RESEARCH ARTICLE | Renal Hemodynamics

Early antihypertensive treatment and ischemia-induced acute kidney injury

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Many patients at risk for AKI receive antihypertensive medication, but data on antihypertensive treatment in the acute phase of AKI are limited. In older patients needing cardiac surgery, risk factors such as hypertension, diabetes, and concomitant vascular disease can increase the AKI risk to 30%. Another patient group at high risk for AKI is recipients of solid organ transplantation. After lung transplantation, an incidence of AKI of 50–60% has been reported and is linked to CKD progression (30). Whether or not antihypertensive drugs, especially angiotensin-converting enzyme (ACE) inhibitors, should be withdrawn is a matter of debate (1, 4, 31). Epoxidecystatrienonic acids (EETs) are synthesized from arachidonic acid (ARA) by cytochrome P-450, and this pathway offers several attractive novel drug targets (16, 17). EETs act as endothelium-derived hyperpolarizing factors on pregglomerular vascular smooth muscle cells (3), increase renal blood flow (16), and exert vasodilatory, anti-inflammatory, antiapoptotic, and proangiogenic effects (15, 16, 21). The degradation of EETs to dihydroxyeicosatrienoic acids and other fatty acid epoxides to their corresponding diols is catalyzed by soluble epoxide hydrolase (sEH), which can be blocked by sEH inhibitors (sEHIs) (27). A potent and highly selective sEH is 1-(4-propanolylperidin-4-yl)-3-[4-(trifluoromethoxy)phenyl]urea (TPPU) (24). TPPU has been shown to decrease reperfusion injury after focal cerebral ischemia (28). Furthermore, administration of sEH reduced renal damage in ANG II-dependent hypertension (11, 29). We have recently shown that CD1 mice develop hypertension secondary to ischemia-induced AKI and developed progressive renal fibrosis and glomerulosclerosis within 2 wk of followup (9). Based on the previously reported beneficial effects of sEH in models of cerebral ischemia and hypertension, we tested whether early antihypertensive therapy with TPPU compared with enalapril as an established antihypertensive drug attenuates AKI after renal ischemia-reperfusion injury (IRI) and improves renal outcome.

METHODS

Animals. The Hannover Medical School Committee on Animal Research approved the present study (33.19-42502-04-14/1657 and 12/0916). The German guidelines are in accordance with National

INTRODUCTION

Hypertension after acute kidney injury (AKI) is common (12) and may be a risk factor for chronic kidney disease (CKD) (5).

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Institutes of Health guidelines for animal welfare. Adult male CD1 mice (30–35 g, 8–10 wk of age) were purchased from Charles River (Sulzfeld, Germany) and used for all experiments. Mice had free access to food (Altromin 1324 standard mouse diet) and domestic quality drinking water and were housed under conventional conditions in individually ventilated cages with a 10:14-h light-dark cycle. Animals were cared for in accordance with our institutional guidelines for experimental animals.

Treatment. The sEH-inhibitor TPPU was administered once daily by gavage. Enalapril treatment via drinking water was calculated based on the medium amount of daily fluid intake (30 mg/kg, day⁻¹). Vehicle treatment with PBS served as the control. All treatments were started 1 day before IRI and continued over the observation period of 2 wk. Vehicle treatment was done in n = 15 mice, and TPPU was administered to n = 7 IRI mice at a dose of 1 mg/kg and to n = 8 IRI mice and n = 4 sham mice at a dose of 10 mg/kg. Plasma levels of TPPU were monitored.

Renal IRI. Mice were anesthetized with isoflurane (3% induction and 1.5% maintenance). Butorphanol (1 mg/kg sc) was given before surgery for analgesia. Renal IRI was induced by left renal pedicle clamping for 35 min (microaneurysm clip, Aesculap). Mice were monitored until fully awake. Physical condition was assessed daily and scored for general appearance and well-being. Organ retrieval was done in deep isoflurane anesthesia (4%), and total body perfusion with ice-cold PBS via the left ventricle caused circulatory arrest. IRI kidneys as well as contralateral kidneys were fixed in 4% parafomaldehyde or shock frozen. Inulin and p-aminophosphorurate clearance were measured surgically to analyze glomerular filtration rate and renal blood flow as described in the Supplemental Methods in the Supplemental Material (Supplemental Material is available online at https://doi.org/10.6084/m9.figshare.12047412.v1).

Functional MRI to measure renal perfusion. Functional MRI was done before surgery at baseline and on days 1 and 14 after IRI using a 7-T small animal scanner (Bruker, Pharmscan) and a circular polarized volume coil (Bruker T10327V3). Mice were anesthetized by isoflurane inhalation. Respiration was monitored and kept between 30 and 60 breaths/min. Respiratory-triggered, fat-saturated T2-weighted sequences were acquired. Kidney volumes were determined by manual segmentation. Renal perfusion was measured without administration of contrast agent using an arterial spin labeling technique as previously described (13). Renal perfusion maps were calculated. Mean local perfusion in the renal cortex was quantified in the IRI previously described (13). Renal perfusion maps were calculated. Functional MRI experiments were done at baseline and on day 1 in n = 7 IRI vehicle-treated and IRI TPPU-treated mice and in n = 4 sham mice treated with TPPU. Baseline measurements were done before treatment and IRI. At day 14 after IRI, n = 4 mice/group were examined, respectively. For day 14, the untouched contralateral control kidney served as control.

Histology and immunohistochemistry. Kidneys were harvested on days 2 and 14 after IRI. The middle part of the kidney was immediately fixed in 4% paraformaldehyde and embedded in paraffin. Two-micrometer paraffin sections were cut and stained with sirius red (collagen type I and type III deposition). Immunohistochemistry to investigate neutrophil infiltration (GR-1) and methanamine silver stain to assess mesangial matrix thickness was done according to standard diagnostic protocols (7) (see Supplemental Methods, available online at https://doi.org/10.6084/m9.figshare.12047412.v1). Interstitial collagen deposition in the cortex and outer medulla was quantified on sections stained with sirius red using ImageJ software (see Supplemental Methods, available online at https://doi.org/10.6084/m9.figshare.12047412.v1). Analysis was performed in a blinded manner using a Leica imaging microscope. Histology for renal morphology and immunohistochemistry was done in n = 4 mice/group at day 2 and n = 7–11 mice/group at day 14 after IRI.

Real-time quantitative PCR. Quantitative PCR was done on a Lightcycler 420 II (Roche Diagnostics, Penzberg, Germany) using Fast-Start SYBR green chemistry as provided in the Supplemental Methods (available online at https://doi.org/10.6084/m9.figshare.12047412.v1). Gene-specific primers for connective tissue growth factor (CTGF; QT00096131), plasminogen activator inhibitor-1 (PAI-1; forward: 5'-AGTGTAGTGCAACCTGGGC-3' and reverse 5'-CTGCTCTTGGTGCGGAAGAC-3'), and IL-6 (QT00098875) were used. Results were normalized to hypoxanthine-guanine phosphoribosyltransferase expression (QT00166768). Quantification was carried out using qgene software.

ELISA to measure systemic chemokine (C-X-C motif) ligand 13 levels. Serum chemokine (C-X-C motif) ligand 13 (CXCL13) levels in mice were measured by ELISA (Quantikine Mouse CXCL13/BLC/BCA-1, Immunoassay catalog no. MCX130) using the Tecan spectra mini ELISA reader (Tecan, Crailtischheim, Germany). CXCL13 levels were quantified by comparison with internal CXCL13 standards.

Flow cytometry. FACS analysis was performed in n = 4 mice/group at day 2 after IRI using the following anti-mouse antibodies: CD45 (clone 30-F11), CD49b (clone DX5), CD11c (clone N418), F4/80 (clone BM8), CD11b (clone M1/70), and fixable viability dye eFluor 506 (65-0866) from eBioscience (Santa Clara, CA) as well as Ly6-C (clone HK1.4) and Ly6G (clone 1A8) from Biolegend (San Diego, CA). The preparation protocol for renal tissue is provided in the Supplemental Methods (available online at https://doi.org/10.6084/m9.figshare.12047412.v1). For flow cytometry, a FACS CantoII (BD Biosciences) was used, and data analysis was done with Kaluza software 1.3 (Beckmann Coulter).

Liquid chromatography mass spectrometry. Liquid chromatography coupled to mass spectrometry (LC-MS) was used to quantify epoxy fatty acid and dihydroxy fatty acid concentrations in plasma and drug concentrations in plasma and renal tissue at day 2 and day 14 in n = 4 animals/group. Quantification of renal tissue and plasma TPPU concentration was carried out using online solid phase extraction (SPE) LC-MS as previously described (26) using a QTRAP triple quadrupole MS (ABSciex, Darmstadt, Germany) (25, 32) or a Micromass LC Quattro instrument (Waters, Eschborn, Germany) (33). The preparation of renal tissue and plasma for LC-MS is provided in the Supplemental Methods (available online at https://doi.org/10.6084/m9.figshare.12047412.v1). Plasma analysis of oxylipins (epoxy fatty acids and dihydroxy fatty acids) was carried out by means of LC-MS following offline SPE using the SepPak LC18 protocol as previously described (23).

Tail-cuff blood pressure measurement. We are aware that radiotlemetry is the gold standard for blood pressure measurements in mouse (20). However, the surgical interventions in our study made sensor placement (particularly in the abdomen) untenable. Carotid artery placement interferes with baroreflex regulation. Systolic blood pressure was measured with a noninvasive tail-cuff blood pressure measurement system (TSE BloodPressure 209000-series, TSE Systems) as provided in the Supplemental Methods (available online at https://doi.org/10.6084/m9.figshare.12047412.v1). Baseline systolic blood pressure was recorded in n = 28 mice before treatment initiation and in n = 7 mice in the vehicle-treated group and TPPU-treated group (1 mg/kg) at different time points after IRI.

Statistical analysis. For statistical analysis, Graphpad prism software (version 5.0c, GraphPad Software, San Diego, CA) was used. For multiple comparisons, ANOVA with post hoc Tukey correction was applied. For comparison of two groups, Student’s unpaired t test was used. Linear regression analysis and Pearson correlation was done for TPPU tissue concentration and renal perfusion. Differences were considered significant at a P value of <0.05. Data are presented as means ± SE.

RESULTS

Antihypertensive treatment normalized blood pressure and attenuated glomerulosclerosis. CD1 mice already had mild hypertension at baseline and showed further blood pressure elevation after unilateral IRI (+20 mmHg after 2 wk in the
vehicle-treated group). Enalapril and TPPU normalized blood pressure during the 2-wk followup (Fig. 1E). Glomerulosclerosis can develop subsequent to hypertension, and a morphological correlate is mesangial matrix expansion. Therefore, mesangial matrix thickness was measured on silver-stained sections (Fig. 1, A–D). Mesangial matrix thickness was significantly increased in the vehicle-treated group 2 wk after IRI. In contrast, glomeruli of TPPU- and enalapril-treated mice showed significantly less expansion of the mesangial matrix than vehicle-treated mice. Notably, the contralateral untouched kidney showed no signs of glomerulosclerosis, although this kidney was also affected by systemic hypertension. Enalapril is known to reduce renal perfusion (10). Therefore, we focused on TPPU for further experiments on renal outcome by functional MRI, inflammation, and fibrosis.

**Inflammation after IRI.** In the early phase after IRI, neutrophils are the first leukocyte subsets infiltrating the kidney. Afterward, macrophages invade the tissue. By flow cytometry, we showed that Ly6G-positive neutrophils and activated Ly6c-positive macrophages were the main leukocyte populations at day 2 after IRI (Fig. 2, A–D). Contralateral untouched kidneys served as controls and had only minor neutrophil and macrophage infiltration similar to sham-operated mice treated with TPPU. GR-1-positive neutrophils were mainly localized in the outer medulla (Fig. 2, E–H). Proinflammatory cytokine expression in IRI kidneys for IL-6 was significantly upregulated in both vehicle- and TPPU-treated groups. In addition, the systemic inflammation marker CXCL13 was significantly increased at day 1 after IRI in the plasma of vehicle- and TPPU-treated mice (Fig. 2J). TPPU treatment did not attenuate early inflammation after IRI.

**Kidney volume and CKD.** Kidney volume was measured by functional MRI longitudinally at baseline and on days 1 and 14 (Fig. 3, A–D). After unilateral IRI, the untouched contralateral kidney showed hypertrophy over time (13). To outweigh this effect, changes in kidney volume after IRI were expressed relatively to the untouched contralateral control kidney at day 1. In the chronic phase after IRI, severe kidney volume loss was detected in all groups as a correlate of CKD. Kidney wet weight was markedly reduced at 14 days after IRI compared with the contralateral control. This effect was even more pronounced in TPPU-treated compared with vehicle-treated controls.

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**Fig. 1. Blood pressure and glomerulosclerosis.** A–D: mesangial matrix thickness was measured at day 14 after ischemia-reperfusion injury (IRI) on methenamin silver-stained tissue samples. Mesangial matrix expansion was enhanced in hypertensive IRI vehicle-treated mice, whereas 1-trifluoromethoxyphenyl-3-(1-propionylpiperidin-4-yl) urea (TPPU)- and enalapril (enal)-treated IRI mice had reduced mesangial matrix expansion. The contralateral kidney served as the control and showed no signs of mesangial matrix expansion. Bar = 10 µm. E: the blood pressure elevation after IRI was attenuated by enalapril and TPPU treatment. F: quantification of mesangial matrix thickness. SBP, systolic blood pressure; d0, day 0; d14, day 14; veh, vehicle. *P < 0.05; **P < 0.01.
IRI kidneys (Fig. 3K). In line with kidney shrinkage, sirius red-positive collagen deposition was enhanced in the IRI groups, indicating progressive renal fibrosis (Fig. 3, E/H11002H). In addition, transcripts for CTGF and PAI-1, both downstream targets of profibrotic transforming growth factor (TGF)-β, were increased after 2 wk in IRI kidneys of both groups; contralateral control kidneys showed normal expression at day 14 (Fig. 3, I–J). TPPU treatment did not attenuate upregulation of profibrotic genes and did not protect from kidney volume loss and renal fibrosis.

TPPU treatment aggravated early renal perfusion impairment after IRI. IRI caused substantial renal perfusion impairment, as measured by arterial spin labeling (Fig. 4, A–E). Surprisingly, TPPU treatment even aggravated renal perfusion...
impairment. Untouched contralateral kidneys served as controls and had perfusion values comparable to baseline perfusion and sham-operated mice treated with TPPU. At day 14, substantial renal perfusion impairment in IRI vehicle- and TPPU-treated mice was present (Fig. 4E). Tissue levels of TPPU were measured by LC-MS and were significantly lower in IRI kidneys compared with contralateral control kidneys at days 2 and 14 (Fig. 4H). Renal perfusion correlated with renal tissue levels of TPPU at day 14, and lower tissue levels were measured in less perfused kidneys (Fig. 4G). To assess renal function after IRI, the healthy contralateral kidney was removed at day 14, and inulin clearance was measured. Renal function was decreased to 20% from normal after IRI and was even further reduced after TPPU treatment (data are expressed as proportion of normal inulin clearance in healthy control mice, i.e., 150 mL/min). IRI resulted in poor renal function in vehicle-treated mice and was even worse in TPPU-treated mice with normal blood pressure (IRI vehicle: 22 ± 4% vs. IRI TPPU: 8 ± 2%; Fig. 4F).

**TPPU treatment affected oxylipin profiles.** Therapeutic plasma levels of TPPU were achieved, and TPPU treatment resulted in increased plasma epoxide-to-diol ratios at days 2 and 14 (Supplemental Fig. S1, available online at https://doi.org/10.6084/m9.figshare.12047247.v2). When we compared day 2 and day 14 under IRI and TPPU treatment, the epoxide-to-diol ratios were observed to be more strongly elevated at day 2 (Supplemental Fig. S1, available online at https://doi.org/10.6084/m9.figshare.12047247.v2). For example, this is shown for the epoxy arachidonic acid ratios of 11(12)-EpETrE to 11,12-DiHETrE and 14(15)-EpETrE to

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**Fig. 3.** Kidney volume and chronic kidney disease at day 14 (d14) after ischemia-reperfusion injury (IRI). A–D: morphometric MRI imaging was done to assess kidney volume longitudinally and showed severe volume loss at day 14 after IRI in vehicle (veh)- and 1-trifluoromethoxyphenyl-3-(1-propionylpiperidin-4-yl) urea (TPPU)-treated mice. E–H: tubulointerstitial fibrosis showed enhanced collagen deposition after IRI in all groups (all images were kept in the same magnification and the stack function was used). Quantification of fibrosis in the outer medulla (OM) is shown. I: connective tissue growth factor (CTGF) mRNA was significantly increased in IRI kidneys compared with contralateral controls without differences between treatment strategies. J: plasminogen activator inhibitor-1 (PAI-1) mRNA expression was even higher in TPPU-treated compared with vehicle-treated IRI kidneys. K: the kidney wet weight decrease was more pronounced in TPPU-treated IRI kidneys compared with vehicle-treated kidneys. *P < 0.05; **P < 0.01; ***P < 0.001.
14,15-DiHETrE, which were significantly increased under TPPU treatment at day 2, whereas this effect was less pronounced at day 14 (Supplemental Fig. S1, available online at https://doi.org/10.6084/m9.figshare.12047247.v2). For plasma epoxide-to-diol ratio measurements, sham-operated mice treated with TPPU served as the control.

**DISCUSSION**

We tested whether or not sEH inhibition with TPPU can normalize blood pressure elevation and attenuate renal damage in the CD1 mouse model of ischemia-induced AKI with progression to CKD. Systolic blood pressure, renal morphology, inflammation, perfusion impairment, and progression to CKD were investigated. We found that both TPPU and our positive control enalapril reduced blood pressure and attenuated glomerulosclerosis. However, aggravation of renal perfusion impairment caused enhanced inflammation and tubulointerstitial fibrosis after TPPU treatment. We have previously characterized CD1 mice in a model of renal IRI (9). CD1 mice developed marked hypertension after ischemia-induced AKI, which was associated with renal perfusion impairment and rarefication of the peritubular capillary network (9). Persistent inflammation and progression to CKD with development of glomerulosclerosis was also seen in CD1 mice after IRI (9). EETs have been protective in experimental models of ANG II-induced hypertension-mediated renal damage (35) and exerted anti-inflammatory (19, 34) as well as antifibrotic effects (18).

Blood pressure elevation was effectively normalized after TPPU or enalapril treatment. We showed that TPPU levels were in the therapeutic range and that target engagement could be demonstrated by oxylipin measurement. TPPU elevated plasma epoxide/diol levels effectively. While in the first days the epoxides of all polyunsaturated fatty acids were strongly elevated, the extent of change in the plasma epoxy-to-diol ratio decreased over the treatment period and reached statistically significance solely for the linoleic acid metabolites by day 14.

This finding indicates a homeostatic adaption to the (sub-
chronic sEH inhibition downregulation the formation of the highly potent epoxy-polyunsaturated fatty acid such as well-characterized ARA-derived EpETs (also known as EETs). This effect has been shown in a large number of studies evaluating the activity of sEH inhibitors showing that linoleic acid metabolites are the most sensitive biomarker effective chronic systemic sEH inhibition (22).

Despite the proven pharmacological effects of TPPU treatment on normalization of systemic blood pressure and severe kidney volume loss, progressive renal fibrosis was present in all IRI AKI groups. By comparing the outcome after enalapril and TPPU treatment following renal IRI, it became apparent that in CD1 mice, the TPPU-mediated elevation of EETs resulted in a similar ability to reduce hypertension as enalapril. Thus, the dominant mechanism of EET stabilization in the context of renal IRI seems to be a reduction of blood pressure, but further specific effects of EET metabolism inhibition on renal IRI cannot be excluded by this study and requires further investigation.

In previous experimental studies, TPPU reduced bleomycin-induced pulmonary fibrosis (36) and sEH deficiency attenuated renal IRI cannot be excluded by this study and requires further specific effects of EET metabolism inhibition on renal IRI cannot be excluded by this study and requires further investigation.

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DISCLOSURES

B.D.H. founded EicOsis, which is developing sEH as therapeutics.

AUTHOR CONTRIBUTIONS


REFERENCES


