-associated AKI is a multifactorial syndrome in which several processes, including inflammation, hemodynamic instability, renal microcirculatory dysfunction and unbalanced renal bioenergetics play a crucial role (Peters et al., 2014). RecAP represents a potential therapy targeting these multiple processes, which was previously shown to exert anti-inflammatory effects on cultured tubular cells (Peters et al., 2015). The effects of recAP on hemodynamics and renal oxygenation were not assessed previously. Here, we demonstrate mild modulatory effects of recAP on these parameters during I/R-induced AKI, while hemodynamics and renal oxygenation are not affected by recAP during endotoxemia-induced AKI. However, in both models, recAP attenuated renal injury and inflammation, confirming the protective effects observed previously during LPS-induced renal inflammation in vivo (Peters et al., 2015).

The kidney is highly susceptible to hypoxic injury as the organ is borderline ischemic and has to meet high energy demands (Ergin et al., 2015b). As such, tissue oxygenation within the kidney is strictly regulated. Under normal conditions, there is a delicate balance between oxygen delivery, mainly determined by RBF, local tissue perfusion and blood oxygen content; and oxygen consumption, influenced by GFR, sodium reabsorption, mitochondrial function and tubular integrity (Evans et al., 2013). I/R-induced injury disturbs this balance by reducing RBF and causing hypoperfusion in the ischemic phase, followed by a decreased renal oxygen delivery and oxygen consumption and increased intrarenal oxygen shunting during the reperfusion phase (Legrand et al., 2009). However, in daily clinical practice, AKI may also occur without clear signs of hypoperfusion (Murugan et al., 2010). Animal and human data suggest that during sepsis, AKI can develop in the setting of sustained or even increased RBF (Langenberg et al., 2005; Langenberg et al., 2006). Due to this discrepancy in kidney perfusion, the effects of recAP were investigated in two distinct models of AKI.

In the I/R model, RBF was impaired during the post-reperfusion period. After recAP treatment, RBF was no longer significantly affected immediately following reperfusion and neither were renal oxygen delivery and consumption at this time-point. Although these microcirculatory improvements were modest and were not maintained until the end of reperfusion, the anti-inflammatory effect of recAP may have reduced arterial to venous shunting, resulting in an improved cortical microvascular oxygen pressure during the 2.5 h reperfusion period.

In contrast, recAP was found not to improve renal oxygenation during endotoxemia. Apart from the timing of the intervention (recAP was administrated 15 min following ischemia and 60 min following LPS administration), the nature of the inflammatory response may account for this observed discrepancy. I/R-induced hypoxia triggers renal

Table 3
Kidney oxygenation during LPS-induced AKI.

		T0	T1.5	T2	T4.5
Cortical μPO_2 (mmHg)	Control	84 [78–95]	74 [68–83]	69 [66–77]‡	65 [60–72]‡
	LPS	88 [82–92]	79 [73–80]	78 [76–80]	56 [53–59]‡,#
	LPS + recAP	88 [84–93]	81 [74–84]	70 [66–75]‡	50 [46–55]‡,#
Medullar μPO_2 (mmHg)	Control	66 [61–71]	56 [52–62]	54 [49–57]‡	50 [44–55]‡
	LPS	68 [64–70]	62 [55–64]	61 [57–62]	42 [39–47]‡,#
	LPS + recAP	65 [64–72]	61 [58–65]	55 [52–56]‡	41 [35–44]‡,#
DO_2 (mL $O_2 \cdot min^{-1}$)	Control	4.1 [3.5–5.0]	3.7 [3.0–4.2]	3.1 [2.8–4.5]	3.2 [2.4–4.4]
	LPS	4.6 [4.3–4.8]	2.1 [2.0–3.0]	6.0 [3.8–8.2]	2.3 [1.3–3.7]
	LPS + recAP	4.0 [3.2–5.5]	2.0 [1.4–2.9]‡,#	4.2 [3.4–5.0]	1.2 [1.0–3.1]‡,#
VO_2 (mL $O_2 \cdot min^{-1}$)	Control	1.9 [1.5–2.3]	1.9 [1.5–2.3]	1.8 [1.5–2.7]	2.3 [1.4–3.4]
	LPS	2.0 [1.9–2.3]	1.3 [1.2–1.4]	3.6 [2.5–4.8]	2.0 [1.1–3.3]
	LPS + recAP	1.9 [1.3–2.5]	1.0 [0.6–1.9]	2.9 [1.8–3.2]	1.0 [0.9–2.8]
ER (%O_2)	Control	44 [43–46]	49 [49–53]	56 [51–62]‡	72 [55–79]‡
	LPS	45 [41–51]	59 [51–61]	67 [57–69]‡	87 [84–91]‡,#
	LPS + recAP	43 [41–48]	52 [44–58]	65 [53–74]	88 [84–93]‡,#

Parameters were determined at baseline (T0) and 1.5 (T1.5), 2 (T2) and 4.5 h (T4.5) after start of LPS infusion. RecAP was administered directly after T1. Data is expressed as median [25th percentile, 75th percentile]. Significant differences within groups were estimated using Friedman test with Dunn's post-test, differences between groups were estimated using two-way repeated measures ANOVA with Bonferonni's post-test on log-transformed data (n = 6 per group).

DO_2, oxygen delivery; ER, oxygen extraction ratio; IR, ischemia-reperfusion; recAP, human recombinant alkaline phosphatase; μPO_2, microvascular oxygen tension; VO_2, oxygen consumption.

‡ $p < 0.05$ compared to baseline within a group.

\# $p < 0.05$ compared to control.

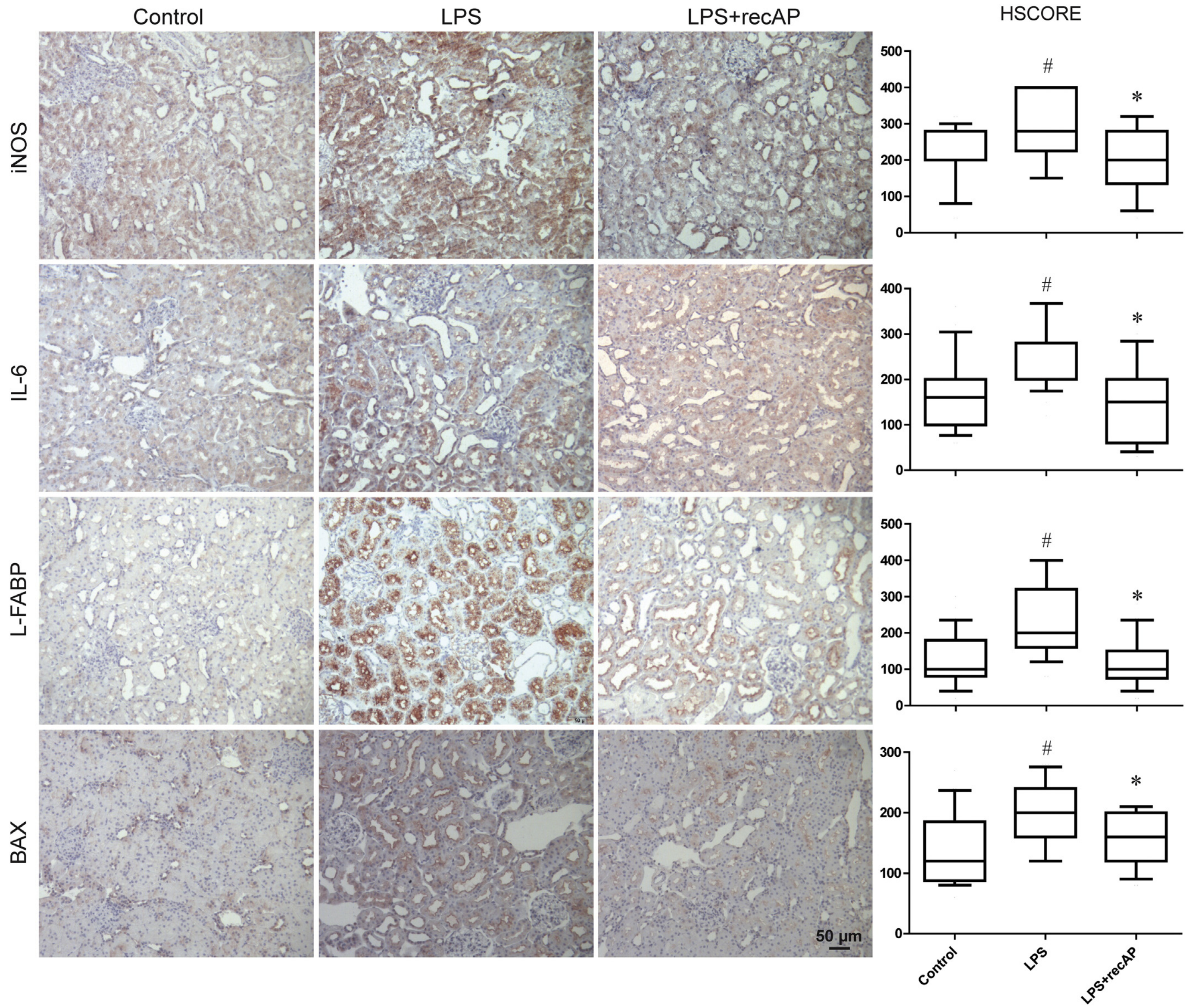

Fig. 3. Immunohistochemical results during LPS-induced AKI. Kidneys were harvested 4.5 h after initiation of LPS treatment. A histological score (HSCORE) value was derived counting cumulative percentages of cells that stained at each intensity multiplied by the weighted intensity of the staining. Data is expressed as box and whiskers plots with 10–90 percentile. Significant differences were estimated using Kruskal-Wallis test with Dunn's post-test (n = 6 per group). #$p < 0.05$ control vs. LPS, *$p < 0.05$ LPS vs. LPS + recAP. Bax, bcl-2-associated X protein; IL-6, interleukin-6; iNOS, inducible nitric oxide synthase; L-FABP, liver-type fatty acid binding protein; LPS, lipopolysaccharide; recAP, human recombinant alkaline phosphatase.

inflammation via several mechanisms, including structural alterations of proteins and cell surfaces, active synthesis of pro-inflammatory mediators and downregulation of anti-inflammatory molecules (Thurman, 2007). In addition, ischemic stress induces the release of cellular factors such as high mobility group 1 protein (Kruger et al., 2009) and heat shock proteins (Smoyer et al., 2000). Toll-like receptor (TLR)-2 and TLR4, pattern recognition receptors (PRR) localized on proximal tubule epithelial cells, are triggered by these endogenous ligands, thereby further exacerbating renal injury together with complement system activation, oxidative and nitrosative stress and mitochondrial dysfunction (Wu et al., 2010; Quintana & Cohen, 2005). This is in contrast to endotoxemia-induced AKI, during which renal inflammation is not only triggered by endogenous PRR ligands, but mainly by the bacterial ligand LPS which induces a profound inflammatory insult upon binding to TLR4 on multiple cells including proximal tubular epithelial cells (Kalakeche et al., 2011). In addition, binding of LPS to TLR4 on immune cells elicits systemic release of inflammatory mediators (Cohen, 2002), to which both vascular endothelium and renal tubular cells respond. In concert with a prolonged leukocyte transit time in dysfunctional capillaries (Holthoff et al., 2012; Goddard et al., 1995), this may further amplify renal inflammation.

RecAP affected the inflammatory response induced by IR, as protein levels of IL-6 and TNF-α were no longer significantly increased and the expression of renal injury parameters was lower, although these were not significantly increased by I/R-injury itself. Possibly, renal injury partially recovered after the 2.5 h reperfusion period in this relatively mild model (Ergin et al., 2015a; Szeto et al., 2011; Weight et al., 1998). Also during endotoxemia, administration of recAP attenuated renal inflammation, as was shown by a less pronounced peritubular influx of leukocytes and IL-6 expression. Moreover, recAP inhibited proximal tubule injury and markers of apoptosis, reflected by L-FABP and Bax immunostaining. Likely, these protective effects may be attributed to dephosphorylation of LPS and the endogenous proinflammatory molecules ATP and APD, released by proximal tubule cells during inflammation (Peters et al., 2015). Of note, and in contrast to the I/R-model, we did not find an increase in renal cytokine protein levels, but did detect a high level of oxidative and nitrosative stress. Despite its anti-inflammatory effects following LPS-induced shock, recAP treatment did not result in improved renal oxygenation in this model. This might be explained by the finding that in a rat model of severe shock recAP cannot restore the imbalance between NO, a major regulator of the microvascular oxygen supply, ROS and oxygen, which is key for an adequate physiological control of renal function (Evans et al., 2013).

Several limitations have to be acknowledged. First, although recAP modulated renal hemodynamics and oxygenation during I/R-induced AKI, these effects were modest and the clinical relevance of these findings might be limited. Second, following endotoxin administration, RBF remained stable over time, thereby limiting the possibility to demonstrate effects of recAP on renal hemodynamics. Although one may argue that the LPS dose administered is not high enough to severely affect renal hemodynamics, we aimed to investigate the effects of recAP in a clinically relevant model. As recently demonstrated in an intensive care-supported model of *E. coli*-induced septic shock in sheep, early AKI is not associated with changes in renal blood flow, oxygen delivery or histological changes, suggesting other mechanisms contributing to septic AKI (Maiden et al., 2016). These findings corroborate previous studies (Langenberg et al., 2005; Langenberg et al., 2006). As our model presents with signs of impaired systemic hemodynamics, renal oxygenation, as well as clear signs of renal injury, we believe that this is a representative model of endotoxemia-induced AKI in which we were able to confirm the renal protective effect of recAP. Third, the fact that recAP is administered not preventively but therapeutically, namely following initiation of the ischemic or endotoxic insult, may have hampered the potential of recAP to modulate renal microcirculatory dysfunction and hemodynamic changes. However, this protocol is more clinically relevant as it is in line with the ongoing phase II trial, in which recAP is administered to patients with confirmed sepsis-associated (Peters et al., 2016). Moreover, previous findings demonstrate that recAP provides renal protection when administered two hours after administration of LPS (Peters et al., 2015), indicating that recAP can still exert protective effects when LPS-induced inflammation is fully developed (Pickkers et al., 2006; Sadeghi et al., 1999). Fourth, the renal protective anti-inflammatory effects were primarily determined by immunostaining, a non-quantitative method. Although these results could have been corroborated by employing quantitative assays (e.g. assessing renal gene expression levels), the primary aim of this study was to investigate the effect of recAP on renal hemodynamics and oxygenation as the renal protective effect of recAP has already been demonstrated in vivo previously (Peters et al., 2015). Finally, as sepsis-associated AKI is a complex syndrome, the potential activity of recAP on other inflammatory components, such as the complement and coagulation pathways, eicosanoids, PRR ligands, and platelet activation factors, would be relevant to assess, but was beyond the scope of the current study. Of interest, the protective potential of AP has been confirmed in several other inflammatory models. AP administration reduced fever after Escherichia coli-induced inflammation in mice (Beumer et al., 2003), improved gas exchange (PaO_2:FiO_2 ratio) during septic shock induced in sheep (Su et al., 2006), prevented liver and lung damage in mice after cecal ligation and puncture (van Veen et al., 2005) and increased thrombocyte counts in piglets following LPS administration (Beumer et al., 2003). Also, AP attenuated circulating cytokine levels in mice (van Veen et al., 2005) and sheep (Su et al., 2006), which was confirmed in a phase 2 clinical study in which bovine intestinal AP more swiftly reduced several systemic inflammatory parameters in patients with sepsis-associated AKI (Pickkers et al., 2012).

In conclusion, recAP can modulate renal oxygenation and hemodynamics immediately following I/R-induced AKI, but did not influence these during endotoxemia-induced AKI. RecAP did exert a clear renal protective anti-inflammatory effect in both models. Whether this renal protective effect is sufficient to improve outcome of patients suffering from sepsis-associated AKI is currently being investigated in a large phase II a/b clinical trial (Peters et al., 2016).

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Transparency document

The Transparency document associated with this article can be found, in online version.

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