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ORIGINAL ARTICLE

Administration of interleukin-1 receptor antagonist ameliorates renal ischemia-reperfusion injury

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Summary

Interleukin (IL)-1 is a major contributor to inflammation and apoptosis during ischemia/reperfusion (I/R) injury. Its deleterious effects are primarily mediated by the activation of nuclear factor-κB (NF-κB). Receptor-binding and signaling of IL-1 can be blocked by the IL-1 receptor antagonist (IL-1ra). The aim of our study was to characterize effects and mechanisms of IL-1ra administration on inflammation, apoptosis, and infiltration in renal I/R injury. Renal ischemia was induced in Lewis rats by clamping of the left renal artery for 45 min. Kidneys were removed for histological and molecular analysis 24 h or 5 days after reperfusion. IL-1ra ameliorated I/R induced renal injury and inflammation. Furthermore, the number of apoptotic tubular cells was lower in IL-1ra-treated animals 24 h after ischemia, which was paralleled by a Bax/Bcl-2 mRNA ratio towards anti-apoptotic effects. IL-1ra reduced the expression of monocyte chemoattractant protein-1 (MCP-1) mRNA at 24 h and 5 days and that of intracellular adhesion molecule-1 (ICAM-1) expression at 24 h in the ischemic reperfused kidneys. Our results indicate that IL-1ra treatment ameliorates renal I/R injury and this protective effect might be mediated by reduced induction of NF-κB mediated MCP-1, ICAM-1, and a decreased ratio between Bax and Bcl-2 mRNA expression.

Introduction

Ischemia/reperfusion (I/R) injury of the kidney is an important cause of renal dysfunction after conditions such as hemorrhagic shock or organ transplantation with a significant impact on patient morbidity and mortality. Cell death through apoptosis as well as inflammatory reactions are important pathogenetic factors in I/R injury.

Interleukin (IL)-1 belongs to a group of cytokines released during the early phase of reperfusion after renal ischemia [1]. It can promote apoptosis and also inflammatory processes [2]. The latter is regulated through an increased expression of various pro-inflammatory cytokines as well as adhesion molecules that mediate the infiltration of leukocytes into the injured tissue [2]. The effects of IL-1 are blocked by the highly competitive endogenous IL-1 receptor antagonist (IL-1ra), which inhibits the binding of IL-1 to the IL-1 receptors I and II, thus, blocking intracellular signaling, which leads to an attenuated inflammatory response as well as a reduced number of apoptotic cells [3].

Recombinant IL-1ra has a substantial anti-inflammatory effect as has been clinically proven in the treatment of rheumatoid arthritis as well as in experimental models of myocardial [4] and cerebral I/R injury [5]. Furthermore, IL-1ra treatment has also been shown to reduce polymorphonuclear infiltration [6] in the ischemic reperfused renal tissue. The mechanism by which the IL-1ra protects against renal I/R injury with respect to apoptosis and expression of apoptosis regulating factors as well as adhesion molecules is not clear so far.

Binding of IL-1 to the IL-1 receptor leads to an activation of the transcription factor nuclear factor-κB

(NF-κB), which results in gene transcription of pro-inflammatory factors, among them monocyte chemoattractant protein-1 (MCP-1) and intracellular adhesion molecule-1 (ICAM-1) [7] which are contributing to the inflammatory reaction that leads to the tissue injury after I/R [8]. MCP-1 and ICAM-1 play a critical role in a key step in the development of tissue damage after I/R, namely leukocyte recruitment and infiltration. NF-κB also affects cell death through apoptosis as it regulates transcription of apoptosis-controlling factors of the Bcl-2 family such as the apoptosis preventing Bcl-2 and the apoptosis promoting Bax.

The aim of our study was to characterize mechanisms involved in the amelioration of renal I/R injury by IL-1ra with respect to the pro-inflammatory and pro-apoptotic effects of IL-1. We hypothesized that IL-1ra decreases post ischemic kidney injury by inhibiting leukocyte infiltration and apoptosis through a reduction of NF-κB regulated MCP-1, ICAM-1, Bcl-2, and Bax expression in the kidney.

Materials and methods

Drug

Recombinant human IL-1ra was obtained from Amgen (München, Germany). The compound is already in clinical use for the treatment of rheumatoid arthritis and was dissolved in phosphate-buffered saline (PBS) and administered intraperitoneally at a dose of 60 mg/kg body weight. Dosage and route of administration were based on previous experiments of the company, which were performed in rats.

Animals and surgery

The abdomen of male Lewis rats was opened under inhalation anesthesia with isoflurane for the induction of renal I/R. The left kidney with the right renal artery, vein, and ureter was prepared. The left renal artery was clamped with an atraumatic vascular clamp for 45 min. During this time the right kidney was removed. Thus, the animals were dependent on the function of the reperfused kidney.

Animals were assigned to two treatment groups (n = 16/group): treatment with IL-1ra (60 mg/kg i.p.; group IL-1ra) or vehicle (PBS) (group VEH). IL-1ra and vehicle, respectively, were applied after the ischemic period on day 0 and day 3. Animals were killed and the remnant kidney was removed for further analysis 24 h after reperfusion. These animals did not receive a second treatment after 3 days. The other set of animals was killed with their kidneys removed 5 days after reperfusion. The principles of NIH Guide for the Care and Use of Laboratory Animals as well as the German Law on the Protection of Animals were followed.

Renal histopathology

Histology was based on paraformaldehyde-fixed, paraffin embedded tissue sections stained with PAS reagent and hematoxylin and eosin (H&E) to evaluate the acute tubular necrosis in the kidney tissues. Samples were coded and examined in a blinded fashion. I/R injury was evaluated on a scale from 0 to 3 [1 = mild (<25 % of section area with tubular necrosis), 2 = moderate (25–50 % of section area with tubular necrosis), 3 = severe; (>50 % of section area with tubular necrosis)]. Two independent observers examined the slides by light microscopy in a blinded fashion.

Immunohistochemistry

For immunohistochemistry, cryostat sections (4 μm) were fixed in acetone, air-dried and stained with primary mouse monoclonal antibodies against monocytes/macrophages (clone ED1) and $CD5^+$ T lymphocytes (clone OX19) (Serotec Labor-Service GmbH, Wiesbaden, Germany). After incubation with primary antibody, sections were incubated with rabbit anti-mouse IgG followed by incubation with the alkaline phosphatase anti-alkaline phosphatase (APAAP) complex (DAKO A/S, Hamburg, Germany). Cells stained positive were counted and the results were expressed as cells per field of view (cells/fv). At least 20 fields of view per section and per specimen were evaluated at 400× magnification.

Terminal deoxynucleotidyl transferase-mediated dUTP nick end-labeling

Terminal deoxynucleotidyl transferase-mediated dUTP nick end-labeling (TUNEL) studies were performed on frozen sections fixed in 4% paraformaldehyde. Sections were incubated with Triton 0.1% and sodium citrate 0.1% at 4 °C, washed and incubated with TUNEL solution containing terminal deoxynucleotidyltransferase (Boehringer Mannheim, Mannheim, Germany). Sections were washed with stop/wash buffer followed by washing and incubation with a rabbit anti digoxygenine antibody (Boehringer Mannheim, Mannheim, Germany). Antibody binding was visualized using fast red chromogene solution. Positive controls were treated with DNAseI and processed as described above. Negative controls were incubated with PBS instead of TUNEL solution. The sections were counterstained with hematoxylin. All positive tubular epithelial cells in each section were counted at a magnification of 100× and related to the number of view fields per section.

Reverse transcription-polymerase chain reaction

Total RNA was isolated from the kidney samples by RNeasy Total RNA Isolations Kit (Qiagen GmbH, Hilden, Germany), according to the instructions of the manufacturer. The quality and quantity of the RNA were photometrically confirmed.

ICAM-1 and MCP-1 mRNA expression in the renal tissue was determined using semi-quantitative reverse transcription-polymerase chain reaction (RT-PCR) performed as previously described [9]. After initial denaturation at 94 °C, 30 cycles of amplification at the accurate annealing temperature for ICAM-1 [10], MCP-1 [11], Bax [12], Bcl-2 [13], and GAPDH were performed. The primer sequences and annealing temperatures are shown in Table 1. PCR products were separated on 2.5% agarose gels containing ethidium-bromide, and visualized under ultraviolet light and the image was taken. Signals were quantified by densitometry and corrected for the GAPDH signal, using an image analysis software program (Gel-Pro Analyser 3.1 Software; Media Cybernetics, Bethesda, MD, USA).

Statistical analysis

Data are expressed as mean ± standard error of mean (SEM). Data were tested using the Chi-Square or Mann–Whitney *U*-test. A *P*-value of <0.05 was considered significant. Histological changes were analyzed by Kruskal–Wallis test followed by multiple pair-wise comparisons according to Dunn's test. Data were analyzed using the SPSS statistical software package (v. 13.0; SPSS GmbH, Munich, Germany).

Results

Morphological evaluation of tissue damage

Acute tubular necrosis was significantly reduced in IL-1ra-treated animals when compared to controls 24 h after induction of ischemia ($P < 0.05$, Fig. 1a and b). After 5 days, the damage was again significantly lower in the IL-1ra-treated animals as compared to controls ($P < 0.05$, Fig. 1a and b).

Apoptosis of tubular cells

The number of apoptotic tubular cells was significantly reduced in IL-1ra-treated animals, 24 h after ischemia as compared to controls (2.47 ± 0.17 vs. vehicle, 5.82 ± 0.71, $P < 0.05$). However after 5 days of reperfusion the number of apoptotic cells was not significantly different between the groups (1.75 ± 0.1 vs. vehicle, 2.1 ± 0.3, $P = 0.753$) (Fig. 2a and b).

Infiltrating leukocytes

Twenty-four hours after ischemia, immunohistochemistry revealed only few infiltrating $CD5^+$ lymphocytes and $CD68^+$ macrophages in the renal tissue (data not shown). Five days after reperfusion the number of infiltrating lymphocytes (3.87 ± 0.56 vs. vehicle, 5.14 ± 0.7, $P < 0.05$) as well as macrophages (2.63 ± 0.23 vs. vehicle, 4.8 ± 0.56, $P < 0.05$) was lower in IL-1ra-treated animals when compared to vehicle-treated controls. (Fig. 3).

mRNA expression of MCP-1, ICAM-1, Bcl-2, and Bax

The mRNA expression of MCP-1 was lower at 24 h (0.5 ± 0.07 vs. VEH 24 h, 2.2 ± 0.12, $P < 0.05$) as well as 5 days after reperfusion (0.6 ± 0.07 vs. VEH 5 days, 1.3 ± 0.24, $P < 0.05$) in the IL-1ra-treated groups when compared to vehicle-treated animals (Fig. 4).

Treatment with IL-1ra was associated with decreased mRNA expression of ICAM-1 mRNA, 24 h after reperfusion (1.2 ± 0.03 vs. VEH 24 h, 1.96 ± 0.08, $P < 0.05$). However, 5 days after reperfusion, there was no difference between the two treatment groups (1.02 ± 0.04 vs. VEH 5 days, 1.15 ± 0.06, $P = 0.564$) (Fig. 5).

Table 1. Primer pairs and annealing temperatures used for RT-PCR analysis.

	Primer pairs	Annealing temperature (°C)	Product length (bp)
MCP-1	Forward: 5′-ATC ACC AGC AGC AGG TGT CCC AAA GAA GCT-3′ Reverse: 5′-AGA AGT GCT TGA GGT GGT TGT GGA AAA GAG-3′	60	258
ICAM-1	Forward: 5′-GGG TTG GAG ACT AAC TGG A-3′ Reverse: 5′-GCA CCG CAG GAT GAG GTT CTT-3′	60	228
Bax	Forward: 5′-AAG AAG CTG AGC CAG TGT CT-3′ Reverse: 5′-CAA AGA TGG TCA CTG TCT GC-3′	59	361
Bcl-2	Forward: 5′-GTA TGA TAA CCG GGA GAT CG-3′ Reverse: 5′-AGC CAG GAG AAA TCA AAC AG-3′	58	612
GAPDH	Forward: 5′-GGTGAAGGTCGGAGTCAACG-3′ Reverse: 5′-CAAAGTTGTCATGGATGACC-3′	56	498

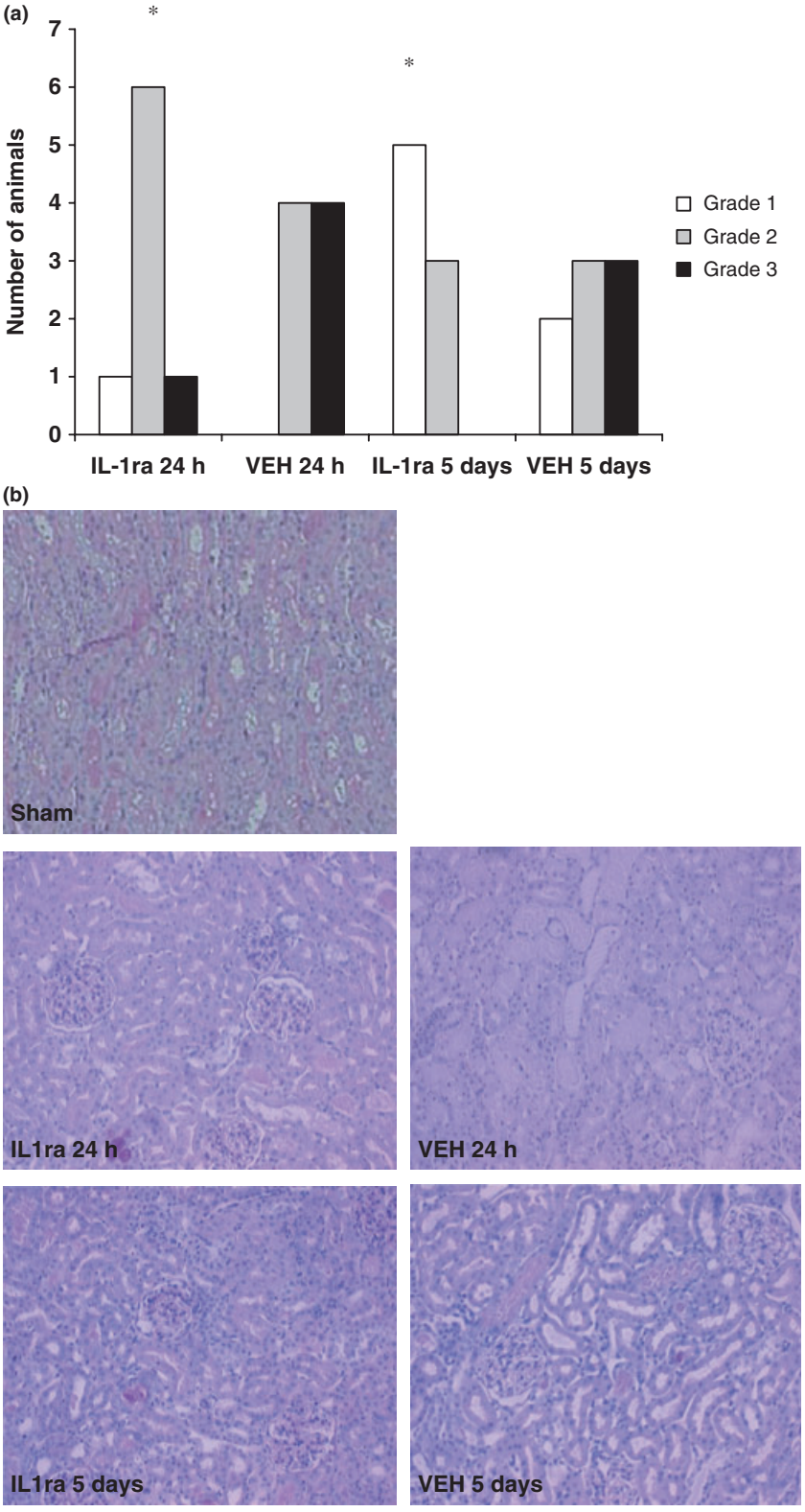

Figure 1 (a) IL-1ra ameliorated renal ischemia/reperfusion (I/R) injury. Grade of I/R injury 24 h and 5 days after renal ischemia in IL-1ra-treated and vehicle-treated animals, $*P < 0.05$ vs. VEH animals. (b) Representative sections demonstrating the morphological damage after 24 h and 5 days (PAS reaction, x100).

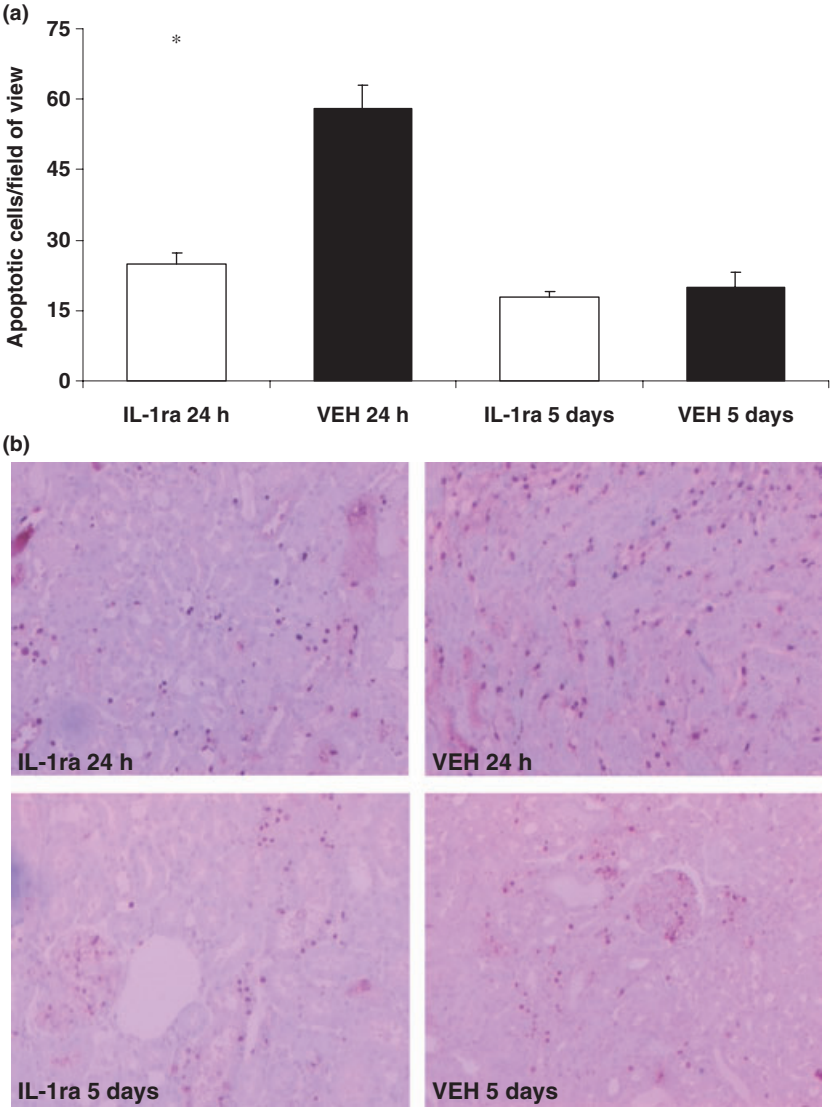

Figure 2 (a) IL-1ra reduced the number of apoptotic cells 24 h after ischemia. The number of TUNEL positive apoptotic cells per field of view was assessed at 24 h or 5 days of reperfusion time after 45-min renal ischemia in IL-1ra-treated and vehicle-treated animals, $*P < 0.05$ vs. VEH 24 h. (b) Representative pictures of TUNEL staining in renal tissues at 24 h and 5 days of reperfusion time after 45-min renal ischemia in IL-1ra-treated and vehicle-treated animals.

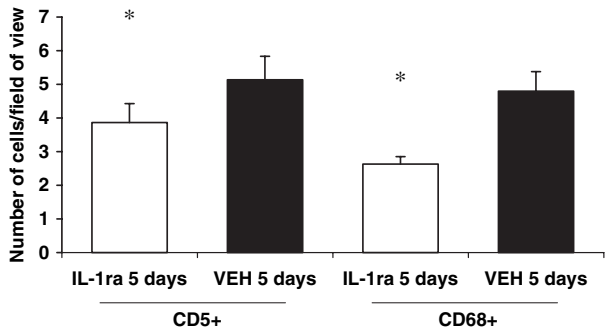

Figure 3 IL-1ra reduced lymphocyte and macrophage infiltration. The number of infiltrating $CD5^+$ lymphocytes and $CD68^+$ macrophages per field of view was determined by immunohisochemistry at 24 h or 5 days of reperfusion time after 45-min renal ischemia in IL-1ra-treated and vehicle-treated animals, $*P < 0.05$ vs. VEH 5 days.

The ratio between apoptosis-promoting Bax and apoptosis-inhibiting Bcl-2 was decreased in IL-1ra-treated animals when compared to controls 24 h after ischemia (0.39 ± 0.03 vs. VEH 24 h, 1.06 ± 0.14, $P < 0.05$), while the ratios were similar between the groups 5 days after reperfusion (0.62 ± 0.05 vs. VEH 5 days, 0.67 ± 0.02, $P = 0.564$, Fig. 6a–c). This points to a shift towards apoptosis-inhibiting factors in IL-1ra-treated animals.

Discussion

In our study the administration of recombinant IL-1ra attenuated renal I/R injury. Our results are in line with those of Furuichi *et al.* [14] who reported that IL-1 α/β knock out mice have a decreased rate of acute tubular necrosis 24 and 48 h after ischemia. In contrast, Haq

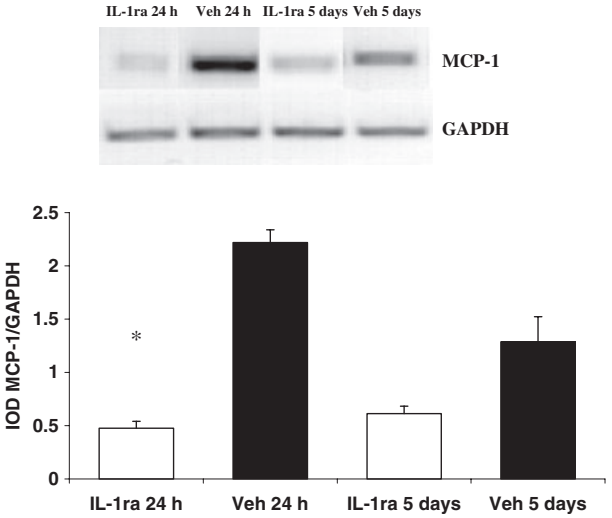

Figure 4 IL-1ra decreased the mRNA expression of monocyte chemoattractant protein-1 (MCP-1). The mRNA expression was determined in the renal tissue of IL-1ra and vehicle treated rats at 24 h and 5 days of reperfusion time after 45 min of left renal ischemia using RT-PCR, $*P < 0.05$ vs. VEH animals.

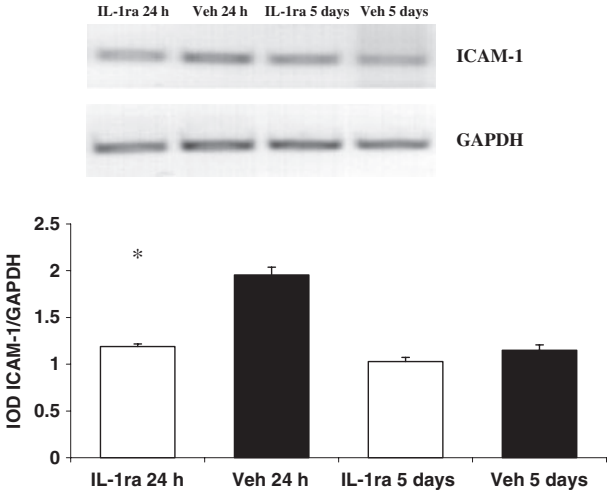

Figure 5 IL-1ra reduced the mRNA expression of intracellular adhesion molecule-1 (ICAM-1). mRNA expression was determined in the renal tissue of IL-1ra and vehicle treated rats at 24 h and 5 days of reperfusion time after 45 min of left renal ischemia using RT-PCR, $*P < 0.05$ vs. VEH animals 24 h.

et al. observed no alteration of acute tubular necrosis following renal I/R injury after recombinant IL-1ra treatment in mice. We suggest that the discrepancy between our results and those of Haq *et al.* [6] might be on account of animal race differences, different doses, and/or modes of administration.

Interleukin-1ra treatment reduced lymphocyte and macrophage infiltration 5 days after ischemia, which is in

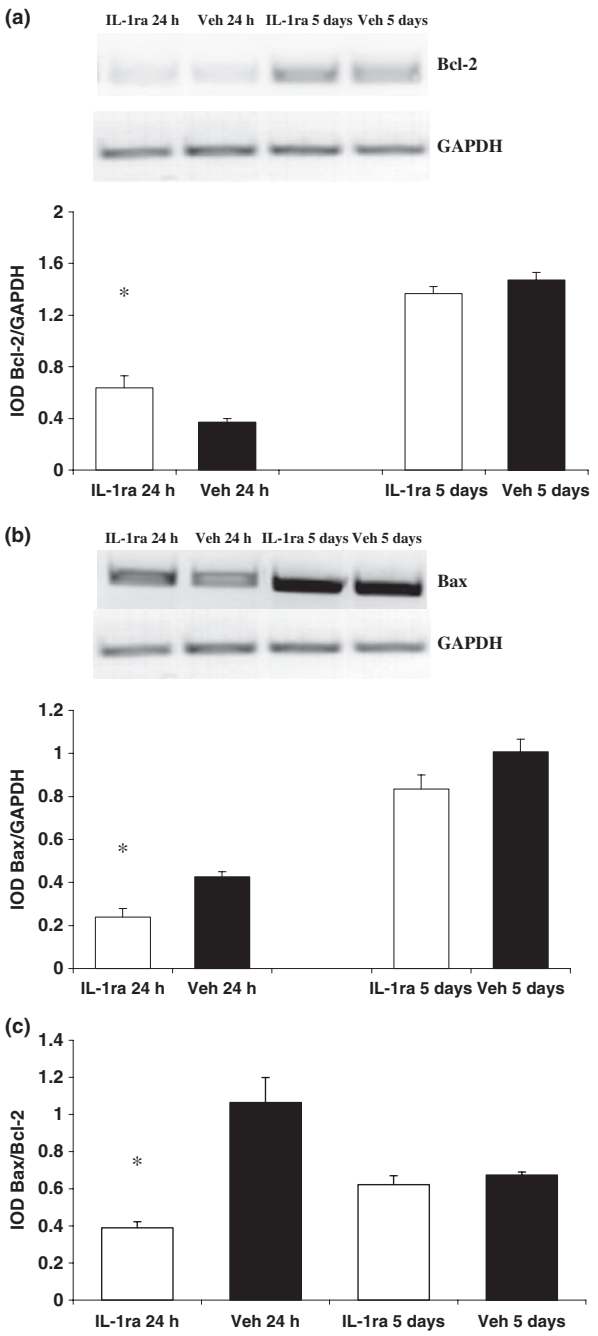

Figure 6 (a) IL-1ra treatment increased the mRNA expression of Bcl-2 24 h after reperfusion but it had no effect on Bcl-2 mRNA expression at 5 days. Bcl-2 mRNA expression was determined in the renal tissue of IL-1ra and vehicle treated animals at 24 h and 5 days of reperfusion using RT-PCR, $*P < 0.05$ vs. VEH 24 h. (b) IL-1ra decreased the mRNA expression of Bax at 24 h of reperfusion time, but Bax mRNA expression was unchanged 5 days after reperfusion. Bax mRNA expression was determined in the renal tissue of IL-1ra and vehicle treated rats at 24 h and 5 days of reperfusion using RT-PCR, $*P < 0.05$ vs. VEH 24 h. (c) IL-1ra reduced the ratio of Bax and Bcl-2 mRNA levels 24 h after reperfusion. $*P < 0.05$ vs. VEH 24 h.

line with observations of Haq *et al.* who reported a reduced polymorphonuclear leukocyte infiltration in IL-1ra-treated mice after I/R injury.

In the kidney, reperfusion following ischemia promotes inflammation and progressive tissue injury [15]. Tissue resident mast cells and macrophages produce reactive oxygen species leading to the activation of endothelial cells which express various chemokines and adhesion molecules leading to the accumulation of leukocytes at the site of injury. IL-1 belongs to the most potent inflammatory cytokines and is one among others, which is secreted by adherent leukocytes. Jo *et al.* [16] reported that expression of IL-1 in the kidney reaches its highest level 24 h after induction of ischemia. IL-1 can induce the expression of adhesion molecules (ICAM-1), cytokines (RANTES), and chemokines (MCP-1) through the activation of NF-κB transcription factor promoting further leukocyte adhesion. IL-1 can increase the rate of apoptosis through an altered expression of Bax and Bcl-2, which can be blocked by NF-κB inhibition [17]. It has been reported that IL-1 can induce cardiac fibroblast migration and also the repair of neurons after ischemic injury in a MAPK-dependent pathway. This suggests a possible involvement of IL-1 in the process of tissue remodeling [18,24].

The IL-1 receptors are expressed on T- and B cells, macrophages, neutrophils, fibroblasts, renal tubular epithelial cells as well as endothelial cells [19–21].

This observation fits to the effects of systemic monocyte-macrophage depletion in a model of renal I/R injury that results in a reduced renal IL-1 expression leading to less severe tubular necrosis, reduced inflammation, and reduced apoptosis of tubular cells [16].

The IL-1 receptor blocking IL-1ra [22] is predominantly expressed in peripheral blood cells, lungs, the spleen and liver [23] and also after experimental ischemia in the brain [24]. Administration of IL-1ra can attenuate a number of systemic, inflammation associated, responses [25]. Our results are in accordance with previous studies demonstrating that IL-1ra reduces ischemic brain, myocardial, and hepatic injury. Adenoviral overexpression of IL-1ra decreased infarct volume [26] and intracerebroventricular injection of IL-1ra reduced cell death in the brain after ischemia [27]. Furthermore, mice lacking IL-1ra had a significantly increased ischemic neuronal injury [28].

In a randomized, double-blind placebo-controlled trial of recombinant IL-1ra in patients with acute stroke, clinical outcomes were improved by treatment with IL-1ra suggesting IL-1ra to be a potential agent for the treatment of ischemic insults also in a clinical setting [29]. Suzuki *et al.* [4] demonstrated that gene transfer of IL-1ra reduced infarct size and neutrophil infiltration. Similarly, IL-1ra gene delivery to the liver by an adenoviral vector also ameliorated ischemic hepatic injury as well as inflammatory cytokine production [30].

In this study, we also aimed to investigate the mechanism by which IL-1ra exerts its protective effects. Both, MCP-1 and ICAM-1 play an important role in leukocyte recruitment to the site of tissue injury. Indeed, previous experiments demonstrated a reduced tissue injury after inhibition or deletion of ICAM-1 [31–34] and inhibition of MCP-1 [35]. Bcl-2 and Bax, both members of the Bcl protein family play a key role in the regulation of apoptosis, where Bcl-2 acts as an inhibitor and Bax as a promoter of apoptosis [36]. We hypothesized that IL-1ra exerts its anti-inflammatory and anti-apoptotic effects by blocking NF-κB mediated gene regulation of MCP-1, ICAM-1, Bcl-2 and Bax.

In our experiment, IL-1ra treatment reduced MCP-1 mRNA expression both at 24 h and 5 days after reperfusion, whereas ICAM-1 mRNA levels were reduced only 24 h after reperfusion while levels were similar to controls 5 days after ischemia. These results suggest that a suppression of the MCP-1 expression by IL-1ra contributes to the protective effect of IL-1ra blockade with a reduction in macrophage infiltration.

We also observed a reduced number of infiltrating lymphocytes in IL-1ra-treated animals 5 days after ischemia. The expression of ICAM-1 which plays a central role in lymphocyte recruitment was also decreased 24 h after reperfusion but was unchanged at 5 days. Thus, the reduced ICAM-1 mRNA expression in the early phase after ischemia could also be responsible for the reduced lymphocyte infiltration 5 days after ischemia, although at this time ICAM-1 mRNA expression was already restored to levels comparable with those of controls. In parallel to the effects on inflammation, IL-Ira also reduced the number of apoptotic cells in the ischemic tissue 24 h after reperfusion, whereas the number of apoptotic cells did not differ 5 days after ischemia. Similarly, the ratio between Bax and Bcl-2 mRNA levels in the renal tissue of IL-1ra-treated animals was shifted towards Bcl-2 mRNA, coding for the anti-apoptotic Bcl-2, 24 h after reperfusion while the ratios were similar when compared to controls, 5 days after ischemia suggesting that IL-1ra influences apoptotic cell death by altering the mRNA expression of Bcl-2 family members. However, based on our observations, it cannot be determined whether this is a direct or an indirect effect of IL-1ra.

In conclusion, treatment with IL-1ra ameliorated renal I/R injury through a reduced inflammatory infiltrate as well as a reduction of apoptosis. IL-1ra seems to contribute to these effects by reducing the expression of MCP-1, ICAM-1 as well as apoptosis regulating factors of the Bcl-2 family. Further studies are needed to show the clinical

relevance of such a therapy in ameliorating renal I/R injury.

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Authorship

KR: performed the research, analyzed the data and wrote the paper. HH: performed the animal surgery. NS, MR and CS: contributed to the histological examinations. UH and JL: designed and conducted the research and took part in the manuscript preparations.

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