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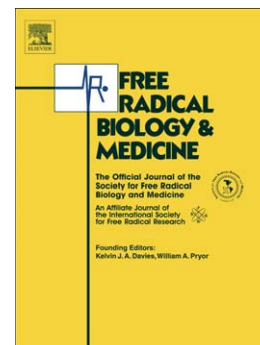
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## **Peroxisome proliferator-activated receptor $\beta/\delta$ agonism protects the kidney against ischemia/reperfusion injury in diabetic rats**

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**ABSTRACT**

Diabetes is an important risk factor for ischemic acute kidney injury, whose pharmacological treatment remains an unmet medical need. The peroxisome proliferator-activated receptor (PPAR) $\beta/\delta$  is highly expressed in the kidney, although its role has not yet been elucidated. Here, we used an in vivo model of renal ischemia/reperfusion (I/R) in streptozotocin-induced diabetic rats (i) to evaluate whether diabetes increases kidney susceptibility to I/R injury, and (ii) to investigate the effects of PPAR $\beta/\delta$  activation. The degree of renal injury (1 h ischemia/6 h reperfusion) was significantly increased in diabetic rats when compared with non-diabetic littermates. PPAR $\beta/\delta$  expression was increased following I/R, with the highest levels in diabetic rats. Administration of the selective PPAR $\beta/\delta$  agonist GW0742 attenuated the renal dysfunction, leukocyte infiltration and formation of interleukin-6 and tumor necrosis factor- $\alpha$ . These effects were accompanied by an increased expression of the suppressor of cytokine signaling (SOCS)-3, which plays a critical role in the cytokine-activated signalling pathway. GW0742 beneficial effects were attenuated by the selective PPAR $\beta/\delta$  antagonist GSK0660. Thus, we report herein that PPAR $\beta/\delta$  activation protects the diabetic kidney against I/R injury by a mechanism that may involve changes in renal expression of SOCS-3 resulting in a reduced local inflammatory response.

Keywords: PPAR $\beta/\delta$ , GW0742, GSK0660, ischemia/reperfusion, kidney, diabetes, SOCS-3

## INTRODUCTION

Acute kidney injury (AKI) is a life-threatening condition associated with high morbidity and mortality. Renal ischemia/reperfusion (I/R) injury is the most common cause of AKI [1]. Despite advances in our understanding of the pathophysiology of AKI, pharmacological interventions are limited and there is currently no successful therapy, except for supportive care. There is good clinical evidence that diabetes is an important risk factor for AKI [2-5] and diabetic patients also are at higher risk of failing to recover from AKI than non-diabetic patients, indicating that either the degree of injury is worse or the repair mechanisms are impaired (or both) [6]. However, the exact mechanisms underlying the increased sensitivity of the diabetic kidney to the injury caused by I/R and other noxious stimuli remain elusive. Inflammation contributes to the pathogenesis of both diabetes and ischemic AKI, with both leukocyte activation and cytokine production playing a pivotal role [7, 8]. Recently, a new class of signaling molecules, the SOCS (suppressor of cytokine signaling) proteins, has been demonstrated to be involved in both inflammation and insulin resistance. In the kidney, SOCS-3 is expressed primarily in glomerular mesangial and proximal tubule cells, where this protein causes feedback inhibition of the formation of both interleukin-6 (IL-6) and tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ) [9].

Peroxisome-proliferator activated receptors (PPARs) are ligand-activated transcription factors belonging to the nuclear receptor superfamily that exerts a critical role in regulating both glucose and lipid metabolism. Three PPARs isoforms have been identified both in rodents and in man [10]. Compared with PPAR $\alpha$  and PPAR $\gamma$ , PPAR $\beta/\delta$ , which is ubiquitously expressed in most adult tissues, is the least understood member of the PPAR family. In the kidney, PPAR $\beta/\delta$  is expressed in all segments of the nephron and is the predominant PPAR isotype expressed in the proximal straight tubule [11]. We and others have reported that activation of PPAR $\alpha$  and PPAR $\gamma$  exerts protective effects in several *in vivo* experimental models of AKI [12-16]. In contrast, there is, to our knowledge, only one report that suggests beneficial effects of PPAR $\beta/\delta$  activation in a mouse model

of ischemic AKI [17] and the molecular mechanisms of the observed beneficial effects are still unknown. Notably, the potential renoprotective effects of PPAR $\beta/\delta$  agonism have never been tested in an animal model of insulinopenic diabetes, where the lack of insulin may drastically influence the susceptibility of the kidney to ischemic damage. Hence, this study was undertaken to investigate (i) the effects of diabetes on the renal injury and dysfunction caused by I/R and (ii) the role of PPAR $\beta/\delta$  on the recovery of renal function following I/R injury in a rat model of insulinopenic diabetes. The highly selective and potent PPAR $\beta/\delta$  agonist, GW0742, [18] and a newly described specific PPAR $\beta/\delta$  antagonist, GSK0660, [19] were used as pharmacological tools for evaluating the potential beneficial effects evoked by PPAR $\beta/\delta$  activation. In order to gain a better understanding of the observed beneficial effects of PPAR $\beta/\delta$  activation in our model, we have carried out mechanistic studies, which focus on the role of the SOCS-3 signaling pathway.

## MATERIALS AND METHODS

### Animals and surgery

Male Wistar rats (Harlan-Italy; Udine, Italy) were provided with a Piccioni pellet diet (n.48, Gessate Milanese, Italy) and water *ad libitum*. Insulinopenic diabetes was induced in 8-week old normal male rats by a single intravenous tail vein injection of STZ (50 mg/kg). A blood sample was collected 4 days after the STZ injection, and plasma glucose was determined using a glucose analyzer (Accu-Chek Compact System, Roche Diagnostics, Basel, Switzerland). Diabetes was defined by a blood glucose >300 mg/dl. The animals were used 6 weeks later without insulin supplements.

Animal care was in compliance with Italian regulations on the protection of animals used for experimental and other scientific purposes (D.M. 116/92). The experimental protocol, approved by the Turin University Ethics Committee, was performed as described elsewhere [13]. Briefly, the rats were anesthetized through i.p. injection (30 mg/kg) of Zoletil<sup>®</sup> (15 mg/kg tiletamine + 15 mg/kg zolazepam; Zoletil 100<sup>®</sup>, 100 mg/ml, Laboratoires Virbac, France). The anesthetized rats were placed onto a thermostatically controlled heating pad, a rectal temperature probe was inserted and body temperature was monitored and maintained at 37 °C. A midline laparotomy was performed and the bladder was cannulated (internal diameter 0.58 mm; Portex) for the collection of urine. The kidneys were located inside the peritoneum, and the renal pedicles, containing the renal artery, vein, and nerves supplying each kidney, were carefully isolated. The rats were subjected to bilateral renal occlusion for 60 min using non-traumatic artery clamps (Dieffenbach Bulldog Clamps, Harvard Apparatus Ltd., Kent, U.K.) to clamp the renal pedicles, followed by reperfusion for 6 h. After the renal clamps were removed, the kidneys were observed for a further 5 min to ensure reflow after which 2 ml saline at 37° C was injected into the abdomen to ensure gut motility. The time of ischemia and reperfusion chosen was based on that found to maximize reproducibility of renal

functional impairment, while minimizing mortality in these animals [20]. Sham-operated rats underwent identical surgical procedures to those undergoing I/R except that artery clamps were not applied. At the end of the reperfusion, the anaesthetized rats were killed by decapitation after aortic exsanguination. The kidneys were isolated, weighed, and rapidly freeze-clamped with liquid nitrogen and stored at  $-80^{\circ}\text{C}$ . For immunohistochemical preparation, kidney slices from all experimental groups were fixed in 4% paraformaldehyde solution overnight. After dehydration through a graded alcohol series, transverse kidney slices were embedded in paraffin and sectioned at  $5\ \mu\text{m}$ .

### Drugs and treatments

Animals were randomly assigned to the following experimental groups:

- Sham and STZ: non-diabetic and STZ-induced diabetic rats were subjected to the surgical procedure alone, without causing ischemia ( $n = 8$  per group).
- I/R and STZ I/R: non-diabetic and diabetic rats were subjected to 60 min ischemia followed by 6 h reperfusion ( $n = 10$  per group).
- STZ I/R + GW0742: diabetic rats, that underwent I/R, were treated with GW0742 (0.03 mg/kg, i.v.) at the beginning of reperfusion ( $n = 10$ ).
- STZ I/R + GSK0660: diabetic rats, that underwent I/R, were treated with GSK0660 (0.1 mg/kg, i.v.) 30 min prior to reperfusion ( $n = 6$ ).
- STZ I/R + GSK0660+ GW0742: diabetic rats, that underwent I/R, were treated with GSK0660 (0.1 mg/kg, i.v.) 30 min prior to reperfusion and with GW0742 (0.03 mg/kg, i.v.) at the beginning of reperfusion ( $n = 8$ ).

The doses of GW0742 and GSK0660 used were chosen based on the  $\text{EC}_{50}$  and  $\text{IC}_{50}$  values for the specific activation of  $\text{PPAR}\beta/\delta$  [18, 19] as well as previous in vivo experiments performed by our group [21].

### **Measurement of biochemical parameters**

At the end of the reperfusion period, 1 mL blood samples were collected and centrifuged (6,000 g for 3 min) to separate the serum, from which biochemical parameters were measured within 24 h. The volume of urine produced was determined using the urine collected during the reperfusion period. Serum and urine creatinine concentrations were measured spectrophotometrically at 490 nm by the Jaffé kinetic reaction [22], using commercially available kits (catalog no. KB02-H1; K002-H1, DetectX, Arbor Assays, Michigan, USA). Renal creatinine clearance was calculated by the standard formula  $C = (U \times V)/P$ , where U is the concentration in urine, V is urine flow rate and P is the plasma concentration. Serum urea and creatinine concentrations and creatinine clearance were used as indicators of impaired renal function, whereas the rise in the serum aspartate aminotransferase (AST) levels was used as an indicator of reperfusion-injury and was measured as previously described [23]. N-acetyl- $\beta$ -glucosaminidase (NAG) was measured in the urine of experimental rats by a colorimetric assay (Roche Diagnostics, Mannheim, Germany) and was used as marker of tubular injury [24].

### **Myeloperoxidase activity**

Myeloperoxidase (MPO) activity, which was used as an indicator of neutrophil infiltration into the kidney, was determined as previously described [25]. Briefly, samples were homogenised in a solution containing 0.5% (w/v) hexadecyltrimethyl-ammonium bromide dissolved in 10 mM potassium phosphate buffer (pH 7) and centrifuged for 30 min at 20,000g at 4°C. An aliquot of the supernatant was then allowed to react with a solution of 1.6 mM tetramethylbenzidine and 0.1 mM H<sub>2</sub>O<sub>2</sub>. The rate of change in absorbance was measured spectrophotometrically at 650 nm. MPO activity was defined as the quantity of enzyme degrading 1  $\mu$ mol of peroxide per min at 37°C and was expressed in milliunits per gram of wet tissue.

### **Western blot analysis**

Western blots were carried out as previously described [26]. Proteins were separated by 8% sodium dodecyl sulphate-polyacrylamide gel electrophoresis and transferred to polyvinylidenedifluoride membrane, which was then incubated with a primary antibody (rabbit anti-PPAR $\beta/\delta$ , dilution 1:200; goat anti-ICAM-1, dilution 1:500; rabbit anti-SOCS-3, dilution 1: 200; rabbit anti-IL-18, dilution 1: 100; goat anti-IL-6, dilution 1: 200; goat anti-TNF- $\alpha$ , dilution 1: 100). Blots were then incubated with a secondary antibody conjugated with horseradish peroxidase (dilution 1:10000) and developed using the ECL detection system. The immunoreactive bands were visualized by autoradiography and the density of the bands was evaluated densitometrically using Gel Pro®Analyzer 4.5, 2000 software (Media Cybernetics, Silver Spring, MD, USA). The membranes were stripped and incubated with  $\beta$ -actin monoclonal antibody (dilution 1:5000) and subsequently with an anti-mouse antibody (dilution 1:10000) to assess gel-loading homogeneity.

#### **Reverse transcriptase-polymerase chain reaction (RT-PCR) analysis**

Total RNA was extracted from the rat kidney by using Omnizol (Euroclone, Milan, Italy) and precipitated with isopropanol. mRNA was reverse-transcribed by using the RevertAid HMinus Synthesis kit and oligo(dT)<sub>18</sub> primers (Fermentas Life Science, Milan, Italy). cDNA were PCR-amplified using EuroTaq (EuroClone) and sequence-specific oligonucleotide primers (Sigma-Genosys, Milan Italy): SOCS-3 (310 bp) forward 5'-CAGCTCCAAGAGCGAGTACCAG-3', reverse 5'-CATGTAGTGGTGCACCAACTTGA-3'; 18S (489 bp) forward 5'-TCAAGAACGAAAGTCGAAGGT-3', reverse 5'-GGACATCTAAGGGCATCACAG-3'; IL-6 (479 bp) forward 5'-CCGGAGAGGAGACTTCACAG-3', reverse 5'-TGGTCTTGGTCCTAAGCCAC-3'; Kim-1 (212 bp) forward 5'-ACTCCTGCAGACTGGAATGG-3', reverse 5'-CAAAGCTCAGAGAGCCCATC-3'; IL-18 (192 bp) forward 5'-CGCAGTAATACGGAGCATAAATGAC-3', reverse 5'-GGTAGACATCCTTCCATCCTTCAC-3'; TNF- $\alpha$  (254 bp) forward 5'-CACGCTCTTCTGTCTACTGA-3', reverse 5'-GTACCACCAGTTGGTTGTCT-3'. Thermal

cycling conditions were as follows: activation of 94°C for 5 min, followed by 39 cycles of amplification at 94°C for 15 s, 60°C for 30 s, 72°C for 30 s (18S); activation of 95°C for 5 min, followed by 35 cycles of amplification at 95°C for 30 s, 60°C for 30 s, 72°C for 30 s (SOCS-3); activation of 95°C for 2 min, followed by 34 cycles of amplification at 95°C for 60 s, 51°C for 60 s, 72°C for 90 s (IL-6); activation of 95°C for 2 min, followed by 34 cycles of amplification at 95°C for 60 s, 53°C for 60 s, 72°C for 90 s (TNF- $\alpha$ ); activation of 95°C for 2 min, followed by 34 cycles of amplification at 95°C for 60 s, 51°C for 60 s, 72°C for 90 s (IL-18); activation of 94°C for 5 min, followed by 34 cycles of amplification at 94°C for 40 s, 56°C for 40 s, 72°C for 60 s (Kim-1). RT-PCR amplicons were resolved in an ethidium bromide-stained agarose gel [1.8% (wt/vol)] by electrophoresis.

#### **Assessment of ED-1 positive cells**

Immunohistochemical detection of monocytes–macrophages was performed on paraformaldehyde (4%)-fixed and paraffin-embedded kidney tissue sections using the mouse monoclonal antibody against ectodysplasin-1 (ED-1) (Serotec, UK). The ED-1 antibody is useful for the detection of a single-chain glycoprotein that is expressed predominantly on the lysosomal membrane of monocytes–macrophages. Briefly, after deparaffinization, endogenous peroxidase activity was blocked with 3% hydrogen peroxide and then sections were incubated with 10% of normal goat serum, followed by incubation with a primary antibody. Specific labeling was detected with a biotin-conjugated rabbit anti-mouse immunoglobulin G and avidin–biotin peroxidase complex (Vector Laboratories, Burlingame, CA, USA). A blinded evaluation of the samples was performed. Five separate fields were randomly selected for each kidney section and the number of labeled cells per field was quantified and expressed as average number per group

#### **Materials**

Unless otherwise stated, all compounds were purchased from the Sigma-Aldrich Company Ltd. (Missouri, USA). GW0742 (PPAR $\beta/\delta$  agonist) and GSK0660 (PPAR $\beta/\delta$  antagonist) were obtained from Tocris Bioscience (Bristol, UK). The BCA Protein Assay kit and SuperBlock blocking buffer were from Pierce Biotechnology Inc. (Illinois, USA). Antibodies were from Santa Cruz Biotechnology (California, USA). Luminol ECL was from Amersham (Buckinghamshire, UK).

### **Statistical analysis**

All values in both the text and figures are expressed as mean  $\pm$  standard error of the mean (S.E.M.) for n observations. One-way analysis of variance with Dunnett's post test was performed using the GraphPad Prism version 4.02 for Windows (GraphPad Software, San Diego, California, USA) and P values below 0.05 were considered as significant.

## RESULTS

### *Body weight and blood glucose levels*

Mean weight  $\pm$  S.E.M. of the non-diabetic rats was  $254 \pm 11$  g ( $n = 18$ ) and STZ-induced diabetes caused a significant decrease in body weight ( $221 \pm 8$  g;  $n = 32$ ;  $p < 0.01$ ). The diabetic rats had a significantly higher non-fasting blood glucose ( $453 \pm 42$  mg/dl;  $p < 0.01$ ) compared to normal controls ( $110 \pm 12$  mg/dl). The acute injection of GW0742 (0.03 mg/kg) did not significantly decrease blood glucose levels at the time of I/R injury ( $403 \pm 28$  mg/dl).

### *Effect of PPAR $\beta/\delta$ agonism on renal dysfunction and injury caused by I/R in diabetic rats*

Rats that underwent renal I/R exhibited a significant increase in serum levels of urea, creatinine, and AST compared with sham-operated rats (Figure 1A, 1B, and 1C, respectively). The serum levels of these markers were significantly higher in diabetic animals exposed to I/R than in their wild-type littermates, thus suggesting an increased severity of I/R-induced renal injury in the presence of diabetes. In order to discount the possibility of a rapid increase in serum creatinine levels due to increased release of creatinine from muscle during I/R, creatinine clearance was also measured (Figure 1 D). At baseline, STZ diabetic rats had a higher creatinine clearance as compared with control non-diabetic animals. I/R exposure led to a drastic decrease in creatinine clearance in both diabetic and non-diabetic rats. Interestingly, administration of GW0742 to diabetic rats subjected to I/R prevented the increase in the serum concentrations of urea, creatinine, and AST and resulted in a significant increase in creatinine clearance (Figure 1A, 1B, 1C and 1D). STZ treatment produced a significant increase in urine volume as compared with non-diabetic animals (Figure 1E). In contrast, I/R caused a decrease in urine flow, and this effect was attenuated by the PPAR $\beta/\delta$  agonist. Renal I/R evoked a significant increase in urinary NAG levels, suggesting

significant tubular dysfunction, which was markedly reduced by GW0742 administration (Figure 1F). Tubular injury was confirmed by measuring renal mRNA levels of the sensitive and early diagnostic marker Kim-1 [27]. As shown in Figure 2, Kim-1 is almost undetectable in the kidney tissue of diabetic sham-operated animals, but it is expressed at very high levels after I/R injury, with the maximum levels being found in the diabetic ischemic group. Treatment of diabetic animals with GW0742 almost completely abolished the I/R-induced increase in the renal content of Kim-1.

In rats pre-treated with the PPAR $\beta/\delta$  antagonist GSK0660 (0.1 mg/kg, i.v.), the beneficial effects of GW0742 against renal dysfunction and injury evoked by I/R were almost abolished (Figure 1 and 2).

In contrast, the administration of the antagonist GSK0660 alone to rats that underwent I/R had no significant effect on any of the measured markers compared to rats that underwent I/R.

#### ***Expression of PPAR $\beta/\delta$ in the rat kidney***

Diabetes did not affect PPAR $\beta/\delta$  expression in sham-operated animals, whereas PPAR $\beta/\delta$  protein levels were markedly increased after I/R injury (Figure 3). Compared to control rats subjected to renal I/R, diabetic rats that underwent I/R exhibited a 50% increase in PPAR $\beta/\delta$  expression. GW0742 administration at the beginning of reperfusion appeared to increase PPAR $\beta/\delta$  protein expression by a further 15%, but this effect was not significant. Similarly, GSK0660 treatment did not significantly affect PPAR $\beta/\delta$  expression.

#### ***GW0742 reduced leukocyte infiltration in the kidney of diabetic rats subjected to I/R***

The beneficial effects of PPAR $\beta/\delta$  activation in our model of renal I/R injury were associated with a reduction in leukocyte infiltration measured in reperfused kidneys at 6 h (Figure 4 and Figure 5).

MPO activity, a marker of neutrophil infiltration, was significantly elevated in diabetic rats subjected to I/R ( $105.61 \pm 6.32$   $\mu$ U MPO/tissue g) in comparison with diabetic sham-operated rats ( $34.37 \pm 2.16$   $\mu$ U MPO/tissue g) (Figure 4A). In GW0742-treated diabetic animals subjected to I/R, the MPO activity was drastically reduced ( $57.53 \pm 4.04$ ).

The adhesion molecule ICAM-1 (Inter-Cellular Adhesion Molecule 1), whose role in the recruitment of neutrophils during postischemic reperfusion injury is widely accepted [28], was scarcely detected in the kidneys of sham-operated diabetic animals and its expression was induced by 6 h reperfusion (Figure 4B). Interestingly, GW0742 administration resulted in a significant reduction of I/R-mediated ICAM-1 expression, as evidenced by Western blot analysis.

As shown in Figure 5, staining for ED-1, a marker for monocytes and macrophages, was not prominent in the kidneys of diabetic sham-operated controls ( $4.8 \pm 0.8$  cells/field), while I/R injury resulted in increased number of ED-1 positive cells in the interstitium and around glomeruli in post-ischemic vehicle-treated diabetic rats ( $23.6 \pm 4.3$  cells/field). Administration of GW0742 at the beginning of reperfusion prevented the increase in monocytes/macrophages infiltration caused by I/R, bringing the number of ED-1 positive cells back to values similar to those measured in diabetic sham-operated animals ( $8.57 \pm 3.2$  cells/field).

#### ***Effects of GW0742 on renal cytokine expression***

As shown in Figure 6 (Panels A, B and C), I/R induced a substantial increase in mRNA levels of IL-6, TNF- $\alpha$ , and IL-18 in the kidneys of diabetic rats, when measured at 6 h reperfusion. Similar results were obtained when protein expression levels of the above mentioned cytokines were measured. Most notably, as shown by both RT-PCR and Western blot experiments, GW0742 administration prevented the I/R-induced rise in IL-6 and TNF- $\alpha$  expression, but it had no effect on I/R-induced IL-18 expression. SOCS-3 is a key signaling molecule involved in negative feedback

loops leading to decreased IL-6 and TNF- $\alpha$  signaling [29]. As shown in Figure 6D, both mRNA and protein levels of SOCS-3 were not affected by I/R injury, while they were drastically upregulated by the administration of GW0742 at the start of reperfusion. In diabetic rats pre-treated with the PPAR- $\beta/\delta$  antagonist GSK0660, the observed effects of GW0742 on IL-6, TNF- $\alpha$ , and SOCS-3 expression were abolished.

## DISCUSSION

In the present study, we report that diabetes significantly aggravates I/R-induced renal injury/dysfunction. Specifically, we demonstrate that diabetes enhances the I/R-induced degree of renal dysfunction and injury, measured as increases in serum creatinine, urea, AST, and decreases in creatinine clearance and urine flow. Tubular injury is suggested by significant increase in urinary NAG excretion and Kim-1 mRNA renal levels in rats subjected to I/R, with the highest levels observed in the diabetic I/R group. NAG is a well known marker of tubular cell dysfunction [24], while Kim-1 is a type I transmembrane glycoprotein, undetectable in the healthy kidney, but expressed at high levels in the epithelial cells of the proximal tubule after ischemic injury, which has been recently qualified by the Food and Drug Administration and the European Medicines Agency as a highly sensitive and specific biomarker of acute kidney tubular injury [30]. The results of this study are in agreement with previous findings showing that hyperglycemia increases renal sensitivity to I/R injury in diabetic animals [31-33]. An association between diabetes and enhanced vulnerability to a renal ischemic insult has been also recently suggested by several clinical studies [2, 4, 5]. Although the mechanisms underling the enhanced renal susceptibility to I/R remain unknown, they have been related to a local inflammatory response [3, 32]. Here, we focus on the potential beneficial role of PPAR $\beta/\delta$  activation against renal I/R injury in the presence of diabetes. Our results clearly demonstrate that a single administration of the selective PPAR $\beta/\delta$  agonist, GW0742, after the ischemic attack attenuates the serum and urine concentrations of biochemical markers of renal dysfunction as well as the renal expression of Kim-1, thus indicating PPAR $\beta/\delta$  as the main pharmacological target for the therapeutic treatment of AKI. The dose of GW0742 used in this study results in the specific activation of the PPAR $\beta/\delta$  isoform *in vivo* and protects other organs (e.g. heart) against I/R [21]. Furthermore, we demonstrate that pretreatment with the selective PPAR $\beta/\delta$  antagonist, GSK0660, abolishes the protection afforded by GW0742, and this finding

implies that the observed beneficial effects of GW0742 are indeed secondary to the selective activation of the PPAR-receptor in question. To date, the renoprotective effects of PPAR $\beta/\delta$  agonists against an ischemic insult have been investigated only in non-diabetic animals pre-treated for 2 days before induction of I/R [17], a treatment paradigm that is unlikely to be of significant clinical relevance for the treatment of AKI. Our results show, for the first time, that the beneficial effects evoked by the acute pharmacological activation of PPAR $\beta/\delta$  can be observed even when the AKI is exacerbated by diabetes. This indicates that the increase in the degree of AKI caused by I/R in the presence of diabetes does not blunt, or even affect, the beneficial effects of renal PPAR $\beta/\delta$  activation. Most notably, in our experimental model, the highly selective PPAR $\beta/\delta$  agonist, GW0742, was applied at the beginning of the reperfusion, indicating that this treatment strategy could potentially be employed in acute situations known to result in AKI including renal transplantation, aortic aneurysm surgery or contrast-induced nephropathy in order to protect or even rescue a kidney previously challenged by ischemia.

The study also explored the mechanisms that may contribute to the observed improvement in renal function. Firstly, we showed that PPAR $\beta/\delta$  is not only expressed in the kidney of both control and diabetic rats, but that its expression is increased following I/R, with the highest levels in the group of diabetic rats. This suggests that the marked effects of GW0742 recorded in the diabetic animals that underwent I/R are related to the increased renal expression of its pharmacological target. We recently observed [26] that 60 min of renal ischemia followed by 6 h reperfusion causes a significant proinflammatory response. During reperfusion, leukocytes are recruited, activated, and adhere to the vascular endothelium. Infiltrating leukocytes produce cytokines, growth factors, proteases, and reactive oxygen species, all of which can injure renal cells. Injured renal cells in turn produce factors that stimulate leukocytes. This chain reaction significantly contributes to the establishment of renal dysfunction. Here, we have demonstrated that I/R injury evokes a strong increase in MPO activity, an indicator of neutrophil infiltration, and ICAM-1 expression, which is

the endothelial ligand for the neutrophil receptor CD11b/CD18. In addition to neutrophils, we also observed that ED-1 positive macrophage staining increases in the kidney of diabetic animals that have sustained I/R injury. Administration of GW0742 attenuated kidney leukocyte infiltration, thus contributing to a reduction of the excessive inflammatory response. Our results are consistent with two recent reports showing the ability of PPAR $\beta/\delta$  agonists to attenuate neutrophil infiltration in *in vivo* mouse models of LPS-induced pulmonary inflammation [34] and cremasteric microcirculation [35]. Interestingly, the decrease in adhesive interactions evoked by the PPAR $\beta/\delta$  agonist was demonstrated to be associated with inhibition of adhesion molecule expression, including ICAM-1 [35]. Infiltrating neutrophils and macrophages may exacerbate tissue injury by producing a variety of cytokines. Previous studies have shown that the concentration of the proinflammatory cytokines IL-6 and TNF- $\alpha$ , produced by both macrophages and neutrophils, is elevated in the ischemic kidney and may have a pathophysiological role following I/R injury [36]. More recently, another pro-inflammatory cytokine, IL-18, has been suggested to contribute to the renal damage observed during I/R injury [37]. In comparison with wild-type mice, both IL-6 and IL-18 transgenic knockout mice subjected to renal I/R injury showed better kidney function and less tubular damage [38-40]. In our model, I/R induced an increase in mRNA levels of IL-6, TNF- $\alpha$ , and IL-18 in the rat kidney at 6 hours after reperfusion. Interestingly, whereas GW0742 inhibited IL-6 and TNF- $\alpha$  production, this drug had no significant effects on the increase of IL-18 in the kidney. Similar results were obtained when protein expression levels of IL-6, TNF- $\alpha$ , and IL-18 were measured by Western blot analysis, thus confirming that a selective modulation of cytokine production may contribute to the protective effects of PPAR $\beta/\delta$  activation in our system. A number of reports have shown that PPAR $\beta/\delta$  can negatively regulate gene expression by inhibiting the activities of specific transcription factor, through a mechanism of transcriptional transrepression. It is known that IL-6 and TNF- $\alpha$  differ from IL-18 in the signal transduction pathways involved in their pro-inflammatory effects. IL-6 and TNF- $\alpha$ , through cell surface receptors, stimulate the signal cascade

including the Janus kinases and the downstream transcription factor signal transducer and activator of transcription 3 (STAT-3) [41, 42]. On the contrary, IL-18 shares a signal transduction pathway with the IL-1 receptor, recruiting MyD88 (an adaptor protein) and IL-1 receptor activating kinase (IRAK) [43]. Therefore, we could speculate that differences in the inhibition of transcription factors may contribute to the selective effect of GW0742 on IL-6 and TNF- $\alpha$  expression. This notion, which is only now beginning to be elucidated, is in keeping with the work of Kino et al [44], who demonstrated that agonist-activated PPAR $\beta/\delta$  suppressed the IL-6-induced activity of STAT-3, thus reducing the hepatic inflammatory reaction. A number of recent studies have shown that both IL-6 and TNF- $\alpha$  activity and the related STAT signaling pathway can be strongly regulated by SOCS-3 proteins [29]. IL-6 signaling is selectively inhibited by SOCS-3 owing to the binding of SOCS-3 to the IL-6 receptor subunit gp130 (Tyr759) [45]. In contrast, to our knowledge, there is no experimental evidence linking SOCS-3 to the IL-18 signaling pathway. Here, we provide evidence for the first time that PPAR $\beta/\delta$  activation, which did not affect IL-18 expression, was associated with increased gene and protein levels of SOCS-3 and reduced expression of IL-6 and TNF- $\alpha$ . These results suggest that SOCS-3 up-regulation evoked by PPAR $\beta/\delta$  agonism may generate a signal that suppresses the expression of IL-6 and TNF- $\alpha$  gene, but not IL-18. It has to be stressed, however, that these effects have been observed only after I/R. Thus, the specific intracellular links between PPAR $\beta/\delta$  agonism and selective cytokine expression certainly warrants further investigation. Whether the actions of GW0742 are predominant on cytokine production or neutrophil/macrophages influx remains to be determined. Nevertheless, our studies suggest that neutrophils and macrophages are major cellular targets for the actions of PPAR $\beta/\delta$  agonists, although a direct effect of GW0742 on other leukocyte subsets cannot be excluded.

In conclusion, our findings provide further support for the role of diabetes as a risk factor for enhanced susceptibility to renal I/R injury. Most notably, the results of this study are the first to directly demonstrate that acute treatment with a PPAR $\beta/\delta$  agonist (GW0742) protects the kidney

against I/R injury in the presence of diabetes, reducing neutrophil infiltration as well as decreasing pro-inflammatory cytokine pathways. These effects of GW0742 were secondary to the activation of PPAR $\beta/\delta$ , as they were counteracted by the specific competitive PPAR $\beta/\delta$  antagonist, GSK0660. Modulation of SOCS-3 protein expression may represent a novel mechanism through which PPAR $\beta/\delta$  activation could selectively affect cytokine bioactivity in AKI.

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**List of Abbreviations**

AKI, acute kidney injury; AST, aspartate aminotransferase; ED-1, ectodysplasin-1; I/R, ischemia/reperfusion; ICAM-1, inter-cellular adhesion molecule 1; IL-18, interleukin-18; IL-6, interleukin-6; KIM-1, kidney injury molecule-1; MPO, myeloperoxidase; PPAR, peroxisome-proliferator activated receptors; SOCS, suppressor of cytokine signaling; TNF- $\alpha$ , tumor necrosis factor- $\alpha$

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## Figure Legends

### Figure 1

**Effects of diabetes and drug treatment on markers of renal dysfunction in rats exposed to renal I/R.** Serum urea (A), creatinine (B), AST (C) levels, creatinine clearance (D), urine flow (E) and urinary NAG levels (F) were measured after sham operation in non-diabetic (Sham) or diabetic (STZ Sham) rats and in non-diabetic and diabetic rats exposed to 1 h ischemia followed by 6 h reperfusion (I/R and STZ I/R, respectively). Further groups of diabetic rats exposed to I/R received GW0742 (0.03 mg/kg, i.v.; STZ I/R+GW) or GSK0660 (0.1 mg/kg, i.v.; STZ I/R+GSK) or GSK0660+GW0742 (STZ I/R+GSK+GW). Data are expressed as mean  $\pm$  S.E.M. ,  $P < 0.01$  vs. I/R, ★  $P < 0.01$  vs. STZ I/R.

### Figure 2

**GW0742 reduced *Kim-1* expression in diabetic rats exposed to renal I/R.** *Kim-1* mRNA expression was analyzed by RT-PCR in the kidney of diabetic (Sham STZ) rats and was compared with *Kim-1* mRNA expression in non-diabetic and diabetic rats that underwent 1 h renal ischemia followed by 6 h reperfusion (I/R and STZ I/R, respectively). Further groups of diabetic rats exposed to I/R received GW0742 (0.03 mg/kg, i.v.; STZ I/R+GW) or GSK0660 (0.1 mg/kg, i.v.; STZ I/R+GSK) or GSK0660+GW0742 (STZ I/R+GSK+GW). Each gel photograph is from a single experiment and is representative of three separate experiments.

### Figure 3

**Effects of diabetes and drug treatment on PPAR $\beta/\delta$  expression in rats exposed to renal I/R.**

Western-blot analysis of PPAR $\beta/\delta$  expression of protein extracts from kidney homogenized of non-diabetic and diabetic rats exposed to sham operation (Sham and STZ Sham) or I/R (I/R and STZ I/R). Further groups of diabetic rats exposed to I/R received GW0742 (0.03 mg/kg, i.v.; STZ I/R+GW) or GSK0660 (0.1 mg/kg, i.v.; STZ I/R+GSK) or GSK0660+GW0742 (STZ I/R+GSK+GW). Densitometric analysis of the related bands is expressed as relative optical density (O.D.), corrected for the corresponding  $\beta$ -actin contents and normalized using the related sham-operated band. Data are means  $\pm$  S.E.M. of three separate experiments. ,  $P < 0.05$  versus I/R.

**Figure 4****Effect of GW0742 on I/R-induced neutrophil infiltration in the kidneys of diabetic rats.**

MPO activity (A) and ICAM-1 expression (B) were measured in kidney homogenates of sham-operated diabetic rats (STZ Sham) and diabetic rats that underwent 1 h ischemia followed by 6 h reperfusion (STZ I/R). GW0742 (0.03 mg/kg, i.v.) was administered to diabetic rats at the beginning of reperfusion (STZ I/R+GW). Densitometric analysis of the ICAM-1 bands is expressed as relative optical density (O.D.) corrected for the corresponding  $\beta$ -actin contents and normalized using the related STZ sham-operated band. Data are means  $\pm$  SEM of three separate experiments for Western blot and four animals per group for MPO.  $\star P < 0.01$  vs. STZ I/R.

**Figure 5****Suppression of monocyte and macrophage infiltration in GW0742-treated rats.**

Representative images show an increase in the staining intensity of ED-1 (a marker of monocytes and macrophages) in the kidney tissue sections of diabetic rats that underwent 1 h ischemia followed by 6 h reperfusion (STZ I/R; panel B) compared to sham-operated diabetic rats (STZ Sham; panel A). GW0742 administration markedly reduced the number of glomerular and tubulointerstitial macrophages (STZ I/R+GW; Panel C). Histograms (Panel D) quantify the number of recruited ED-1 positive monocytes and macrophages in the kidney tissue sections from diabetic rats exposed to sham operation (STZ Sham) or I/R (STZ I/R) or GW0742 treatment (STZ I/R+GW). Data are expressed as means  $\pm$  S.E.M.  $\star P < 0.01$  vs. STZ I/R.

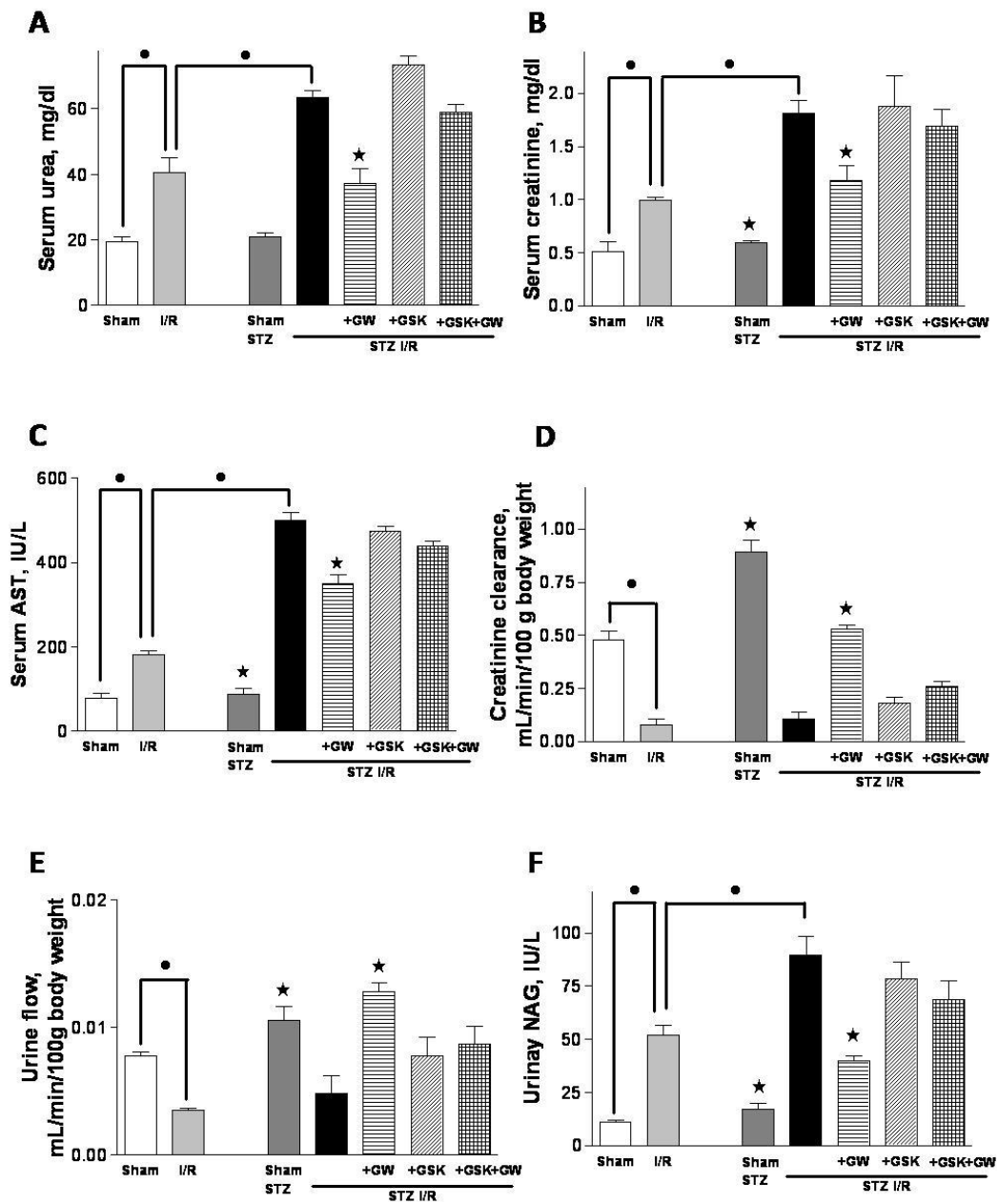
**Figure 6****Effects of drug treatment on IL-6, TNF- $\alpha$ , IL-18, and SOCS-3 mRNA and protein levels in diabetic rats exposed to renal I/R.**

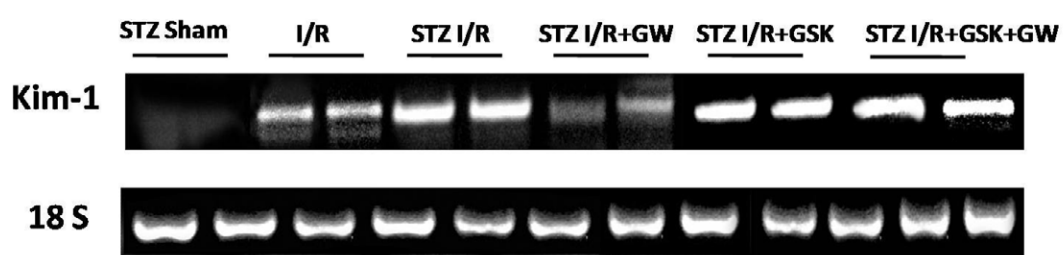
Gel photographs and autoradiograms depict, respectively, mRNA and protein levels of IL-6 (A), TNF- $\alpha$  (B), IL-18 (C), and SOCS-3 (D) in the kidney of diabetic animals that underwent sham operation (STZ Sham) or 1 h ischemia followed by 6 h reperfusion (STZ I/R). Further groups of diabetic rats exposed to I/R received GW0742 (0.03 mg/kg, i.v.; STZ I/R+GW) or GSK0660 (0.1 mg/kg, i.v.; STZ I/R+GSK) or GSK0660+GW0742 (STZ I/R+GSK+GW). Each gel photograph and autoradiogram is from a single experiment and is representative of three separate experiments. Densitometric analysis of the bands from Western blot experiments is expressed as relative optical density (O.D.) of protein expression, corrected for the corresponding  $\beta$ -actin content and

normalized using the related STZ Sham-operated band. Data are means  $\pm$  S.E.M. of three separate experiments. ★  $P < 0.01$  vs. STZ I/R.

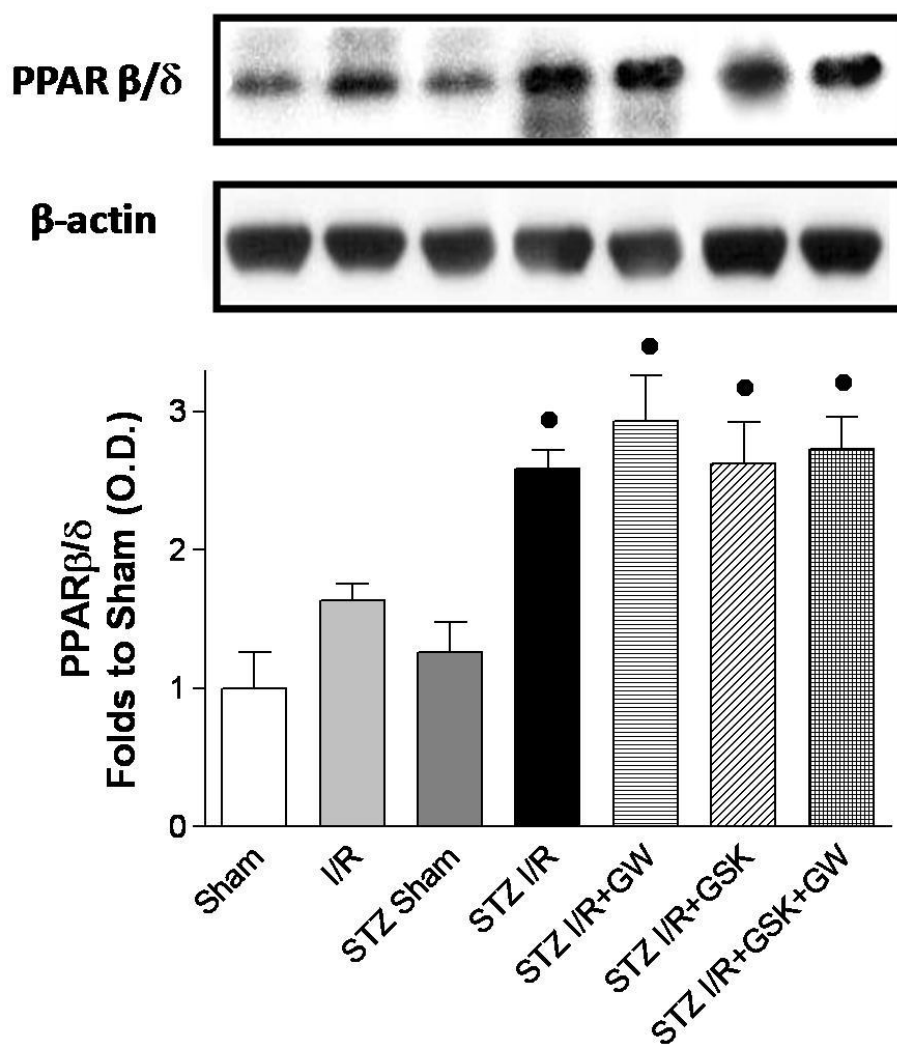
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Figure 1



**Figure 2**

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**Figure 3**

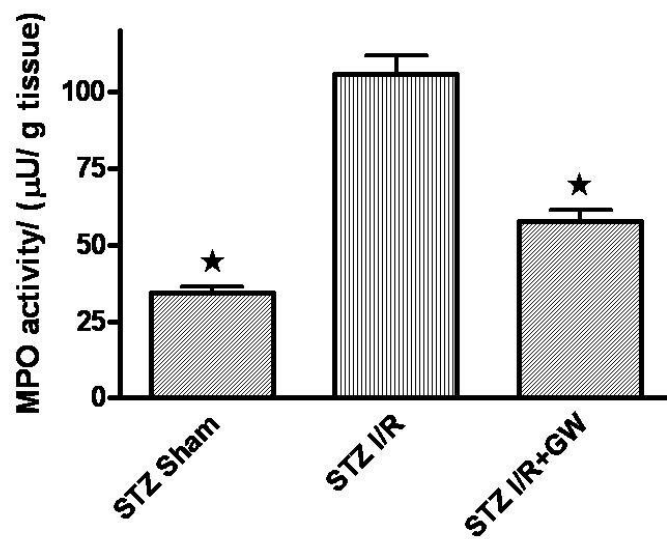
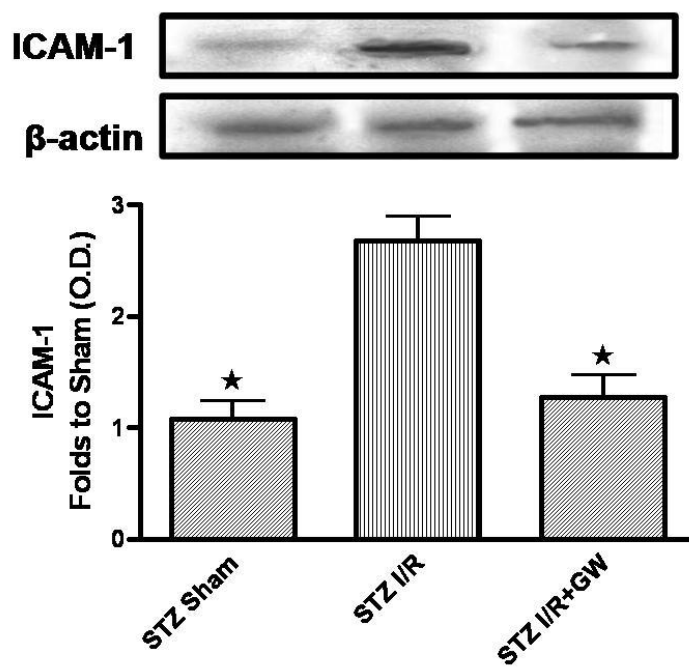
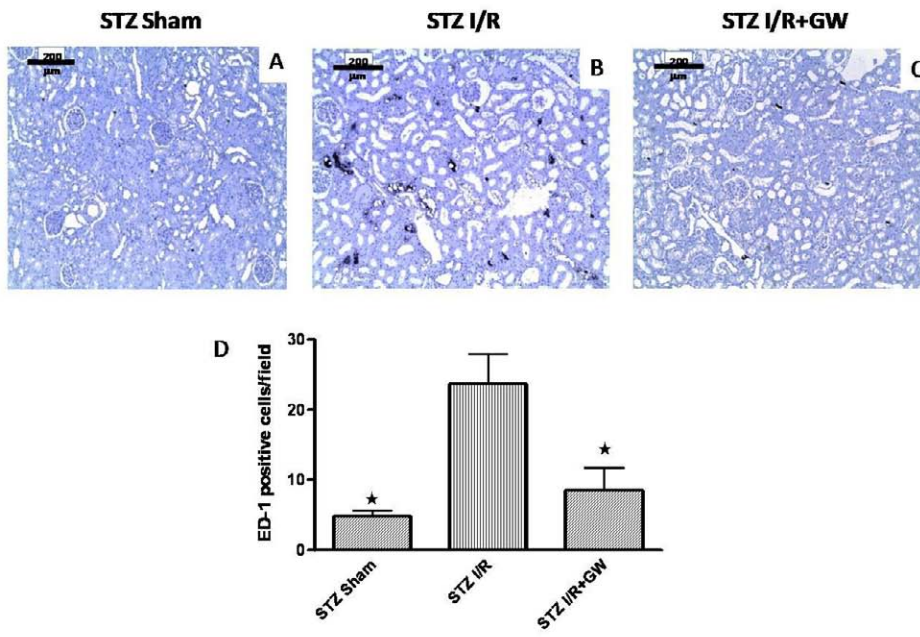
**A****Figure 4****B**

Figure 5



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Figure 6

