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Sulfenic acid modification of ET-B receptor is responsible for the benefit of a non-steroidal MR antagonist in renal ischemia

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ABSTRACT

Acute kidney injury is associated with high mortality rates and chronic kidney disease development. Ischemia/reperfusion (IR) is an important cause of acute kidney injury. Unfortunately, there is no available pharmacological approach to prevent/limit renal IR injury in common clinical practice. Renal IR is characterized by diminished nitric oxide bioavailability and a reduction in the renal blood flow; however, the mechanisms leading to these alterations are poorly understood. In a rat model of renal IR, we show that the administration of the novel non-steroidal MR antagonist BR-4628 can prevent or treat the renal dysfunction and tubular injury induced by IR. Renal injury induced by ischemia was associated with increased oxidant damage, which leads to a cysteine sulfenic acid modification in ET_B receptor and consequently decreased eNOS activation. These modifications were efficiently prevented by non-steroidal MR antagonism. Furthermore, we demonstrate that the protective effect of BR-4628 against IR was lost when a selective ET_B receptor antagonist was co-administered. These data describes a new mechanism for reduced eNOS activation during renal IR that can be blocked by MR antagonism with BR-4628.

Acute kidney injury (AKI) is one of the most common complications in hospitalized and intensive care unit patients and is associated with adverse outcomes such as increased mortality rates and chronic kidney disease development.¹ An important cause of AKI is renal ischemia/reperfusion (IR).² Currently, there are not any effective pharmacological approaches to treating or preventing AKI despite recent advances in the understanding of the underlying mechanisms involved.^{3,4} Ischemic AKI is a complex entity in which multiple processes are involved including: tubular cell injury, endothelial alterations, increased oxidant stress and inflammation.^{5,6} One of the hallmarks of ischemic AKI is the reduction in the renal blood flow.⁷ This alteration is mediated by numerous factors such as an increase in the production of vasoconstrictor mediators and/or a reduction in the levels of vasodilator agents.⁵ A reduction in the NO bioavailability is proposed to play an important contribution to the sustained vasoconstriction observed in renal IR, which perpetuates the injury as a consequence of worsened renal ischemia.⁸

Of particular interest is the role of mineralocorticoid receptor (MR) activation in modulating the blood flow during renal ischemia: previous studies have shown that MR antagonism with spironolactone protects against renal IR injury by preventing the reduction in the renal blood flow.^{9,10} The mechanisms by which MR antagonism leads to kidney protection remain unclear. Although the blockade of MR may be a promising therapeutic approach, the use of spironolactone in renal ischemia is limited the increased risk of hyperkalemia especially in patients with compromised renal function.^{11,12} Recently novel non-steroidal MR antagonists (MRA) such as BR-4628 have been described. BR-4628 is derived from the dihydropyridine and displays a greater selectivity and potency for MR inhibition.¹³ Furthermore, the risk of hyperkalemia may be less with this class of agents, at least in the chronic setting.¹⁴

In this study we tested the efficiency of the non-steroidal MRA BR-4628 to protect against renal injury induced by IR and provide insights into the mechanism of kidney protection conferred by MRA. First, we evaluated whether BR-4628 can prevent or treat renal injury induced by IR. AKI induced by bilateral renal IR was assessed by the increase in plasma levels of creatinine (fig. 1A) and urea (fig. 1B). These alterations were prevented by BR-4628 when administered before or up to 3 hours after the ischemic insult. We determined the effects on sensitive biomarkers of tubular injury such as Hsp72, Kim-1 and NGAL. The increased urinary Hsp72 induced by IR was significantly reduced in rats treated with BR-4648 (fig. 1C). A similar pattern was observed for renal Kim-1 (fig. 1D) and NGAL (fig. 1E) mRNA levels. The tubular lesions induced by IR were documented by histological analysis. Kidneys from rats subjected to IR displayed tubular casts, tubular dilation and cell detachment (fig. 1H). These structural alterations were also prevented by BR-4628 administration before (fig. 1I) or after (fig. 1J) the induction of the renal ischemia. This effect was corroborated by the quantification of injured tubules (fig. 1F). **As an indicator of apoptosis in the kidneys with bilateral renal IR, we analyzed the protein levels of cleaved caspase-3. Renal IR was characterized by an increase in cleaved caspase-3 levels, which was prevented by MR antagonism (Supplementary fig. 1). To evaluate if the treatment with BR-4628 reduced inflammation induced by renal IR, we performed immunohistochemistry for CD68 positive cells to evaluate macrophage infiltration in renal sections. As shown in supplementary figure 2 renal IR was associated with a marked increase in macrophage infiltration, an effect that was efficiently prevented by the administration of BR-4628 either before or after the induction of the renal injury. The levels of potassium remained unchanged either by IR or by MR antagonism with BR-4628 (Supplementary fig. 3).** Therefore, the antagonism of MR by BR-4628 prevented or treated the renal dysfunction and tubular lesions induced by IR. These functional and histological benefits were associated to the prevention of renal oxidant stress.

Indeed, the production of reactive oxygen species is a hallmark of IR and may induce damage to biomolecules such as proteins and alter their function.¹⁵ In addition to the ROS released upon renal reperfusion, it has been shown that MR activation contributes to increased ROS production by activation of the NADPH oxidase in both endothelial and smooth muscle cells.^{16,17} Moreover in endothelial cells aldosterone reduced the expression of glucose-6-phosphate dehydrogenase, a key regulator of the redox balance, therefore increasing oxidative stress.¹⁸ Renal IR increased the amount of oxidized proteins in the kidney, an effect that is prevented by MR antagonism before or after renal IR (Supplementary fig. 4A). BR-4628 administration also prevented the IR-induced increase in the urinary levels of hydrogen peroxide, a marker of oxidant stress (Supplementary fig. 4B).

The underlying mechanisms of the benefit of MRA in renal IR are unclear; we hypothesized that the improvement in renal blood flow may be associated with the effects of ET-1 on renal hemodynamics. ET-1 is one of the vasoactive substances that are altered during renal IR and that may contribute to the enhanced vasoconstriction.¹⁹ ET-1 induces constriction when it binds on the ET_A receptor in the smooth muscle cells. On the contrary, when it binds the ET_B receptor in endothelial cells, it exerts vasodilator actions through increased NO production by eNOS.^{20,21} As expected, renal IR induced an increase in mRNA levels of pre-pro-endothelin-1 (Fig 2A). This was not prevented by MR antagonism suggesting that the effect of MRA is downstream of ET-1 production (Fig. 2A). The mRNA levels of ET_A receptor were not altered (data not shown). However, ET_B receptor expression was reduced in the setting of IR (Fig. 2B). This effect was prevented by BR-4628 administration before or after IR. To analyze whether the mechanism of kidney protection from IR by MR antagonism is dependent on the ET_B receptor, we included another groups of rats in which a selective ET_B receptor antagonist (BQ-788)²² was co-administered with BR-4628. Renal IR was accompanied by increased plasma creatinine (Fig. 3A) and renal vascular resistance (Fig. 3B),

significantly reduced renal blood flow (Supplementary fig. 5A) and tubular injury (Fig. 3C). No changes were observed for the mean arterial pressure among the groups (Supplementary fig. 5B). As previously reported,²³ ET_B antagonism had no effect on renal IR severity (similar levels of creatinine, renal blood flow and renal injury in the IR+ BQ-788 group when compared to untreated IR rats) (Figs. 3 A-C). Again, MR antagonism with BR-4628 was associated protection against IR (Figs. 3 A-C). Importantly, ET_B receptor antagonism fully blunted the protective effect of BR-4628: creatinine levels remains elevated, renal vascular resistance was augmented and histology analysis indicated similar degree of renal injury than in the non-treated IR group, showing that the protective effect of MRA is lost when an ET_B antagonist is co-administered.

To understand the underlying mechanisms linking the ET_B receptor and the benefit of MR antagonism in IR-induced renal injury, we evaluated the presence of a cysteine sulfenic acid modification, a post-translational modification of ET_B. It has been previously reported that Cysteine (Cys405) of ET_B can be modified, preventing its activation and therefore preventing increased eNOS activity mediated by the ET_B receptor when stimulated by ET-1.²⁴ In cultured pulmonary artery endothelial cells, aldosterone induces inactivation of the ET_B receptor through a mechanism involving MR activation, increased oxidant stress and sulfenic acid modification in Cys405, thus leading to reduced NO production.²⁵ We hypothesized that this mechanism could be translated *in vivo* and underlies the benefit of the BR-4628 MRA in IR. ET_B sulfenic acid modification was analyzed in kidneys following IR, treated or not with the BR-4628 MRA. Renal IR induced ET_B receptor sulfonation *in vivo* (Fig. 4A). These data suggest that NO bioavailability may be reduced during renal IR due to ET_B inactivation by a cysteine sulfenic acid modification. To support this idea we analyzed the phosphorylation of eNOS at Serine residue 1177 (S1177) that is known to activate the production of NO by eNOS,²⁶ as well as urinary nitrites and nitrates excretion as an indirect

measure of NO production.^{27,28} During renal IR, phosphorylation of the S1177 residue is decreased (Fig. 4B), and this effect **was** associated with a reduction in urinary nitrites and nitrates excretion (Fig. 4C). MR antagonism with BR-4628 prevented the sulfenic acid modification of the ET_B receptor (Fig. 4A), and prevented the decrease of S1177 eNOS phosphorylation (Fig. 4B). Moreover, the reduction of urinary nitrites and nitrates was prevented by MRA given before or after the induction of renal IR (Fig. 4C).

Therefore, during renal IR there is an increase in the ROS, which in turn induces a sulfenic acid modification in ET_B receptor, preventing its ability to stimulate eNOS and NO production. This results in renal vasoconstriction and decreased blood flow (Fig. 4E). On the other hand, when MR is antagonized with BR-4628 the oxidant injury and the sulfenic acid modification on ET_B receptor are prevented, thus maintaining normal eNOS activation and NO balance which appears to blunt the deleterious effects of IR (Fig. 4D).

In summary, we report that BR-4628, a novel non-steroidal MR antagonist is a novel pharmacological approach for prevention or treatment of AKI in a rat model of renal IR injury. Moreover, we provide evidence that during renal IR, the ET_B receptor undergoes sulfenic acid modification of a critical cysteine residue that decreases the activation of eNOS and hence, NO production. Finally we demonstrated that the benefit of the non-steroidal MR antagonist BR-4628 against renal IR is dependent on ET_B receptor activation and increased NO bioavailability. Altogether, these data supports the use of non-steroidal MR antagonists in the clinical setting for the prevention and/or treatment of ischemic AKI.

CONCISE METHODS

Experimental protocols

All the experiments involving animal manipulation were performed according to the ethical guidelines of INSERM for the care and use of laboratory animals. Male Wistar rats (Janvier Labs, France) weighting 270-300 g were included in the study. The BR-4628 was administered at a dose of 10 mg/kg by oral gavage in vehicle (40% kolliphor, 10% ethanol and 50% water); the dose was selected according to previous studies performed in rats.²⁹ In the first set of experiments 20 rats were divided in 4 groups: sham-operated rats, rats with 25 min of bilateral ischemia (IR), rats treated with BR-4628 at day -2 and 1h before the induction of IR (BR-4628 pre) and rats receiving BR-4628 (10 mg/kg) 3 hours after the IR (BR-4628 post). For the second set of experiments 30 rats were divided in 5 groups: sham, rats underwent 25 min of bilateral ischemia (IR), rats receiving the ET_B antagonist BQ-788 for 2 days before IR (ET_BA), rats receiving BR-4628 for 2 days before the induction of IR (MRA) and rats receiving BR-4628 and BQ-788 2 days before renal ischemia (ET_BA + MRA). Rats were sacrificed 24h after IR.

Kidney ischemia/reperfusion injury model

The rats were anesthetized by an intra-peritoneal injection of sodium pentobarbital (30 mg/kg) and placed on a heating pad with a rectal probe to maintain a constant body temperature around 37 °C. An abdominal incision was performed and both renal pedicles were exposed and dissected. Renal ischemia was induced by placing non-traumatic vascular clamps over the pedicles and was visually verified by change in kidney color. After 25 min the clamps were released and the reperfusion was corroborated by the return of oxygenated blood to the kidney. The abdominal incision was closed in two layers with 3-0 sutures and the reperfusion was allowed for 24 h.

Evaluation of functional parameters

After 24 h of reperfusion the rats were anesthetized with an intra-peritoneal injection of sodium pentobarbital (30 mg/kg) and placed on a heating pad. **The right femoral artery was catheterized with polyethylene tubing (PE-50) in order to monitor the mean arterial pressure.** The left renal artery was dissected and the renal blood flow was recorded by placing an ultrasound flow probe (Transonic) filled with ultrasonic coupling gel around the artery. A blood sample was taken and the plasma creatinine and urea concentrations were determined by an automatic analyzer (Konelab 20i, Thermoscientific). **The levels of potassium were analyzed by flame photometer.** At the end of the experiment the right kidney was removed and fixed in Bouin Fixative solution and the left kidney was quickly frozen for molecular studies.

Histological analysis

After fixation of the tissue, the kidney slices were dehydrated and embedded in paraffin. Sections of 4 μm were made and stained by hematoxylin and eosin. For each rat, 10 sub-cortical fields were visualized and analyzed on a Leica DM4000 microscope at a magnification of 200 X. The percentage of injured tubules was blindly analyzed. **Tubular injury was assessed in Hematoxylin and eosin stained sections in at least 10 cortical fields per rat. The number of tubules displaying epithelial cell necrosis and detachment, cast formation or tubular dilation was determined. The percentage of injured tubules was calculated against the total number of tubules per field. For CD68 immunohistochemistry studies, 4 μm sections from paraffin embedded tissues were de-waxed and probed against CD68 [ED1] (Abcam antibody ab31630). Ten cortical fields (magnification 400X) were randomly recorded from**

each kidney slide. The number of cells that were positive for CD68 were counted and reported as CD68 positive cells per high power field.

RNA extraction and real time PCR

Total RNA extraction was carried out from kidney cortex by using the TRIZOL reagent (Life Technologies) according to the manufacturer's instructions. The reverse transcription was performed with 1 µg of RNA and the Superscript II Reverse transcriptase Kit (Life Technologies, Carlsbad, CA, USA). Transcript levels of genes were analyzed by real-time PCR (fluorescence detection of SYBR green) in an iCycler iQ apparatus (Bio-rad). The mRNA levels were normalized by the amount of 18S as an endogenous control. The primer sequences of the analyze genes are listed in the supplementary table 1.

Oxyblot

The protein carbonylation levels were assessed with the Oxyblot protein oxidation detection kit, following the manufacturer's instructions (Chemicon, Millipore). For each assay, 10 µg of protein were used. To quantify protein oxidation, we defined an oxidation index as the ratio of the densitometric value of the Oxyblot bands versus β-actin levels (Las3000 DarkBox, Fuji Photo Film Europe, GMBH).

Western blot analysis

Total renal proteins were isolated from cortexes from each group and homogenized in 1 % SDS homogenization buffer with protease inhibitor (Roche). Protein samples containing 20 µg of total protein were diluted in Laemmli buffer and heated at 95 °C. The proteins were resolved in 4-15% Mini-PROTEAN TGX precast polyacrylamide gels (Bio-rad) and transferred onto polyvinylidenedifluoride membranes (Bio-rad). Membranes were then blocked with 5 % blotting-grade non-fat dry milk. Membranes were then incubated in 0.1 %

blotting-grade non-fat dry milk with their respective antibodies. Specific antibodies against ET_B (Santa Cruz Biotechnology 1:500), Caspase-3 (Santa Cruz Biotechnology 1:200) or pS1177-eNOS (Abcam 1:5000) were used. After incubation with primary antibody, membranes were washed and incubated with their respective secondary antibody. As a loading control, membranes were incubated overnight at 4 °C with goat anti-actin antibody (Santa Cruz Biotechnology, 1:5000 dilution). Proteins were detected with an enhanced chemiluminescence kit (Bio-rad). For urinary Hsp72 detection by western blot, urine was diluted 1:100 in 0.9% saline solution, and 10 µL of each dilution was loaded and resolved by 8.5% SDS-PAGE electrophoresis and electroblotted, as previously described. Membranes were then blocked with 5% blotting-grade non-fat dry milk and incubated in 0.1% blotting-grade non-fat dry milk with anti Hsp72 antibody (Santa Cruz Biotechnology, 1:10000).

Immunoprecipitation

For IP analysis the tissues from each group were pooled and the proteins were extracted in dimedone lysis buffer. ET_B receptor was immune-precipitated using protein A agarose beads (Thermo Scientific) and 4 µg of anti ET_B antibody (Santa Cruz Biotechnology). The immune precipitated proteins were eluted by boiling in Laemmli buffer, blotted and probed for anti-cysteine sulfenic acid (Millipore) and ET_B.

Urinary hydrogen peroxide and nitrates and nitrites excretion

The urinary levels of hydrogen peroxide were detected with the Amplex® Red kit (Life Technologies) according to the manufacturer's instructions. For the detection of urinary nitrates and nitrites the colorimetric nitric oxide assay kit (Oxford Biomedical Research) was used. The data were normalized by the urinary creatinine.

Statistics

The results are represented as means \pm SEM. The statistical differences among the groups were determined by Kruskal-Wallis test using the Dunn's post hoc test for multiple comparisons in the GraphPad Prism 6 software. The p value < 0.05 was defined as statistically significant.

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DISCLOSURE

Peter Kolkhof is an employee at BAYER HealthCare Pharmaceuticals.

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FIGURE LEGENDS

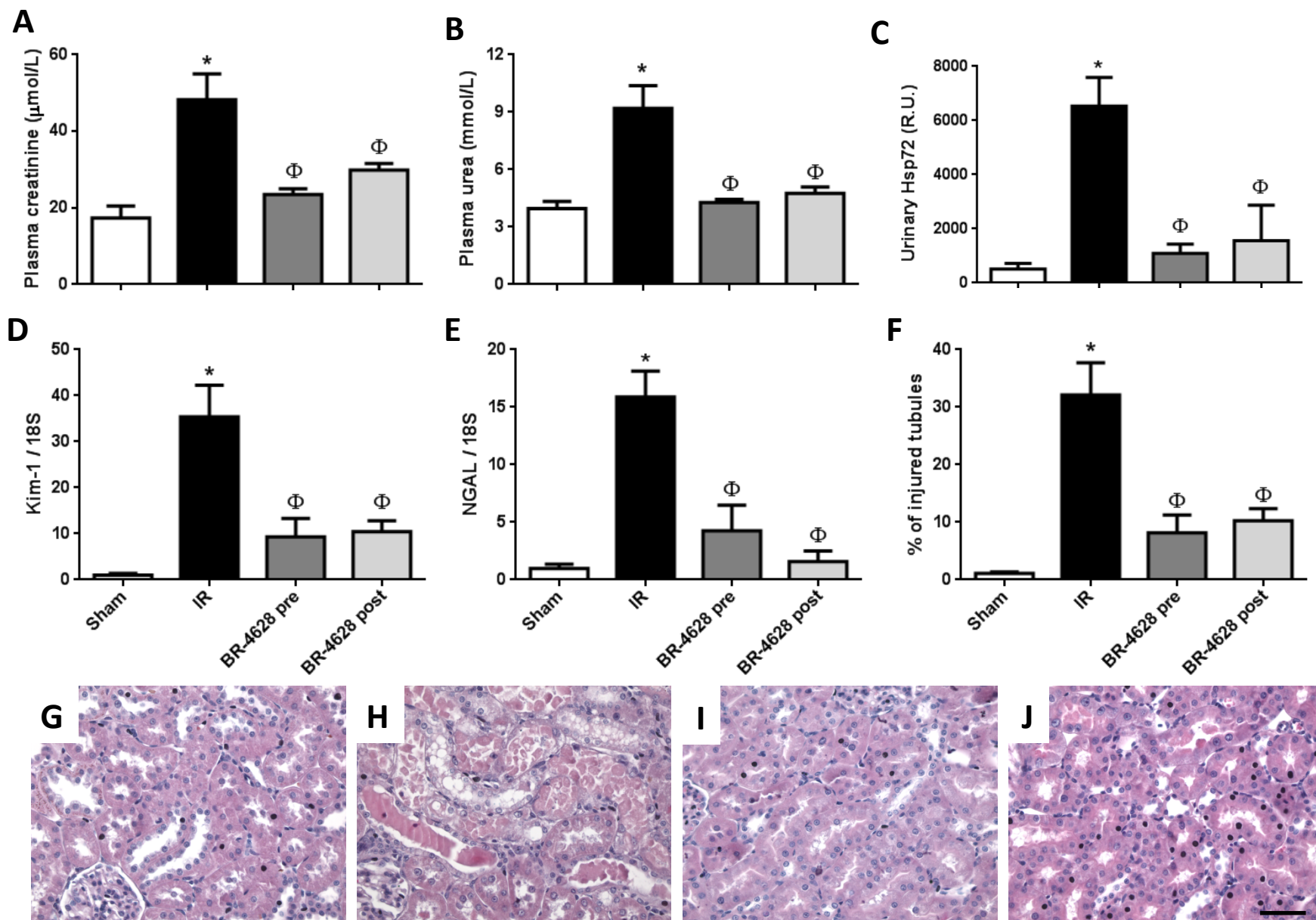
Fig 1. Benefit of BR-4628 (10 mg/kg) administration against renal injury induced by IR. Renal function was assessed by quantification of the plasma levels of (A) creatinine and (B) urea. As indicators of tubular injury: (C) the urinary levels of Heat shock protein 72 were determined by western blot and the bands scanned for densitometric analysis and the mRNA levels in kidney of kidney injury molecule-1 (D) and Neutrophil Gelatinase-Associated Lipocalin (E) were determined by RT-PCR. (F) The percentage of injured tubules was blindly quantified in 10 fields per rat. Representative images from the H&E staining are shown for sham (G), IR (H), BR-4628 pre (I) and BR-4628 post (J). Scale bar= 20 μ m. n=5 per group. *p<0.05 vs. sham and Φ p<0.05 vs. IR.

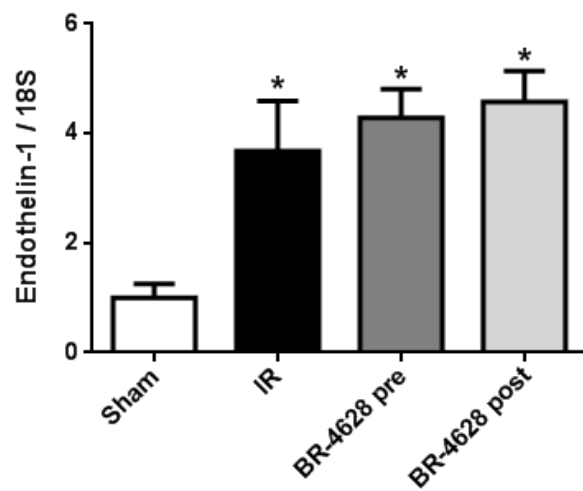
Fig 2. Effect of renal IR on ET-1 and ET_B receptor. (A) mRNA levels of endothelin-1. (B) The upper inset shows a representative western blot analysis for the levels of ET_B receptor in the kidney cortex and the lower inset the densitometric analysis. n=5 per group. *p<0.05 vs. sham and Φ p<0.05 vs. IR.

Fig 3. The benefit of BR-4628 is prevented by ET_B receptor antagonism. (A) Plasma creatinine levels as an indicator of renal function. (B) **The renal vascular resistance was determined by dividing the mean arterial pressure versus the renal blood flow in the left renal artery.** (C) The percentage of injured tubules was quantified in H&E stained slides. Representative images from the H&E staining are shown for IR (D), IR + ET_BA (E), IR + MRA (F) and IR + ET_BA + MRA (G). ET_BA = ET_B receptor antagonism. MRA =

Mineralocorticoid receptor antagonism. Scale bar= 20 μm . n=5 per group. * $p < 0.05$ vs. sham and $\Phi p < 0.05$ vs. IR.

Fig 4. Effect of renal IR on ET_B receptor sulfenic acid modification and eNOS phosphorylation. (A) ET_B receptor was immunoprecipitated from kidney proteins extracted in dimedone lysis buffer and immunoblotting was performed to detect the protein sulfenic acid levels (R-SOH). (B) The activation of eNOS was determined by the levels of phosphorylation in the S1177 residue of eNOS determined by western blot analysis. (C) The urinary levels of nitrites and nitrates were quantified and normalized by urinary creatinine. (D) Schematic representation of ET-1 signaling through ET_B receptor in the endothelial cells on normal conditions. (E) Schematic representation of altered ET_B signaling during renal ischemia leading to reduced NO production in the endothelial cell. n=5 per group. * $p < 0.05$ vs. sham and $\Phi p < 0.05$ vs. IR.



A**B**