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Selective deletion of the endothelial sphingosine-1-phosphate 1 receptor exacerbates kidney ischemia-reperfusion injury

Ahrom Ham[1], **Mihwa Kim**[1], **Joo Yun Kim**[1], **Kevin M. Brown**[1], **Marcus Fruttiger**[2], **Vivette D. D'Agati**[3], and **H. Thomas Lee**[1]

[1]Department of Anesthesiology, College of Physicians and Surgeons of Columbia University, New York, NY, USA

[2]UCL Institute of Ophthalmology, University College London, London, United Kingdom

[3]Department of Pathology, College of Physicians and Surgeons of Columbia University, New York, NY, USA

Abstract

The role for the endothelial sphingosine-1-phosphate 1 receptor (S1P1R) in acute kidney injury (AKI) remains unclear as germline endothelial S1P1R deletion is embryonically lethal. Here, we generated conditional endothelial S1P1R deficiency by crossing mice with floxed S1P1R with mice expressing a tamoxifen-inducible form of Cre recombinase under the transcriptional control of the platelet-derived growth factor-β gene. Mice with tamoxifen-induced deletion of endothelial S1P1R had increased renal tubular necrosis, inflammation, impaired vascular permeability as well as exacerbated renal tubular apoptosis after ischemic AKI compared to tamoxifen-treated wild type mice. Moreover, endothelial S1P1R deletion resulted in increased hepatic injury after ischemic AKI. As a potential mechanism for exacerbated renal injury, conditional endothelial S1P1R null mice had markedly reduced endothelial HSP27 expression compared to wild type mice. Cultured glomerular endothelial cells treated with a specific S1P1R antagonist (W146) for 3 days also showed reduced HSP27 expression compared to vehicle treated cells. Finally, mice treated with W146 for 3 days also showed reduced endothelial HSP27 expression as well as exacerbated renal and hepatic injury after ischemic AKI. Thus, our studies demonstrate a protective role for endothelial S1P1R against ischemic AKI most likely by regulating endothelial barrier integrity and endothelial HSP27 expression.

Keywords

Apoptosis; Cre-recombinase; inflammation; necrosis; tamoxifen

Corresponding Author: H. Thomas Lee, M.D., Ph.D., Professor, Director of Transplantation Anesthesiology, Department of Anesthesiology, Anesthesiology Research Laboratories, Columbia University, P&S Box 46 (PH-5), 630 West 168th Street, New York, NY 10032-3784, Tel: (212) 305-1807, Fax: (212) 305-8980, tl128@columbia.edu.

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Introduction

Renal ischemia and reperfusion (IR) injury is a major cause of perioperative acute kidney injury (AKI) for patients undergoing major vascular, cardiac or transplantation surgery and is directly associated with high mortality, morbidity and cost.[1,2] Furthermore, patients who suffer from AKI also frequently develop extra-renal organ dysfunction including hepatic dysfunction, intestinal barrier disruption, respiratory failure and the systemic inflammatory response syndrome with the eventual development of sepsis and multi-organ failure.[3,4] These extra-renal systemic complications secondary to AKI are the leading causes of mortality in the intensive care unit.[5] Unfortunately, the severity and incidence of AKI has been increasing without effective therapy or improvements in patient survival for the past 60 years.[6]

Sphingolipids are integral components of the plasma membrane and modulate diverse pathways of cell death including necrosis, apoptosis, inflammation, and immunity.[7,8] In particular, sphingosine-1-phosphate (S1P), produced by phosphorylation of sphingosine by sphingosine kinases, is the natural ligand for a family of five lysophospholipid targeted G-protein coupled receptors ($S1P_{1–5}Rs$) and is a powerful modulator of cell death and stress.[8] Encouraging preclinical studies suggest that modulation of S1PRs in renal proximal tubule cells reduces ischemic AKI. Specifically, we and others showed previously that activation of $S1P_1R$ or inhibition of $S1P_2R$ in renal proximal tubules provided powerful renal protection against ischemic AKI in mice.[9,10]

However, unlike the well characterized role for proximal tubule $S1P_1R$ modulation against ischemic AKI, the exact role of endothelial $S1P_1R$ in ischemic AKI remains unclear as germline global endothelial $S1P_1R$ deletion results in embryonic lethality. Furthermore, the role for S1P in modulating endothelial cell function and survival remain controversial. Some studies suggest that endothelial S1P serves to strengthen the vascular endothelial cell barrier whereas other studies suggest detrimental, pro-inflammatory roles for S1P in endothelial cells.[11–13]

In this study, we aimed to test whether endothelial $S1P_1Rs$ play a protective role against AKI due to renal IR injury. In order to do this, we generated mice with an inducible deletion of endothelial $S1P_1R$ by crossing mice with floxed $S1P_1R$ ($S1P_1R^{f/f}$) with mice that express a tamoxifen-inducible form of Cre recombinase ($iCreER^{T2}$) under the transcriptional control of the Platelet-derived growth factor-b (Pdgfb) gene. We subjected these mice as well as wild type ($S1P_1R^{f/f}$) mice to mild (20 min) renal IR injury. We also explored the potential mechanisms for exacerbated renal injury in endothelial $S1P_1R$ null mice subjected to renal IR injury. Since $S1P_1R$ modulates heat shock protein 27 (HSP27) expression in some cell lines[14,15] and since HSP27 is a well-known cytoprotective protein in endothelial cells[16], we also tested the hypothesis that genetic deletion or chronic blockade of $S1P_1R$ decreases endothelial HSP27 expression.

Results

Generation of conditional endothelial $S1P_1R$ deficient mice

In order to conditionally delete $S1P_1R$ in endothelial cells of adult mice, we crossed floxed $S1P_1R$ ($S1P_1R^{f/f}$) mice with mice that express a Platelet-derived growth factor-b (Pdgfb) promoter driven by a tamoxifen-inducible form of Cre-recombinase ($iCreER^{T2}$) in vascular endothelial cells ($Pdgfb^{iCreER}$ mice).[17,18] Tail PCR provided genotypes of conditional endothelial $S1P_1R$ null ($S1P_1R^{f/f}$ $Pdgfb^{iCreER}$) mice and wild type ($S1P_1R^{f/f}$) mice generated from our breeding (Figure 1A). Since the $Pdgfb^{iCreER}$ mice also express an internal ribosomal entry site element and a sequence coding for enhanced green fluorescent protein (EGFP), we detected kidney endothelial cell EGFP expression in $S1P_1R^{f/f}$ $Pdgfb^{iCreER}$ mice with fluorescent microscopy (Figure 1B).[17] EGFP expression was strong in kidney endothelial cells from $S1P_1R^{f/f}$ $Pdgfb^{iCreER}$ mice but was not visible (beyond faint background auto-fluorescence from external elastic membrane) in kidney sections from $S1P_1R^{f/f}$ mice.

$S1P_1R^{f/f}$ $Pdgfb^{iCreER}$ mice were treated daily with 5 mg/kg tamoxifen (i.p.) for 3 days for Cre-recombinase mediated deletion of endothelial $S1P_1R$. $S1P^{f/f}$ mice were also treated with tamoxifen for 3 days and used as controls. We confirmed deletion of endothelial $S1P_1R$ in $S1P_1R^{f/f}$ $Pdgfb^{iCreER}$ mice with RTPCR. $S1P_{1–5}R$ mRNA levels in freshly isolated endothelial cells (prepared with magnetic bead coupled antibody capture) and proximal tubules (prepared with percoll gradient separation) were measured with conventional and real time RTPCR. A representative gel image of PCR products and the quantified mRNA levels (Figure 2) show a drastically decreased $S1P_1R$ mRNA (∼90%) in endothelial cells isolated from kidneys of $S1P_1R^{f/f}$ $Pdgfb^{iCreER}$ mice compared to the wild type ($S1P_1R^{f/f}$) mice endothelial cells. Other S1PR subtypes ($S1P_{2–5}R$) did not change with conditional endothelial $S1P_1R$ deletion (Figure 2). Figure 2 also shows that renal proximal tubular $S1P_{1–5}R$ mRNA expression was similar between tamoxifen-treated $S1P_1R^{f/f}$ $Pdgfb^{iCreER}$ mice and $S1P_1R^{f/f}$ mice.

We also performed immunohistochemistry for kidney $S1P_1R$ and for CD34 (an endothelial cell marker) in tamoxifen-treated $S1P_1R^{f/f}$ $Pdgfb^{iCreER}$ and $S1P_1R^{f/f}$ mice. Figure 3 show representative images for $S1P_1R$ and CD34 immunoreactivity in the kidney. Figure 3A shows images from kidney blood vessels and Figure 3B shows immunohistochemistry images for peritubular capillaries and glomeruli. As shown in Figure 3A, $S1P_1R$ immunoreactivity was markedly attenuated in kidney blood vessels from tamoxifen-treated $S1P_1R^{f/f}$ $Pdgfb^{iCreER}$ mice when compared to $S1P_1R^{f/f}$ mice. CD34 immunoreactivity was similar between tamoxifen-treated $S1P_1R^{f/f}$ $Pdgfb^{iCreER}$ mice and $S1P_1R^{f/f}$ mice in kidney blood vessels. Furthermore, Figure 3B shows that $S1P_1R$ is robustly expressed in peritubular capillaries and glomeruli from $S1P_1R^{f/f}$ mice but not from $S1P_1R^{f/f}$ $Pdgfb^{iCreER}$ mice. Equivalent CD34 immunoreactivity was detected in peritubular capillaries and glomeruli from $S1P_1R^{f/f}$ mice and $S1P_1R^{f/f}$ $Pdgfb^{iCreER}$ mice. These finds are consistent with previous studies that pericytes and smooth muscle cells do not produce Pdgfb *in vivo*.[17] $S1P_1R$ immunoreactivity was positive in kidney leukocytes from conditional endothelial $S1P_1R$ null mice (Figure 3).

Endothelial $S1P_1R$ deletion exacerbates renal and hepatic injury after ischemic AKI in mice

Next, we tested whether endothelial $S1P_1R$ protects against ischemic AKI and remote hepatic injury in mice. We previously showed that ischemic AKI also results in hepatic necrosis, vacuolization and elevated plasma transaminase levels in mice.[19] Plasma creatinine and ALT values were similar between sham-operated (anesthesia, laparotomy, right nephrectomy and recovery) tamoxifen-treated $S1P_1R^{f/f}$ $Pdgfb^{iCreER}$ mice and $S1P_1R^{f/f}$ mice (Figure 4A). As expected plasma creatinine and ALT levels increased mildly but significantly in tamoxifen-treated wild type ($S1P_1R^{f/f}$) mice 24 hr after mild (20 min) renal IR. However, conditional endothelial $S1P_1R$ deficient ($S1P_1R^{f/f}$ $Pdgfb^{iCreER}$) mice subjected to renal IR had significantly increased renal and hepatic injury as evidenced by significantly higher plasma creatinine and ALT levels compared to $S1P_1R^{f/f}$ mice subjected to renal IR.

We also assessed renal function by estimating glomerular filtration rate 24 hours after renal IR injury in mice with the FITC-inulin clearance technique as described by Qi *et al*.[20] The glomerular filtration rate in tamoxifen-treated $S1P_1R^{f/f}$ $Pdgfb^{iCreER}$ mice subjected to renal IR was lower (0.22±0.08 ml/min/100g body weight, N=3) than the glomerular filtration rate in tamoxifen-treated $S1P_1R^{f/f}$ mice subjected to renal IR (0.66±0.21 ml/min/100g body weight, N=3).

Chronic $S1P_1R$ blockade exacerbates renal and hepatic injury after ischemic AKI in mice

We then tested the effects of chronic $S1P_1R$ blockade (W146 treatment daily for 3 days) in C57BL/6 mice subjected to sham-operation or to 20 min renal IR injury. Again, sham-operated vehicle-treated and W146-treated mice had similar baseline renal and hepatic markers of injury (Figure 4B). Vehicle-treated mice showed increased renal and hepatic injury after 20 min renal IR. However, chronic W146-treated mice had significantly elevated plasma markers of renal and hepatic injury.

Endothelial $S1P_1R$ deficient mice have increased kidney necrosis, neutrophil infiltration, and apoptosis and liver necrosis and vacuolization after renal IR

Figure 5A shows representative H&E images (from 6 experiments) of tamoxifen-treated mice subjected to sham-operation or to 20 min renal ischemia and 24 hr of reperfusion (magnification 200X). Sham-operated tamoxifen-treated wild type ($S1P_1R^{f/f}$) mice and tamoxifen-treated endothelial $S1P_1R$ deficient mice had normal renal histology (Figure 5A). Compared to sham-operated $S1P_1R^{f/f}$ mice, the kidneys of tamoxifen-treated $S1P_1R^{f/f}$ mice subjected to renal IR showed moderate tubular vacuolization, proteinaceous casts with increased tubular dilatation and congestion (Figure 5A). Consistent with the plasma creatinine data, tamoxifen-treated endothelial $S1P_1R$ deficient ($S1P_1R^{f/f}$ $Pdgfb^{iCreER}$) mice had increased renal tubular necrosis, congestion and cast formation. The Jablonski scale[21] renal injury score (scale: 0–4) for histology grading was used to grade renal tubular necrosis 24 hr after 20 min renal IR (Figure 5B). Twenty min of renal ischemia and 24 hr of reperfusion resulted in mild to moderate acute tubular necrosis in tamoxifen-treated wild type ($S1P_1R^{f/f}$) mice. In contrast, tamoxifen-treated endothelial $S1P_1R$ deficient mice had significantly higher renal injury scores (Figure 5B).

Figure 5C shows representative images (from 4–6 experiments) of immunohistochemistry of neutrophil infiltration (dark brown) in the kidneys (corticomedullary junction) of tamoxifen-treated mice subjected to sham-operation or to 20 min of renal ischemia and 24 hr of reperfusion (magnification 200X). In sham-operated tamoxifen-treated wild type ($S1P_1R^{f/f}$) mice, we were unable to detect any neutrophils in the kidney. However, few neutrophils were detected in sham-operated tamoxifen-treated endothelial $S1P_1R$ deficient mice. Neutrophil infiltration increased in tamoxifen-treated wild type mice concentrated near the corticomedullary junction (Figure 5C). Again, tamoxifen-treated endothelial $S1P_1R$ deficient mice had increased neutrophil infiltration after 20 min of renal IR and 24 hr of reperfusion (Figure 5C and Figure 5D).

TUNEL staining detected apoptotic renal tubular cells in kidneys of mice (representative of 5 experiments). In tamoxifen-treated sham-operated mice, very few TUNEL positive cells were detected in the kidneys of both wild type and endothelial $S1P_1R$ deficient mice (Figure 5E, 100X). TUNEL staining showing renal tubule cell apoptosis increased in tamoxifen-treated wild type mice subjected to 20 min of renal IR (Figure 5E and Figure 5F). We show here that tamoxifen-treated endothelial $S1P_1R$ deficient mice had significantly increased number of apoptotic TUNEL-positive cells in the kidney 24 hr after IR.

Liver histology was also assessed by H&E staining of liver sections (representative of 5–6 slides). As shown in Figure 5G, sham-operated wild type mice had normal liver histology. Twenty min renal ischemia and 24 hr reperfusion resulted in mild-moderate nuclear and cytoplasmic degenerative changes, cellular vacuolization, congestion as well as individual hepatocyte necrosis and focal apoptotic, pyknotic nuclei were observed (Figure 5G). The severity of liver injury was increased for conditional endothelial $S1P_1R$ deficient mice consistent with higher plasma ALT and manifested by increased of cellular degenerative changes including increased individual hepatocyte necrosis, apoptosis and vacuolization. Some degree of centrilobular necrosis was observed in endothelial $S1P_1R$ deficient mice. Vascular congestion and leukocyte (mainly neutrophil) infiltration were also detected after renal IR in endothelial $S1P_1R$ deficient mice.

Chronic $S1P_1R$ blockade increases renal tubular necrosis, neutrophil infiltration, and apoptosis and liver necrosis and vacuolization after renal IR

Figure 6A shows representative H&E images (from 5–6 experiments) of vehicle or W146-treated C57BL/6 mice subjected to sham-operation or to 20 min of renal ischemia and 24 hr of reperfusion (magnification 200X). Sham-operated vehicle-treated and W146-treated C57BL/6 mice had normal renal histology (Figure 6A). Compared to sham-operated mice, the kidneys of vehicle-treated C57BL/6 mice subjected to 20 min renal IR showed increased renal injury (increased tubular vacuolization, proteinaceous casts with increased tubular dilatation and congestion, Figure 6A). Furthermore, C57BL/6 mice treated with W146 for 3 days mice had increased renal tubular necrosis, congestion and cast formation after 20 min renal IR. The Jablonski scale renal injury scores again show that 20 min of renal ischemia and 24 hr of reperfusion resulted in mild to moderate acute tubular necrosis in vehicle-treated C57BL/6 mice. In contrast, W146-treated C57BL/6 mice had significantly higher renal injury scores (Figure 6B).

Figure 6C shows representative images (from 4–6 experiments) of immunohistochemistry of kidney neutrophil infiltration (dark brown) of vehicle- or W146-treated C57BL/6 mice subjected to sham-operation or to 20 min of renal ischemia and 24 hr of reperfusion (magnification 200X). In sham-operated mice, we were unable to detect any neutrophils in the kidney. Neutrophil infiltration increased in vehicletreated mice subjected to renal IR (Figure 6C). Chronic W146-treament further increased neutrophil infiltration after 20 min of renal ischemia and 24 hr of reperfusion (Figure 6C and Figure 6D).

Chronic $S1P_1R$ antagonist treatment also increased renal apoptosis after IR. In sham-operated C57BL/6 mice, very few TUNEL positive cells were detected in the kidneys from vehicle- and W146-treated mice (Figure 6E, 100X, representative of from 4–6 experiments). TUNEL staining increased in vehicle-treated mice subjected to 20 min renal IR (Figure 6E and Figure 6F, N=4–6). Furthermore, W146-treated mice had significantly increased numbers of apoptotic TUNEL-positive cells in the kidney 24 hr after IR.

Liver histology was also assessed by H&E staining of liver sections. As shown in Figure 5G (representative of 5–6 slides), sham-operated C57BL/6 mice had normal liver histology. Vehicle-treatment and 20 min renal ischemia and 24 hr reperfusion resulted in mild-moderate hepatic injury (nuclear and cytoplasmic degenerative changes, cellular vacuolization, congestion as well as individual hepatocyte necrosis and focal apoptotic, pyknotic nuclei, Figure 5G). Similar to conditional endothelial $S1P_1R$ deficient mice, the severity of liver injury was increased for W146-treated C57BL/6 mice subjected to renal IR injury (increased hepatocyte degenerative changes including increased individual hepatocyte necrosis, apoptosis and vacuolization). Some degree of centrilobular necrosis was again observed in W146-treated mice subjected to 20 min renal IR injury.

Endothelial $S1P_1R$ deficiency increases pro-inflammatory gene expression in the kidney after IR

We measured the expression of pro-inflammatory cytokine mRNAs in the kidney (TNF-α, ICAM-1, MCP-1 and MIP-2) 24 hr after renal IR by RTPCR (primer sequences listed in Table 1). We show that tamoxifen-treated wild type mice subjected to renal IR had significantly increased expression of all pro-inflammatory mRNAs examined compared to the tamoxifen-treated sham-operated wild type mice (Figure 7). Moreover, tamoxifen-treated endothelial $S1P_1R$ deficient mice had even greater increases in TNF-α, MCP-1 and ICAM-1 expression without any changes in MIP-2 expression compared to tamoxifen-treated wild type mice subjected to renal IR.

Endothelial $S1P_1R$ deficiency increases kidney vascular permeability after renal IR

We measured kidney vascular permeability after 20 min renal IR with EBD injection. EBD binds to plasma proteins and its appearance in extravascular tissues reflects an increase in vascular permeability. Analysis of EBD extravasations in kidneys from tamoxifen-treated wild type ($S1P_1R^{f/f}$) or endothelial $S1P_1R$ deficient mice subjected to either sham-operation or to renal IR is shown in Figure 8. We show that renal IR caused significant increases in kidney vascular permeability. However, tamoxifen-treated endothelial $S1P_1R$ deficient mice had significantly increased EBD extravasation after sham-operation or after renal IR.

Conditional endothelial $S1P_1R$ deficiency or chronic $S1P_1R$ blockade decreases endothelial HSP27 mRNA expression in mice

We probed for the potential mechanisms for increased renal injury and increased impairment of vascular permeability with endothelial $S1P_1R$ deficiency or antagonism. Since $S1P_1R$ induces HSP27 expression in certain cell types[14,15] and since HSP27 is a well-known cytoprotective protein in endothelial cells[16], we hypothesized that deletion or chronic blockade of $S1P_1R$ decreases endothelial HSP27 expression. Figure 9A and Figure 9B show that freshly isolated renal endothelial cells from naïve conditional endothelial $S1P_1R$ deficient mice express significantly reduced HSP27 mRNA compared with tamoxifen-treated wild type ($S1P_1R^{f/f}$) mice without any changes in HSP32 or HSP70 expression. Furthermore, we show that HSP27, HSP32 and HSP70 mRNA levels in freshly isolated proximal tubule cells were similar between conditional $S1P_1R$ deficient mice and wild type mice. Finally, we determined that endothelial cells isolated from C57BL/6 mice chronically treated with W146 (0.1 mg/kg for 3 days) also express significantly reduced HSP27 mRNA compared to vehicle-treated C57BL/6 mice (Figure 9C). However, HSP27 mRNA expression did not change in endothelial cells isolated from kidney of C57BL/6 mice treated with W146 (0.1 mg/kg) for 1 hr compared to vehicle-treated mice (Figure 9D). Consistent with this, W146 treatment for 1 hour did not exacerbate ischemic AKI in C57BL/6 mice (Cr=1.44±0.14 mg/dL, N=4) compared to vehicle-treated mice (Cr=1.30±0.09 mg/dL, N=4).

$S1P_1R$-mediated modulation of cultured endothelial cell HSP27 mRNA expression

We next tested whether $S1P_1R$ antagonism or activation modulates HSP27 mRNA expression in cultured endothelial cells. Human umbilical vein endothelial (EA.hy926) cells (Figure 10A) or mouse glomerular endothelial cells (Figure 10B) were treated with a selective $S1P_1R$ antagonist (1 μM W146) or agonist (1 μM SEW2871) for 3 days and probed for HSP27 mRNA expression with RTPCR. Therefore, Figure 10 and Figure 10B show that human and mouse endothelial cells treated with W146 for 3 days had significantly reduced HSP27 mRNA expression. Conversely, chronic $S1P_1R$ activation with SEW2871 significantly increased HSP27 mRNA expression in human and mouse endothelial cells. In contrast, W146 treatment for 1 hr did not change HSP27 mRNA expression in human endothelial cells (Figure 10C).

Chronic $S1P_1R$ antagonism or HSP27 knockdown decreases endothelial cell viability

We next tested whether chronic (3 days) $S1P_1R$ antagonism or HSP27 knockdown modulates endothelial cell viability in culture. MTT assay was performed to measure endothelial cell viability. Human umbilical vein endothelial cells treated with W146 for 3 days had significantly reduced endothelial cell viability (∼83% of vehicle-treated group) whereas 1 hr treatment had no effect (Figure 11A). We then tested whether knockdown of HSP27 expression also modulates endothelial cell viability in culture. Figure 11B shows that HSP27 siRNA treatment for 48 hr significantly attenuated HSP27 mRNA expression in endothelial cells compared to control siRNA-treated cells. Furthermore, HSP27 siRNA induced HSP27 knockdown significantly reduced endothelial cell viability in culture (∼73% of control siRNA group). Therefore, from these studies, we conclude that downregulation of HSP27 expression decrease endothelial cell survival.

Discussion

Acute kidney injury (AKI) remains a significant clinical complication with high mortality and morbidity despite more than 6 decades of research.[1,4] Due to extreme susceptibility to ischemia induced necrosis and apoptosis, renal proximal tubular injury has been considered the hallmark of AKI pathology.[22] However, it is becoming increasingly clear that complex and close orchestration of signaling events between proximal tubule cells, endothelial cells, pericytes and resident dendritic cells play a major role in ischemic AKI.[23,24] In particular, endothelial cell injury after ischemic AKI maintains and even extends renal dysfunction and may play a critical role in the development of chronic kidney disease.[25] Indeed, renal vascular endothelial dysfunction results in reduction in post-ischemic peritubular capillary blood flow as well as in increased coagulation, leukocyte infiltration and tubular inflammation. Therefore, therapeutic strategies to protect against endothelial dysfunction would reduce the extent and maintenance phase of AKI after renal IR injury.

Sphingosine-1-Phosphate (S1P) is an endogenous lysophospholipid ligand that regulates diverse cellular function in many organs by binding to cell surface G-protein coupled high affinity S1P receptors.[26,27] There are 5 identified S1PRs that produce distinct as well as overlapping intracellular signaling events. $S1P_1R$, $S1P_2R$ and $S1P_3R$ in particular are widely expressed in many cell types including the brain, liver, heart, kidney and the immune system.[26] Of 5 S1P receptor subtypes, the $S1P_1R$ has been the most extensively studied. Activation of the $S1P_1R$ protects against cardiac and hepatic IR injury and reduces systemic and tissue inflammation by directly affecting lymphocyte egress.[28–30] In the kidney, activation of the $S1P_1R$ with selective agonists (SEW2871 or FTY720) protects against renal IR injury by reducing inflammation, attenuating pro-inflammatory T-lymphocyte function as well as by eliciting direct cytoprotective effects on renal proximal tubule cells.[28,31,32]

Although the renal tubular protective role for $S1P_1R$ activation is well established, the role for endothelial $S1P_1R$ in modulating ischemic AKI is not entirely clear.[10,28,32] This is mainly because germline global or conditional endothelial $S1P_1R$ deletion results in embryonic lethality due to immature formation of blood vessels and excessive hemorrhage.[18,33] In addition, conflicting data exists in the literature regarding the role of S1P in endothelial cell function and survival. While many studies implicate a cytoprotective and barrier-strengthening role for endothelial S1P and $S1P_1R$ activation, other studies suggest that S1P promotes inflammation and leukocyte adhesion in endothelial cells.[11–13] For example, S1P promotes adhesion molecule VCAM-1 and E-selectin induction in endothelial cells via NF-kB activation.[34,35] Furthermore, chronic overexpression of sphingosine kinase-1 and increased S1P synthesis in cultured endothelial cells increases VCAM-1 expression and neutrophil adhesion in response to TNF-α stimulation.[36]

In this study, we crossed floxed $S1P_1R$ mice with mice that express a tamoxifen-inducible form of Cre recombinase under the transcriptional control of Pdgfb to selectively delete endothelial $S1P_1R$ in adult mice.[17,18] $Pdgfb^{iCreER}$ mice express a tamoxifen inducible form of Cre recombinase ($iCreER^{T2}$) under the transcriptional control of the Pdgfb gene. As Pdgfb is predominantly expressed in endothelial cells, use of $Pdgfb^{iCreER}$ mice would result

in more selective expression of Cre recombinase in endothelial cells when compared to Tie-2^{Cre} mice.[17] Although Tie-2^{Cre} mice are widely used to delete the gene of interest in endothelial cells as Tie-2 expression was thought to be restricted to endothelial cells, recent studies suggest that certain myeloid cells also express Tie-2.[37] Indeed, Cre driven by Tie-2 will recombine genomic DNA in both endothelial and myeloid leukocytes as some leukocytes, in particular monocytes and eosinophils, also express Tie-2.[38,39] We performed qRTPCR for $S1P_{1–5}R$ subtypes in splenocytes and peritoneal macrophages and determined that conditional endothelial $S1P_1R$ deletion did not change expression of $S1P_{1–5}R$ subtypes in leukocytes (data not shown). Furthermore, Pdgfb^{iCreER} mice has the added advantage as they express Cre recombinase fused to a mutant form of the human estrogen receptor that is insensitive to endogenous estrogen but is responsive to tamoxifen.[17]

With immunohistochemistry as well as RTPCR, we showed that $S1P_1R^{f/f}$ Pdgfb^{iCreER} mice have selectively reduced $S1P_1R$ expression in endothelial cells after 3 days of tamoxifen treatment. We also complemented our inducible endothelial $S1P_1R$ studies by treating C57BL/6 mice with a selective $S1P_1R$ antagonist (W146). We demonstrate in this study that either inducible deletion of endothelial $S1P_1R$ or chronic pharmacological blockade of $S1P_1R$ exacerbated renal IR injury and remote hepatic dysfunction in mice. Furthermore, inducible genetic deletion of endothelium or chronic $S1P_1R$ antagonist treatment increased renal tubular necrosis, neutrophil infiltration, and apoptosis. These data prove that endothelial $S1P_1R$ activation serves a protective role against ischemic AKI.

In addition to increased kidney neutrophil infiltration, we determined that inducible endothelial $S1P_1R$ null mice had increased expression of several key pro-inflammatory mRNAs including ICAM-1, MCP-1 and TNF-α, in the kidney after renal IR. ICAM-1 is an important mediator of neutrophil trans-endothelial migration and MCP-1 acts as a chemoattractant for monocytes and lymphocytes to areas of injury. [26,27] These findings suggest that endothelial $S1P_1R$ serves to protect against pro-inflammatory leukocyte attachment and transmigration after renal IR injury.

Since heat shock proteins are well recognized cytoprotective proteins and regulate endothelial cell survival, cytoskeleton structure and differentiation, we tested whether endothelial $S1P_1R$ deficiency leads to depletion of one of these cytoprotective chaperone heat shock proteins.[16,40] We showed in this study that selective endothelial $S1P_1R$ deletion or chronic $S1P_1R$ blockade with W146 decreased endothelial HSP27 expression *in vivo*. Indeed, $S1P_1R$ activation has been shown to modulate HSP27 expression in other cell types. S1P has been shown to induce HSP27 via the p38 MAP-kinase and the PI3-kinase/Akt pathways in osteoblasts.[14,15] We propose that marked reduction in endothelial HSP27 may be responsible for increased renal and hepatic injury after ischemic AKI in endothelial $S1P_1R$ null mice. Further study will be necessary to determine the precise relationship between $S1P_1R$ and HSP27 in mediating endothelial protection after ischemic AKI.

In contrast to increased renal and hepatic injury with downregulation of endothelial HSP27 in endothelial $S1P_1R$ null mice, we showed previously that overexpression of sphingosine kinase-1 and enhanced activation of $S1P_1R$ induced HSP27 in renal proximal tubules in mice and in HK-2 cells. Furthermore, we recently demonstrated that HSP27 overexpression

protected mice against hepatic IR induced renal endothelial cell apoptosis by promoting F-actin cytoskeletal preservation.[41,42] HSP27 is a member of a family of chaperone proteins that are up-regulated in response to a wide range of cellular stresses including hypoxia, ischemia and exposure to toxic drugs.[43,44] Increased expression of HSP27 serves to defend a cell against injury or death by acting as chaperones facilitating proper polypeptide folding and aberrant protein removal.[45–47] In addition, HSP27 is a potent anti-apoptotic protein and is a key stabilizer of the actin cytoskeleton; both of these cellular effects lead to increased resistance against cell death.[40,48] Furthermore, HSP27 is directly responsible for increased tolerance for endothelial cell death against apoptosis and heat stress.[16,40] We also provide evidence for a direct link between HSP27 expression and endothelial viability as knockdown of HSP27 expression with HSP27 siRNA or with chronic W146 treatment reduced viability of human endothelial cells in culture whereas short term (1 hr) W146 treatment did not reduce HSP27 expression and failed to reduce endothelial cell viability. Collectively, these findings suggest an important role for HSP27 in $S1P_1R$-mediated endothelial cell protection.

In addition to $S1P_1R$-mediated endothelial cell protection, additional mechanisms may regulate endothelial function during and after ischemic AKI. Grenz *et al*.[49] eloquently demonstrated that increased adenosine generation and transport via transcriptional regulation of equilibrative nucleoside transporters regulate post-ischemic no-reflow phenomenon by modulating renal endothelial A_{2b} adenosine receptors. Interestingly, we also recently showed that increased adenosine generation after ischemic AKI enhances sphingosine kinase-1 activity leading to increased S1P generation and $S1P_1R$ activation in the kidney.[50] Future studies are required to determine whether increased vascular adenosine by transcriptional regulation of equilibrative nucleoside transporters modulate endothelial $S1P_1R$ activity to regulate endothelial function after ischemic AKI.

Ischemia and reperfusion injury is characterized by intensive tissue hypoxia followed by profound inflammation.[51] Previous studies have shown that hypoxia inducible factor (HIF)-1α dependent transcriptional regulation plays a critical role in modulating tissue hypoxia and inflammation as well as promoting cellular repair.[51] Interestingly, S1P signaling can directly modulate HIF-1α activity.[52] It remains to determined in future studies whether endothelial $S1P_1R$ signaling can directly modulate HIF-1α signaling to protect against ischemic AKI induced tissue hypoxia and inflammation.

Plasma creatinine measurement as an index of renal function after AKI has several limitations especially in non-steady state conditions due to renal tubular secretion of creatinine and plasma non-creatinine chromagen interference.[53,54] In fact, Eisner *et al*. demonstrated that ∼35–50% of creatinine clearance is mediated by renal tubular secretion of creatinine rather than glomerular filtration in mice.[54] Their findings indicate that utilizing creatinine would lead to overestimation of renal glomerular filtration rate. Their findings also suggest that renal tubular damage due to IR injury would decrease renal tubular creatinine secretion in mice leading to even greater increase in plasma creatinine levels. Therefore, we also estimated glomerular filtration rate with FITC-inulin technique.[20] These 2 indices of renal function together with assessment of renal tubular necrosis, inflammation and apoptosis allow us to support our conclusion that mice with conditional deletion of endothelial $S1P_1R$ have increased renal injury after ischemic AKI.

One of the most exciting aspects of sphingolipid research is small molecule therapy targeting $S1P_1R$ to protect renal proximal tubules and endothelial cells against ischemic AKI. However, multiple S1PR subtypes are expressed in a single cell type including renal tubules and endothelial cells that may produce divergent and undesirable side effects. Therefore, specific targeting of $S1P_1R$ will be critical to prevent undesirable S1PR receptor signaling that may limit therapeutic potential of $S1P_1R$ activation. Several compounds specifically targeting $S1P_1R$ have been already developed and are being tested in preclinical and clinical trials.[55]

In summary, we demonstrate in this study that inducible and selective endothelial $S1P_1R$ deletion in adult mice exacerbates renal and hepatic injury after ischemic AKI by exacerbating kidney vascular permeability impairment, renal tubular necrosis, apoptosis and inflammation. We also propose that a selective downregulation of endothelial HSP27, a key cytoprotective protein, may explain increased organ injury in endothelial $S1P_1R$ null mice.

Methods

Generation of mice with conditional tamoxifen inducible deletion of endothelial $S1P_1R$

Mice with global or endothelial specific deletion of $S1P_1R$ are embryonically lethal due to immature formation of blood vessels and excessive hemorrhage.[33] Therefore, we generated conditional endothelial $S1P_1R$ deficient mice by crossing mice with floxed $S1P_1R$ ($S1P_1R^{f/f}$, provided by Dr Rick L. Proia (NIH)) with mice that express a tamoxifen-inducible form of Cre recombinase ($iCreER^{T2}$) under the transcriptional control of the Pdgfb gene.[17,18] Resulting $S1P_1R^{f/f}$ $Pdgfb^{iCreER}$ (endothelial $S1P_1R$ deficient) or $S1P_1R_{f/f}$ (wild type) mice on a C57BL6/129 background were genotyped by tail biopsy PCR using Pdgfb-Cre transgene and $S1P_1R$ loxP-specific primers (Table 1). Pdgfb-Cre primers generated a 450-bp fragment. $S1P_1R$ loxP primers amplified a 250-bp fragment for the $S1P_1R$ floxed allele and a 200-bp fragment for the $S1P_1R$ wild-type allele. Adult (~20g) mice were injected with 5 mg Tamoxifen in filter-sterilized olive oil (i.p.) daily for 3 days to conditionally delete endothelial $S1P_1R$.

EGFP detection in $S1P_1R$ $Pdgfb^{iCreER}$ mice

$Pdgfb^{iCreER}$ mice also express an internal ribosomal entry site element and a sequence coding for enhanced green fluorescent protein (EGFP).[17] To confirm that Pdgfb-iCreER transgene expression is specific for endothelial cells in the kidney, we detected EGFP expression in kidney cryosections from $S1P_1R^{f/f}$ $Pdgfb^{iCreER}$ or $S1P_1R_{f/f}$ mice. Kidneys were fixed in 30% sucrose solution for 4 hr and embedded into optimal cutting temperature compound (Tissue-Tek, Sakura Finetek USA, Trannce, CA). The 8 μm cryosections were mounted in ProLong Gold antifade reagent-containing DAPI from Molecular Probes (Invitrogen, Carlsbad, CA) and imaged under a fluorescence microscope.

Murine model of renal IR injury

After Institutional Animal Care and Use Committee approval, we subjected adult (~20g) male mice to 20 min. of renal IR as previously described.[56,57] Sham-operated animals underwent the same surgical procedures (anesthesia, laparotomy, bowel manipulations and

wound closure) without renal ischemia. We collected kidney, liver and plasma 24 hr after renal IR or sham-operation to examine the severity of renal and hepatic dysfunction. We also collected kidney 5 hr after renal IR or sham-operation in a separate cohort of mice to measure impairments in kidney vascular permeability.

Analyses for plasma markers for renal and hepatic injury

Plasma creatinine was measured by an enzymatic creatinine reagent kit according to the manufacturer's instructions (Thermo Fisher Scientific, Waltham, MA). This method of creatinine measurement largely eliminates the interferences from mouse plasma chromagens well known to the Jaffe method.[58] Renal function was also assessed by estimating glomerular filtration rate 24 hours after renal IR injury. The glomerular filtration rate was estimated with fluorescein isothiocyanate (FITC-inulin) clearance technique as described by Qi *et al*.[20] The plasma alanine aminotransferase (ALT) activities were measured by using the Infinity ALT assay kit according to the manufacturer's instructions (Thermo Fisher Scientific, Waltham, MA).

Histological detection of kidney and liver injury

Kidney and liver morphological assessment was performed by an experienced pathologist (V.DA.) who was unaware of the treatment that each animal had received. An established grading scale of necrotic injury (0–4, Renal Injury Score) to the proximal tubules was used for the histopathological assessment of IR-induced damage as outlined by Jablonski *et al*.[21] and as described previously in our studies.[59,60] Liver H&E sections were graded for hepatocyte hypereosinophilia, cytoplasmic vacuolization, coagulation necrosis, sinusoidal congestion and sinusoidal inflammation as described by us previously.[19]

Histological detection of kidney apoptosis and neutrophil infiltration

We detected apoptosis after renal IR with TUNEL staining as described using a commercially available in situ cell death detection kit (Roche, Indianapolis, IN) according to the instructions provided by the manufacturer.[29,61] Apoptotic TUNEL positive cells were quantified in 5–7 randomly chosen 100X microscope images fields in the corticomedullary junction and results were expressed as apoptotic cells counted per 100X field. Renal inflammation after IR injury was determined by detecting neutrophil infiltration with immunohistochemistry 24 hr after renal IR as described previously.[29,61] Neutrophils infiltrating the kidney were quantified in 5–7 randomly chosen 200X microscope image fields in the corticomedullary junction and results were expressed as neutrophils counted per 200X field.

Kidney vascular permeability after ischemic AKI

Changes in the kidney vascular permeability 5 hr after renal IR were assessed by quantifying extravasations of Evans blue dye (EBD) into the tissue as described by Awad *et al*. with some modifications as described by us. [10,61]

Immunohistochemistry for $S1P_1R$ and CD34

$S1P_1R$ and CD34 immunohistochemistry were performed in paraffin-embedded kidney sections from tamoxifen-treated conditional endothelial $S1P_1R$ deficient mice and wild type ($S1P_1R^{f/f}$) mice with rabbit anti-mouse $S1P_1R$ (Cayman, Ann Arbor, MI, USA, 1:100) or with rat anti-mouse CD34 (Cedarlane labs, Burlington, NC, USA, 1:100). A primary antibody that recognized IgG_{2a} (Serotec, Raleigh, NC, USA) was used as a negative isotype control in all experiments.

Isolation of mouse kidney proximal tubule cells and endothelial cells

Adult $S1P_1R^{f/f}$ $Pdgfb^{iCreER}$ or $S1P_1R_{f/f}$ mice were pretreated with tamoxifen for 3 days. Mouse kidney proximal tubules were isolated using Percoll density gradient separation as described previously.[9,62] Renal endothelial cells were isolated with CD144 antibody (eBioscience inc, San Diego, CA) capture and magnetic bead separation (Dynabeads®, Invitrogen) according to the manufacturer's instructions (Invitrogen, Carlsbad, CA) and as described by us previously.[9]

Cell culture

Immortalized mouse glomerular endothelial cells were obtained from Dr. M. Madaio (Georgia Regents University) and grown in low glucose DMEM/Ham's F12 medium plus 10% FBS, 2 mM l-glutamine, and 10 mM HEPES.[63] Immortalized human umbilical vein endothelial cells (EA.hy926, American Type Culture Collection, Manassas, VA) cells were grown in high-glucose DMEM plus 10% FBS. Endothelial cells were pretreated with vehicle (1% DMSO), 1 μM W146 (3-amino-4-(3-hexylphenylamino)-4-oxobutyl phosphonic acid, a selective $S1P_1R$ antagonist) or SEW2871 (5-[4-phenyl-5-(trifluoromethyl)-2-thienyl]-3-[3-(trifluoromethyl)phenyl]-1,2,4-oxadiazole, a selective $S1P_1R$ agonist) for 3 days to chronically antagonize or activate $S1P_1R$ *in vitro*. Some EA.hy926 cells were also pretreated for 1 hr with W146 before harvest.

HSP27 siRNA Transfection

Immortalized human umbilical vein endothelial cells were transfected with 20–100 nM HSP27 siRNA (sc-29350) or with control siRNA (sc-36869) from Santa Cruz Biotechnology (Santa Cruz, CA) with siRNA transfection reagent (sc-29528) in siRNA transfection medium (sc-36868) for 48 hr according to the manufacturer's instructions.

Reverse transcription PCR analyses (RTPCR)

Kidney inflammation after renal ischemia was also determined by measuring mRNA encoding markers of inflammation, including keratinocyte derived cytokine (KC), intercellular adhesion molecule-1 (ICAM-1), monocyte chemoattractive protein-1 (MCP-1), macrophage inflammatory protein-2 (MIP-2), and tumor necrosis factor-alpha (TNF-α) 24 hr after renal IR. We also performed RTPCR for heat shock proteins (HSP27, heme oxygenase-1 [HO-1 or HSP32] and HSP70) and $S1P_{1–5}R$ in kidneys isolated from naive mice. Finally, we measured HSP27 mRNA expression in cultured endothelial cells with RTPCR. RTPCR was performed as described previously with the primers listed in Table 1.[29,61]

Measurement of endothelial cell viability

Endothelial cell viability was tested with a 3-[4,5-dimethyl(thiazol-2-yl)-3,5-diphery]tetradium bromide (MTT) cytotoxicity assay as described.[64] The MTT cytotoxicity assay measures cell viability by quantifying the mitochondrial formation of formazan product (dark blue) via dehydrogenase-mediated reduction of the tetrazolium ring of MTT.

Statistical analysis

The data were analyzed with Student's *t*-test when comparing means between two groups or one-way ANOVA plus Tukey's *post hoc* multiple comparison test when comparing multiple groups. The ordinal values of the renal and hepatic injury scores were analyzed by the Mann–Whitney nonparametric test. In all cases, a probability statistic $P<0.05$ was taken to indicate significance. All data are expressed throughout the text as means ± SEM.

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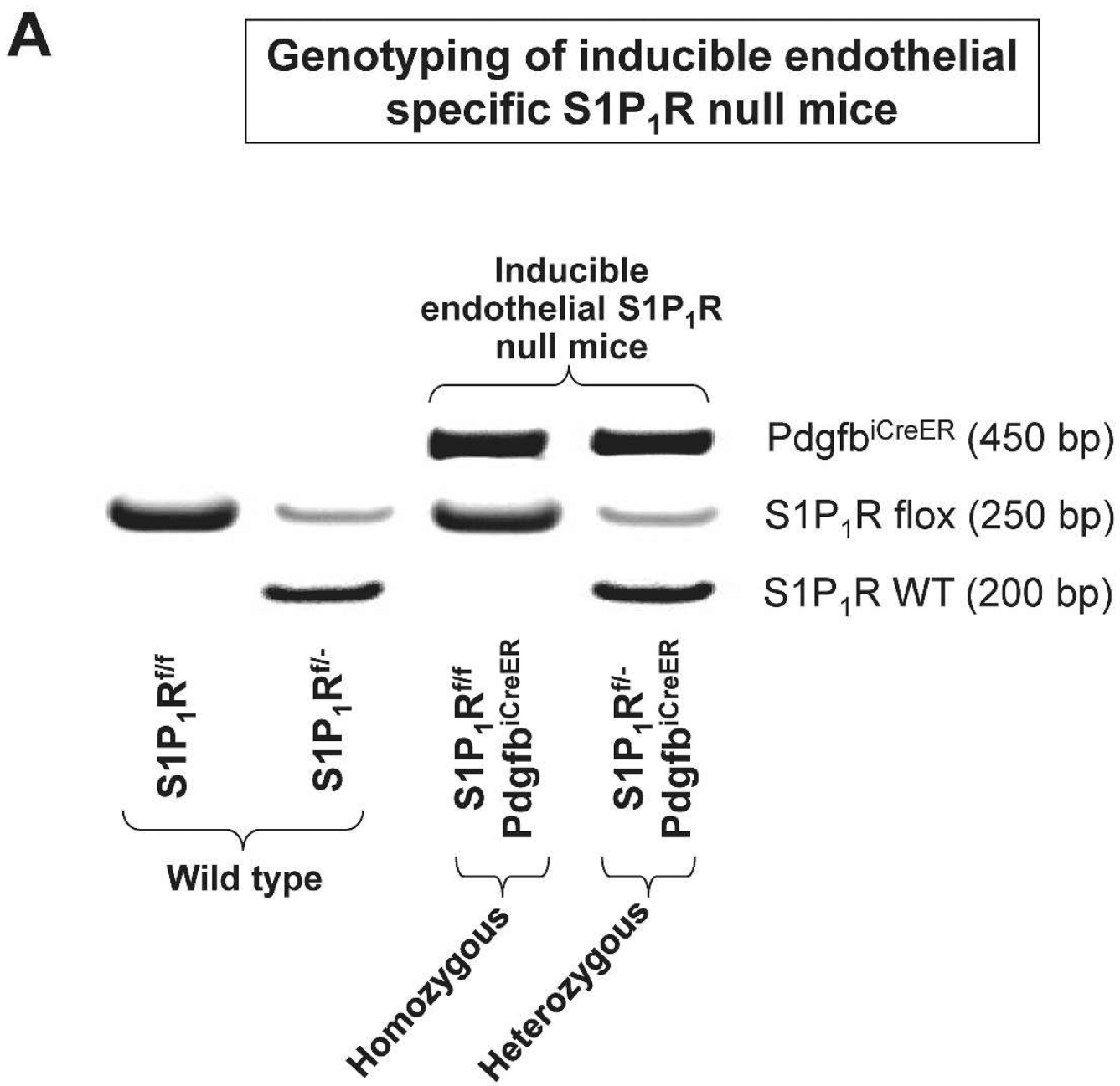


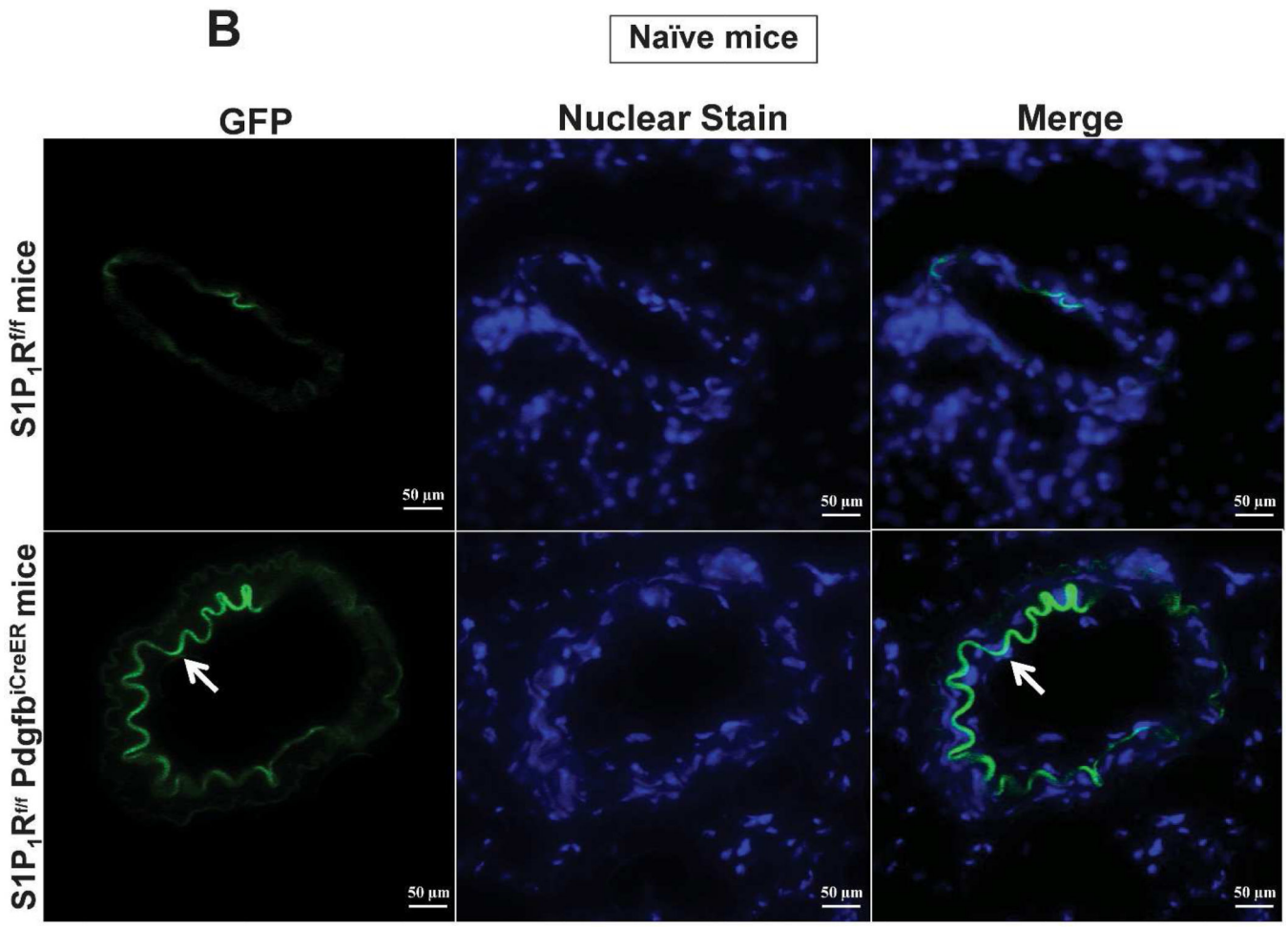
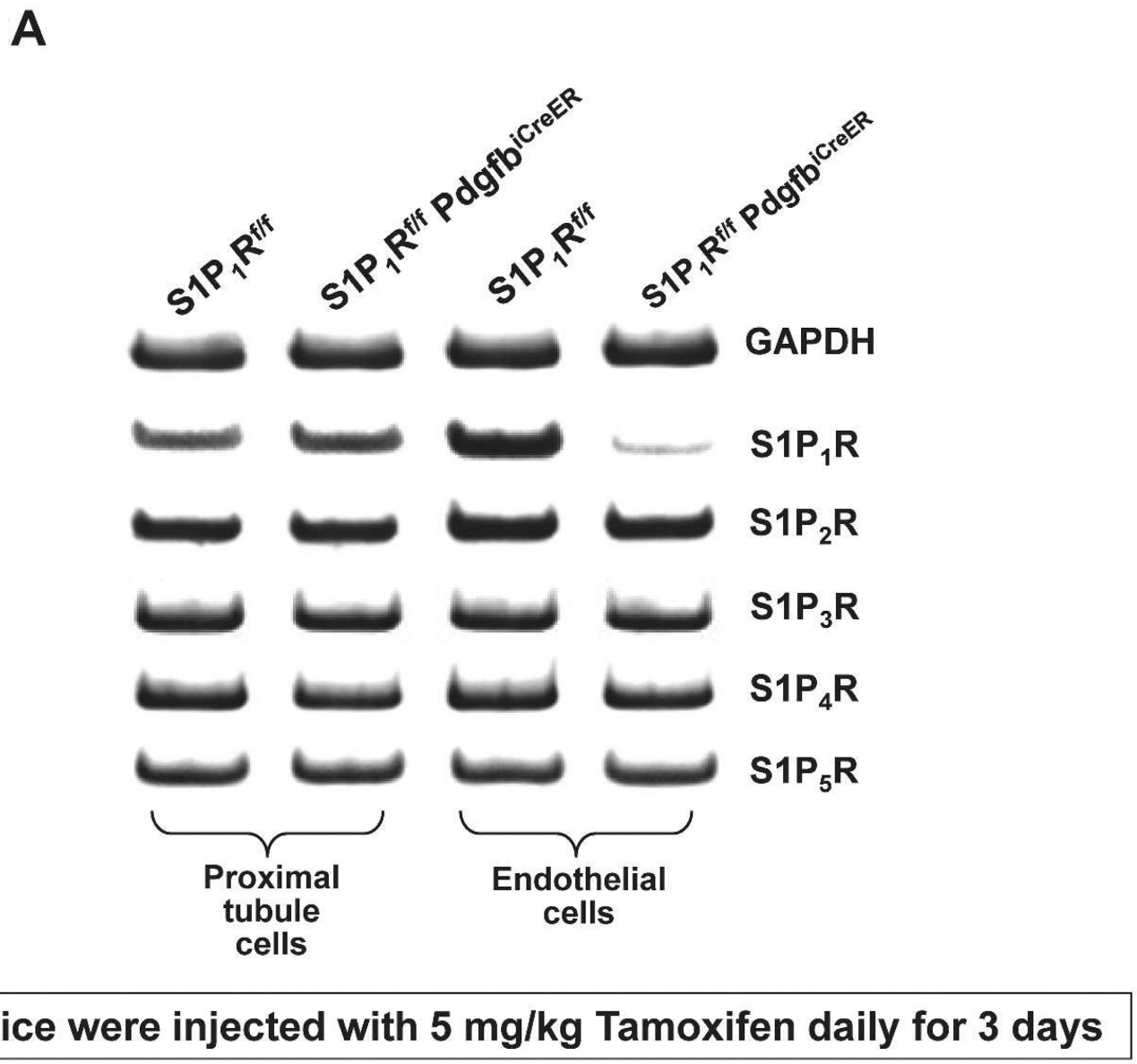


Figure 1. Generation of mice with conditional endothelial specific $S1P_1R$ deletion

A. Tail PCR confirming the genotypes of conditional endothelial specific $S1P_1R$ null ($S1P_1R^{f/f}$ $Pdgfb^{iCreER}$ or $S1P_1R^{f/-}$ $Pdgfb^{iCreER}$) mice and the wild type ($S1P_1R^{f/f}$ or $S1P_1R^{f/-}$) mice. B. As $Pdgfb^{iCreER}$ mice express an internal ribosomal entry site element and a sequence coding for enhanced green fluorescent protein (EGFP), we detected the expression of EGFP in kidney endothelial cells from in $S1P_1R^{f/f}$ $Pdgfb^{iCreER}$ mice with fluorescent microscopy. EGFP expression was strong in endothelial cells from kidneys of $S1P_1R^{f/f}$ $Pdgfb^{iCreER}$ mice (arrow). Besides faint auto-fluorescence from external elastic membrane, EGFP expression was not detected in $S1P_1R^{f/f}$ mice kidney endothelial cells. We also performed DAPI nuclear staining in both $S1P_1R^{f/f}$ $Pdgfb^{iCreER}$ mice and in $S1P_1R^{f/f}$ mice (blue).



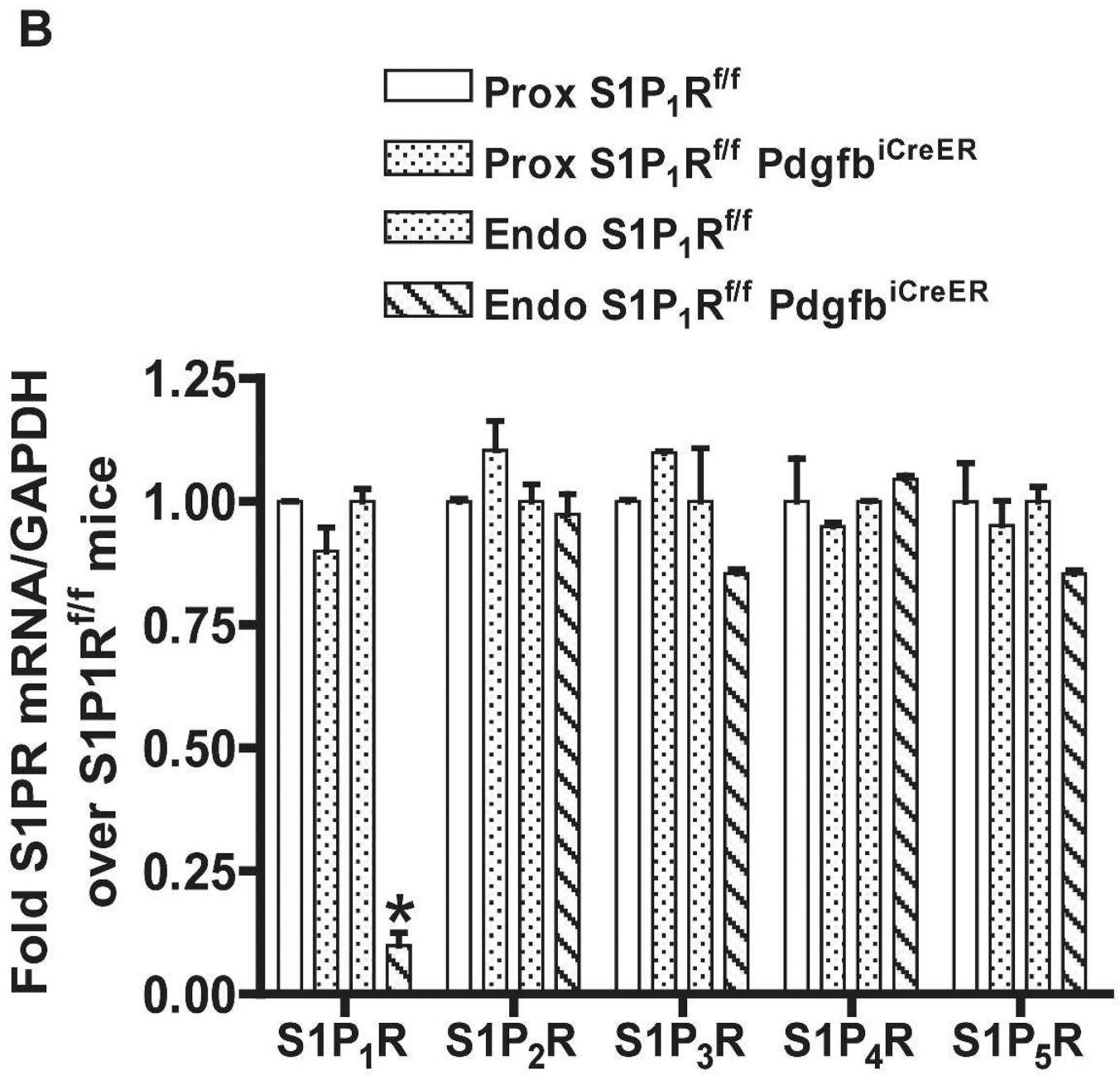
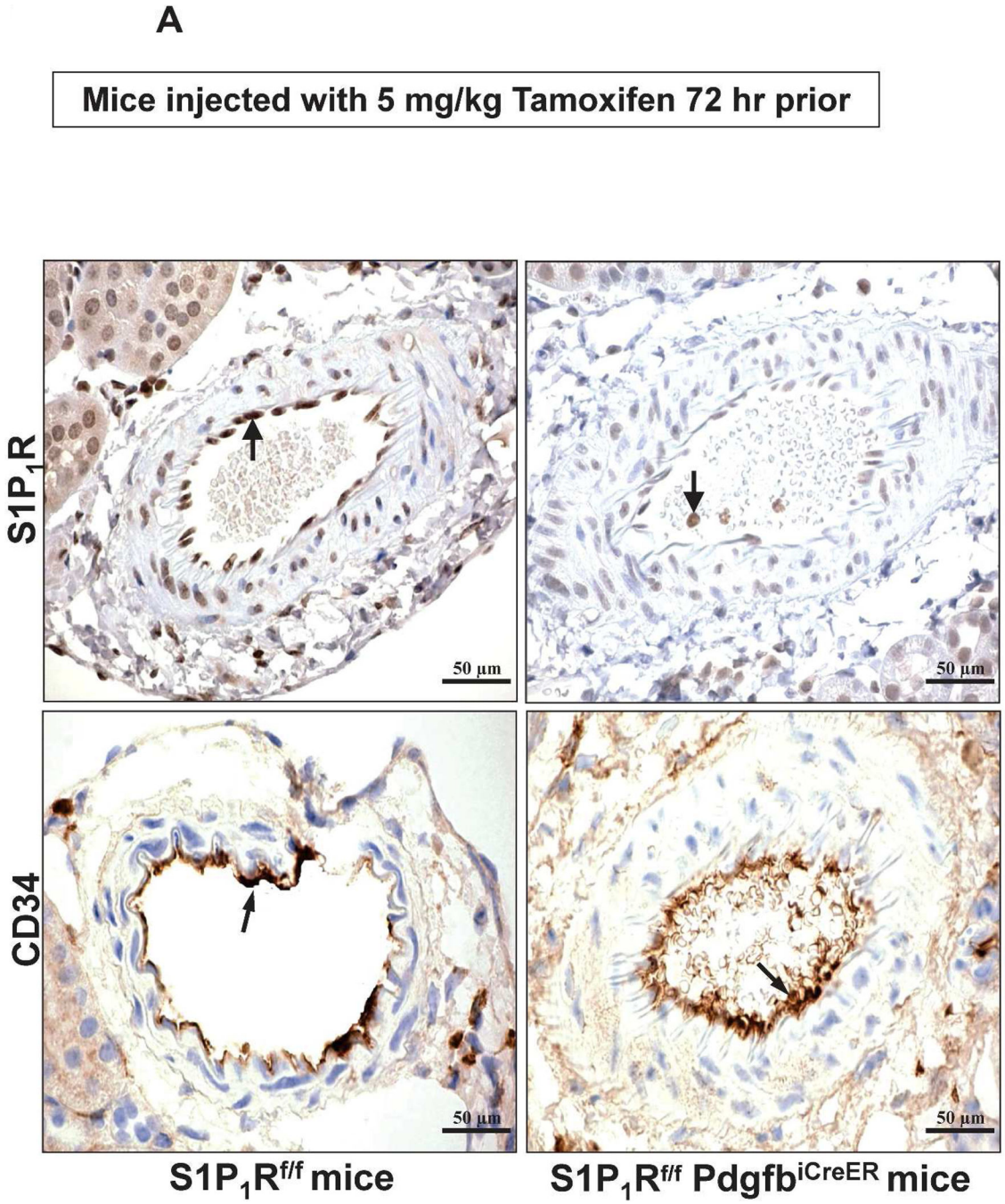


Figure 2. Conditional deletion of renal endothelial $S1P_1R$ in $S1P_1R^{f/f}$ $Pdgfb^{iCreER}$ mice

$S1P_{1–5}R$ mRNA levels measured with RTPCR in freshly isolated renal endothelial cells (with Dynabeads coupled to CD144 antibody) and renal proximal tubules (with Percoll density gradient separation) from conditional $S1P_1R$ null mice ($S1P_1R^{f/f}$ $Pdgfb^{iCreER}$) and wild type mice ($S1P_1R^{f/f}$). Representative gel image of PCR products (A) and densitometric quantification of relative band intensities normalized to GAPDH (B, N=4). Endothelial cells from conditional endothelial $S1P_1R$ null mice show significantly (~90%) reduced $S1P_1R$ mRNA expression compared to wild type mice without any changes in other S1PR subtype ($S1P_{2–5}R$) mRNA expression (N=4). Furthermore, proximal tubule $S1P_1R$ mRNA levels

were similar between conditional endothelial $S1P_1R$ null mice and wild type mice. *$P<0.05$ vs. $S1P_1R^{f/f}$ mice.



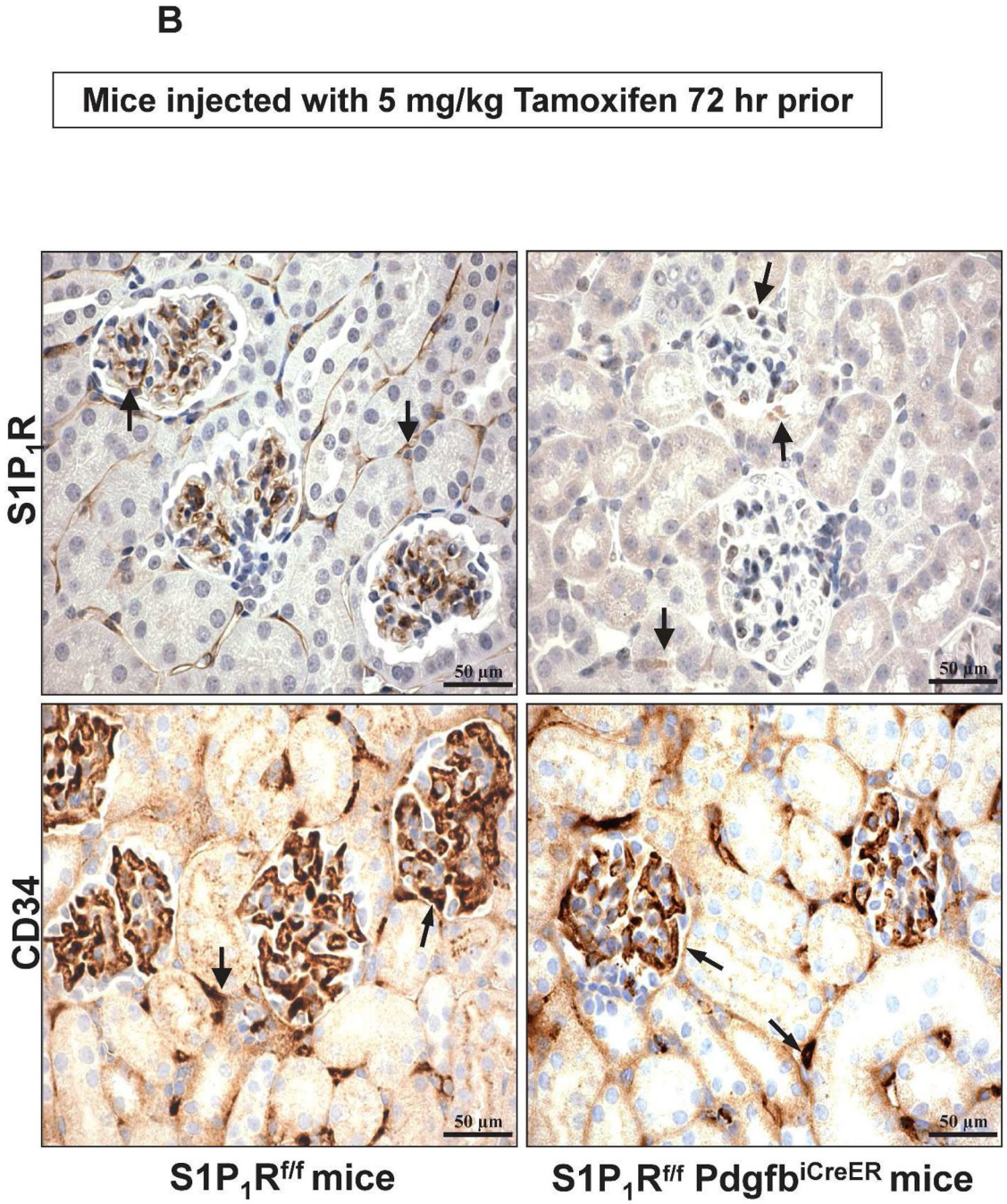
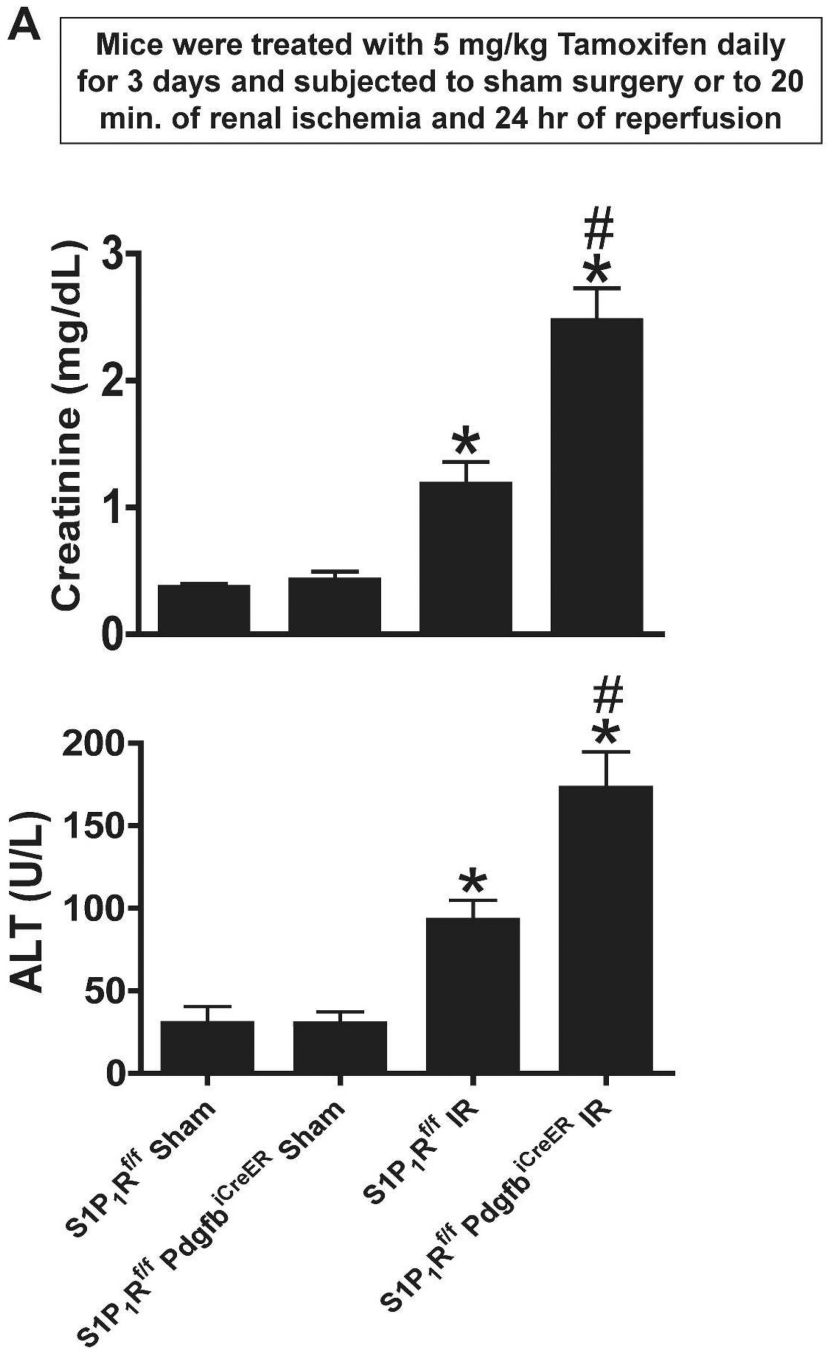


Figure 3. Reduced kidney endothelial $S1P_1R$ protein expression in conditional endothelial $S1P_1R$ null ($S1P_1R^{f/f}$ $Pdgfb^{iCreER}$) mice

A. Representative images (magnification 600X) for $S1P_1R$ and CD34 immunoreactivity (brown stain, arrows) in kidney blood vessels from tamoxifen-treated $S1P_1R^{f/f}$ $Pdgfb^{iCreER}$ and $S1P_1R^{f/f}$ mice. $S1P_1R$ immunoreactivity was drastically reduced (near absent) in tamoxifen-treated $S1P_1R^{f/f}$ $Pdgfb^{iCreER}$ mice when compared to $S1P_1R^{f/f}$ mice. $S1P_1R$ immunoreactivity was still visible from intravascular leukocytes in conditional endothelial $S1P_1R$ null mice. CD34 immunoreactivity was similar between tamoxifen-treated $S1P_1R^{f/f}$ $Pdgfb^{iCreER}$ mice and $S1P_1R^{f/f}$ mice. Representative of 4 experiments. B. Representative

images (magnification 600X) for $S1P_1R$ and CD34 immunoreactivity (brown stain, arrows) in peritubular capillaries and glomeruli from tamoxifen-treated $S1P_1R^{f/f}$ $Pdgfb^{iCreER}$ and $S1P_1R^{f/f}$ mice. $S1P_1R$ immunoreactivity was absent in peritubular capillaries and glomeruli from tamoxifen-treated $S1P_1R^{f/f}$ $Pdgfb^{iCreER}$ mice when compared to $S1P_1R^{f/f}$ mice. Again, leukocyte $S1P_1R$ immunoreactivity was visible in kidneys from conditional endothelial $S1P_1R$ null mice. CD34 immunoreactivity was similar between tamoxifen-treated $S1P_1R^{f/f}$ $Pdgfb^{iCreER}$ mice and $S1P_1R^{f/f}$ mice. Representative of 4 experiments.



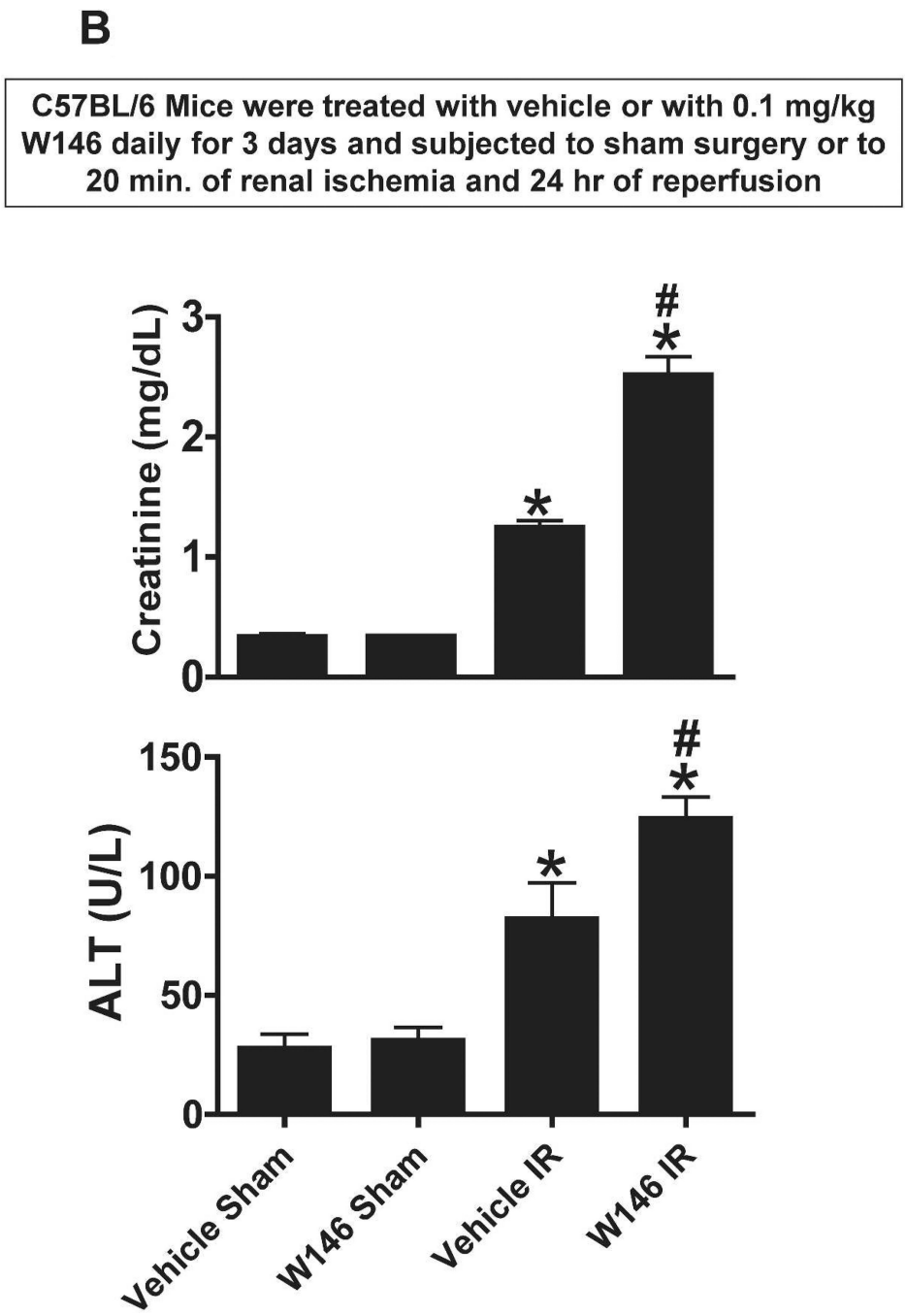


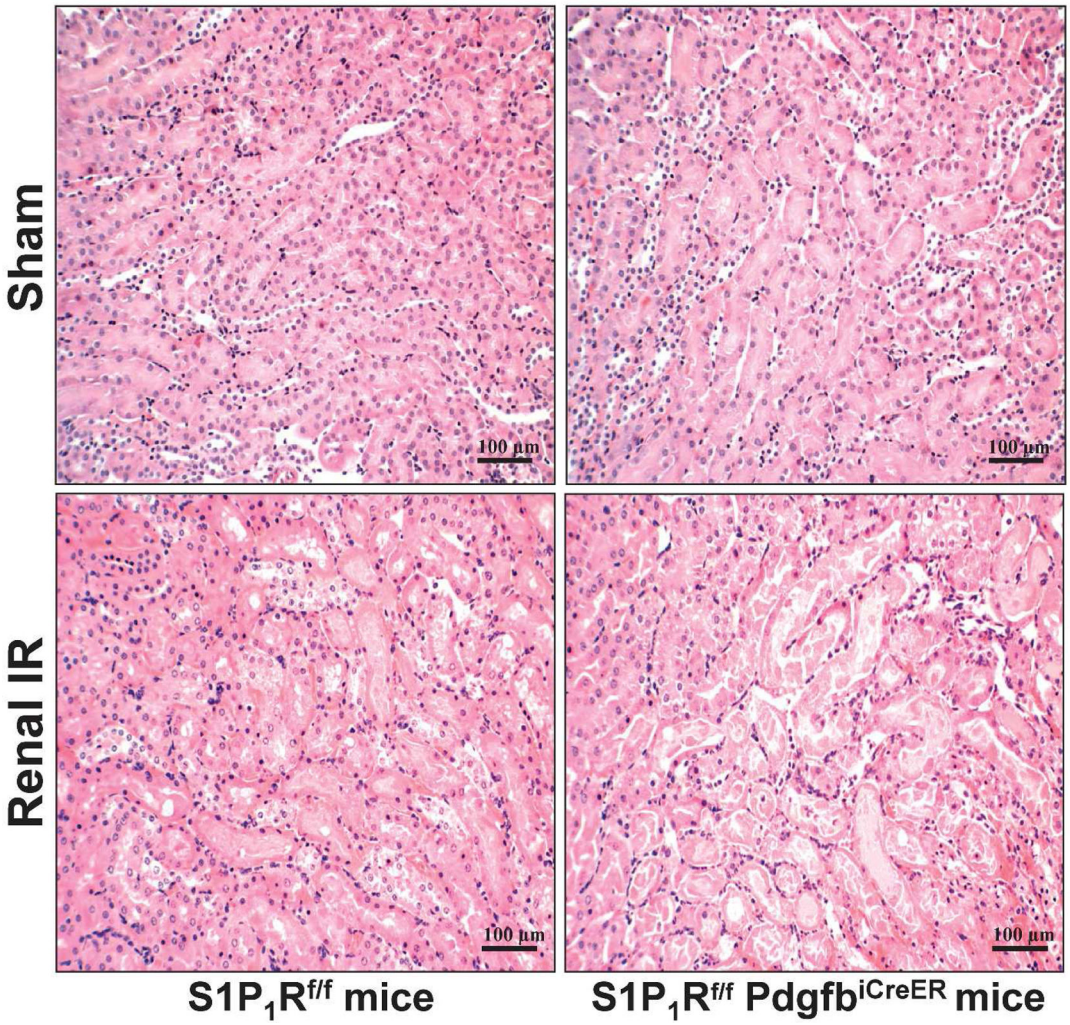
Figure 4. Endothelial $S1P_1R$ deficiency or chronic $S1P_1R$ antagonist treatment exacerbates renal and hepatic injury after renal IR

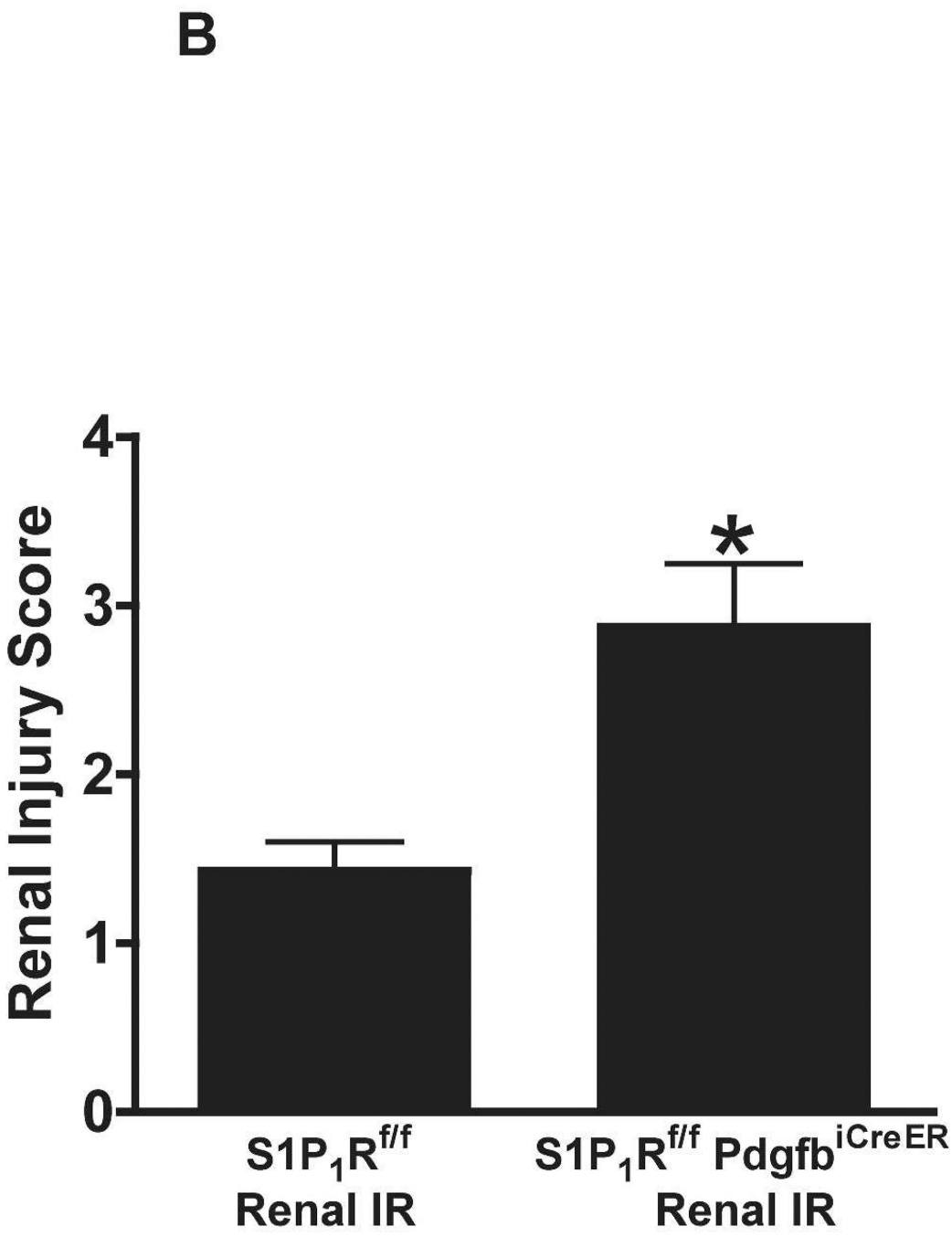
A. Plasma markers for renal and hepatic injury from tamoxifen-treated endothelial $S1P_1R$ null ($S1P_1R^{f/f}$ $Pdgfb^{iCreER}$) mice or wild type ($S1P_1R^{f/f}$) mice subjected to sham-operation or to 20 min of renal ischemia and 24 hr reperfusion (N=5–7). Endothelial $S1P_1R$ deletion significantly increased renal and hepatic injury in mice. B. Plasma markers for renal and hepatic injury from vehicle-or W146-treated (0.1 mg/kg daily for 3 days) C57BL/6 mice subjected to sham-operation or to 20 min of renal ischemia and 24 hr of reperfusion (N=6). Chronic $S1P_1R$ antagonist treatment increased renal and hepatic injury after renal IR injury.

*P<0.05 vs. respective sham-operated mice. #P<0.05 vs. wild type ($S1P_1R^{f/f}$) or vehicle-treated C57BL/6 mice subjected to renal IR injury.

A

Mice were treated with 5 mg/kg Tamoxifen daily for 3 days and subjected to sham surgery or to 20 min. of renal ischemia and 24 hr of reperfusion





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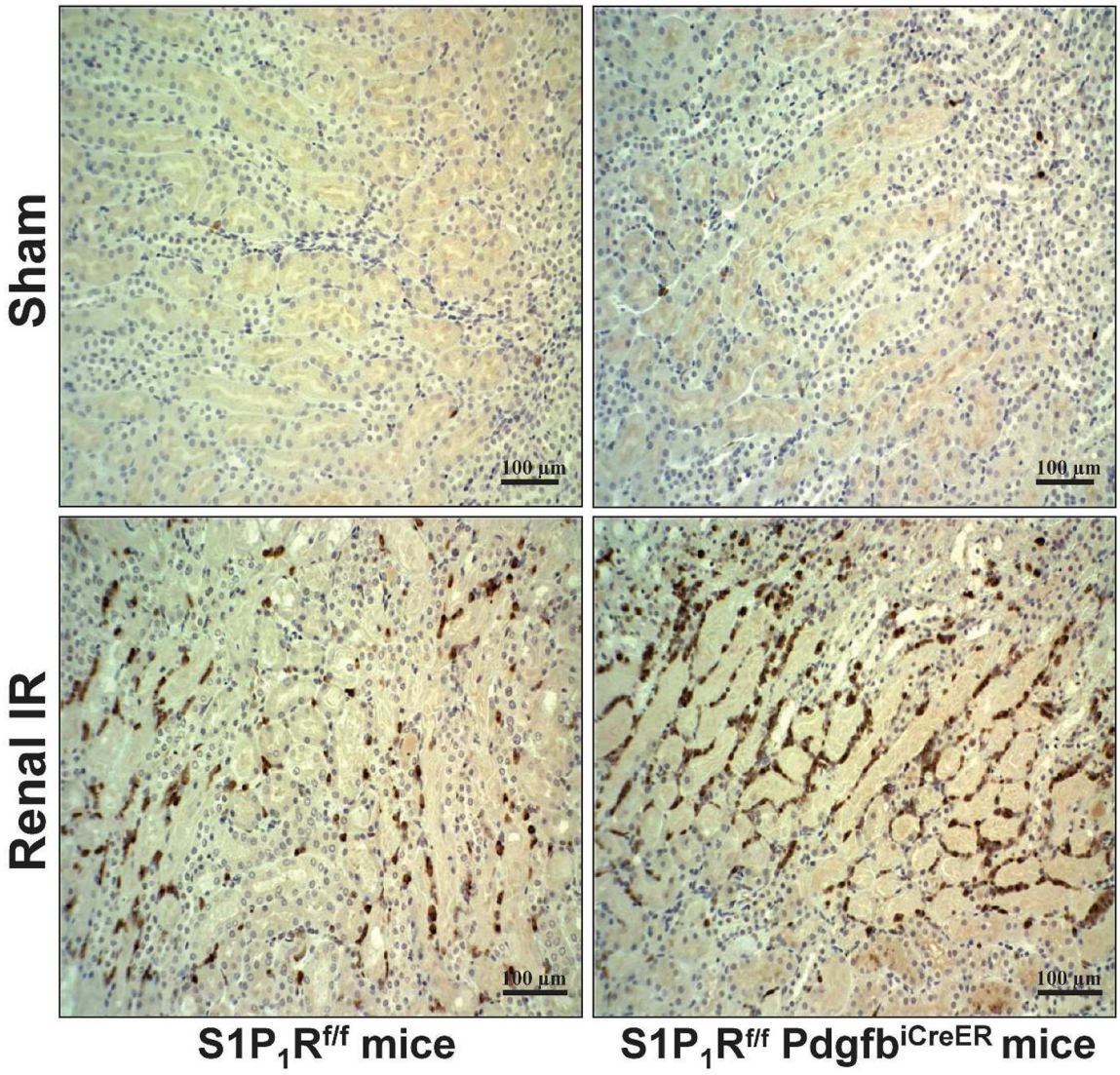
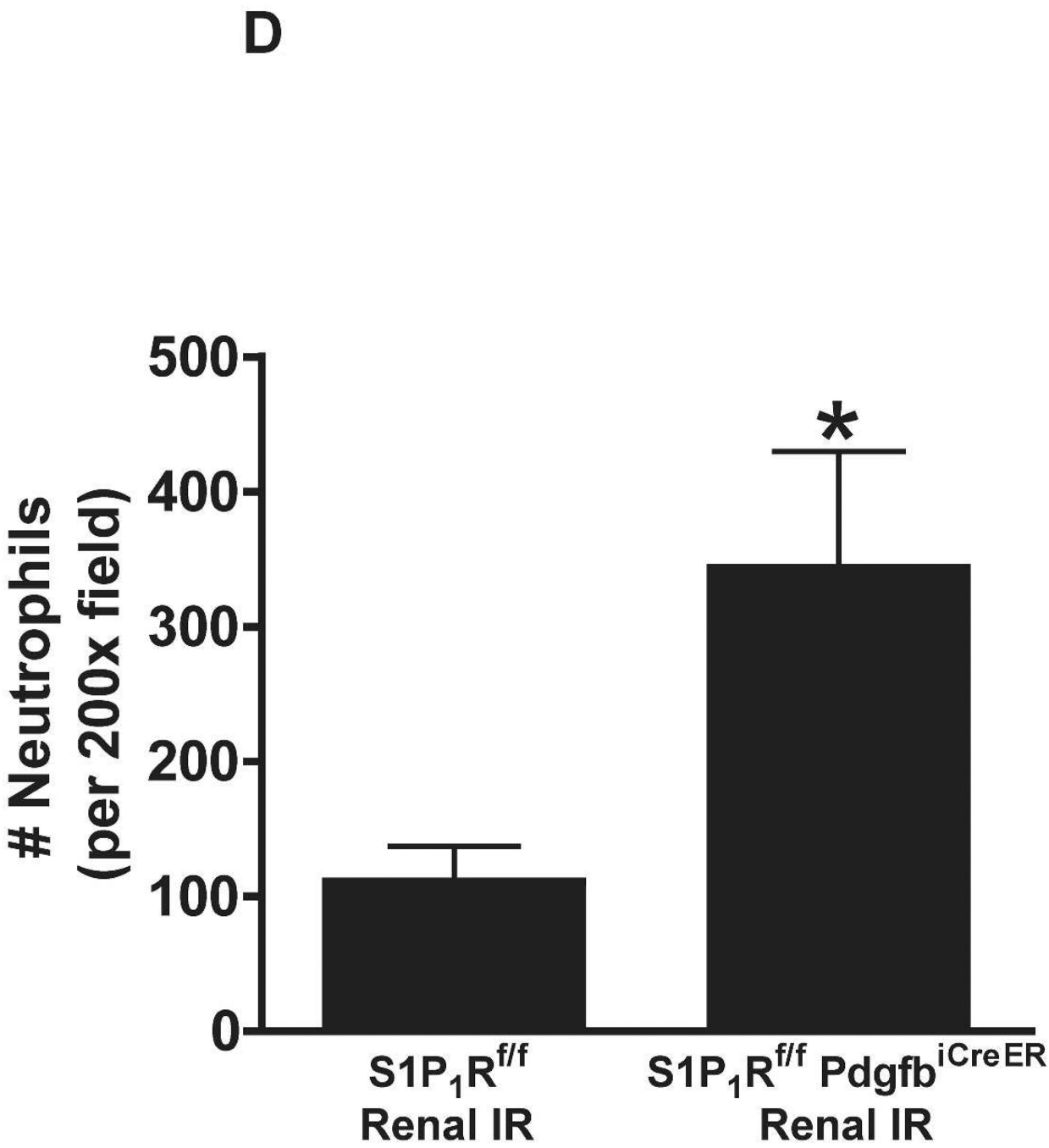
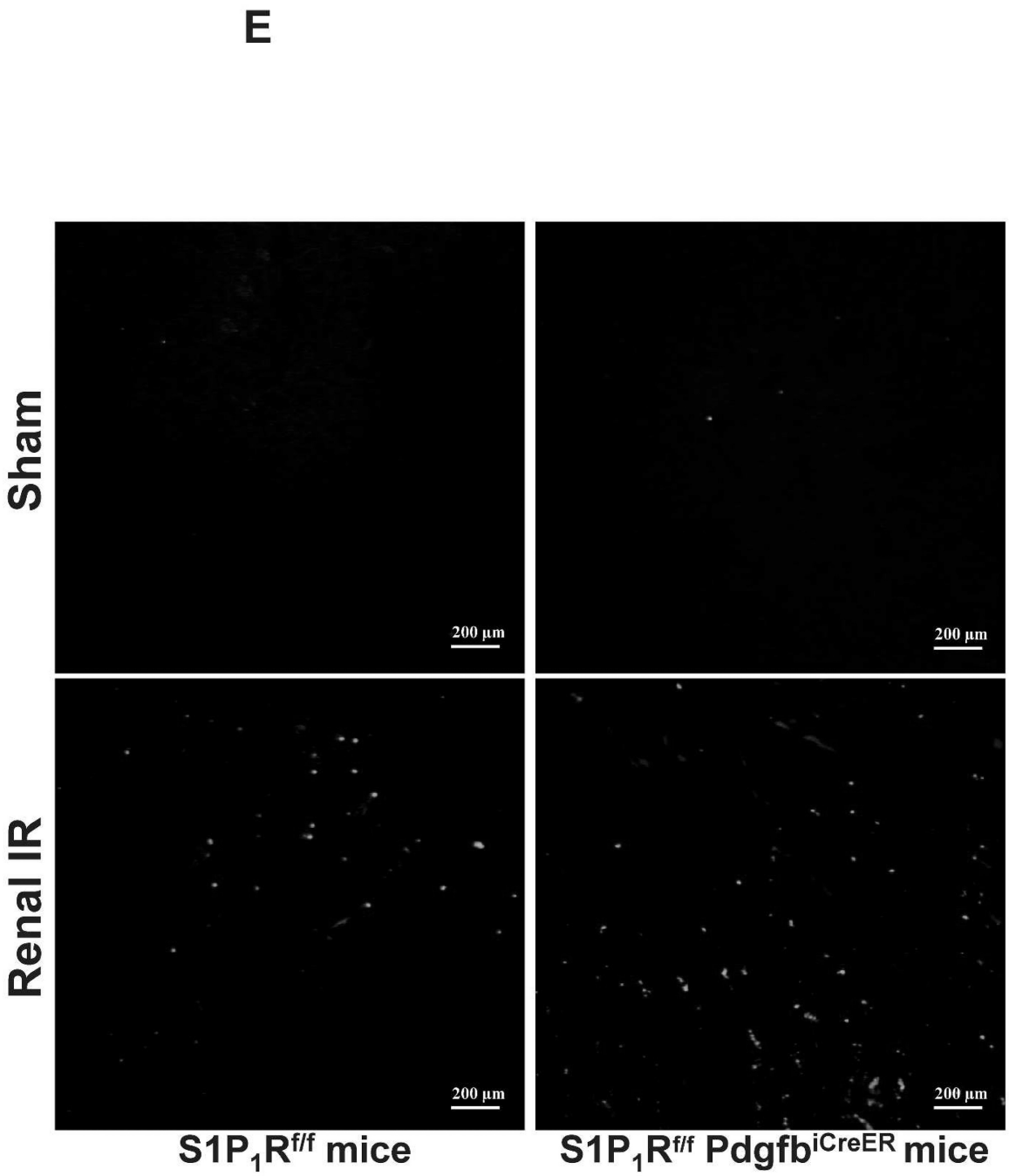
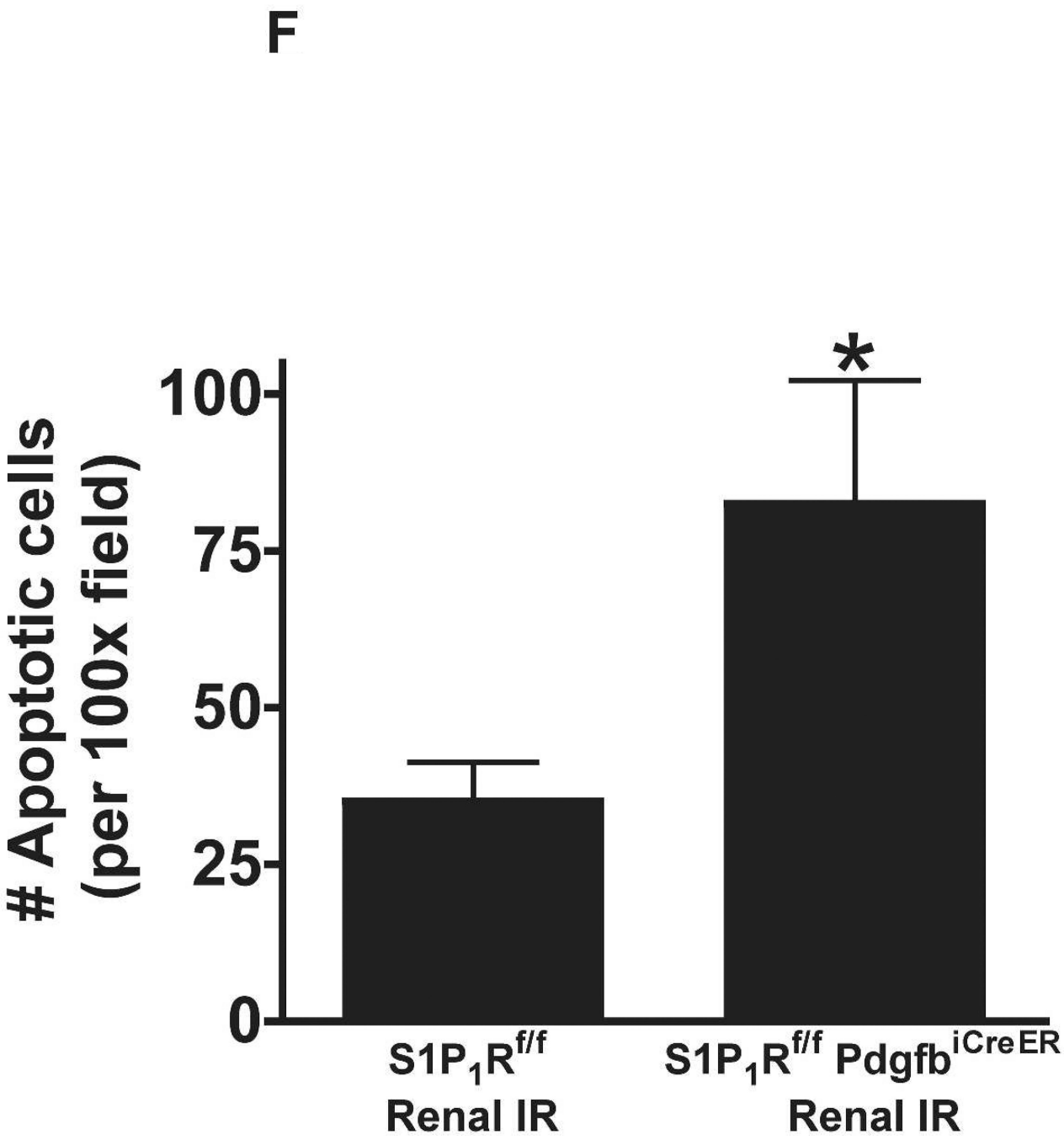








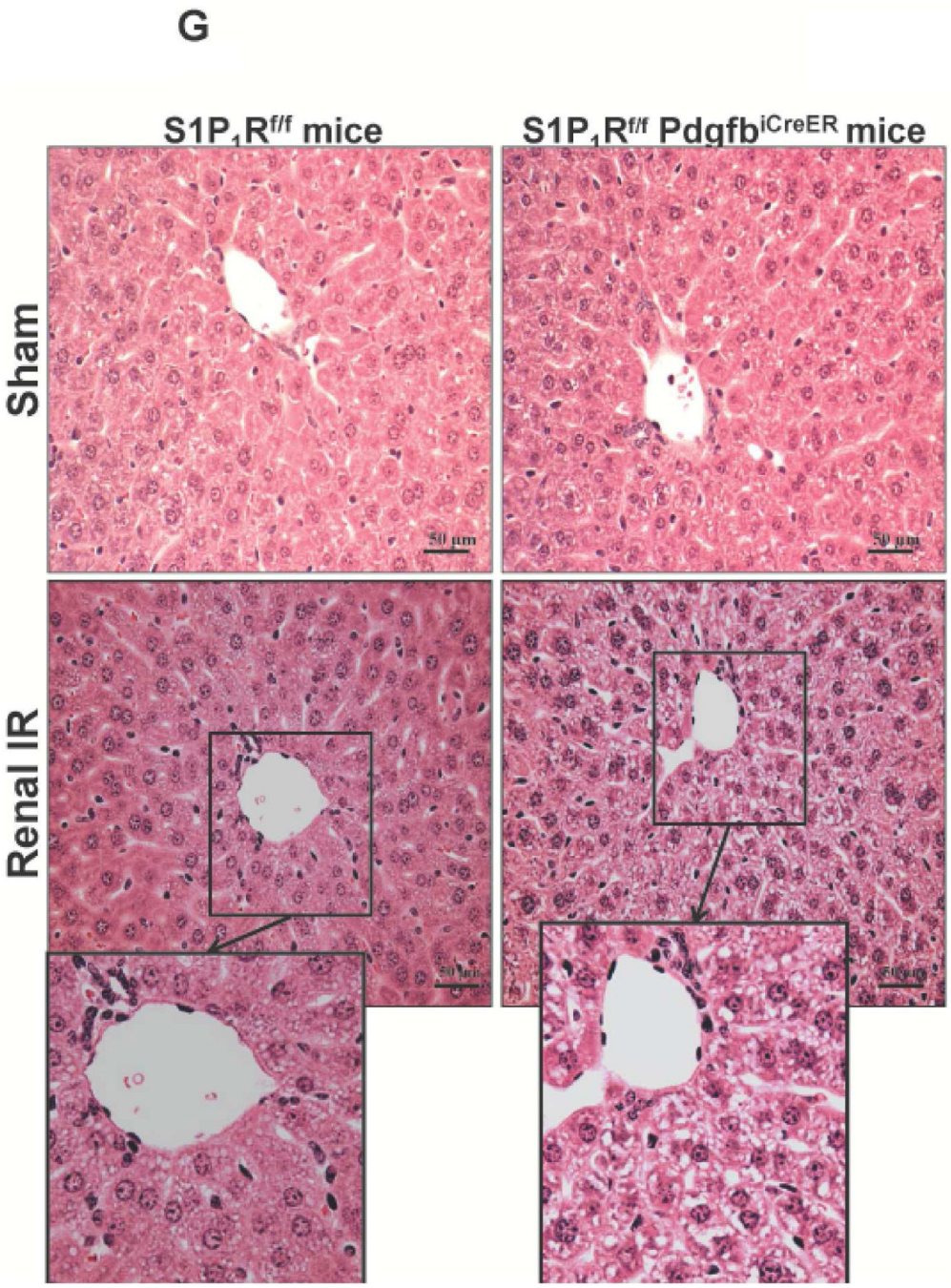
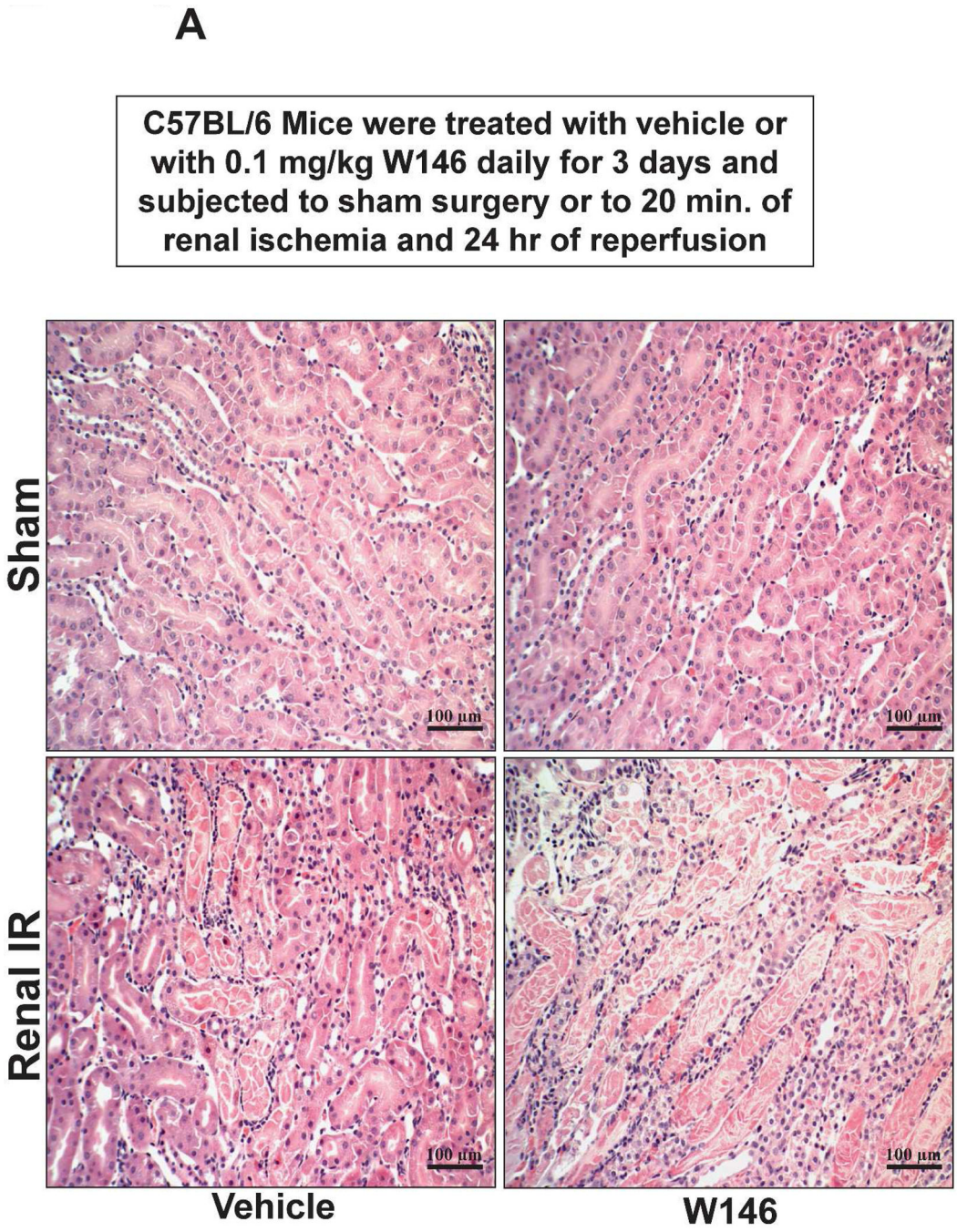
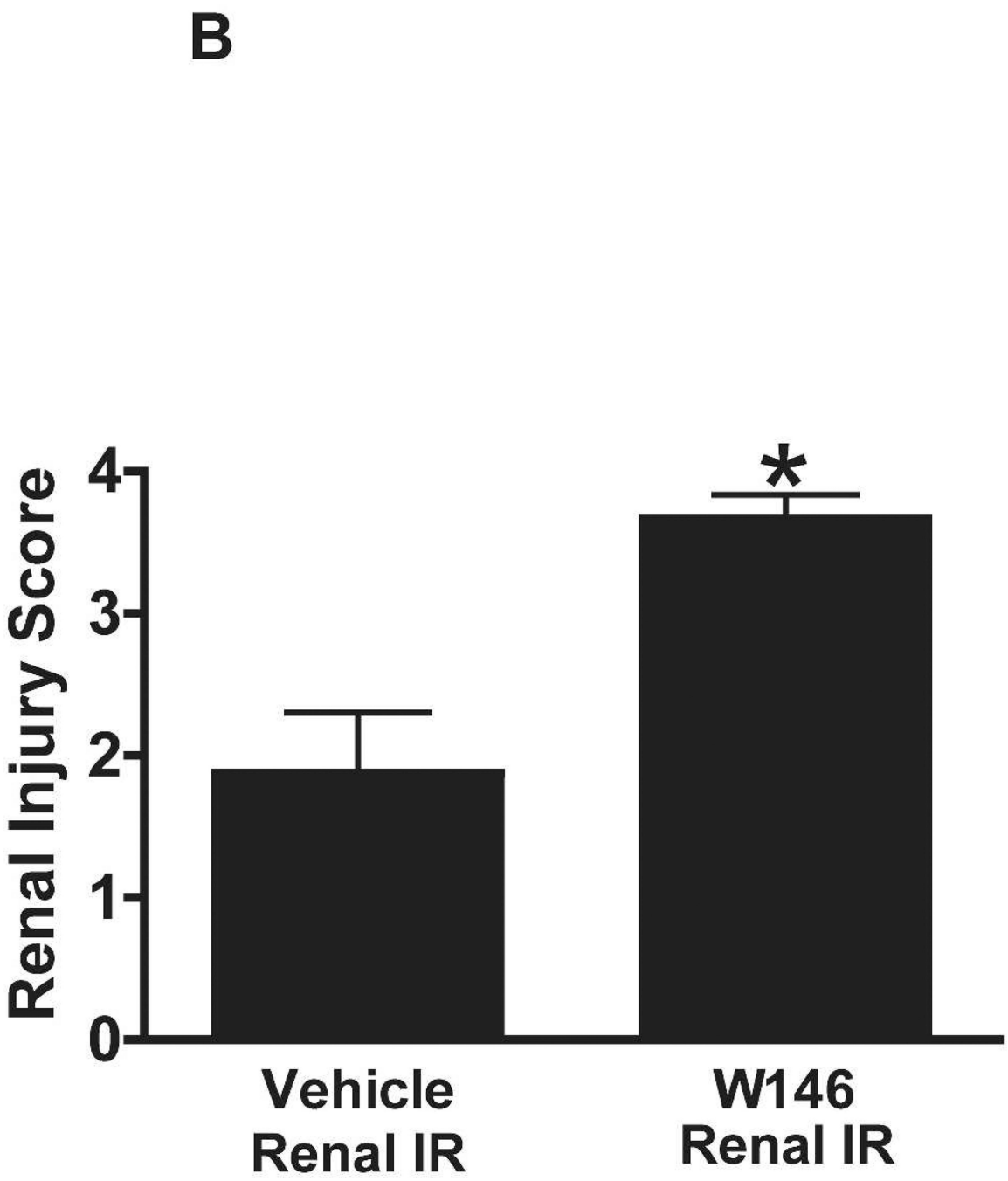


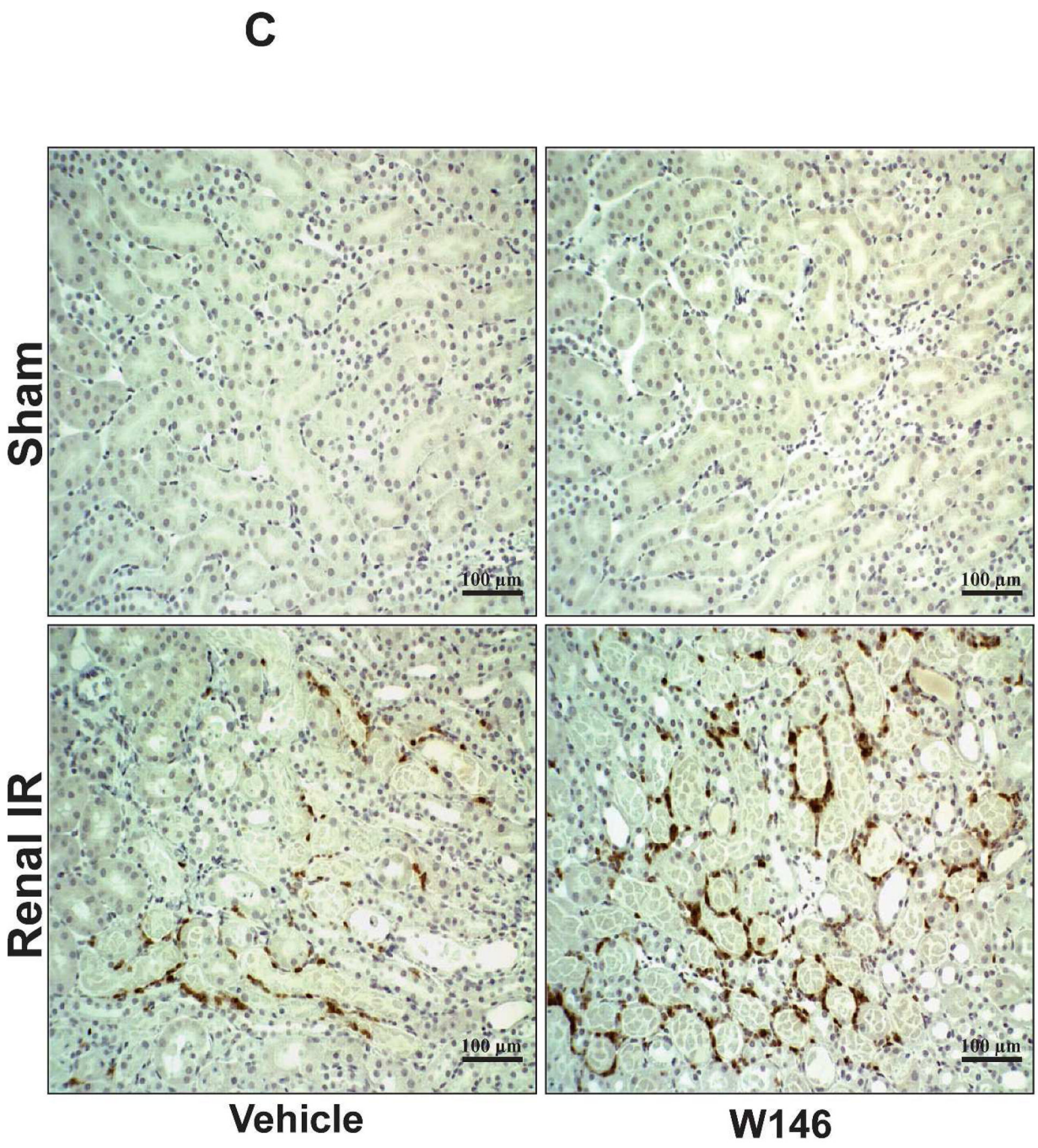
Figure 5. Endothelial $S1P_1R$ deletion increases renal necrosis, neutrophil infiltration and apoptosis as well as hepatic vacuolization after renal IR

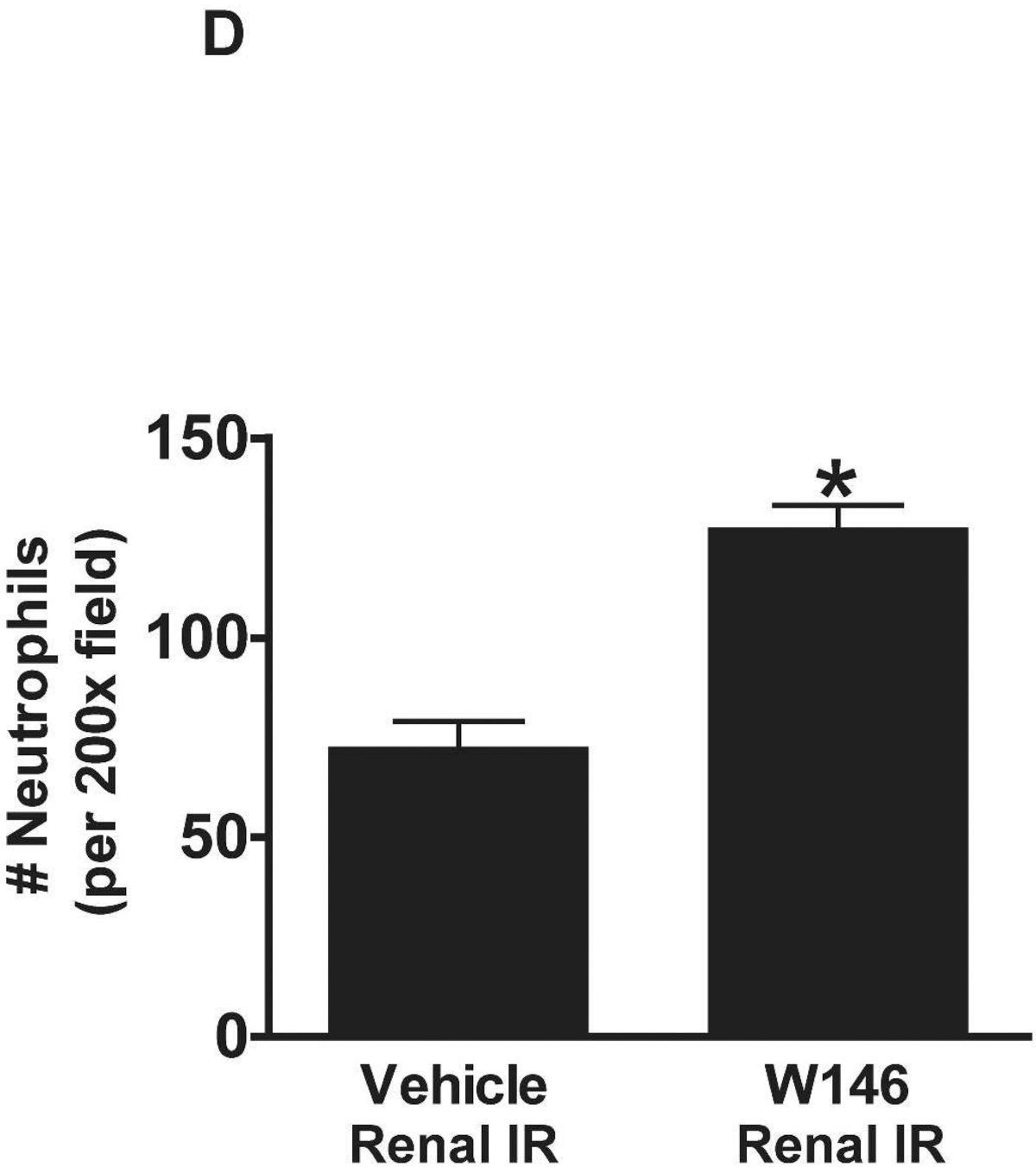
A. Representative hematoxylin and eosin staining (magnification 200X) of kidney sections of tamoxifen-treated (5 mg/kg i.p. daily for 3 days) endothelial $S1P_1R$ null ($S1P_1R^{f/f}$ $Pdgfb^{iCreER}$) mice or wild type ($S1P_1R^{f/f}$) mice subjected to sham-operation or to 20 min of renal ischemia and 24 hr of reperfusion. Photographs are representative of 6 independent experiments. B. Summary of Jablonski scale renal injury scores (scale 0–4) for mice subjected to renal IR. Tamoxifen-treated wild type ($S1P_1R^{f/f}$) mice showed increased renal tubular necrosis after renal IR. Endothelial $S1P_1R$ deficiency further exacerbated renal

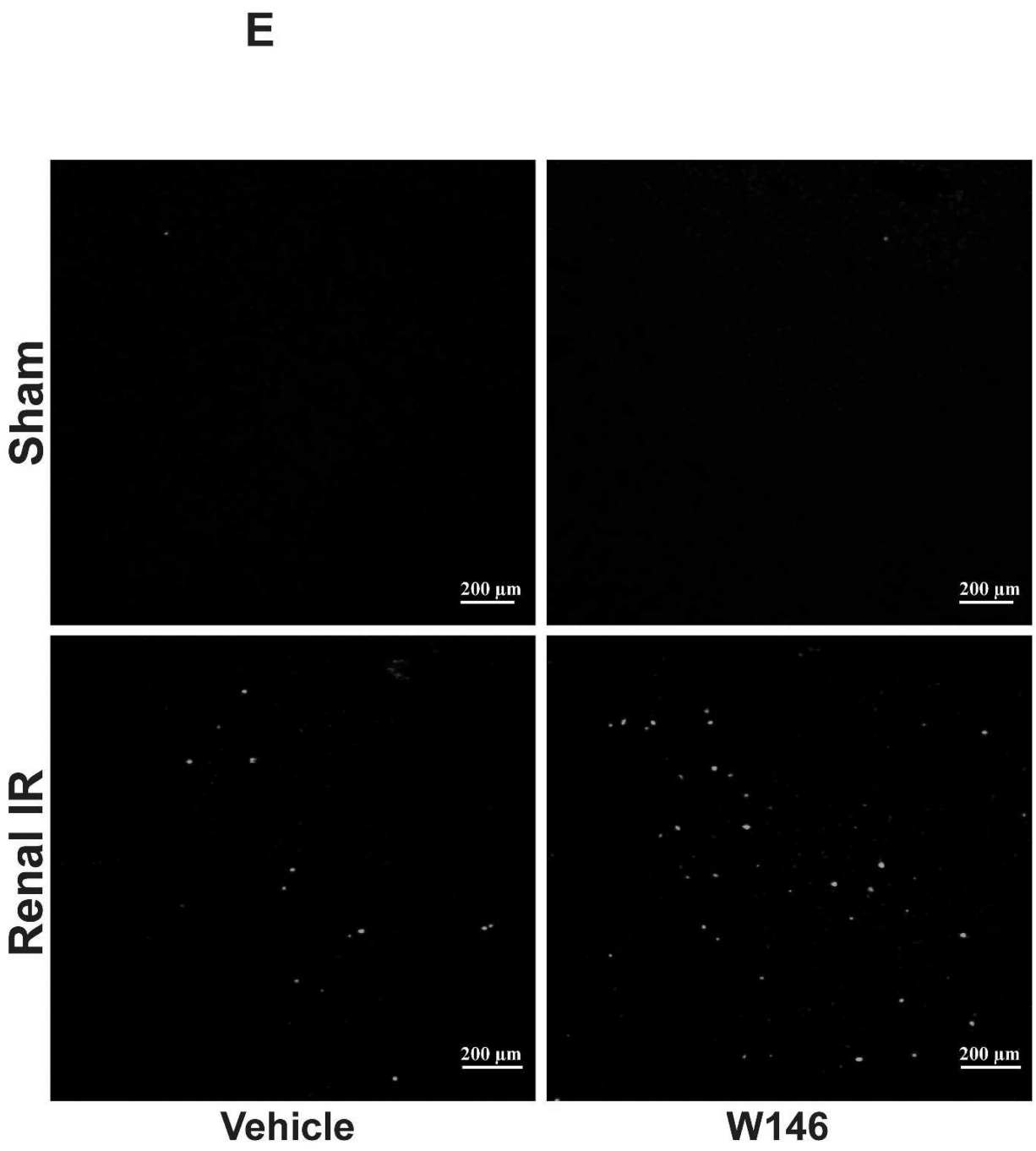
tubular necrosis in mice subjected to renal IR injury. C and E. Representative photomicrographs for immunohistochemistry (brown staining) for neutrophil infiltration (C, magnification 200X) and TUNEL staining (E, representing apoptotic nuclei, magnification 100X) from kidneys of tamoxifen-treated endothelial $S1P_1R$ null ($S1P_1R^{f/f}$ $Pdgfb^{iCreER}$) mice or wild type ($S1P_1R^{f/f}$) mice subjected to sham-operation or to 20 min of renal ischemia followed by 24 hr of reperfusion (IR). Tamoxifen-treated wild type mice showed extensive neutrophil infiltration and TUNEL positive cells after 20 min of renal IR. Tamoxifen-treated endothelial $S1P_1R$ null ($S1P_1R^{f/f}$ $Pdgfb^{iCreER}$) mice had increased neutrophil infiltration after renal IR. Few neutrophils were also visible in tamoxifen-treated sham-operated endothelial $S1P_1R$ null mice. D and F. Quantifications of infiltrated neutrophils per 200X field (D, N=4–6) and apoptotic cells per 100X field (F, N=5) in the kidneys of tamoxifen-treated endothelial $S1P_1R$ null ($S1P_1R^{f/f}$ $Pdgfb^{iCreER}$) mice or wild type ($S1P_1R^{f/f}$) mice subjected to 20 min of renal ischemia followed by 24 hr of reperfusion. *$P<0.05$ vs. $S1P_1R^{f/f}$ mice subjected to renal IR. Error bars represent 1 SEM. G. Liver histology was assessed by H&E staining (representative of 5–6 slides, 400X). Sham-operated wild type mice had normal liver histology. Twenty min of renal ischemia and 24 hr of reperfusion resulted in mild to moderate nuclear and cytoplasmic degenerative changes, cellular vacuolization, congestion as well as individual hepatocyte necrosis and focal apoptotic, pyknotic nuclei were observed. The severity of liver injury was increased for conditional endothelial $S1P_1R$ deficient mice manifested by increased of cellular degenerative changes including increased individual hepatocyte necrosis, apoptosis and vacuolization.

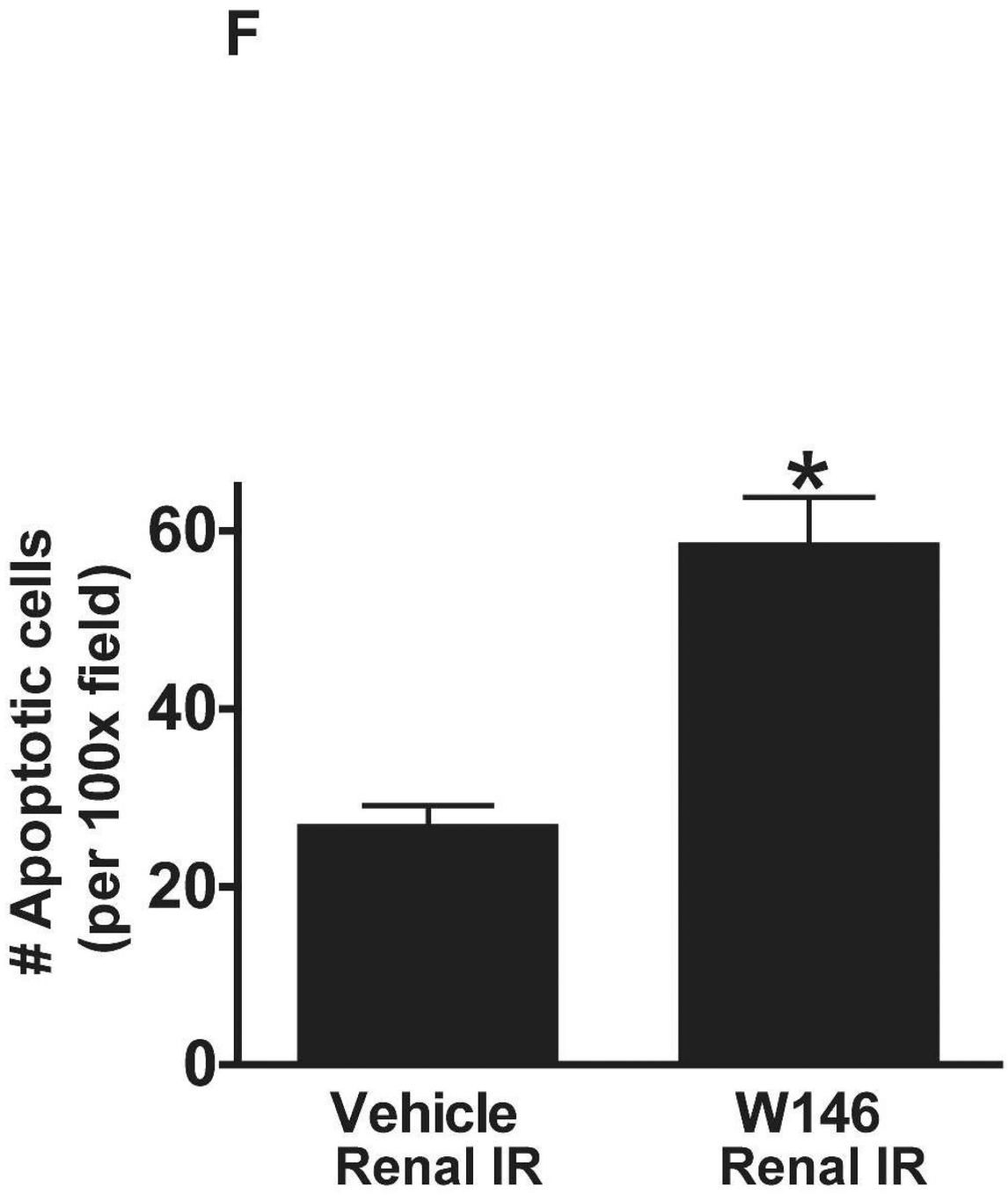












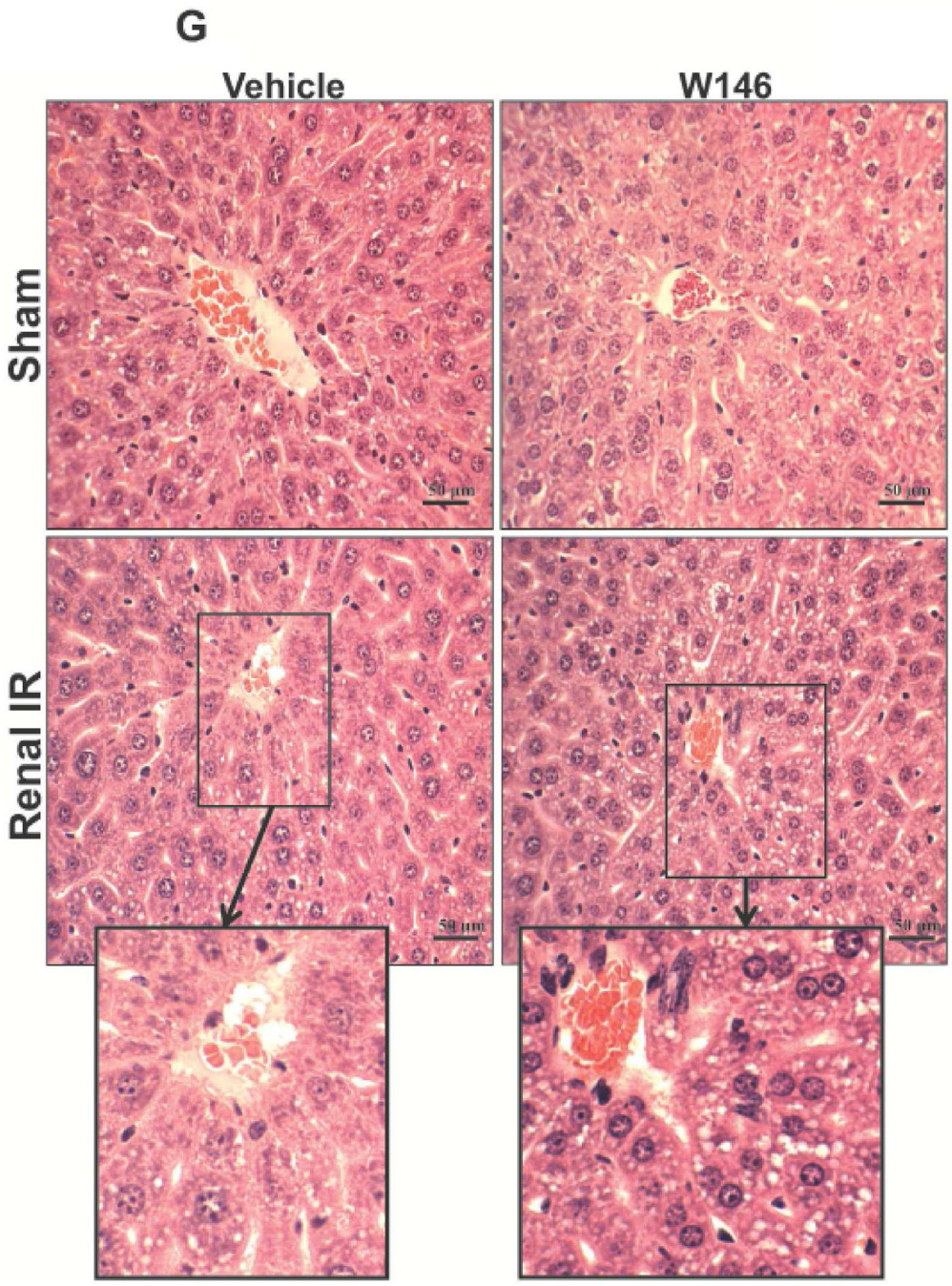
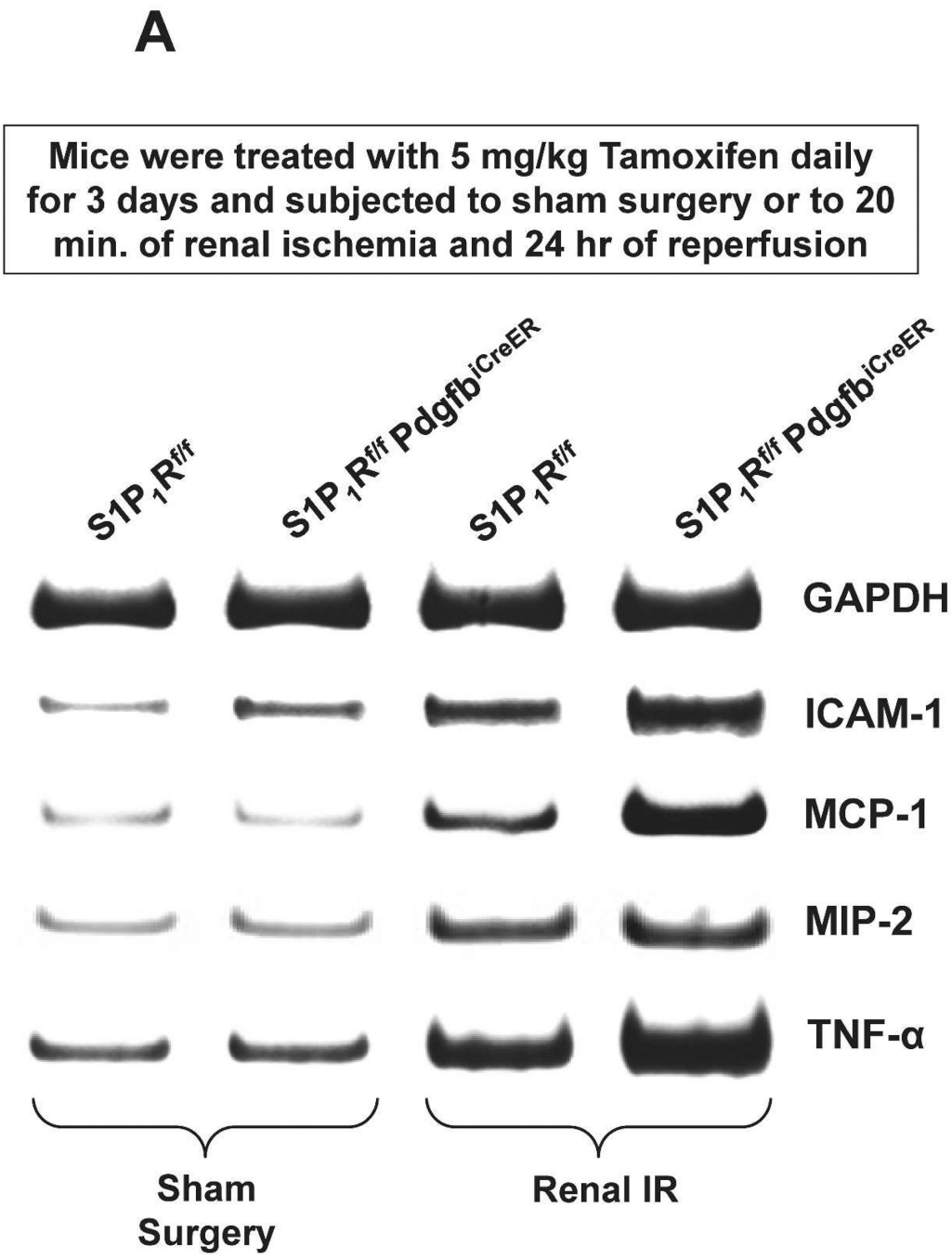


Figure 6. Chronic S1P$_1$R antagonist treatment in C57BL/6 mice increases renal necrosis, neutrophil infiltration and apoptosis as well as hepatic vacuolization after renal IR

A. Representative hematoxylin and eosin staining (magnification 200X) of kidney sections of vehicle (1% DMSO)- or W146-treated (0.1 mg/kg i.p. daily for 3 days) C57BL/6 mice subjected to sham-operation or to 20 min of renal ischemia followed by 24 hr of reperfusion. Photographs are representative of 5–6 independent experiments. B. Summary of Jablonski scale renal injury scores (scale 0–4) for mice subjected to renal IR. W146-treated mice showed increased renal tubular necrosis after renal IR compared to vehicle-treated C57BL/6 mice subjected to renal IR injury. C and E. Representative photomicrographs for

immunohistochemistry (brown staining) for neutrophil infiltration (C, magnification 200X) and TUNEL staining (E, representing apoptotic nuclei, magnification 100X) from kidneys of vehicle-treated or W146-treated C57BL/6 mice subjected to sham-operation or to 20 min of renal ischemia followed by 24 hr of reperfusion (IR). Vehicle-treated C57BL/6 mice had numerous neutrophil infiltration and TUNEL positive cells after 20 min renal IR. W146-treated C57BL/6 mice had even higher number of neutrophil infiltration and TUNEL-positive cells after renal IR. D and F. Quantifications of infiltrated neutrophils per 200X field (D, N=4–6) and apoptotic cells per 100X field (F, N=4–6) in the kidneys of vehicle- or W146-treated C57BL/6 mice subjected to 20 min of renal ischemia followed by 24 hr of reperfusion. *P<0.05 vs. vehicle-treated C57BL/6 mice subjected to renal IR. Error bars represent 1 SEM. G. Liver histology was assessed by H&E staining. Sham-operated C57BL/6 mice had normal liver histology (representative of 5–6 slides, 400X). Vehicle-treatment and 20 min renal ischemia and 24 hr reperfusion in C57BL/6 mice resulted in mild to moderate hepatic injury (nuclear and cytoplasmic degenerative changes, cellular vacuolization, congestion as well as individual hepatocyte necrosis and focal apoptotic, pyknotic nuclei). Similar to conditional endothelial $S1P_1R$ deficient mice, the severity of liver injury was increased for W146-treated C57BL/6 mice subjected to renal IR injury (increased hepatocyte degenerative changes including increased individual hepatocyte necrosis, apoptosis and vacuolization).



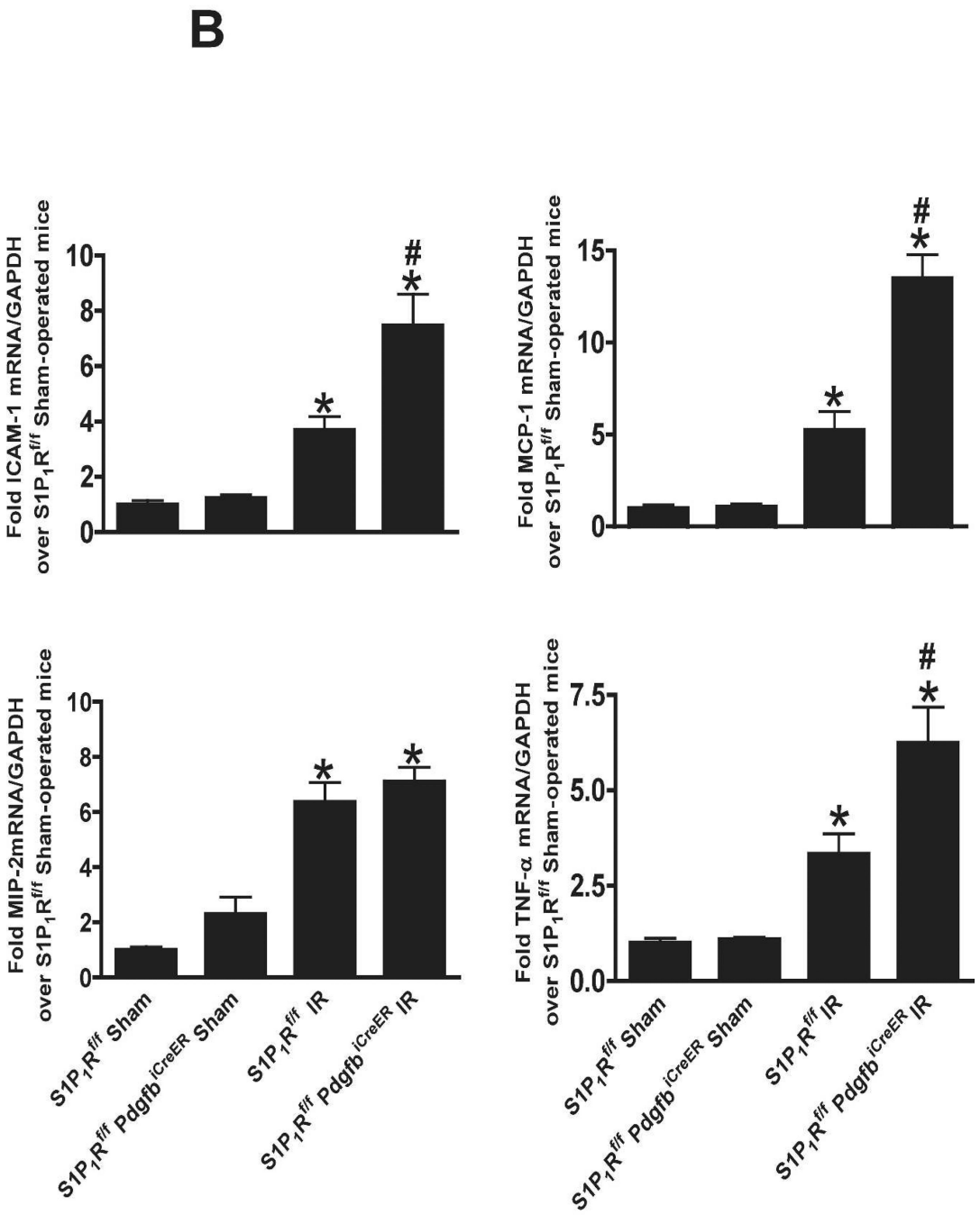


Figure 7. Endothelial $S1P_1R$ deficiency increases pro-inflammatory gene expression in the kidney after renal IR

Representative gel images (A) of RTPCR and densitometric quantification of relative band intensities normalized to GAPDH (B) of pro-inflammatory markers TNF-α, ICAM-1, MCP-1 and MIP-2 in kidneys from tamoxifen-treated (5 mg/kg i.p. daily for 3 days) endothelial $S1P_1R$ null ($S1P_1R^{f/f}$ $Pdgfb^{iCreER}$) or wild type ($S1P_1R^{f/f}$) mice. Mice were subjected to sham-operation or to 20 min of renal ischemia followed by 24 hr of reperfusion (N=4–5 per group). Conditional deletion of endothelial $S1P_1R$ significantly increased the expression of TNF-α, MCP-1 and ICAM-1 mRNAs examined compared to the wild type

mice subjected to renal IR. *P<0.05 vs. Sham-operated mice. #P<0.05 vs. wild type mice subjected to renal IR. Error bars represent 1 SEM.

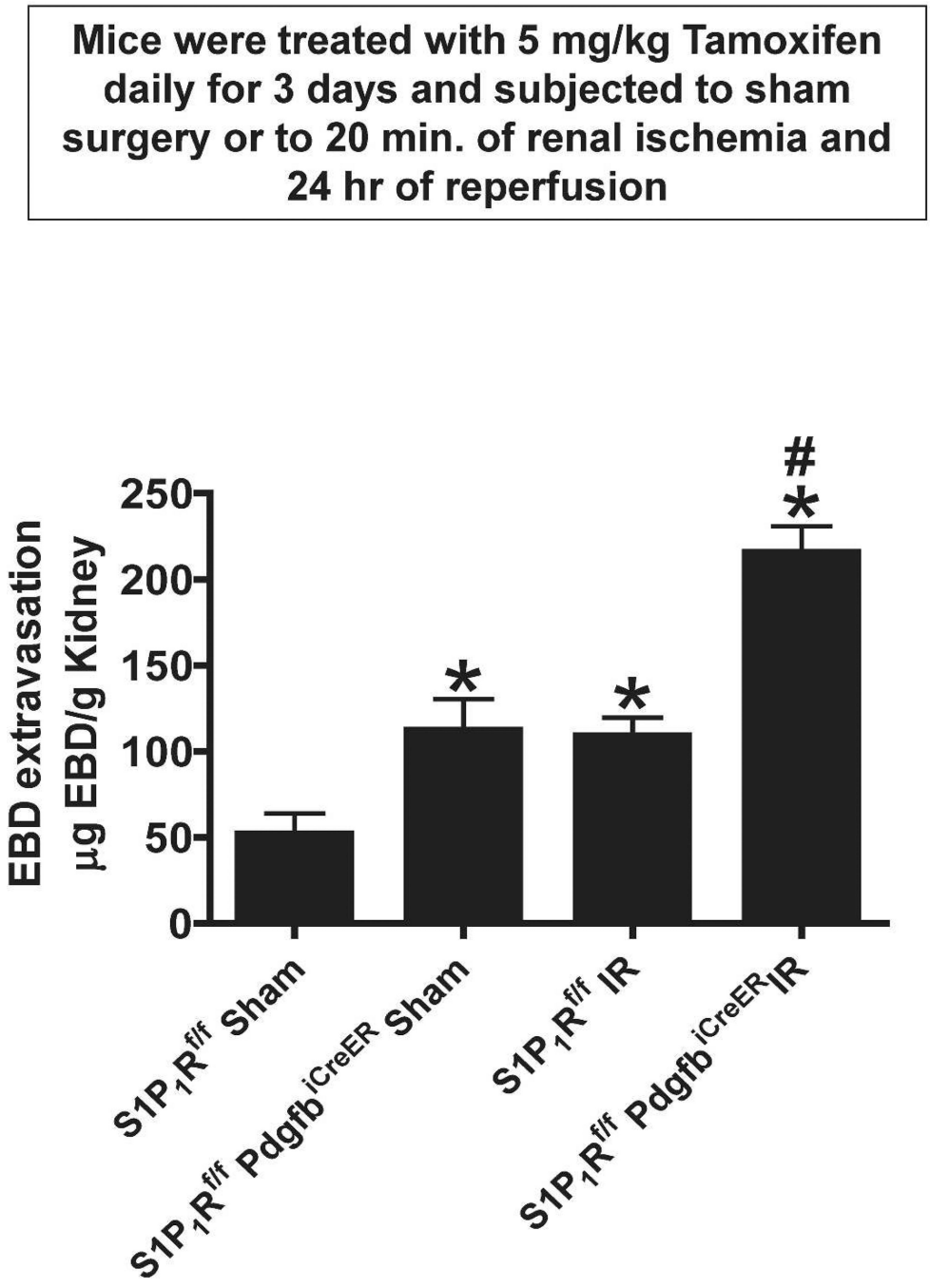
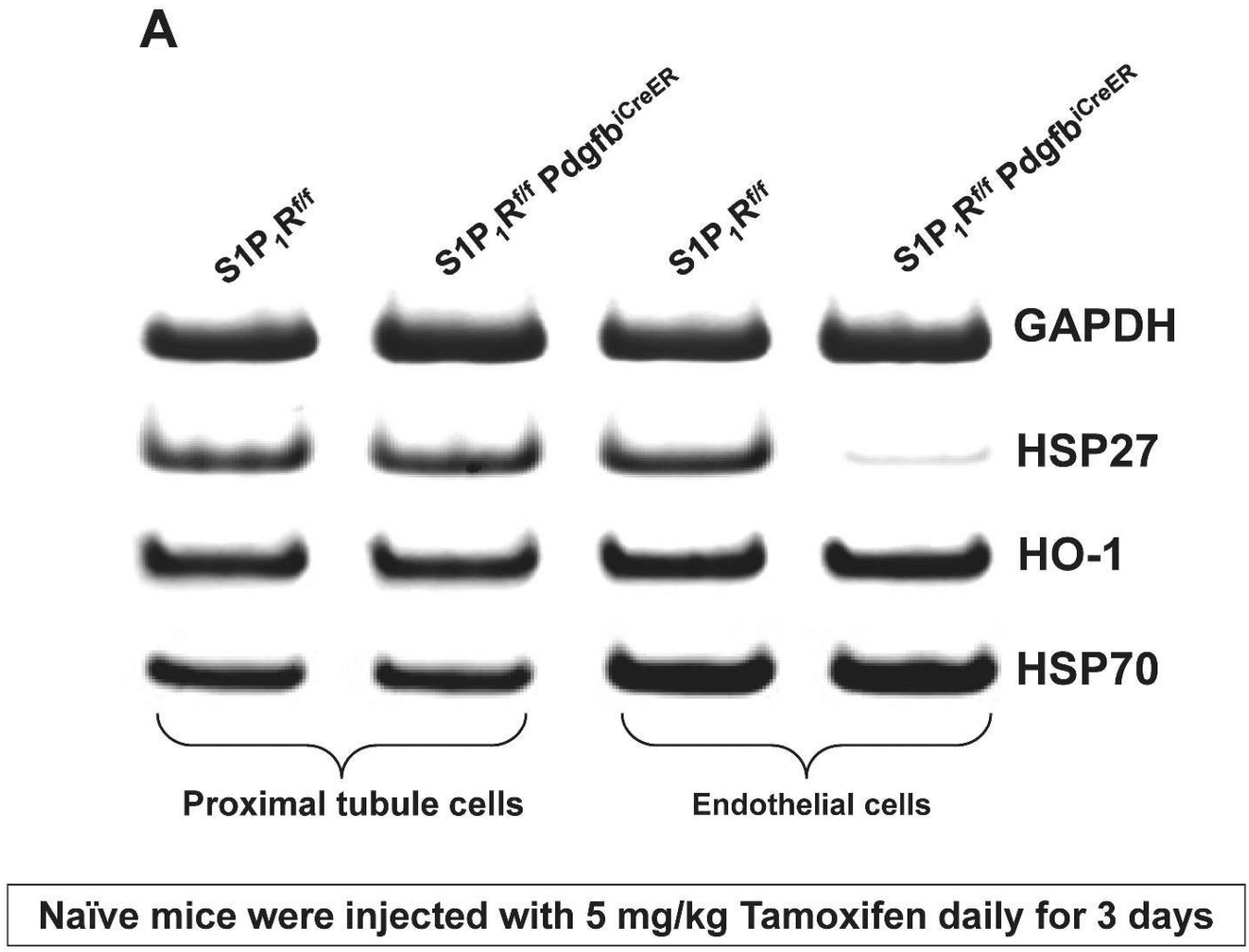
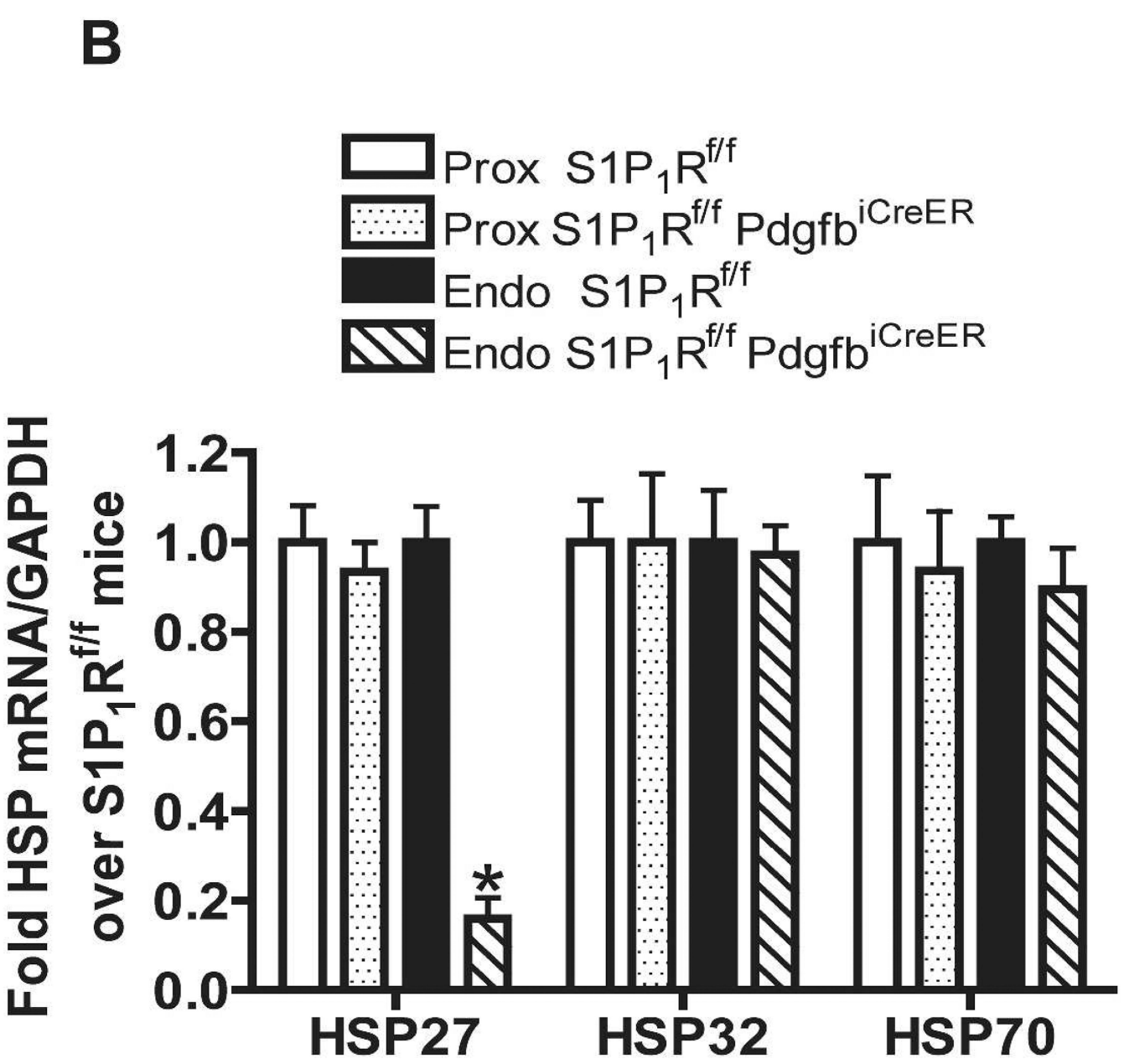


Figure 8. Endothelial $S1P_1R$ deficiency increases kidney vascular permeability after IR

Quantification of EBD extravasations as indices of kidney vascular permeability from tamoxifen-treated wild type ($S1P_1R^{f/f}$) or endothelial $S1P_1R$ null mice subjected to either sham-operation or to renal IR 5 hr prior (N=5). Wild type mice subjected to renal IR had increased kidney vascular permeability. Moreover, tamoxifen-treated endothelial $S1P_1R$ null mice had significantly increased kidney EBD extravasation after sham-operation or after renal IR. Data are presented as means ± SEM. *P<0.05 vs. sham-operated wild type ($S1P_1R^{f/f}$) mice. $^{\#}$P<0.05 vs. wild type ($S1P_1R^{f/f}$) mice subjected to renal IR.





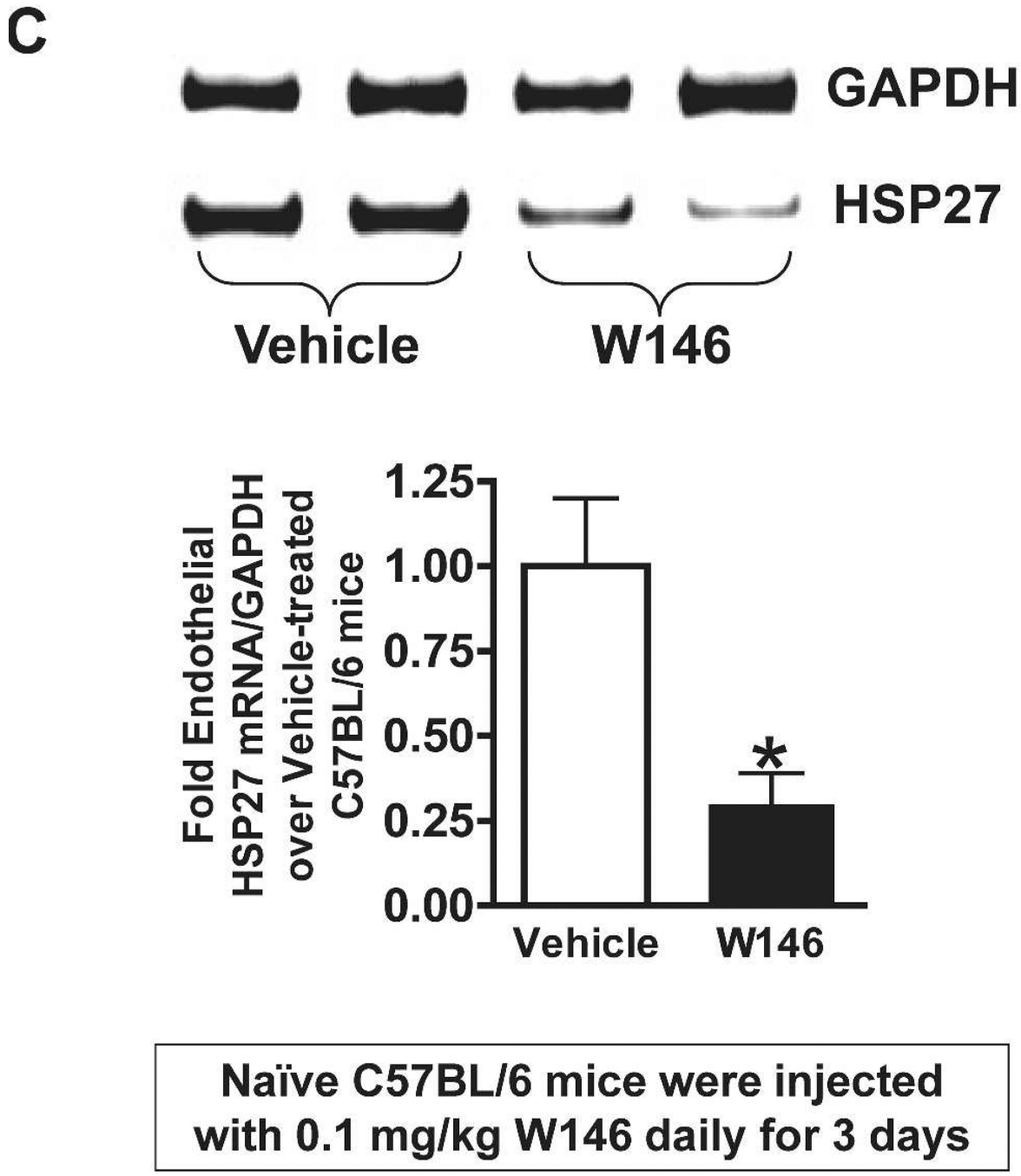


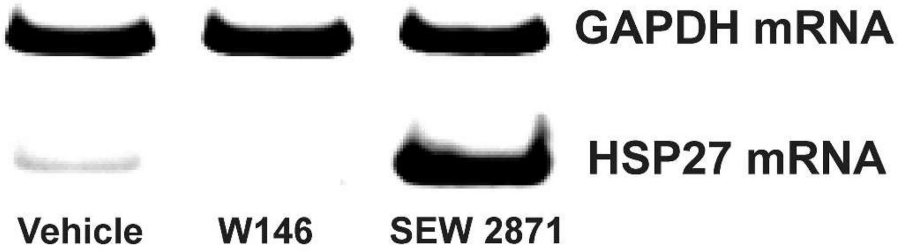
Figure 9. Endothelial $S1P_1R$ deficiency or chronic $S1P_1R$ blockade decreases endothelial HSP27 mRNA expression in mice

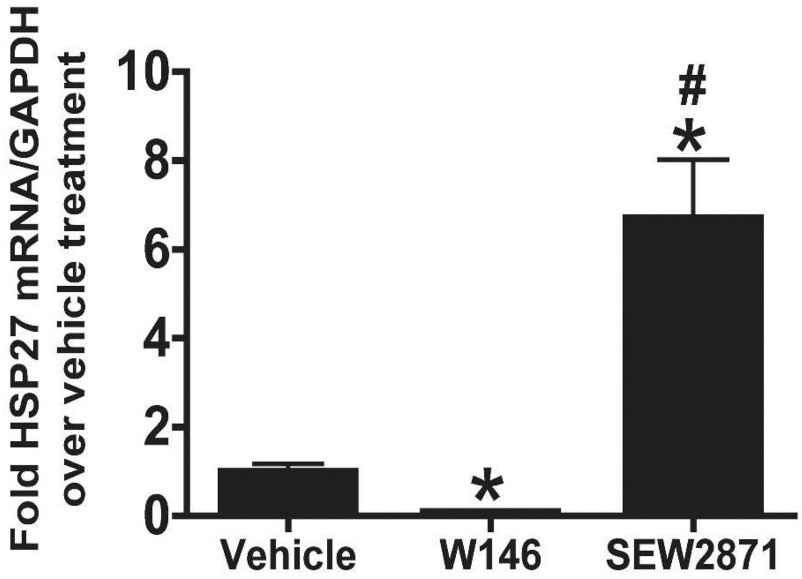
Representative gel images of RTPCR (A) and densitometric quantification of relative band intensities normalized to GAPDH (B) of HSP27, HO-1 (HSP32) and HSP70 mRNA expression in freshly isolated endothelial and proximal tubule cells from conditional endothelial $S1P_1R$ null mice or from wild type ($S1P_1R^{f/f}$) mice. Renal endothelial cells from endothelial $S1P_1R$ null mice express significantly reduced HSP27 mRNA compared wild type mice without any changes in HO-1 or HSP70 expression. Furthermore, we show that HSP27, HSP32 and HSP70 mRNA levels in freshly isolated proximal tubule cells were

similar between endothelial $S1P_1R$ null mice and wild type mice. C and D. Representative gel images of RTPCR (top) and densitometric quantification of relative band intensities normalized to GAPDH (bottom) of HSP27 mRNA expression in freshly isolated endothelial cells from C57BL/6 mice treated for 3 days (C) or for 1 hr (D) with vehicle or with W146 (a selective $S1P_1R$ antagonist, 0.1 mg/kg). $S1P_1R$ antagonist treatment significant reduced endothelial HSP27 expression only after treatment for 3 days with W146 (C). W146 treatment for 1 hr had no effect on endothelial HSP27 expression (D). *$P<0.05$ vs. wild type ($S1P_1R^{f/f}$) mice or vehicle-treated C57BL/6 mice.

A

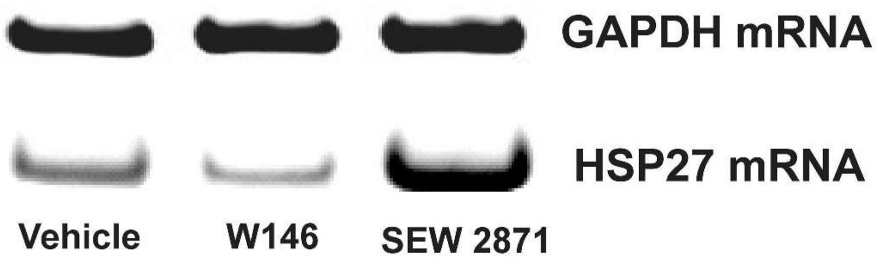
Human umbilical vein endothelial cells were treated with vehicle, 1 μM W146 or 1 μM SEW2871 daily for 3 days

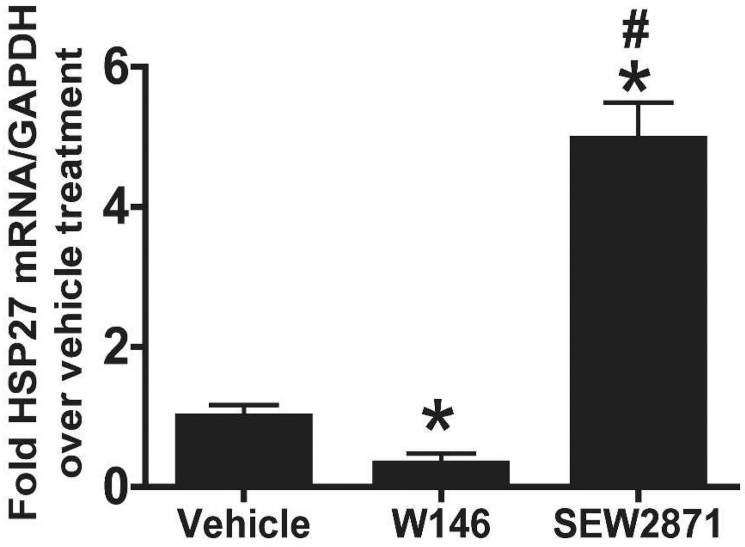




B

Mouse glomerular endothelial cells were treated with vehicle, 1 μM W146 or 1 μM SEW2871 daily for 3 days





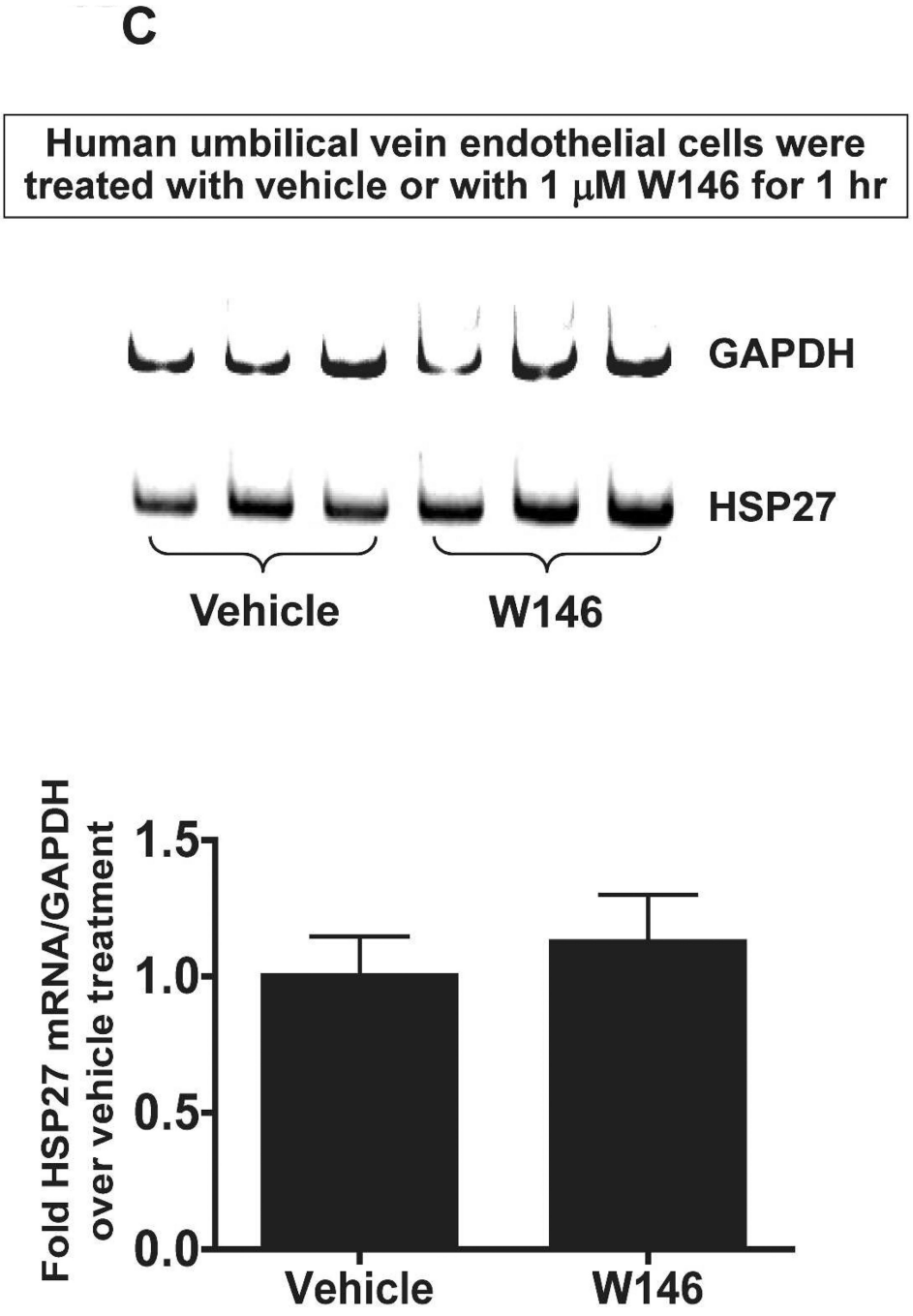
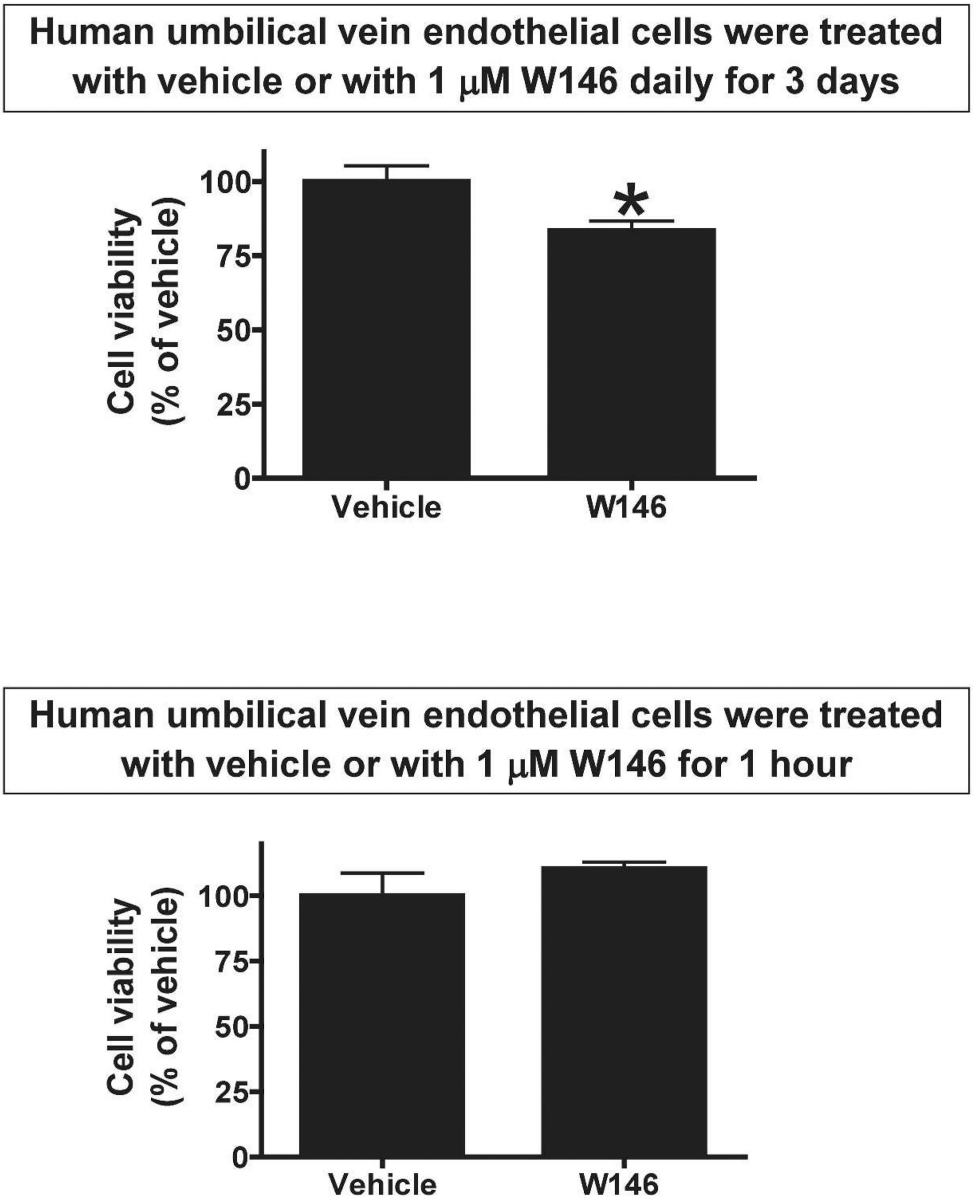


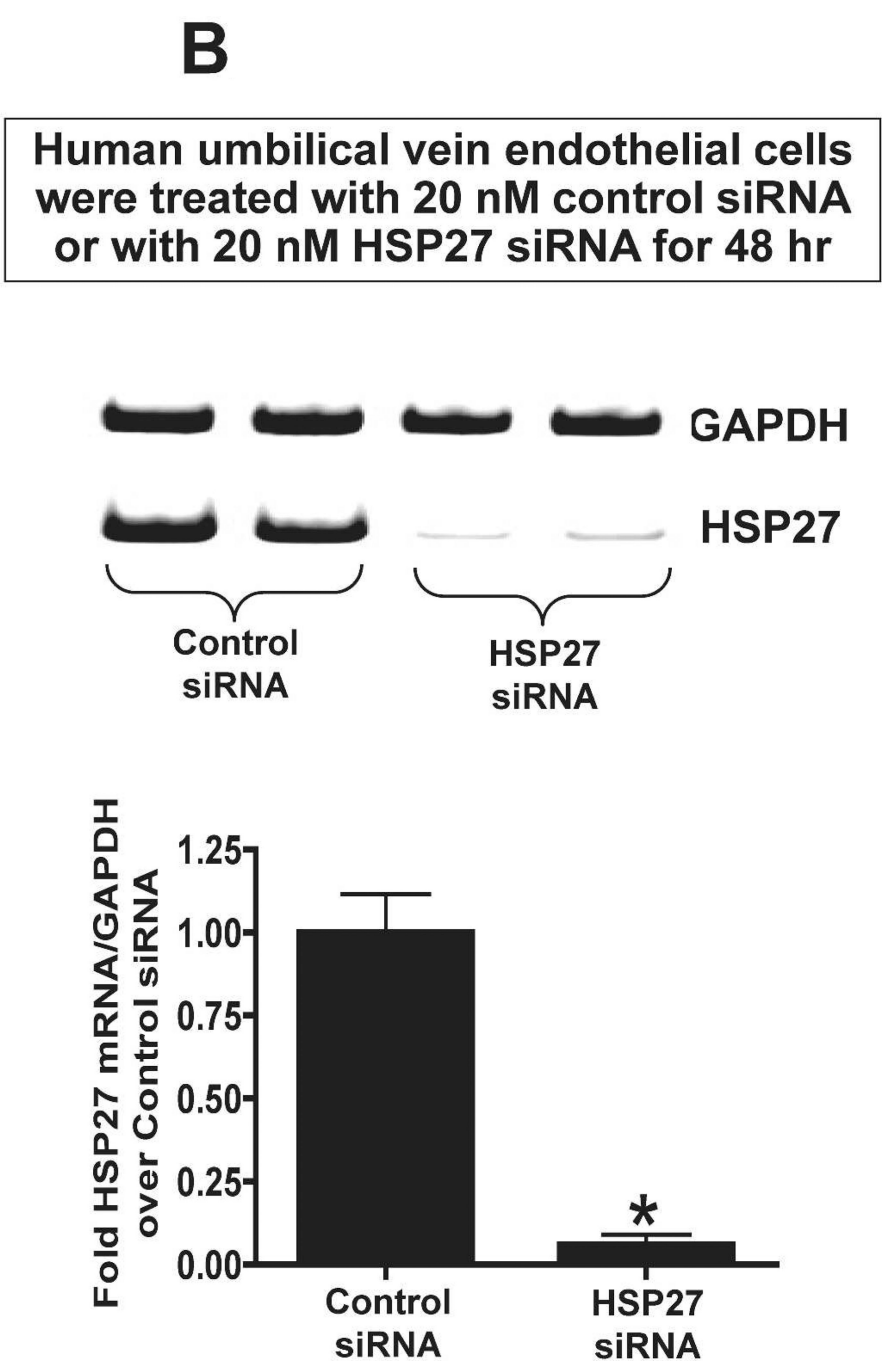
Figure 10. S1P$_1$R modulates HSP27 mRNA expression in cultured endothelial cells

A and B. Representative gel images of RTPCR (top) and densitometric quantification of relative band intensities normalized to GAPDH (bottom) of HSP27 mRNA expression in human umbilical vein endothelial cells (A, N=6) or mouse glomerular endothelial cells (B, N=5) treated with vehicle (1% DMSO), with a selective S1P$_1$R agonist (1 μM SEW2871) or with a selective antagonist (1 μM W146) for 3 days. Endothelial cells treated with W146 for 3 days had significantly reduced HSP27 mRNA expression. In contrast, chronic S1P$_1$R activation with SEW2871 significantly increased HSP27 mRNA expression in human and

mouse endothelial cells. C. Representative gel images of RTPCR (top) and densitometric quantification of relative band intensities normalized to GAPDH (bottom) of HSP27 mRNA expression in human umbilical vein endothelial cells (N=4) treated with vehicle (1% DMSO) or with a selective antagonist (1 μM W146) for 1 hr. W146 treatment for 1 hr did not change endothelial HSP27 expression. *P<0.05 vs. vehicle-treated cells.

A





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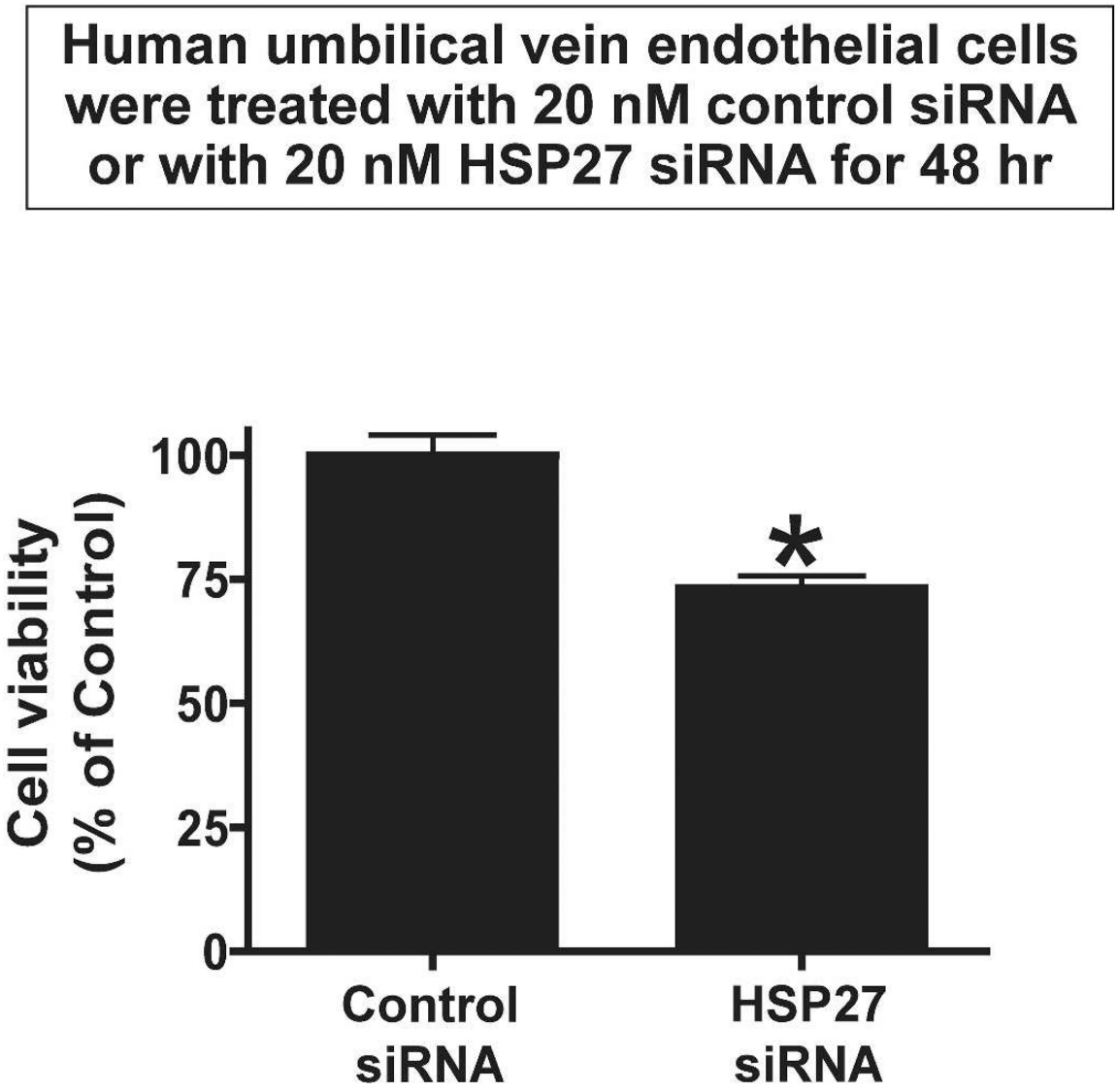

Figure 11. Chronic $S1P_1R$ antagonism or HSP27 knockdown decreases endothelial cell viability

MTT assay measured endothelial cell viability. A. Human umbilical vein endothelial cells were treated with vehicle (1% DMSO) with a selective $S1P_1R$ antagonist (1 μM W146) for 3 days (N=4, top) or for 1 hr (N=4, bottom). Treatment with W146 for 3 days significantly reduced endothelial cell viability whereas 1 hr treatment had no effect. B. Representative gel images of RTPCR (top) and densitometric quantification of relative band intensities normalized to GAPDH (bottom) of HSP27 mRNA expression in human umbilical vein endothelial cells (N=4) treated with 20 nM control siRNA or with 20nM HSP27 siRNA for

48 hr (N=4). HSP27 siRNA treatment significantly attenuated HSP27 mRNA expression in endothelial cells. C. siRNA induced HSP27 knockdown significantly reduced endothelial cell viability in culture (N=4). *P<0.05 vs. vehicletreated cells.

Table 1

Primers used to amplify cDNAs based on published GenBank sequences for mice. bp, base pairs; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; ICAM-1, intercellular adhesion molecule-1; iCreER, tamoxifen inducible Cre recombinase, MCP-1, monocyte chemoattractive protein-1; MIP-2, macrophage inflammatory protein-2; Pdgfb, platelet derived growth factor-b; S1PR, sphingosine 1-phosphare receptor; SK, sphingosine kinase; TNF-α, tumor necrosis factor-alpha; HSP, heat shock protein; HO-1, heme oxygenase-1.

Primers	Species	Sequence (Sense/Antisense)	Product Size (bp)	Cycle Number	Annealing Temp (°C)
$S1P_1R$	Mouse	5′-CGGTGT AGACCC AGAGTCCT-3′ 5′-AGC AGC AGATGAGAATGAAC-3′	393	20	64
$S1P_2R$	Mouse	5′- AAAACC AACC ACTGGCTGTC-3′ 5′-GAGTGGAACTTGCTGTT-3′	317	25	60
$S1P_3R$	Mouse	5′-AAGCCTAGCGGGAGAGAAAC-3′ 5′-GGCAATC AAAACC ATCAGGT-3′	452	22	64
$S1P_4R$	Mouse	5′-GC AGAAGTCTCC ACGTCCTC-3′ 5′-GCTGAGTGACCGAGAAGTCC-3′	403	23	62
$S1P_5R$	Mouse	5′-ACACCAAATGCCCAGCTTAC- 3′ 5′- ACC AAGAGC AC AGCC AAGTTC-3′	335	32	62
$S1P_1R$ FLOX	Mouse	5′-GAGCGGAGGAAGTTAAAAGTG- 3′ 5′-CCTCCTAAGAGATTGCAGCAA- 3′	Flox 250, Cont 200	45	56
$Pdgfb^{iCreER}$	Mouse	5′-CCAGCCGCCGTCGCAACT- 3′ 5′-GCCGCCGGGATCACTCTCG- 3′	450	35	60
TNF-α	Mouse	5′-TACTGAACTTCGGGGTGATTGGTCC-3′ 5′-C AGCCTTGTCCCTTGAAGAGAACC-3′	290	24	65
ICAM-1	Mouse	5′-TGTTTCCTGCCTCTGAAGC-3′ 5′-CTTCGTTTGTGATCCTCCG-3′	409	21	60
MCP-1	Mouse	5′- ACCTGCTGCTACTC ATTC AC-3′ 5′-TTGAGGTGGTTGTGGAAAAG-3′	312	22	60
MIP-2	Mouse	5′-CC AAGGGTTGACTTC AAGAAC-3′ 5′-AGCGAGGC AC ATC AGGTACG-3′	282	28	60
HSP27	Mouse	5′-CCTAAGGTCTGGC ATGGT A-3′ 5′-AGGAAGCTCGTTGTTGAAGC-3′	373	25	66
HSP27	Human	5′-GTCCCTGGATGTC AACC AC-3′ 5′-GACTGGGATGGTGATCTCG-3′	286	15	65
HSP32 (HO-1)	Mouse and human	5′-TGAAGGAGGCC ACC AAGGAG-3′ 5′-GTGGGCC ACC AGC AGCTC-3′	320	15	65
HSP70	Mouse and human	5′-GATC ACC ATC ACC AACGAC AA-3′ 5′-TTGTCC AGC ACCTTCTTCTTGTC-3′	218	15/18	65(H)/ 60(M)
GAPDH	Mouse and human	5’-ACCACAGTCCATGCCATCAC-3’ 5’-CACCACCCTGTTGCTGTAGCC-3’	450	15	65

Respective anticipated PCR product size (bp, base pairs), PCR cycle number for linear amplification, and annealing temperatures used for each primer are provided.