Aus der Klinik für Urologie
der Medizinischen Fakultät Charité – Universitätsmedizin Berlin

DISSERTATION

ROLE OF 20-HYDROXYEIKOSATETRAENOIC ACID IN EXPERIMENTAL ACUTE KIDNEY INJURY

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1. Introduction

Ischemia-reperfusion (IR) injury is a complex inflammatory process that involves the vascular endothelium and activated leukocytes. It develops as an effector phase of ischemic injury hours or days after the initial insult [1]. During ischemia, the endothelium is primed to produce free radicals and to secrete chemo-attractants which upon reperfusion sequester and activate neutrophils [2]. White blood cells carried to the area by the restored blood flow release multiple inflammatory factors such as interleukins and free radicals. Inflammatory response increases metabolic demands of injured tissue and exacerbates IR injury. The restored blood flow introduces oxygen into cells that damages cellular proteins, DNA, and the plasma membrane. [3].

Repair and regeneration processes occur simultaneously to cellular apoptosis, autophagy, and necrosis; the fate of an organ depends on whether cell death or regeneration prevails [1].

Ischemia-reperfusion: role in the kidney

Renal ischemia-reperfusion injury is an inevitable consequence of transplantation and also results from systemic and renal hypo-perfusion following aortic crossclamping [2]. IR-induced morphological changes, referred to as acute tubular necrosis, are believed to be responsible for causing delayed graft function in human kidney grafts. Depending on the severity of IR injury, tubular cell death may be caused by either apoptosis or necrosis. Severe IR injury of kidney grafts, often caused by prolonged cold storage, with subsequent delayed graft function is supposed to contribute to acute rejection [4]. Furthermore, early nonspecific IR injury is thought to reduce long-term graft survival by enhancing host alloresponsiveness thus accelerating chronic allograft nephropathy [5], [6].

Ischemia-reperfusion: cellular events (see Fig. 1)

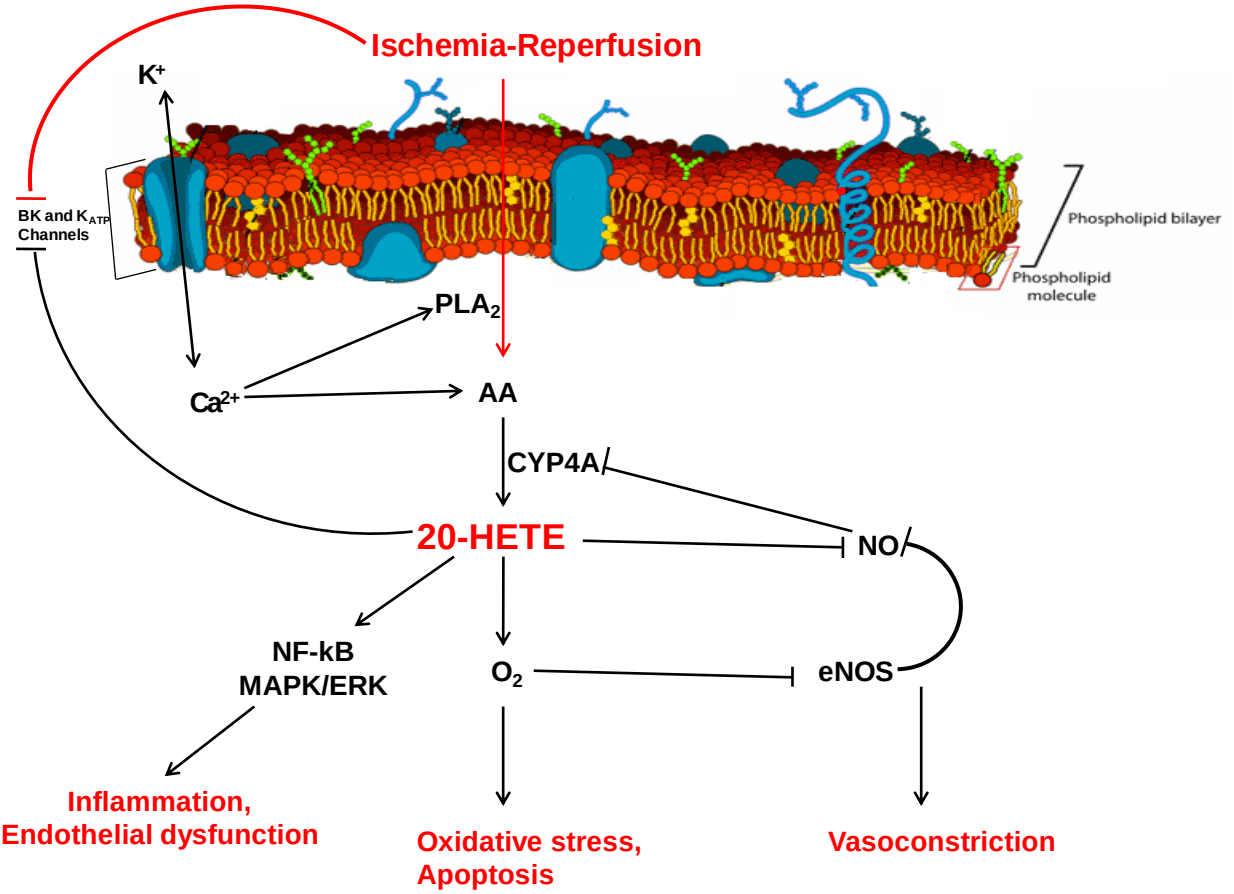


Fig. 1 Intracellular metabolism of 20-HETE during ischemia-reperfusion

Picture of the cell membrane: UNSW Cell Biology 2008 ANAT3231 Lecture 03 - Compartments and Membranes

Most of the metabolic energy of cells is expended on maintaining ion gradients across the cell membrane. A calcium/potassium (Ca^{2+}/K^{+}) pump, such as Adenosine TriPhosphate(ATP)-sensitive potassium channel (KATP channel) and BK (Maxi-K^{+}) channels, keep extracellular potassium low and extracellular calcium high compared to intracellular concentrations. The extracellular concentration of calcium ions under physiological conditions is 10 000 higher than within the cytoplasm. KATP channel is driven by the energy stored in ATP. Within two minutes without blood flow cells lose the energy to power the calcium/potassium pump. Potassium ions rush out of the cell while calcium and chloride ions rush inwards as the cell membranes depolarize. High levels of intracellular calcium ion activate proteolytic enzymes (known as nuclear endonucleases, calpains) which break down many cell proteins, particularly those in the cytoskeleton (spectrin, neurofilament- and microtubule-associated protein) and begin the process of apoptosis [7].

Role of arachidonic acid and 20-HETE in ischemia-reperfusion injury

The influx of calcium ions into cells after a few minutes of ischemia also activates phospholipase enzymes which attack cell membrane phospholipids causing the release of arachidonic acid [8]. In particular, cytoplasmic PhospoLipase A_2 ($cPLA_2$) activated by Ca^{+2} preferentially releases oxidized arachidonic acid (AA) [9]. AA is metabolized to 20-hydroxyeicosatetraenoic acid (20-HETE) via Cytochrome P450 (CYP 450) [10]. It was found that 20-HETE is also able to inactivate Ca^{2+}/K^{+} channels and to facilitate Ca^{2+} overload of the cell, thus exacerbating IR injury and leading to a vicious circle [11].

20-HETE is a major vasoconstrictor eicosanoid in the microcirculation, its overexpression leads to hypertension and endothelial dysfunction [12] by diminishing the release and actions of nitric oxide (NO) [13].

NO is a vasodilator, derived from the oxidation of L-arginine (L-arg) under the control of three nitric oxide synthase (NOS) isoforms [14]. Two of the NOS enzymes are constitutively expressed in endothelial cells (eNOS) or neurons (nNOS), while the third is inducible (iNOS) following cytokine or inflammatory mediator activation. All three NOSs have been identified in the kidney [15]. NO plays a major role in renal homeostasis by antagonizing the vasoconstrictive effect of angiotensin II on the afferent arteriole [16], maintaining renal blood flow and glomerular filtration rate and regulating renal sodium excretion [17]. During ischemia-reperfusion injury, the production of NO by the endothelium is decreased [18] leading to microvascular constriction and reduction in blood flow following reperfusion.

20-HETE inhibits the release and action of NO via eNOS uncoupling [19]. In turn, eNOS uncoupling causes additional O_2 formation and thereby promotes oxidative stress. Given its property as a vasoconstrictor, it is interesting to note that 20-HETE production is maximal in the nephron segment that has most direct access to the systemic circulation and may contribute to regulation of renovascular tone [20].

Although it has long been known that under physiological conditions NO and NO donors inhibit the catalytic activities of CYP450, thus decreasing 20-HETE generation [21], in the environment of IR the release and function of 20-HETE in the vasculature outweigh the beneficial effects of NO [19].

20-HETE has been also shown to be an endothelial cell activator [22]. The activation of endothelial cells is characterized by the enhanced production of adhesion

molecules and cytokines that culminate in the increased adhesion of leukocytes and the transformation of the endothelium to an inflammatory phenotype which eventually leads to endothelial dysfunction [22].

Endothelial dysfunction (ED) is a widely used term to describe any form of abnormal activity of the endothelium, encompassing both dysfunctional production of messenger molecules and expression of proinflammatory adhesion molecules [23]. ED is deleterious and is implicated as a key factor in the initiation and progression of various pathophysiological processes including peripheral ischemia, hypertension and inflammation [24 - 25].

20-HETE by itself is also known as a proinflammatory agent that activates nuclear factor-kB (NF-kB) and mitogen-activated protein kinase/extracellular signal-regulated kinase (MAPK/ERK) signaling pathways leading to increased transcription of numerous proinflammatory genes and upregulation of intercellular adhesion molecule (ICAM). 20-HETE induces the production of the proinflammatory lipid mediator prostaglandin E2 (PGE2) and stimulates the expression of distinct inflammatory cytokines, most prominently IL-8 and IL-13. [26 - 27].

Role of 20-HETE in ischemia-reperfusion injury of different organs

The role of 20-HETE in IR injuries was widely studied in brain and heart. Plasma levels of 20-HETE increase in rats after transient occlusion of the middle cerebral artery (t-MCAO) [28] and vice versa 20-HETE acts as a potent constrictor of cerebral arteries [29]. 20-HETE also increases the production of reactive oxygen species [19, 30] and activates a number of intracellular signaling pathways [31 - 32] involved in apoptosis and cell death.

During cardiac IR injury unesterified arachidonic acid [33 - 34] and 20-HETE accumulate intracellularly [35 - 36]. CYP450-mediated release of 20-HETE has been shown to exacerbate myocardial injury and significantly increase the infarct size [37].

Role of 20-HETE in the kidney

CYP450 monooxygenases are expressed in renal vascular and tubular structures and have diverse physiological and pathophysiological functions [38 - 39].

On the one hand there are a number of diseases originating from renal dysfunction that have been associated with pathological changes in 20-HETE production, including cyclosporine-induced nephrotoxicity [40] and altered pressure-natriuresis, leading to systemic hypertension [41]. Ward et al. [42] found that elevated urinary 20-HETE excretion was associated with increased oxidative stress in kidneys of patients with essential hypertension. Recently, it also has been indicated that 20-HETE increases free radical formation in endothelial cells in the kidney by NOS uncoupling [43], which promotes renal epithelial cell damage during IR injury [44]. 20-HETE has been shown to have proapoptotic effects on renal epithelial cells during the recovery phase following an ischemic episode [45]. In the renal vasculature, as well as in other organs, 20-HETE acts as a potent vasoconstrictor, impairing vascular relaxation and reducing NO availability in hypertension. It contributes to superoxide production, endothelial dysfunction and inflammation [1, 19, 22] (Fig. 1).

On the other hand, 20-HETE has been shown to inhibit Na-K-ATPase and sodium reabsorption in both the proximal tubule and medullary thick ascending loop of Henle [46 - 47] and to increase sodium excretion in urine without changing glomerular filtration rate (GFR) and mean arterial blood pressure [48]. Inhibition of tubular sodium retention attenuates medullary hypoxia by reducing oxygen demand during the ischemic period, which may ameliorate renal IR damages. Thus, similar to other inhibitors of tubular electrolyte transport, 20-HETE may have a light renoprotective effect [48 - 49].

Hypothesis (Fig. 2)

We aimed at attenuating potential adverse effects of 20-HETE during renal IR by either blocking the enzyme CYP4A, that is responsible for 20-HETE synthesis, or using an antagonist to 20-HETE directly and exclusively in the renal vasculature.

We hypothesize that pharmacological blocking of 20-HETE before an ischemic insult improves renal function and structural damage in a rat model of warm ischemia.

2. Materials and methods

2.1 Experimental design

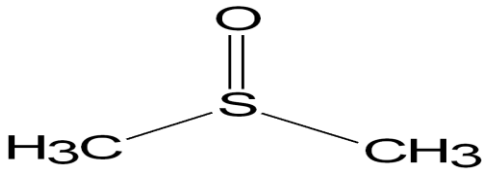
2.1.1 Animals

Male Lewis rats (weight, 200-300 g; Harlan-Winkelmann GmbH, Borchen, Germany) were maintained under standard laboratory conditions, and fed with rat chow and water ad libitum. All experiments were approved by a governmental committee on animal welfare (Project G 0306/06, LaGeSo, Berlin).

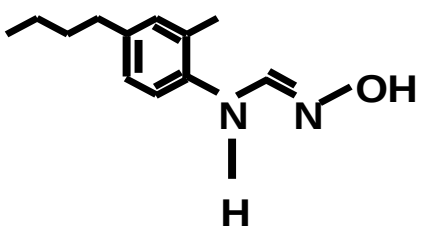
2.1.2 Substances

All animals were divided into groups (6 animals per group) according to the substance used for pretreatment during the operation:

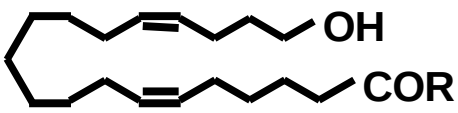
1. Vehicle: $(CH_3)_2SO$ - dimethyl sulfoxide (DMSO) ex tempore melted and diluted 1:100 (1µl DMSO in 100µl 37^0C warmed aqua ad injectabile), kept for 3 min on a "Vortex-genie-2" (Scientific Industries, INC, Bohemia, N.Y., USA) for better dilution.



2. HET0016: CYP4A-inhibitor of 20-HETE synthesis [N-hydroxy-N'-(4-butyl-2 methylphenyl) formamidine] (Taisho Pharmaceutical Co. Ltd. (Saitama, Japan))
   Basis-solution: 50 µg/µl HET0016 in 1µl DMSO; stored in – 80^0C; before injection, ex tempore melted and diluted 1:100 in 37^0C warmed aqua ad injectabile, kept for 3 min on a "Vortex-genie-2" (Scientific Industries, INC, Bohemia, N.Y., USA) for better dilution.



3. 20HEDE: antagonist of 20-HETE action, 6,15-20-HEDE [20-hydroxyeicosa-6(Z),15(Z)-dienoic acid] (DKB-III-91-32, J. Falck, USA). Basis-solution: 20 µg/µl 6,15-20-HEDE in 1µl DMSO; stored in – 80^{0}C; before injection , ex tempore melted and diluted 1:100 in 37^{0}C warmed aqua ad injectabile, kept for 3 min on a "Vortex-genie-2" (Scientific Industries, INC, Bohemia, N.Y., USA) for better dilution.



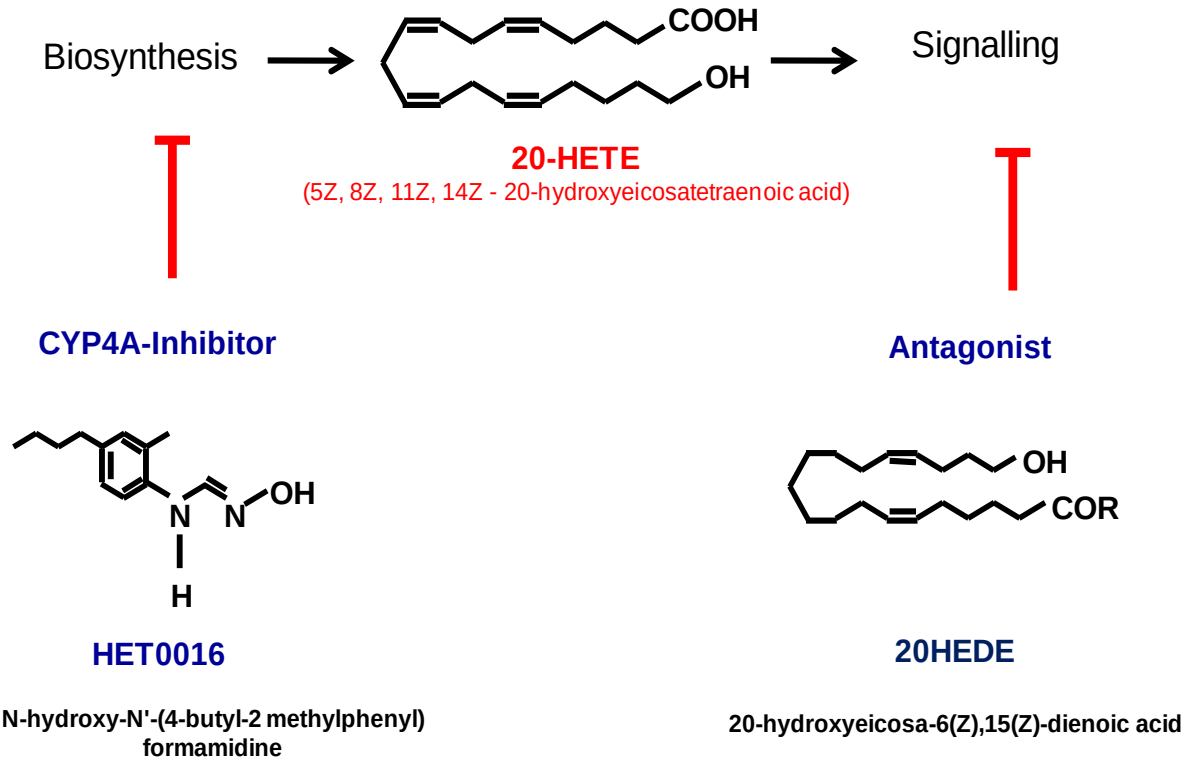


Fig. 2 Mechanism of action of HET0016 and 20HEDE

HET0016 blocks the synthesis of 20-HETE and 20HEDE inhibits its action.

2.1.3 Animal surgery

Native male Lewis rats served as controls.

2.1.3.1 Sham group

Operation procedure was performed under inhalation anesthesia with isoflurane (“Forene”, Abbott GmbH & Co., KG Wiesbaden). Animals underwent a midline laparotomy. The aorta and the v.cava were freed from surrounding tissue above and under the left renal pedicle. Fluid resuscitation for the peri- and immediate post-transplant period was achieved by instillation of 5 ml of normal saline. The laparotomy incision was closed, and animals were allowed to wake up [50]. After recovery from surgery and anesthesia, rats were transferred to the housing facility for postoperative follow up.

2.1.3.2 Clamping experiment (Fig. 3)

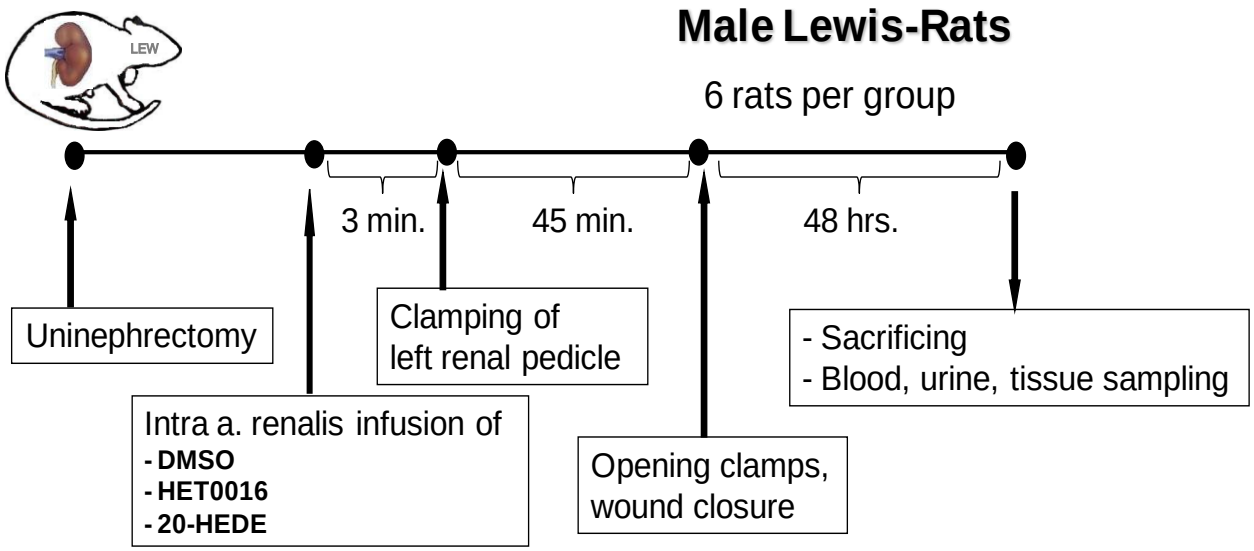


Fig. 3 Design of clamping experiment

The animals were placed on a heated surgical table to maintain rectal temperature at 37°C. Operation procedure was performed under inhalation anesthesia with isoflurane ("Forene", Abbott GmbH & Co., KG Wiesbaden). The animals underwent a midline laparotomy followed by right nephrectomy: the pedicle of the right kidney (right renal vessels and right ureter) was ligated and dissected, and the kidney was taken out of the abdomen. The abdominal aorta and the vascular pedicle of a left kidney were freed from surrounding tissue. The abdominal aorta was occluded with atraumatic clamps above and under the left renal artery. 0.5 µl of a vehicle or a substance, warmed to 36^0C, (see 2.1.2) was injected into the abdominal aorta between clamps, close to the renal artery. The upper clamp was opened, to let the blood and the injected substance flow into the kidney. After the normal color of kidney was restored (after 1 min), the lower clamp was opened. After 3 minutes of perfusion the vascular pedicle was occluded with a microvascular clamp (Yasargil Aneurysm Clip, mini temporary, Rudolf, Fridingen) for 45 min. After ischemia, the clamps were withdrawn. Fluid resuscitation for the peri- and immediate post-transplant period was achieved by instillation of 5 ml of normal saline into the peritoneal cavity before abdominal closure. The laparotomy incision was closed, and animals were allowed to wake up. After recovery from surgery and anesthesia, rats were transferred to the housing facility for postoperative follow up.

2.1.4 Postoperative follow-up and tissue sampling

24 hours after surgery animals were placed into metabolic cages for 24 hours. Twenty-four-hour urine samples were tested for urine creatinine, urea and protein excretion. These measurements were determined with automated methods. Serum creatinine, urine creatinine and urea were measured.

48 hours after the operation rats were sacrificed by exsanguination under deep general anesthesia. Blood samples were collected for measurement of serum creatinine and urea concentrations. These chemistries were determined with automated methods. The left kidney was harvested and weighed, one half of it was snap-frozen in liquid nitrogen and than stored in -80^0C, and the other half was put into 4% paraformaldehyde ("Roti-Histofix 4%", Carl Roth GmbH & Co., Karlsruhe) for subsequent hematoxylin-eosin staining and histological examination.

2.2 20-HETE production in the isolated perfused kidney

The left kidney of inbred Lewis rat (n = 6 per group) was isolated, the renal artery was cannulated and the organ was put on a plastic grid into an organ chamber and perfused at constant flow (5 ml/min) at 37°C using a peristaltic pump (WPI, Germany).

The oxygenated (95% O_2–5% CO_2) perfusion buffer contained either vehicle (0.1 % DMSO) or HET0016 (200 nM). After 20 min of equilibration period perfusion was stopped for 20 min to mimic warm ischemia and then followed by 30 min of reperfusion. The total renal outflow was collected every 5 min before and up to 30 min after inducing ischemia over a period of 1 min each. The perfusate-samples were snap frozen in liquid nitrogen and stored at -80°C.

20-HETE concentration in the perfusate was measured with high performance liquid chromatography mass spectrometry (HPLC-MS) (LC-MS/MS; Lipidomix GmbH, Berlin).

2.3 Vascular reactivity in the isolated perfused kidney

2.3.1 Dissection and tissue preparation

Under Isoflurand narcosis Lewis rats were uninephrectomized and received injection of HET0016 or vehicle into a. renalis prior to 45 minutes of warm ischemia and 30 minutes after reperfusion as described above (n = 6 per group). Kidneys of the initially uninephrectomized Lewis rats served as controls. The left kidney was isolated and left renal artery was cannulated via a polyethylene catheter. Then they were removed, and transferred into a warmed Plexiglass chamber.

2.3.2 Perfusion

The kidney was perfused continuously with warmed (37^0 C) and aerated (95% O2 and 5% CO2 gas mixture) PSS solution, which contained either vehicle (0.1 % DMSO) or HET0016 (200 nM) by using a peristaltic pump (WPI, Germany) delivering a constant flow (5 ml/min) throughout the experiment. Drugs were given as a bolus injection delivered into the silicone rubber perfusate tubing close to the kidney.

2.3.3 Measurement of vascular reactivity

After an equilibration period of 20 min the renal vascular bed was challenged with rising concentrations of Angiotensin II (1 to 1000 nM) or phenylephrine (10 to 1000 nM) to obtain vasoconstrictor response.

Perfusion pressure was monitored by a pressure transducer (Living Systems Instrumentation, Burlington, VT, USA) and recorded on a polygraph. Since the flow was maintained at a constant rate, changes of the outflow pressure indicated either vasoconstriction or vasodilatation.

The means for changes in perfusion pressure of six experiments in each group were then arranged together to obtain a final mean ± SEM.

2.4 Histopathology

The formalin-fixed renal tissues were gradually dehydrated: 2 hours in 70% ethanol then 2 hours in 90% ethanol, overnight in 90% ethanol, then 3 times for 2 hours in 100% ethanol, and finally all 2 times for 20 minutes in xylol. Dehydrated tissue was embedded 2 times for 2 hours in soft paraffin and at the end overnight in hard paraffin. Paraffin-blocks were cut with the microtome (Leitz 1512) into 2 to 4 µm thick sections.

2.4.1 Conventional histology

2.4.1.1 Hematoxylin and eosin (HE) staining

Solutions and reagents

Eosin-Phloxine B Solution: Eosin Y (1%), Phloxine B (1%), ethanol (95%), glacial acetic acid, distilled water.

Hematoxylin solution (Mayer): potassium, hematoxylin, sodium iodate, citric acid, distilled water.

Procedure

Deparaffinized sections, were placed into 2 changes of xylene, 10 minutes each, re-hydrated in 2 changes of absolute alcohol, 5 minutes each, then 95% alcohol for 2 minutes and 70% alcohol for 2 minutes, washed briefly in distilled water, counterstained in Mayer hematoxylin solution for 8 minutes, washed in warm running tap water for 10 minutes, rinsed first in distilled water, then in 95% alcohol, 10 dips. Then slides were

stained in eosin-phloxine B solution for 30 seconds to 1 minute, dehydrated through 95% alcohol, 2 changes of absolute alcohol, 5 minutes each. Stained slides were cleared in 2 changes of xylene, 5 minutes each, and mounted with xylene-based mounting medium.

The kidneys preserved in 4% paraformaldehyde were dehydrated step by step: 2 hours in 70% ethanol then 2 hours in 90% ethanol, overnight in 90% ethanol, then 3 times for 2 hours 100% ethanol, and finally 2 times for 20 minutes xylol. Dehydrated tissue was put two times for two hours in soft paraffin and at the end embedded overnight in hard paraffin.

2.4.1.2 Periodic acid-Schiff (PAS) staining

Solutions and reagents

0.5% periodic acid solution, Schiff reagent, Mayer's hematoxylin solution.

Procedure

Deparaffinized sections were hydrated, oxidized in 0.5% periodic acid solution for 5 minutes, rinsed in distilled water, placed in Schiff reagent for 15 minutes, washed in lukewarm tap water for 5 minutes, counterstained in Mayer's hematoxylin for 1 minute. Stained slides were washed in tap water for 5 minutes, dehydrated and coverslipped using a synthetic mounting medium.

2.4.1.3 Acute tubular necrosis score (ATN Score)

Sections stained with hematoxylin and eosin or with periodic acid-Schiff (PAS) were analysed for assessment of acute tubular lesions. Quantitative analysis of renal tubular necrosis was performed by optical microscopy using Acute Tubular Necrosis Score (ATN Score). Briefly, 100 intersections were examined for each kidney and a score from 0 to 3 was given for each tubular profile involving an intersection:

0 - normal histology;

1 - tubular cell swelling, brush border loss, nuclear condensation, with up to one third of tubular profile showing nuclear loss;

2 - as for score 1, but greater than one third and less than two thirds of tubular profile shows nuclear loss;

3 - more than two thirds of tubular profile shows nuclear loss.[51]

2.4.2 Immunohistochemical staining

Macrophage infiltration was assessed by ED1 immunohistochemical staining. It was performed with the ED-1 for monocyte/macrophages mouse monoclonal primary antibodies (Serotec, Oxford, UK). The standard avidin-biotin-complex method with peroxidase labeled secondary antibody and diaminobenzidine detection (DAB, Vector) alkaline phosphatase antialkaline phosphatase complex technique with Fast Red Kit detection (APAAP, Dako) was used for immunohistochemical detection of ED-1 cells on paraffin sections. Sections were deparaffinized and rehydrated through xylene (2 ×10 minutes), 100% ethanol (2 ×10 minutes), 95%, 70%, and 50% ethanol and phosphate-buffered saline (PBS) (each 1 × 5 minutes). Antigen retrieval was induced by citrate boiling in a pressure device for 3 minutes. To block endogenous peroxidase, sections were immersed in 0.3% H_2O_2 for 20 minutes. Then incubation with CAS solution (Zymed, San Francisco, CA, USA) was carried out for 30 minutes to block unspecific antibody binding and followed by incubation with primary antibodies diluted in PBS (pH 7.6) for 1 hour. Sections were then rinsed twice with PBS. Visualization was performed using the horseradish peroxidase–labeled secondary antibody for 30 minutes and diaminobenzidine (DAB) or amino ethyl carbazole color detection (Dako, Glostrup, Denmark). Alkaline phosphatase antialkaline phosphatase complex technique with Fast Red Kit detection (APAAP, Dako) was used for immunohistochemical detection of ED1+ cells on paraffin sections. Immunoreactivity was visualized with an alkaline phosphatase antialkaline phosphatase (APAAP) complex (Dako). Negative control staining was performed by incubation with corresponding isotype controls instead of primary antibody.

Morphometric analysis was conducted in a blinded fashion. Expression of ED1+ monocyte/macrophages and CD8+ lymphocyte was analyzed semi quantitatively (positively stained cells). For each parameter 10 randomly chosen HPFs in cortex and outer medulla were evaluated. The means for 6 rats at any time point were then grouped together to obtain a final mean ± SD.

2.4.3 Apoptosis detection

Renal apoptosis was examined using the *In situ* Cell Death Detection Kit (Roche Diagnostics GmbH, Mannheim, Germany) based on the TdT-mediated dUTP nick end labeling (TUNEL) method. Paraffin-embedded renal cross sections were treated

according to the manufacturer's instructions and stained with Fluorescein. TUNEL-positive apoptotic cells were visualized under a fluorescence microscope (Zeiss Axio Imager A1, Jena, Germany). and quantitated using a digital imaging system (Zeiss Axiocam HR with Axiovision 4.4 software). In each microcompartment, 10 randomly chosen fields of view (FoV) per section were evaluated. The means of six samples were grouped together to obtain a final mean ± SD. Positive cell staining was recorded digitally and expressed as the percentage of TUNEL-positive area per FoV at 400× magnification.

2.5 Analytical procedures

2.5.1 Creatinine clearance (CCl)

was counted considering body and kidney weight using the following formula [2]:

CCl = (UC x UV / SC x 1440 min) / KW

UC - urine creatinine (mg/ml)
SC - serum creatinine (mg/ml)
UV - urine volume (ml)
KW - kidney weight (g)

2.5.2 Fractional excretion of sodium (FE_{Na})

was estimated using the following equation [2]:

$$FE_{Na} = \frac{SC \times U_{Na}}{S_{Na} \times UC} \times 100\ \%$$

SC - serum creatinine (mg/ml)
UC - urine creatinine (mg/dl)
U_{Na} - urine sodium (mmol/l)
S_{Na} - serum sodium (mmol/l)

2.5.3 Urinary sodium excretion ($U_{Na}V$)

was counted considering kidney weight using the following formula [52]:

$$U_{Na}V = U_{Na} \times D / KW$$

U_{Na} - urine sodium (mmol/l)
D - diuresis (ml/min)
KW- kidney weight (g)

2.5 Statistical analysis

Unpaired Student's *t* test and one-way analysis of variance (ANOVA) were performed for comparison of data between groups. A *P* value of less than 0.05 was considered statistically significant. All statistical tests were performed using SPSS 11.5 for Windows (SPSS, Inc., Chicago, IL)

3. Results

3.1 Ischemia-reperfusion (IR) - induced 20-HETE release in isolated perfused kidneys

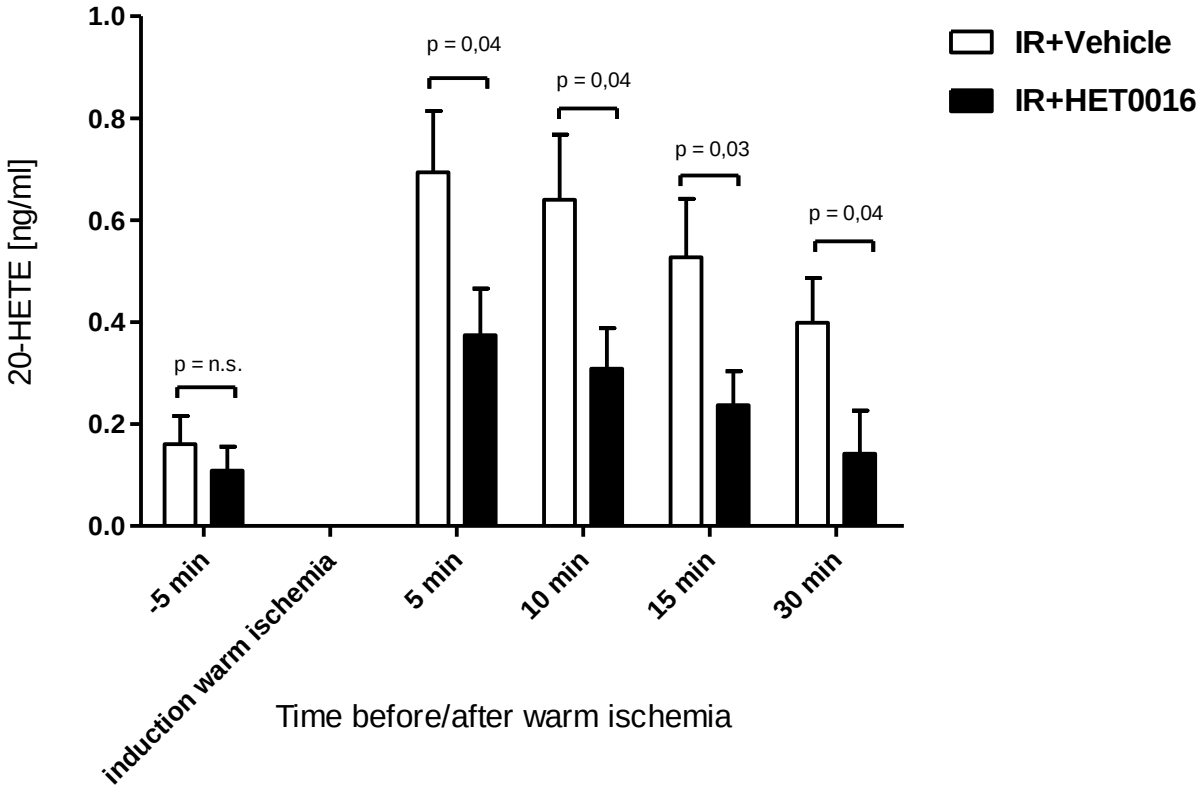


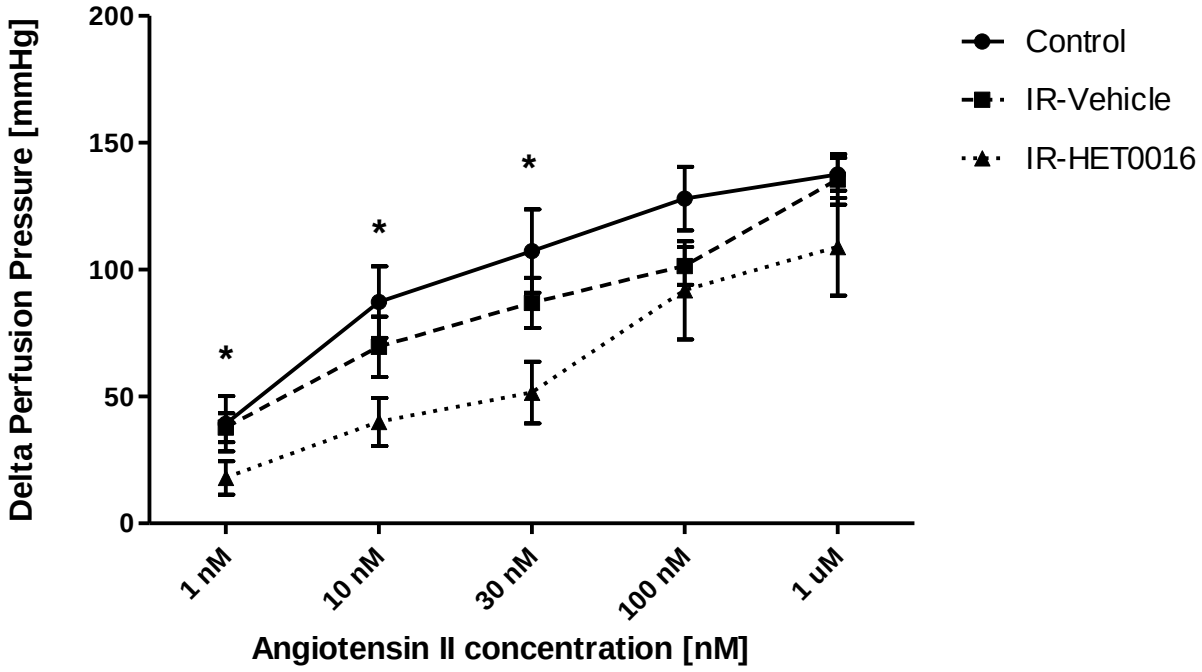
Fig. 4 Influence of IR on 20-HETE release and blocking of 20-HETE by HET0016 in isolated perfused kidneys

IR induced 20-HETE overproduction that was partially blocked by HET0016. Data are given as mean ± SEM (n=6 per group).

We used isolated perfused rat kidneys to analyze the effect of IR on renal 20-HETE production. The experiments were performed in the absence (0.1 % DMSO as vehicle control) or presence of the CYP4A-inhibitor HET0016 (200 nM) to estimate the contribution of 20-HETE de novo synthesis to total 20-HETE release. Under basal conditions, vehicle-perfused kidneys constantly released low amounts of 20-HETE (0.16 ± 0.06 ng/ml) corresponding to about 0.8 ng/min at a flow rate of 5 ml/min. 20 minutes

of non-flow global ischemia induced a significant and sustained overproduction of 20-HETE (See Fig. 4). 20-HETE release was increased more than 4-fold during the first 5 min of reperfusion to 0.69 ± 0.12 ng/ml ($p<0.001$ vs. basal levels; n=6) and remained significantly elevated until the end of the experiment (0.40 ± 0.08 ng/ml). HET0016 did not significantly reduce the basal 20-HETE release but partially blocked IR-induced 20-HETE overproduction. HET0016 reduced 20-HETE release to about 50 % in the initial period of reperfusion (0.32 ± 0.08 ng/ml at 5 min; $p<0.05$ vs. vehicle control; n=6 per group) and mediated a complete decline to basal levels after 30 min of reperfusion.

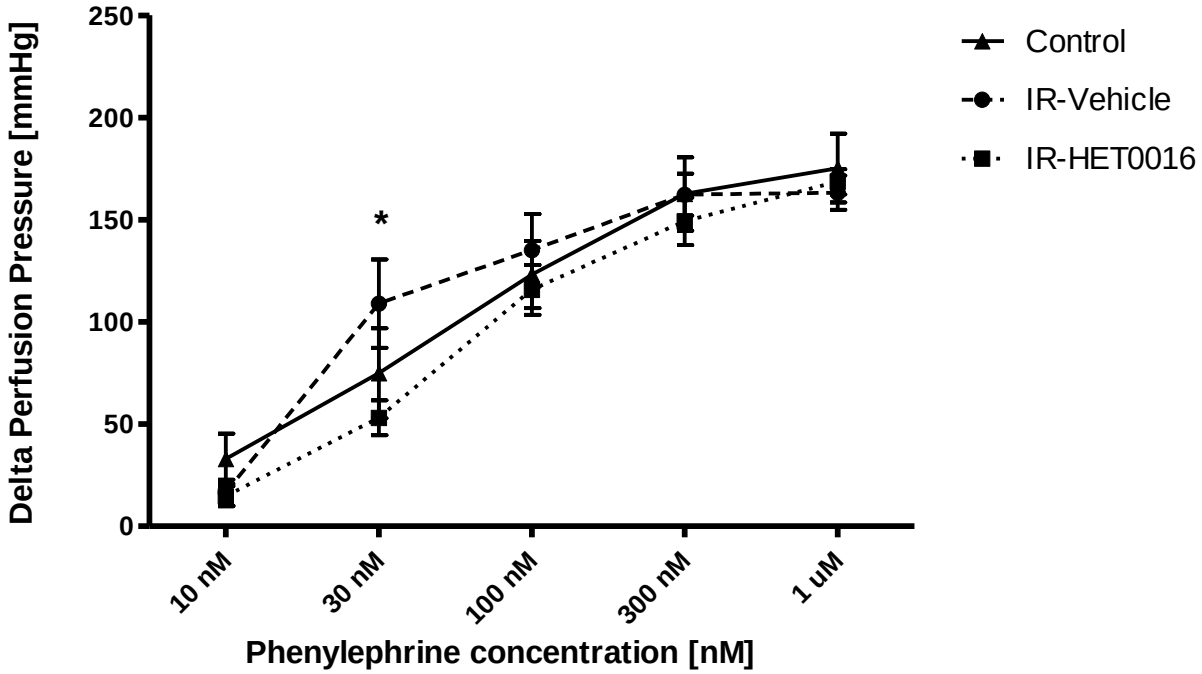
3.2 Vascular reactivity in isolated perfused kidneys



***** - $p < 0,05$ IR-Vehicle vs. IR-HET0016

Fig. 5a Effects of Angiotensin II-induced vasoconstrictor response on the vasculature of isolated perfused rat kidneys (IR+Vehicle vs. IR+HET0016) vs. controls

Angiotensin II, applied at concentrations of 1 nM – 1 uM, caused dose-dependent constrictions in perfused renal vascular beds. HET0016 tended to reduce vasoconstrictory influence of Angiotensin II in kidneys after IR, this tendency reached a significant difference at lower concentrations of Angiotensin II, namely at 1 nM, 10 nM and 30 nM (Fig 5a).



***** - p < 0,05 IR-Vehicle vs. IR-HET0016

Fig. 5b The effects of Phenylephrine-induced vasoconstrictor responses on the vasculature of isolated perfused rat kidneys (IR+Vehicle vs. IR+HET0016) vs. controls

When applied by bolus injection at different doses (10 nM – 1 uM), phenylephrine induced dose-dependent vasoconstriction in the perfused kidneys. IR tended to aggravate vasoconstriction, caused by phenylephrine, however this tendency was not significant in any concentration of phenylephrine. Addition of HET0016 ameliorated phenylephrine-induced vasoconstriction. However this effect was significant only at 30 nM concentration of Phenylephrine (Fig 5b).

3.2 Sham group

Comparing kidney function parameters of native male Lewis rats with sham operatedones, we did not find significant differences: serum creatinine (SCr) 0.31 ± 0.03 mg/dl vs. 0.33 ± 0.06 mg/dl ($p > 0.05$), and creatinine clearance (CCl) 1.44 ± 0.04 vs. 1.37 ± 0.09 ml/min/g. This implies that the surgical procedure has no influence on renal function.

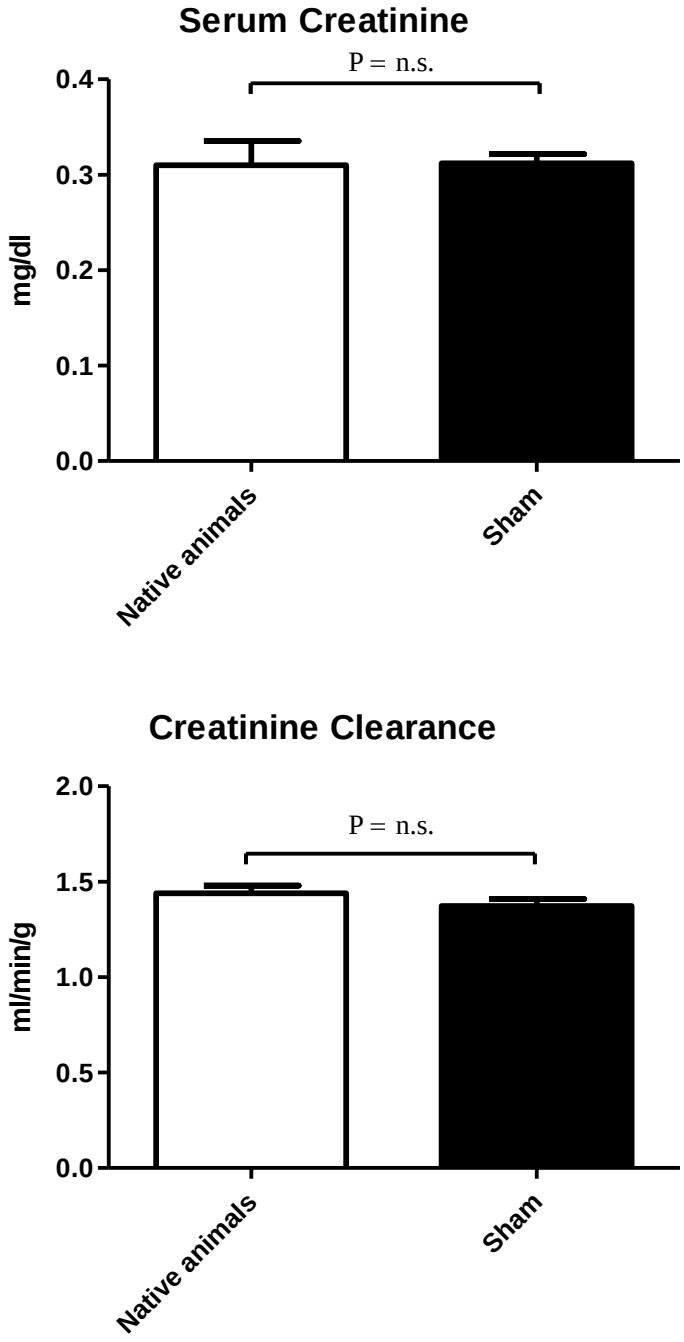


Fig. 6 Serum-creatinine and creatinine clearance levels of native and sham-operated animals, postoperative day 2 (n=3/group)

Differences between groups were not significant.

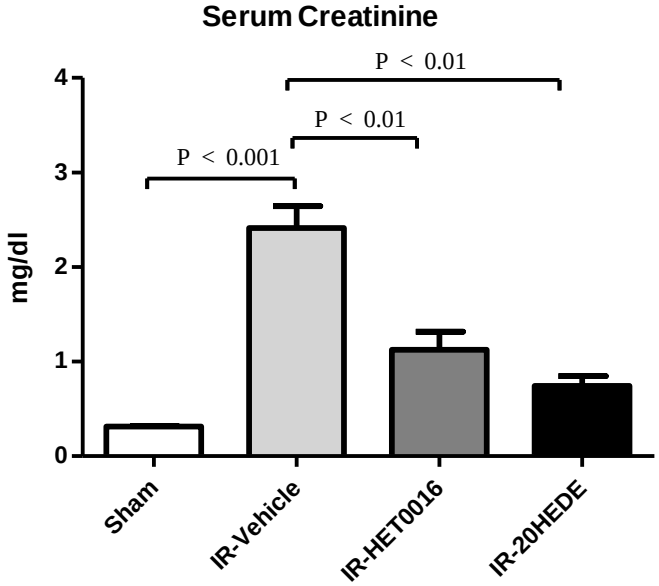
Kidneys of the sham operated animals displayed no morphological abnormalities.

3.3 Ischemia-reperfusion experiments

3.3.1 Renal function

Increased serum creatinine and urea levels, as well as decreased creatinine clearance indicate a significant decline in renal function representative for our model of acute kidney IR injury (Fig. 7). In vehicle-treated animals, 45 min of warm ischemia induced an 8-fold increase in the serum creatinine and urea levels and reduced creatinine clearance to 20% compared to the sham-operated uninephrectomized controls (0.31 ± 0.02 vs. 1.37 ± 0.04, p<0.001, n=6 per group). The Vehicle itself (100 µl of saline containing 1 % DMSO injected via the renal artery 5 min before inducing ischemia) had no effect on the renal function; there were no differences in the kidney function parameters between vehicle and sham groups (creatinine: 2.41 ± 0.23 versus 2.54 ± 0.77 mg/dl; urea: 287.8 ± 34.8 versus 303.2 ± 76.13 mg/dl; creatinine clearance: 0.26 ± 0.02 versus 0.25 ± 0.09 ml/min; all comparisons were n.s.).

IR-induced impairment of renal function (Fig. 7) was significantly ameliorated by pretreatment with HET0016 (50 µg injection via the renal artery) or 6,15-20-HEDE (20 µg injection). The creatinine clearance was improved to 0.63 ± 0.12 ml/min with HET0016 (p<0.01 vs. vehicle; n=8 per group) and to 0.76 ± 0.12 ml/min with 6,15-20-HEDE (p<0.01 vs. vehicle; n=8 per group).



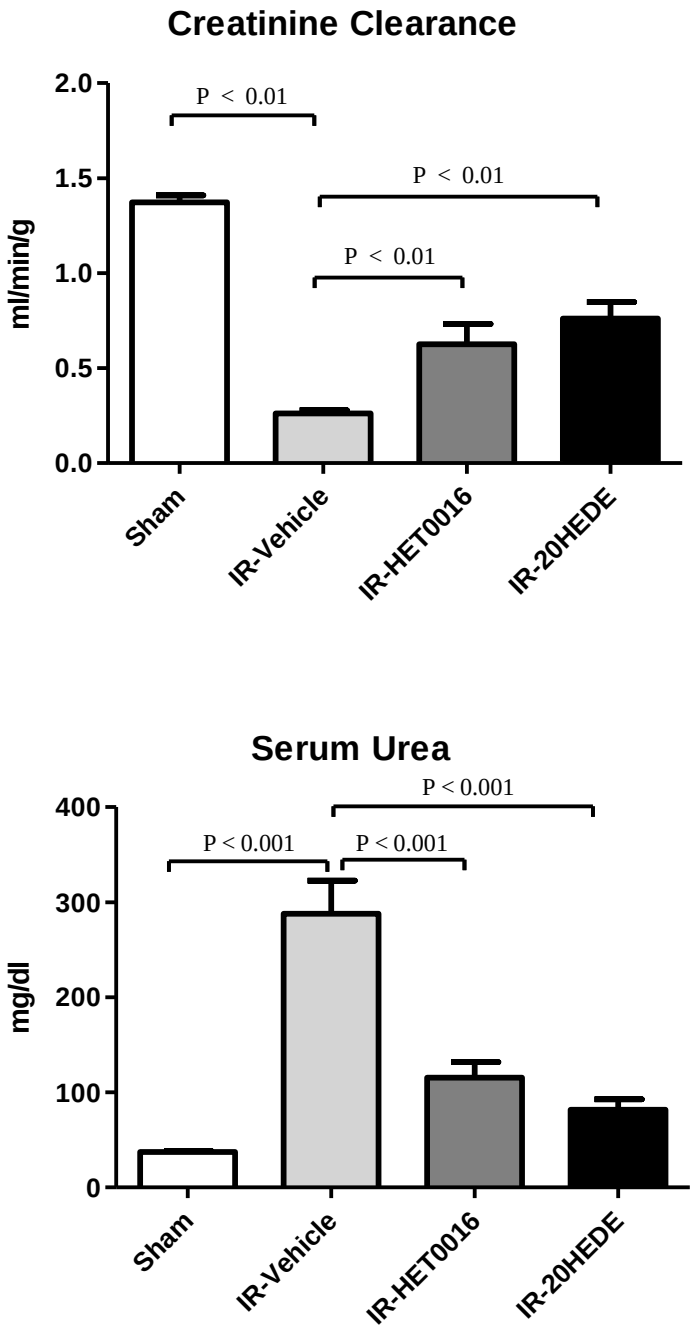


Fig. 7 Serum-creatinine, creatinine clearance and serum urea levels of sham-operated rats and animals after induction of 45 min of warm ischemia, day 2 after operation (n=6/group)

Differences between sham-operated rats and animals that underwent 45 min of warm ischemia were significant, irrespectively of the substance used for pretreatment. One-way analysis of variance revealed significant differences between vehicle-pretreated animals and those pretreated with the 20-HETE blocker or the antagonist.

3.3.2 Urinary sodium excretion, fractional excretion of sodium

IR damage leads to a significant decrease in urine sodium excretion and increase in fractional excretion of sodium. Injection of 20-HETE inhibitors prior to IR does not influence sodium excretion.

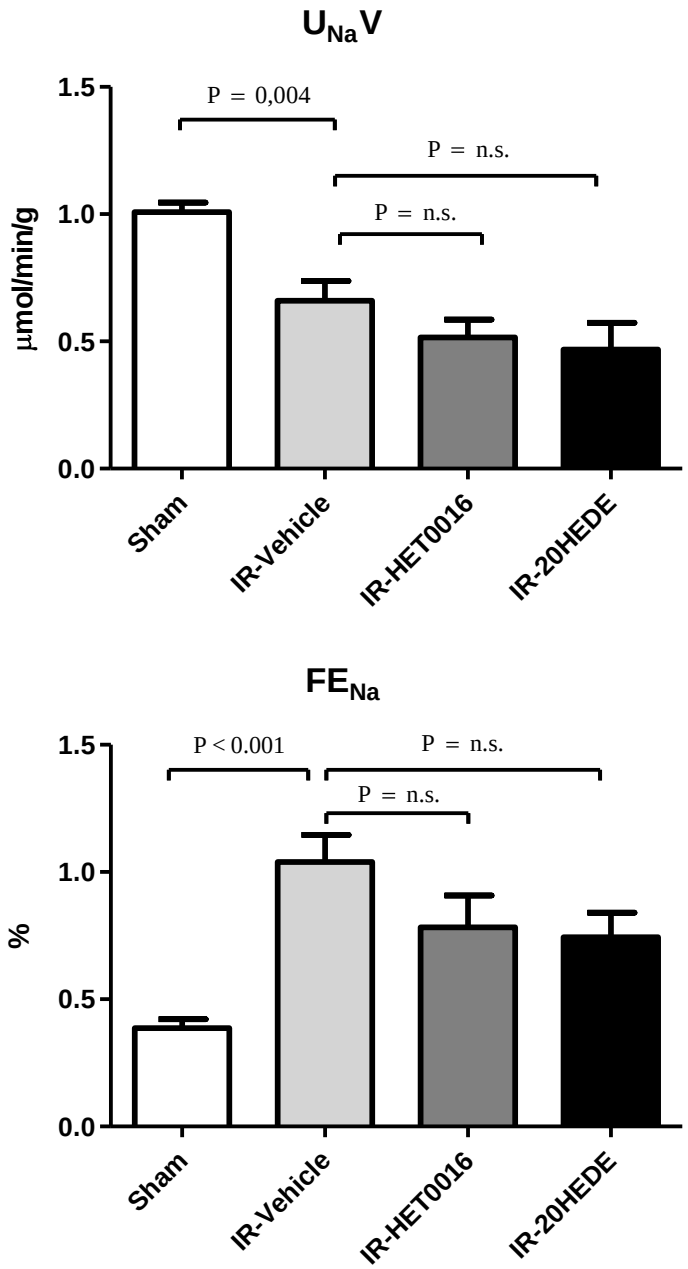


Fig. 8 Urinary sodium excretion and fractional excretion of sodium in sham operated rats and those, exposed to 45 min of warm ischemia, day 2 after renal pedicle clamping (n=6/group) Significant differences are present between sham operated animals and those exposed to IR independent of pretreatment. The differences between IR groups with different pretreatment were not significant.

3.3.3 Renal morphology

IR induced severe renal tubular damage (Fig. 9, 10). Typical signs of the injury were the occurrence of flattened tubular epithelium, exfoliated tubular epithelial cells, widened tubular lumina, hyaline cast formation, and necrotic tubules as shown in a representative image of HE-stained sections of the outer medulla of vehicle-treated animals (Fig. 9). The degree of necrotic renal injury was significantly higher in the outer medulla (ATN score: 2.50 $\pm$ 0.20) compared to the renal cortex (ATN score: 1.33 $\pm$ 0.12) (Fig. 10). The ATN scores were significantly reduced by administration of HET0016 (outer medulla: 1.04 $\pm$ 0.31, $p<0.001$ vs. vehicle; cortex: 0.58 $\pm$ 0.27, $p=0.05$ vs. vehicle). Also the 20-HETE antagonist 6,15-20-HEDE exerted a strong protective effect and reduced the ATN scores to 0.92 $\pm$ 0.21 in the outer medulla and to 0.41 $\pm$ 0.16 in the renal cortex (both $p<0.001$ vs. vehicle).

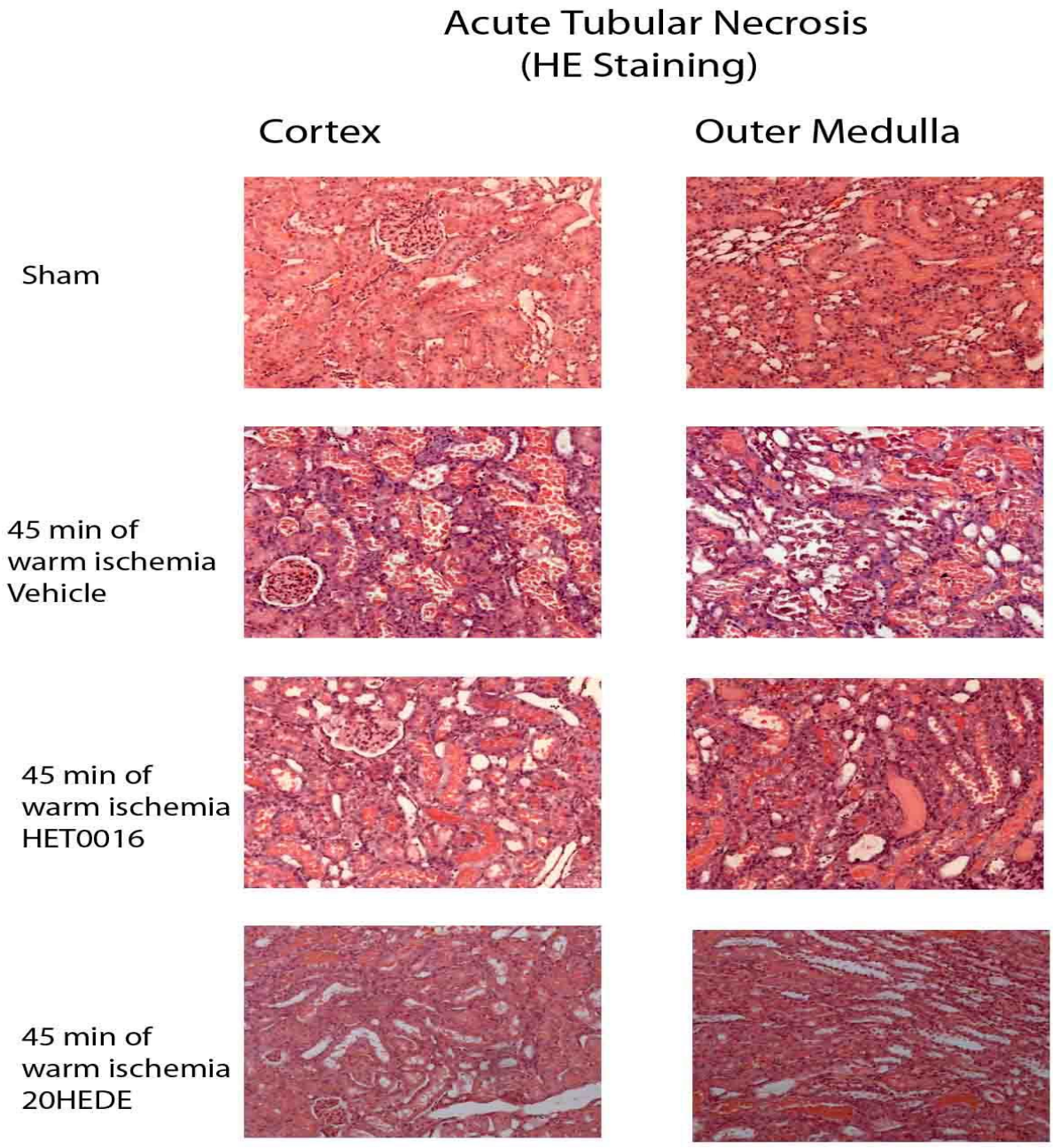


Fig. 9 Renal histology (hematoxylin and eosin staining) showing features of acute tubular necrosis (ATN) in kidneys of animals after sham and clamping operation. Postoperative day 2 Kidney histology of sham-operated animals is normal. Comparison of kidneys after IR and vehicle pretreatment versus IR and HET0016 or 20HEDE-pretreatment shows more widespread tubular injury with complete epithelial cell necrosis and denuded basement membranes. IR induced more pronounced tubular damage in the outer medulla. Magnification X 400.

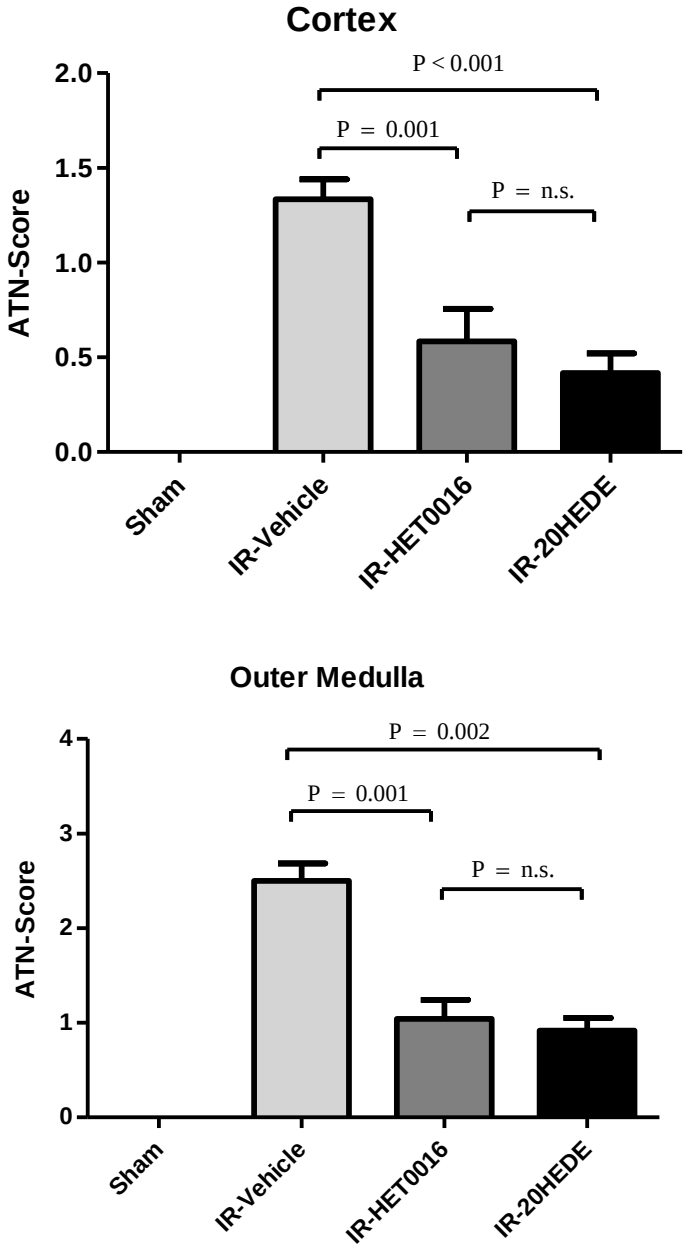


Fig. 10 Acute tubular necrosis score. Kidneys of the animals after sham and clamping operations. Postoperative day 2 (n=6/group)

Kidneys of sham operated animals displayed no acute tubular necrosis. Forty-five min of warm ischemia caused severe tubular damage that was significantly lower in animals pretreated with 20-HETE-inhibitors compared with vehicle. There was no significant difference in ATN-Score between animals pretreated with HET0016 or 20HEDE. IR induced tubular damage was more pronounced in the outer medulla than in the cortex irrespectively of the substance used for pretreatment.

3.3.4 Inflammatory cell infiltration

IR induced renal inflammation as indicated by a dense infiltration of monocytes/macrophages into the damaged zones of the outer medulla and renal cortex. Representative immunohistochemical staining for ED1+ cells is shown in Figs. 11 and 12. Monocytes/macrophages were predominantly located in the tubulointerstitium adjacent to injured tubules and around small arteries. Morphometric quantification displayed higher cell counts in the outer medulla (53.4 $\pm$ 1.6 cells per field of view (FoV)) compared to renal cortex (46.8$\pm$ 2.0 cells per FoV) of vehicle treated animals (Fig. 11). Application of HET0016 attenuated the response by about 50% (outer medulla: 24.7 $\pm$ 4.7 and cortex: 15.2 $\pm$ 1.7 cells per FoV, both $p < 0.001$ vs. vehicle control). The 20-HETE antagonist 6,15-20-HEDE also markedly reduced ED1+ infiltrates to 19.0 $\pm$ 5.9 in the outer medulla and to 15.5 $\pm$ 4.4 cells per FoV in the cortex (both $p<0.001$ vs. vehicle).

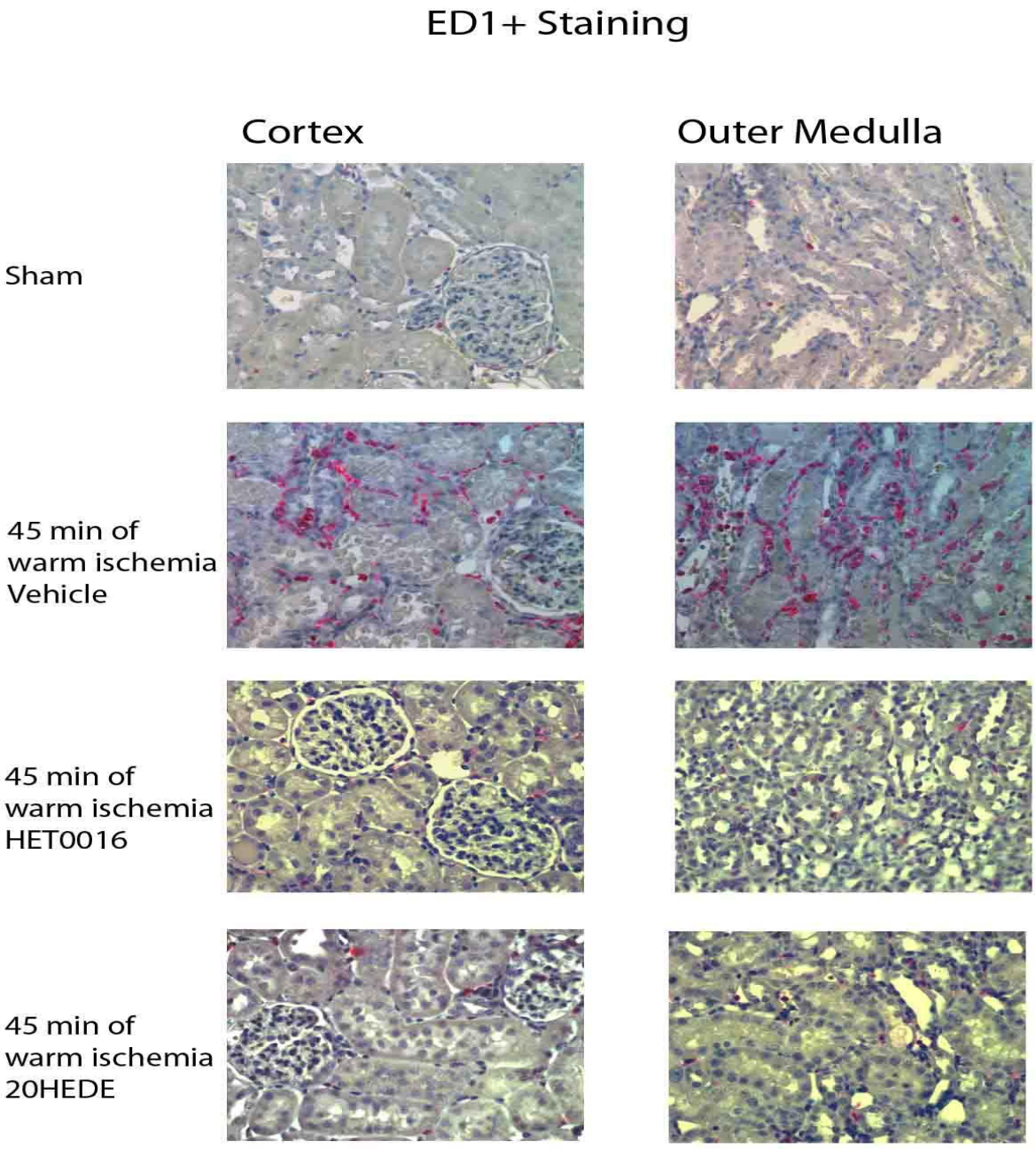


Fig. 11 ED1-positive monocytes/macrophages in representative cross-sections of rat kidneys after sham and clamping operation. Postoperative day 2

Kidneys of sham operated animals indicate the presence of smaller numbers of monocytes/macrophages than kidneys after IR in cortex and outer medulla, although the infiltration of ED1+ positive cells is larger in the outer medulla of all groups. Kidneys after IR injury and vehicle-pretreatment show the highest monocytes/macrophages density. Magnification X 400.

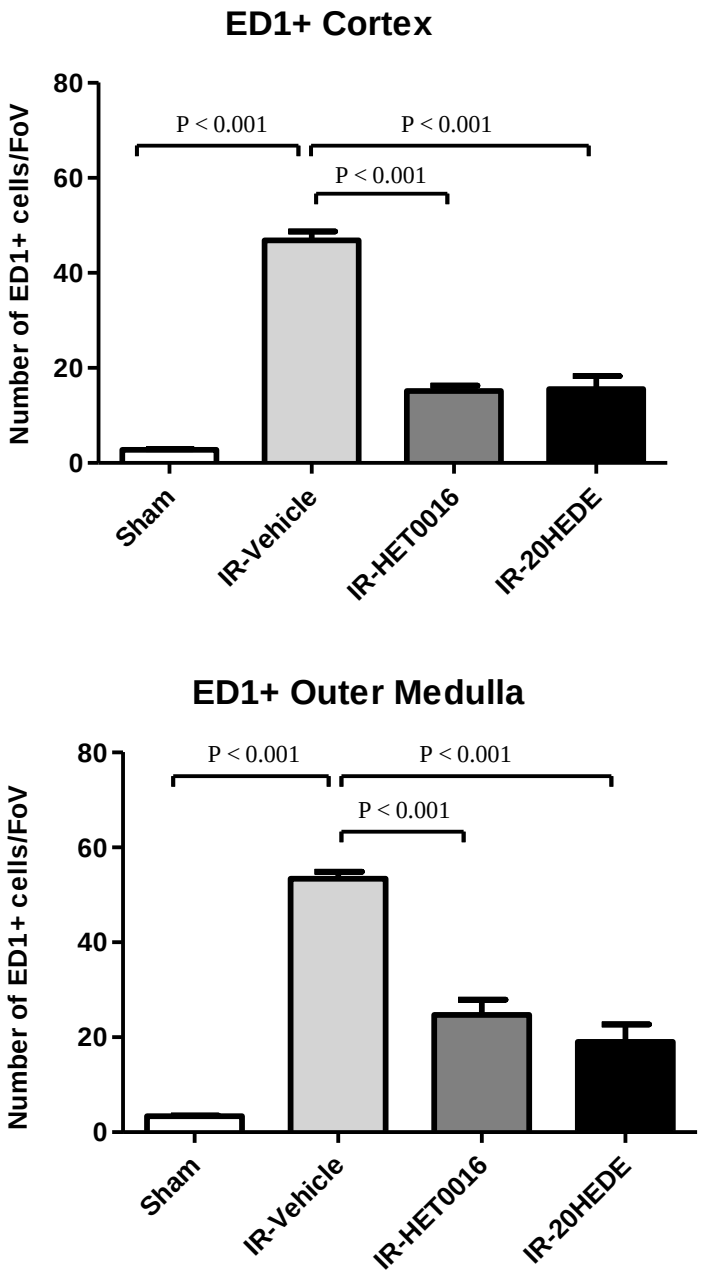


Fig. 12 Infiltration of ED1-positive monocytes/macrophages in kidneys of animals after sham vs. clamping operation. Postoperative day 2 (n=6/group)

Kidneys after warm ischemia showed a significant increase in the number of monocytes/macrophages per field of view. Both HET0016 and 20HEDE significantly reduced the number of ED1+ cells compared to vehicle treatment. There was no significant difference in the number of inflammatory cells per field of view between the animals pretreated with HET0016 or 20HEDE. The number of ED1+ positive cells was higher in the outer medulla than in the cortex in all groups.

3.3.5 Tubular cell apoptosis

IR-induced renal injury resulted in a marked increase in apoptotic tubular epithelial cells as assessed by TUNEL staining (Fig. 13, 14). Semi-quantitative evaluation showed that apoptosis, as well as acute tubular necrosis, was more pronounced in the outer medulla (4.10 $\pm$ 0.26 % per FoV) than in the renal cortex (1.87 $\pm$ 0.27 % per FoV). The number of apoptotic cells was significantly reduced by pretreatment with HET0016 (outer medulla: 1.37 $\pm$ 0.27 %, cortex: 0.53 $\pm$ 0.12 % per FoV; both $p < 0.001$ vs. vehicle control) or 6,15-20-HEDE (outer medulla: 0.94 $\pm$ 0.42 %, cortex 0.52 $\pm$ 0.21 % per FOV; both $p < 0.001$ versus vehicle).

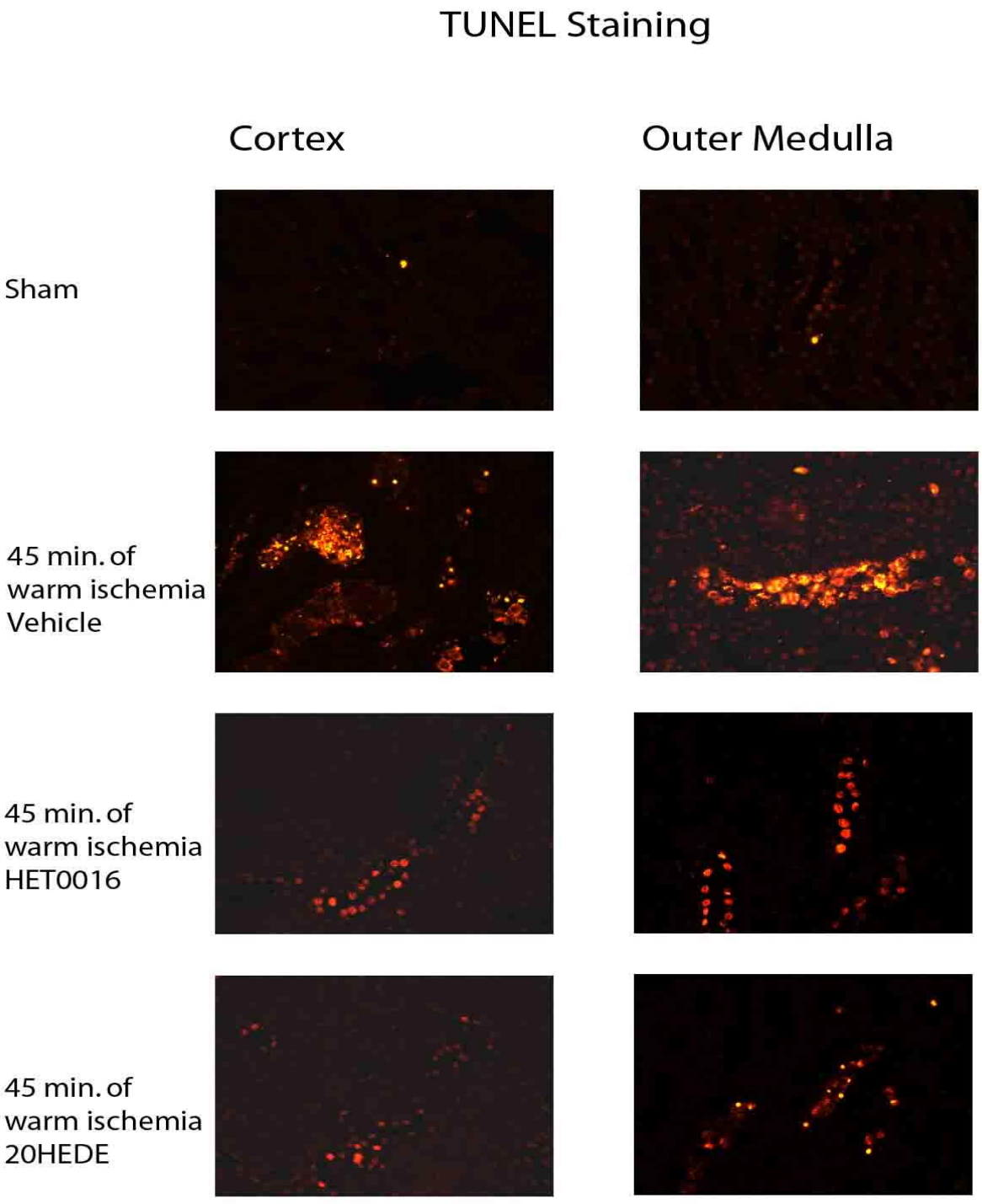


Fig. 13 Fluorescein-stained apoptotic cells in representative cross-sections of rat kidneys after sham and clamping operation. Postoperative day 2

Areas of confluent fluorescence in kidneys after ischemia-reperfusion indicate the presence of large numbers of TUNEL-positive apoptotic cells, while kidneys of animals after sham operation show little fluorescence. Magnification X 400.

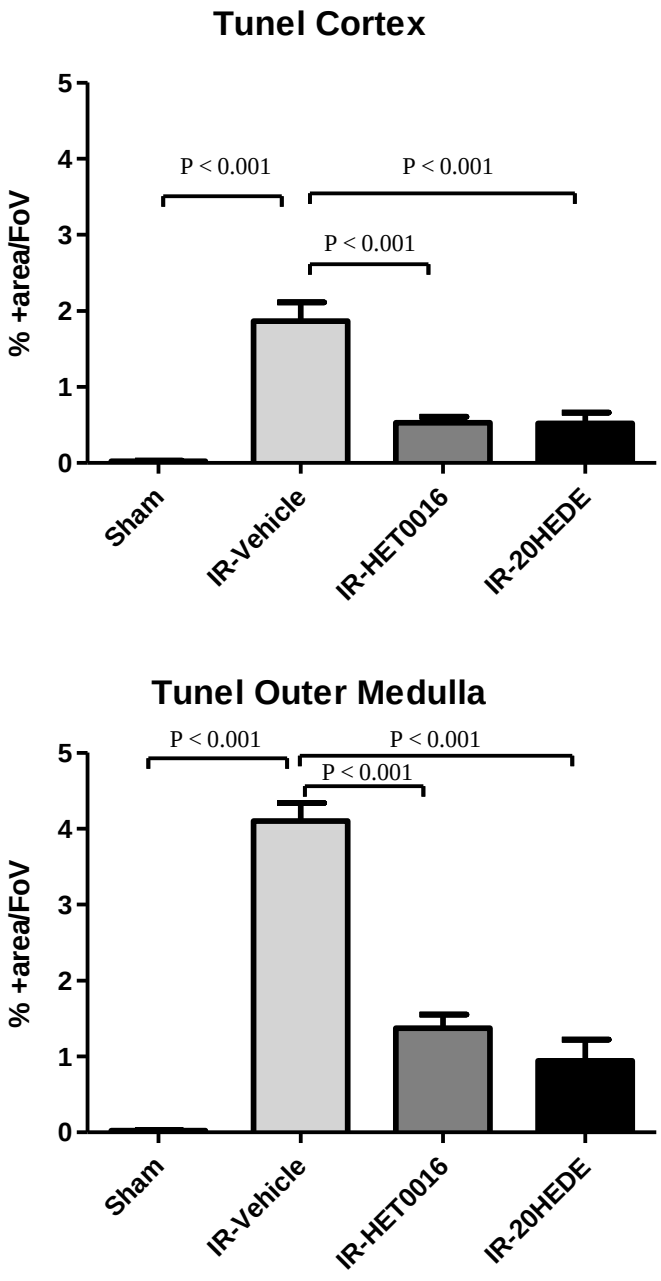


Fig.14 TUNEL-positive apoptotic cells in rat kidneys after sham operation and renal pedicle clamping. Postoperative day 2. (**n=6/group)**

IR caused a significant increase of apoptotic cells in kidney. HET0016 or 20HEDE-pretreatment before induction of warm ischemia significantly reduced the number of apoptotic cells in the cortex and medulla, while there was no significant difference in the number of apoptotic cells between groups pretreated with HET0016 or 20HEDE.

4. Discussion

Ischemia-reperfusion injury is a major problem in kidney surgery that can cause acute renal failure and impair renal function after kidney transplantation or nephron-sparing surgery.

The detrimental role of 20-HETE in IR Injury of the heart and brain was scientifically proven in numerous studies [19, 29, 34, 53] but the reports of its role in the kidney remain controversial [38 - 39].

We hypothesized that inhibiting diverse effects of the vasoconstrictor 20-HETE may improve the function and morphology of rat kidneys subjected to warm ischemia.

Our clamping model with 45 min of warm ischemia is life supporting, it imitates the clinical situation in the operating room: time of renal ischemia during partial resection of the kidney is approximately 45 min [54], time of vessel anastomosis during kidney transplantation also averages 45 minutes [55].

Different attempts were made to establish an animal model for nephron-sparing surgery [56 - 57]. Partial nephrectomy model without removal of the contralateral kidney mimics the clinical situation, but the important limitation of this model is the disability to assess a split renal function due to compensatory functioning of the remaining contralateral kidney [57 - 58]. Unilateral nephrectomy accompanied by partial nephrectomy with application of ischemia of the remaining kidney on animals has a substantial death rate greater than 90% three to four days after surgery due to renal insufficiency [58]. The benefits of the clamping model used in our study are a high survival rate (about 100 %) and the possibility to measure the influence of the investigated substances on both the function and the morphology of the ischemic kidney.

In the kidney, cytochrome P450-dependent metabolism of arachidonic acid may cause divergent reactions under IR conditions. In the proximal tubule and medullary ascending loop of Henle 20-HETE promotes natriuresis and ameliorates IR injury [20], while in the vasculature 20-HETE causes vasoconstriction of the renal arteries and arterioles, thus aggravating IR damage [36, 59 - 60]. Therefore, it was important to achieve a direct and selective inhibition of 20-HETE in the kidney vasculature by

injecting 20-HETE inhibitors directly into the renal artery. Furthermore, our route of administration of 20-HETE inhibitors directly into the renal artery avoided its systemic vasodilatory effect.

Eicosanoids are 20 carbon fatty acid derivatives (eicosa, Greek for 20) produced from arachidonic acid through three major enzymatic pathways: cyclooxygenase (COX), lipoxygenase (LO), and cytochrome P450 monooxygenase (CYP450). [61 - 62].

Cytochrome P450 monooxygenase (CYP450) oxidizes free arachidonic acid to produce hydroxy (HETE)- and epoxy (EET)-arachidonic acid derivates. The major CYP450-catalyzed reactions in most tissues are mediated by epoxygenase and o-hydroxylase activities of the CYP450 family, which are responsible for biosynthesis of epoxyeicosatrienoic acids EETs and 20-HETE, respectively [36, 62] (See Fig.15).

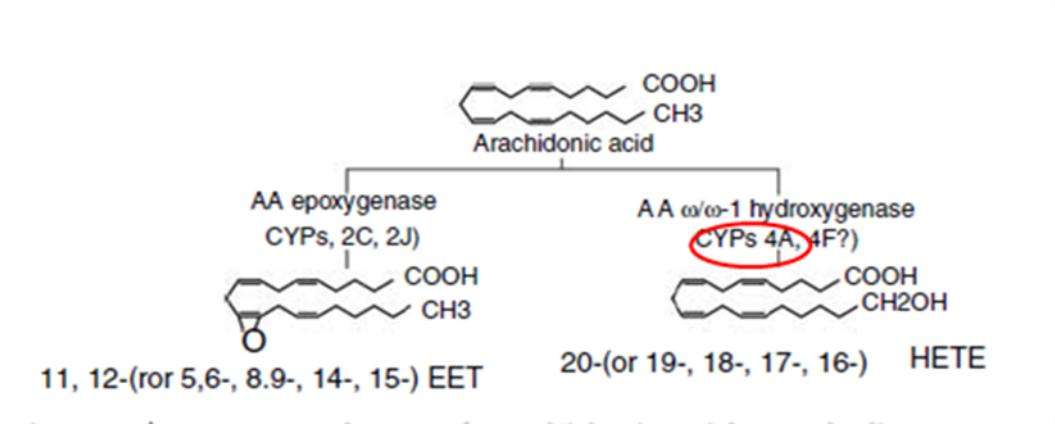


Fig. 15 CYP450 pathway of arachidonic acid metabolism [61].

CYP450 monooxygenases have diverse physiological and pathophysiological functions in renal vascular structures [38 - 39]. 20-HETE is an endogenous constrictor of renal arteries and preglomerular arterioles [36, 59]. In contrast EET, which is also produced in the vascular endothelium, is a potent vasodilator [36, 38 - 39, 61]. We used inhibitors of CYP4A, thus selectively inhibiting 20-HETE production.

A variety of CYP4A-inhibitors have been synthesized in order to block 20-HETE production, for instance 12,12-dibromododec-11-enamide (DBDD) and N-methylsulfonyl-12, 12-dibromododec-11-enamide (DDMS) as a second generation of inhibitors to selectively block the formation of 20-HETE [11, 63]. These compounds competitively inhibit the formation of 20-HETE by renal microsomes at concentrations of 10 μM, whereas epoxygenase activity is also reduced by 10–20% [11]. At higher

concentrations (50 μM), they are equally effective at inhibiting the formation of EET and 20-HETE. The main limitation of using these inhibitors for *in vivo* studies is that they are fatty acids that readily bind to plasma proteins. This property limits their ability to diffuse into tissues and inhibit the formation of 20-HETE when given via a blood-borne route. Therefore, these compounds are only effective at inhibiting the formation of 20-HETE when added to protein-free solutions *in vitro* or when directly applied to tissues *in vivo*.

More recent studies have applied a new inhibitor of the formation of 20-HETE, N-hydroxy-N'-(4-butyl-2 methylphenyl) formamidine (HET0016) [64]. HET0016 appears to be the most specific inhibitor of the synthesis of 20-HETE currently available (Fig.16). This compound selectively inhibits the formation of 20-HETE at a low concentration (10 nM). It has no effect on epoxygenase, cyclooxygenase (COX), or lipoxygenase activity at concentrations up to 1 μM [64]. At this concentration, it also has minimal effects on the activity of other CYP isoforms (2CYP2C9, 2D6, 3A4) involved in drug metabolism in humans [64]. We therefore chose 50 μg/μl as our standard dose of HET0016. We showed that 50 μg/μl HET0016 strongly inhibits 20-HETE synthesis in isolated perfused kidneys (Fig. 4).

A number of analogs of 20-HETE, such as 5-, 15-, and 19-HETE, block the vasoconstrictive actions of 20-HETE in renal arteries [65]. The most effective analog appears to be 20HEDE – 20-hydroxyeicosa-6,15-dienoic acid. Unlike the other similar compounds (5-, 15-, and 19-HETE), it is not metabolized by COX. It completely blocks the vasoconstrictive response of the renal arterioles to 20-HETE at a small concentration (0.5 μM) [36, 65]. This led to our decision to use 20HEDE as an optimal inhibitor of 20-HETE action (Fig. 16).

Our pharmacological interventions that aimed at inhibiting CYP-dependent 20-HETE synthesis and blocking 20-HETE action were similarly effective in preserving renal function. The data on necrosis, inflammation, and apoptosis suggests that blockade of 20-HETE action may be more promising in preventing renal injury than inhibition of 20-HETE synthesis. We assume that HET0016-mediated inhibition of 20-HETE synthesis only partially reduced the total release of 20-HETE upon IR, whereas 6,15-20-HEDE had the capacity to antagonize the action of total free 20-HETE, independent of its origin. In agreement with this assumption, HET0016 significantly but only partially blocked the release of 20-HETE from isolated perfused kidneys after hypoxia (Fig. 4). We speculated that the additional source of free 20-HETE was

probably a phospholipase-mediated release of preformed 20-HETE from membrane stores where this eicosanoid is esterified to the sn-2 position of phospholipids [66].

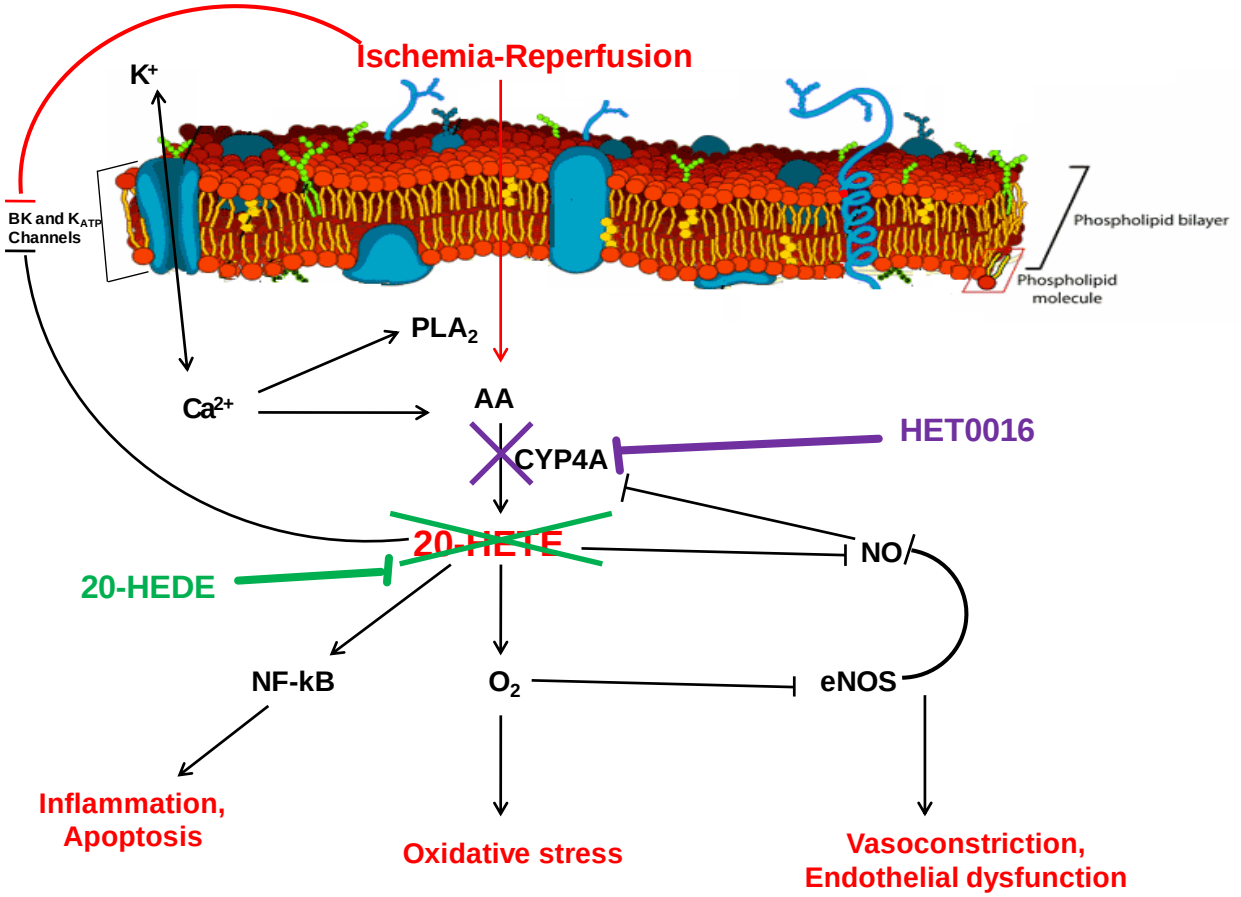


Fig. 16 Mechanism of action ot 20-HETE inhibitors

Picture of the cell membrane: UNSW Cell Biology 2008 ANAT3231 Lecture 03 - Compartments and Membranes

Increased levels of CYP4A isoforms have been found in diverse pathophysiological mechanisms accompanying kidney diseases. The expression of CYP4A enzymes and formation of 20-HETE is elevated in diabetic kidney [67 - 68], cyclosporin-induced and cisplatin-induced nephrotoxicity [40, 67 - 69]. The renal excretion of 20-HETE is elevated in Bartter's syndrome and in hepatorenal syndrome [68]. Considerable evidence indicates that overproduction of 20-HETE in kidney in several models of hypertension contributes to end-organ damage by enhancing renal

hypertension [70]. So far the function of CYP4A metabolites and 20-HETE in acute kidney injury has not been well characterized [36].

We have demonstrated that injection of 20-HETE inhibitors prior to IR significantly improves early outcomes after clamping of the renal pedicle for 45 min (Fig. 7). We have confirmed that 20-HETE suppression has an immediate renoprotective effect leading to an early recovery of kidney function, as seen by an improvement in serum creatinine and creatinine clearance.

In analogy to our study, Dolegowska et al. examined the dynamics of 20-HETE changes during the first 5 min of kidney allograft reperfusion in humans and analyzed whether the observed alterations were associated with post-transplant graft function. The analysis revealed that throughout the reperfusion period, 20-HETE concentrations were significantly lower in the early graft function group compared with the delayed graft function group [71].

Nakamura et al. [72] investigated the effects of cisplatin treatment on the kidney and found that it significantly elevated the level of CYP4A, the promoter of 20-HETE, and that the subsequent enhancement of 20-HETE expression proportionally correlated with increased levels of blood urea nitrogen and creatinine.

20-HETE production has been shown to be increased in rats treated with cyclosporine A [69]. Coincidentally, the side-effects of cyclosporine A, when used as an immunosuppressant, include reduced glomerular filtration rate and afferent arteriolar constriction with subsequent hypertension, effects that are also caused by 20-HETE. There is a strong correlation between blood pressure and proteinuria, indicating that renal pathological changes evoked by cyclosporine A are tied to vasoconstriction and blood pressure changes. This is consistent with our results that inhibition of 20-HETE with HET0016 lowers blood pressure and improves renal function and morphology.

In the study of polycystic kidney disease on a rat model [73] Park et al. showed that treatment with HET0016 not only decreases the size of the kidney-cysts but also reduces blood urea nitrogen and plasma creatinine concentration, which is consistent with out functional data (Fig. 7).

In contradiction to our results, Regner et al. [48] found that systemic pretreatment of rats with 20-HETE analogs (s.c. injection of vehicle or HET0016, 30 min before induction of renal ischemia or 1 min thereafter) significantly attenuated IR injury by increasing urine sodium excretion without changing glomerular filtration rate (GFR) or

mean arterial blood pressure. Contradictions in our findings may be related to either the use of different models or treatment forms. We used uninephrectomized Lewis rats with unilateral clamping of the left renal vessels and local low-dose drug application, whereas Regner et al. used Sprague-Dawley rats with bilateral clamping of two kidneys and systemic high-dose drug administration. Moreover, Regner et al. only used 20-HETE agonists and did not compare their effects with those of 20-HETE antagonists. A specific concern associated with high dose systemic levels of HET0016 may arise from the potential of this CYP-inhibitor to inhibit not only 20-HETE synthesis, but also the ω-hydroxylation and inactivation of leukotriene B4 that functions as neutrophil chemoattractant and thereby aggravates ischemic renal damage [74]. However, these contradictory findings may also reflect the complex vascular and tubular roles of 20-HETE that must be carefully considered in any study on the function of this CYP eicosanoid in the kidney. This very problem also led to apparently contradictory findings on the pro- and antihypertensive roles of 20-HETE. Spontaneously hypertensive rats and salt-sensitive Dahl rats develop hypertension that is associated with either overproduction or deficiency of 20-HETE [75 - 76]. This apparent contradiction has been largely resolved recognizing that vascular overproduction of 20-HETE induces vasoconstriction, endothelial dysfunction and hypertension, whereas tubular deficiency of 20-HETE impairs salt excretion thus causing hypertension. This dual and site-specific role of 20-HETE is similarly reflected by the opposite effects of HET0016 in different models, namely reduction of blood pressure in androgen- and cyclosporine A-hypertensive rats versus induction of salt-sensitive hypertension in normal Sprague-Dawley rats [12, 69, 77]. In our study 20-HETE plays different roles in the acute phase of IR kidney injury. We intentionally injected low doses of HET0016 (50 µg) and of the 20HEDE (20 µg) directly into the renal artery just before occlusion, given the short biological half-life of HET0016 (about 50 min *in vivo*) [78]. We intended to achieve effective drug levels during ischemia (Fig. 4) and to allow a rapid washout upon reperfusion. In contrast, Regner et al. used high doses of HET0016 (5 mg/kg) and 5,14-20-HEDE (10 mg/kg) and applied the compounds subcutaneously 30 min prior to 45 min of warm ischemia, which could limit 20-HETE inhibition during the reperfusion in their experimental model. In support, they showed that 20-HETE agonist was also effective when applied immediately after reperfusion. Taken together, the apparent contradiction between our results and those of Regner et al. indicates the existence of rather strict therapeutic windows in terms of dosage and timing that determine the

outcome of pharmacological interventions targeting the complex CYP/20-HETE-sytem in the kidney.

In addition Regner et al. attributed the renoprotective effect of 20-HETE analogs in IR injury to the inhibition of tubular sodium reabsorption and subsequent moderation of renal medullary hypoxia in a manner similar to that of other inhibitors of tubular electrolyte transport. Active reabsorption of sodium is a process that requires a large amount of oxygen, that is why decreases under IR conditions of the kidney [82]. Insufficient reabsorption of sodium by renal tubule due to insufficient oxygenation activates (distally, at the macula densa) signals that constrict the glomerulus, reducing filtration and therefore the delivery and reabsorption of tubular solute. This mechanism explains the reduction of absolute sodium excretion ($U_{Na}V$) in the rats that underwent IR (Fig. 8).

Thus, hypoxic impairment of the Na-reabsorption in the medullary thick limbs reduces glomerular filtration, which relieves medullary oxygen insufficiency whenever the workload exceeds capacity and this might attenuate medullary hypoxia. However, the selective dopamine receptor-1 agonist Fenoldopam, that also reduces sodium reabsorption, but in contrast to 20-HETE is a vasodilator, has been clinically tested for the prevention or treatment of acute kidney injury without a positive feedback [48], suggesting that the tubular renoprotective effects of 20-HETE, presented by the inhibition of tubular sodium reabsorption, are of less importance in the pathomechanism of IR in the kidney then its deleterious role in the renal vasculature.

Commonly used tests for the investigation of renal sodium excretion are the fractional excretion of sodium and urinary sodium excretion. Fractional excretion of sodium (FE_{Na}) characterizes the excreted fraction of filtered sodium [83]. Urinary sodium excretion ($U_{Na}V$) is the absolute amount of sodium excreted with urine per period of time (minute), measured taking into account a kidney weight.

We showed that the pretreatment of the animals with 20-HETE inhibitors only led to a mild, not to a significant decrease of $U_{Na}V$ and FE_{Na} (Fig. 8).

Previous studies have demonstrated a detrimental role of 20-HETE and CYP4A in ischemic injury of the heart and brain. In this regard, selective inhibition of CYP4A isoforms protected myocardium from ischemic injury. Nithipatikom et al. [37] in their study on dogs revealed that 20-HETE pretreatment produces a detrimental effect on

ischemia-reperfusion injury in canine hearts, and the inhibition of 20-HETE markedly reduces myocardial infarct size. The inhibition of 20-HETE production counteracted cerebral vasospasm and reduced infarct size in animal models of ischemic stroke [19], [29, 34]. For instance Poloyac et al. [78] revealed that 20-HETE inhibitor HET0016 improved cerebral blood flow and significantly reduced cerebral infarct size after temporary middle cerebral artery occlusion in rats.

Kucuk et al. [79] in their study on rats showed that pretreatment of animals with glyceryl trinitrate significantly reduced ATN and improved renal function after 45 min of experimental warm ischemia.

In our present study clamping of the renal pedicle induced severe acute tubular necrosis in the kidney. We here showed that inhibition of 20-HETE in ischemic kidney reduced the acute tubular necrosis score (ATN) (Figs. 9, 10). Our results are in line with those of Mahmoud et al. [2] who studied IR injury of the rat kidney on a model analogous to ours. They induced IR injury in rats by clamping of the left renal artery for 45 min and removal of the right kidney. Renal function and histology were assessed on day 2 postoperatively. The animals received an intravenous bolus of 2 vasodilators: Prostaglandin E1 and NO precursor L-Arginine. The study revealed that the pretreatment of the rats with both vasodilators significantly improved renal function and decreased acute tubular necrosis score compared to those of control animals. Similar to our findings pretreatment with L-Arginine (Prostaglandin E1 and NO precursor) also caused a reduction in FE_{Na} that was increased after IR, although it did not reach a significant difference.

In our study acute tubular necrosis was most prominent in the outer medulla. In accordance with our results Fuller et al. [80] in their experimental model of kidney transplantation on rats have revealed that grafts transplanted after 24 hrs of cold storage showed more severe tubular injury in the outer medulla of the kidney than in the cortex on post-transplantation day 3.

Apoptosis of tubular epithelial cells is a common feature of ischemic and nonischemic renal injury and typically occurs at an earlier time point than tubular cell necrosis [16]. We have shown that apoptosis, similar to acute tubular necrosis, was more pronounced in the outer medulla compared to the cortex. We demonstrate here a strong antiapoptotic effect of 20-HETE inhibitors, when given to donor kidneys before ischemic injury (Figs. 13, 14).

Nilakantan et al. demonstrated that *in vitro* continual production of 20-HETE increases cytotoxicity in renal epithelial cell culture following a simulated IR injury model. The increased cell death was associated with increased levels of apoptosis-promoter caspase-3, which can be blocked by a specific inhibitor of 20-HETE synthesis, HET0016. This data implicate the possibility of direct cytopathic proapoptotic effects of 20-HETE on renal epithelial cells during IR [45].

A study on the heart indicated that 20-HETE acts as an inhibitor of ATP-sensitive potassium (K_{ATP})-channels [86]. This mechanism may link 20-HETE overproduction to hypoxic cell injury. Opening of mitochondrial and plasmalemmal K_{ATP}-channels is a key component of survival mechanisms in several cell types [87] and also provides a basis for pharmacological interventions to improve IR-induced renal injury [88]. Thus, 20-HETE is apparently involved as a mediator in many of the pathophysiological mechanisms leading to acute kidney injury and apoptosis. However, a more detailed understanding of this pleiotropic detrimental role of 20-HETE overproduction is hampered by the fact that the primary cellular target, the putative 20-HETE receptor, is thus far unknown.

In vivo experiments on a rat myocardial infarction model [89] revealed that 20-HETE suppression not only decreases the infarct size, but also diminishes the number of apoptotic cells in the infarction area.

20-HETE blocker HET0016 not only has a renoprotective effect, but also has a vasodilatory action, as we demonstrated in the isolated perfused kidneys of rats (Figs. 5a, 5b). In keeping with our findings, another major vasodilator, sildenafil, improved renal function after experimental renal IR injury [90]. Reduction of tubular damage and number of apoptotic cells, achieved by pretreatment of the animals with sildenafil, as well as with 20-HETE inhibitors in our study, paralleled the improvement of serum creatinine levels [90].

CYP4A enzymes are involved in the pathomechanism of inflammation in the rat kidney [91]. In the present study, experimental IR injury caused marked infiltration of kidney tissue with ED1+ inflammatory cells. We found a higher number of monocytes/macrophages in the outer medulla compared to the cortex. Pretreatment with HET0016 and 20HEDE significantly reduced the monocyte/macrophage infiltration (Figs. 11, 12).

Ishizuka et al. showed that *in vitro* treatment of endothelial cells with 20-HETE markedly increased levels of prostaglandins E2 and F2 alpha, markers of inflammation, and interleukin-8, a potent neutrophil chemotactic factor whose overproduction by the endothelium causes vascular injury [22]. 20-HETE activates the pro-inflammatory transcription factor NF-κB, the major promoter of inflammation, and thus initiates endothelial activation including oxidative stress as well as enhanced cytokine and adhesion molecule expression [92]. This cascade elicits the infiltration of inflammatory cells that in turn aggravate hypoxic tubular injury during the development of acute kidney injury [93 - 94]. Likewise, 20-HETE activates the MAPK/ERK proinflammatory-pathway by stimulating phosphorylation of ERK1/2. In keeping with these findings we have demonstrated a decrease of number of proinflammatory ED1+ cells in kidneys, pretreated with 20-HETE inhibitors.

Ysebaert et al. observed a higher number of monocytes/macrophages infiltration in uninephrectomized rats exposed to warm renal ischemia for 45 minutes compared to uninephrectomized controls. ED1+ cell infiltration could be seen already 24 hours after IR and was most prominent within the first 5 postoperative days. Administration of mycophenolate mofetil (MMF), known for its anti-inflammatory properties [95], significantly diminished the number of monocyte/macrophage infiltration 2 days after IR [96]. We have shown a significant reduction of proinflammatory ED1+ cells 48 hours after IR in the rats pretreated with 20-HETE blockers, underlining the antiinflammatory properties of 20HEDE and HET0016.

Translation of the experimental findings into clinical applicability

Nephron sparing partial resection of the kidney with crossclamping of the renal pedicle results in renal IR injury, as simulated in our clamping model. In human kidney transplantation, the use of marginal donor kidneys that underwent severe IR damage often results in poorer long-term outcomes [97]. Pharmacological inhibition of 20-HETE in the kidney before severe ischemic injury may confer renoprotection and help to preserve kidney function after renal transplantation and partial resection of the kidney with crossclamping of the renal vessels.

During the past few years knowledge of the mechanisms mediating ischaemia-reperfusion injury in the general setting, and more specifically in kidney transplantation, has greatly expanded. Systematic screening of genes that are upregulated or

downregulated during ischaemia-reperfusion damage has further indicated the complexity of factors and signals involved in postischemic renal injury. Targeting of a single molecule is thought unlikely to provide complete amelioration of injury. Multidrug approach or single drugs targeting multiple signals are expected to be the next step in an attempt to reduce post-ischemic injury and delayed graft function [97]. In this context 20-HETE inhibitors might be beneficial for the treatment of delayed graft function given their ability to moderate different pathways of IR injury in kidney.

Conclusions

Our data could offer a therapeutic perspective in IR-induced acute kidney injury after surgery with temporary occlusion of renal arterial blood, for example during partial nephrectomy, and after kidney transplantation. However, further studies are required to strictly define the therapeutic window for 20-HETE antagonists or agonists.

5. Summary

20-Hydroxyeicosatetraenoische Säure (20-HETE) kann den Ischämie-Reperfusion (IR)-Schaden durch die vasokonstriktorischen und proinflammatorischen Effekte verschlimmern.

Hypothese. In der vorliegenden Arbeit sollte gezeigt werden, dass die Synthesehemmung oder Blockade der Wirkung von 20-HETE den IR-induzierten akuten Nierenschaden verringert.

Methoden und Ergebnisse. Ex vivo Versuche an isoliert perfundierten Nieren zeigten, dass IR die 20-HETE Freisetzung verstärkt. Dies konnte durch Vorbehandlung mit HET0016 - einem Inhibitor der Cytochrom P450 (CYP)-abhängigen 20-HETE-Synthese – reduziert werden. Angiotensin II und Phenylephrin führten zu einer Konzentrations-abhängigen Steigerung des Perfusionsdruckes in ex vivo perfundierten Nieren. Behandlung mit HET0016 antagonisierte diesen Effekt bei physiologischen Konzentrationen von Angiotensin II und Phenylephrin. Uninephrektomierte männliche Lewis Ratten wurden für 45 Minuten einer warmen Ischämie durch Abklemmen der linken Nierengefäße unterzogen. Die Tiere erhielten 5 Minuten vor der Ischämie N-hydroxy-N'-(4-butyl-2methylphenyl) Formamidin (HET0016; 20-HETE Syntheseinhibitor), 20-Hydroxyeicosa-6(Z),15(Z)-diensäure (20HEDE; 20-HETE-Antagonist) oder Vehikel (DMSO) durch die Nierenarterie infundiert. Die Organe wurden 2 Tage nach Reperfusion entnommen. Vorbehandlung mit HET0016 oder 20HEDE reduzierten die renale Dysfunktion signifikant (Serum-Kreatinin, Kreatininclearance und Serum-Harnstoff). Außerdem verringerte die Vorbehandlung mit HET0016 und 20HEDE deutlich den akuten Tubulusschaden, die Apoptose der Tubuluszellen und die inflammatorische Infiltration im Vergleich zur Vehikel-Gabe. Nach Schädigung durch IR kam es zu einer Reduktion der Natrium-Ausscheidung im Urin und Steigerung der fraktionellen Natriumexkretion. Dies wurde von beiden 20-HETE-Inhibitoren nicht signifikant beeinflusst.

Zusammenfassung. Unsere Ergebnisse konnten zeigen, dass der Arachidonsäuremetabolit 20-HETE - ein potenter Vasokonstriktor – bei der Pathogenese des IR-induzierten akuten Nierenschadens beteiligt ist. Die Blockade von 20-HETE vor der renalen Ischämie wirkte renoprotektiv.

6. Literature

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7. Curriculum Vitae

Mein Lebenslauf wird aus datenschutzrechtlichen Gründen in der elektronischen Version meiner Arbeit nicht veröffentlicht.

AWARDS

1. „Students Research Award“, 7th International Conference of medical students and young doctors in the field of surgery, May 2005, Katowice, Poland
2. 1st Preis „Charité-Allianz Research Award“, 17th European Students’ Conference October 2006, Berlin, Germany
3. Bernd Schönberger-Preis, 17. Jahrestagung des Arbeitskreises Nierentransplantation der Fort - und Weiterbildungskommission der Deutschen Urologen, November 2009, Lübeck, Germany

PUBLICATIONS

1. Chaykovska L., Deger S., Wille A., Friedersdorff F., Kasper A., Dragun D., Liefeldt L., Miller K., Giessing M., Fuller T.F., Kidney transplantation into urinary conduits with ureteroureterostomy between transplant and native ureter: single-center experience. Urology. 2009 Feb;73(2):380-5. Epub 2008 Nov 20

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Lyubov Chaykovska

9. Erklärung

„Ich, Lyubov Chaykovska, erkläre, dass ich die vorgelegte Dissertation mit dem Thema: „Role of 20-hydroxyeikosatetraenoic acid in experimental acute kidney injury“ selbst verfasst und keine anderen als die angegebenen Quellen und Hilfsmittel benutzt, ohne die (unzulässige) Hilfe Dritter verfasst und auch in Teilen keine Kopien anderer Arbeiten dargestellt habe.“

Datum Unterschrift