Early Diabetes Aggravates Renal Ischemia/reperfusion-induced Acute Kidney Injury

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Abstract

Acute kidney injury (AKI) due to ischemia and reperfusion (IR) can be associated with the progression of chronic kidney injury. In addition, studies suggest that chronic diabetes is an independent risk factor for AKI; however, the impact of early diabetes on the severity of AKI remains unknown. We investigated the effects of early diabetes on the pathophysiology of renal IR-induced AKI. C57BL/6J mice were randomly assigned into the following groups: 1) sham-operated; 2) renal IR; 3) streptozotocin (STZ - 55 mg/kg/day) and sham-operated; and 4) STZ and renal IR. On the 12th day after treatments, the animals were subjected to bilateral IR for 30 minutes followed by reperfusion for 48 hours, and the mice were euthanized by exsanguination. Renal function was assessed by analyzing the plasma creatinine and urea concentrations with biochemical methods. Proteinuria was evaluated using a commercial kit. Kidney tissue was used to evaluate the morphology, gene expression by qPCR, and protein expression by Western blotting. Compared to the sham operated, renal IR resulted in increased plasma creatinine and urea levels, decreased nephrin mRNA expression, increased tubular cast formation, and Kim-1, Ki-67, pro-inflammatory and pro-fibrotic factor mRNA expression. Compared with the sham treatment, STZ treatment resulted in hyperglycemia, but did not induce changes in kidney function or pro-inflammatory or pro-fibrotic factors. However, STZ treatment aggravated renal IR-induced AKI by exacerbating glomerular and tubular injury, inflammation, and the profibrotic response. Early diabetes constitutes a relevant risk factor for renal IR-induced AKI.

Introduction

Acute kidney injury (AKI) is a common disorder worldwide that is associated with high morbidity and mortality [1]. AKI due to an renal ischemia and reperfusion (IR) occurs during various clinical conditions, such as cardiac and hepatic surgeries, shock, sepsis, vascular occlusions and kidney transplantation [2]. Under these circumstances, a rapid decline in kidney function occurs and is closely related to high plasma creatinine levels, renal epithelial and vascular cell injury, interstitial innate immune cell infiltration; these phenomena lead to interstitial fibrosis and often to the development of chronic kidney disease (CKD), culminating in end-stage renal disease (ESRD) [3, 4]. Despite recent advances in understanding the pathophysiological mechanisms associated with AKI, there is still no effective therapy for the prevention or treatment of AKI.

Diabetes mellitus is a metabolic syndrome characterized by elevated blood glucose levels. Type 1 diabetes mellitus (T1DM) occurs due to an autoimmune disease characterized by the selective destruction of the β-pancreatic cells that synthesize and secrete insulin, a hormone responsible for the maintenance of glycemia under physiological conditions [5]. Diabetic kidney disease (DKD) is closely related to the progression of CKD and consequent loss of kidney function [6]. DKD is initially characterized by hyperfiltration, microalbuminuria and inflammatory cell infiltration. In no longer reversible states, podocyte injury and thickening of the glomerular basement membrane occur, with a consequent decline in glomerular filtration rate and macroalbuminuria [6].

It is known that diabetes is an important risk factor for AKI [7], mainly in cardiac patients [8] or patients with partial nephrectomy [9], after renal infarction [10] and septic shock [11]. In addition, it has been shown that diabetic patients who suffer episodes of AKI are more likely to develop CKD [12]. However, the mechanisms by which diabetes influences the severity of renal IR injury remain unknown, although some of the pathological findings in diabetic nephropathy, including interstitial inflammation and fibrosis, are also related to IR-induced AKI [13].

Given the increased incidence of diabetes and its contribution as a risk factor for a variety of surgical complications [14, 15], more research is required to understand the molecular and cellular pathophysiological mechanisms underlying IR-induced AKI in diabetic conditions. In addition, studies with experimental models have associated the initial phase of ischemic AKI with chronic diabetes [16–18], and little is known about the effects of AKI in the early stages of diabetes. Thus, the aim of the current study was to investigate the interaction between early diabetes and renal IR-associated AKI. Here, we established a renal IR model in streptozotocin (STZ)-induced diabetic mice to investigate whether hyperglycemia aggravates renal IR-induced AKI during early diabetes and the possible cellular mechanisms involved in this maladaptive process. In particular, we focused on kidney function, tubular and glomerular injury, autophagy, and the expression of genes related to inflammatory and fibrosis responses. In this study, we intend to contribute to the identification of possible therapeutic targets for the treatment of renal IR-induced AKI in early diabetic conditions.

Methods

Animals

Male C57BL/6J mice, acquired from the animal care facility of the University of Sao Paulo Medical School (Sao Paulo, Brazil), were housed at the facility of the Department of Physiology and Biophysics, Institute of Biomedical Sciences, University of Sao Paulo (Sao Paulo, Brazil). The animals were maintained under temperature - controlled conditions - and a 12-hour light-dark cycle and were given free access to water and food. All the presented protocols were approved by the Ethics Committee on the Use of Experimental Animals (12/2017) and are in accordance with the ethical principles adopted by the Brazilian Society of Laboratory Animal Science and in compliance with the ARRIVE guidelines.

Thirty mice (aged 8 weeks, weighing approximately 22 grams) were randomly assigned to four groups: sham-operated (sham); renal IR (IR); streptozotocin (STZ); and STZ followed by renal IR (STZ /IR).

Streptozotocin treatment

According to the Ethics Committee, nonsedated animals received a daily intraperitoneal injection (55 mg/kg) [19] of STZ (Sigma-Aldrich, St. Louis, MO, USA), diluted in citrate buffer (0.1 M, pH 4.5), for five consecutive days. Notably, the control and IR groups received only citrate buffer (Vehicle). To favor drug metabolism, all the treatments were performed in animals that had been fasted for four hours. On the fifth day after the last injection of STZ, mice with blood glucose ≥ 250 mg/dL (evaluated by Kit Accu-

Chek Perform, Sao Paulo, SP, Brazil) and asymptomatic mice were considered diabetic and, therefore, allowed to continue the study.

Renal ischemia surgery and reperfusion

On the twelfth day after the last injection of STZ, the animals were weighed, and the blood glucose levels were evaluated. Next, the animals were intraperitoneally anesthetized with ketamine (100 mg/kg) and xylazine (20 mg/kg, Virbac, Jurubatuba, SP, Brazil) and immediately placed on a hot plate to maintain a constant temperature at 37 °C. After total loss of pain reflexes, an abdominal incision was made in the white line to expose the kidneys. The blood flow of both the renal arteries was interrupted by the use of mini-clamps (RS-5426, Roboz Surgical Instrument Company, Inc, Gaithersburg, MD, USA) for 30 minutes [20]. Renal ischemia was assessed based on the change in kidney color. Then, the mini-clamps were carefully removed to allow renal reperfusion, which was confirmed by the return of oxygenated blood to the kidneys. Subsequently, the abdominal muscles and skin were sutured, and asepsis was performed. The control and STZ groups underwent sham surgery. For this sham surgery, an abdominal incision was made in the white line, and the kidneys were exposed, but the blood flow through the renal arteries was not interrupted. After surgery, the animals received saline solution intraperitoneally to prevent dehydration.

Plasma, urine and kidney collection

At the end of 48 hours of kidney reperfusion, the animals were weighed, and blood glucose was evaluated. The animals were then anesthetized as previously described. After the complete loss of pain reflexes, cardiac puncture was performed to collect blood samples (approximately 1 mL), and the mice were euthanized by exsanguination. Immediately, an abdominal incision was made using a scalpel, and the urinary content of the bladder was collected. Next, the left kidney was removed, weighed, and cut into two sections. One section was quickly frozen and pulverized in liquid nitrogen for further analysis by RT-PCR and the remaining section was crushed in PBS solution (0.15 M NaCl containing 10 mM sodium phosphate buffer, pH 7.4) with protease inhibitors (Sigma Aldrich), centrifuged (4000×g for 10 minutes at 4 °C) and frozen at -80 °C for protein analysis by Western Blotting. The right kidney was perfused with PBS solution, removed, cross-sectioned, inserted into properly labelled histological cassettes and fixed in 4% paraformaldehyde solution. After dehydration, the slices were embedded in paraffin for morphological studies as previously described [21].

Kidney function analysis

The plasma creatinine and urea levels as well as urine creatinine levels were assessed using colorimetric tests (Labtest, Lagoa Santa, MG, Brazil) according to the manufacturer's instructions. Urine osmolarity was measured using an osmometer (Micro osmometer, Precision Systems, Natick, MA, USA). The urinary albumin concentration was determined using a SilverQuest Silver Staining Kit (Sigma-Aldrich) according to the modified Oakley method [22]. Briefly, the urine samples from the bladders were separated by SDS polyacrylamide gel electrophoresis (10%). Next, silver staining was performed on the gels, and the albumin (bovine serum albumin – BSA; 66 kDa) bands were identified using a molecular weight marker.

The experiments were carried out following the manufacturer's instructions, and the urine protein concentration was normalized to the urine creatinine concentration. The bands were assessed by optical densitometry using ImageJ software (National Institutes of Health (NIH), Bethesda, MD, USA) [23].

Morphological studies

As previously described [21, 23] and summarized here, fixed 4-μm kidney slices were deparaffinized for histological studies. Next, the histological slices were stained with hematoxylin-eosin (HE) and examined in a blinded manner by one independent investigator using a light microscope (Eclipse 80i, Nikon, Tokyo, Japan) in order to evaluate the tubular and glomerular morphology and interstitial conditions. For glomerular area analysis, glomeruli were manually surrounded, and the images were evaluated using NIS-Elements (Nikon) software, which provided the area values for each glomerulus. All the glomeruli per slice were analyzed in a blinded manner by different investigators.

Total renal tissue gene expression studies

As previously described [21, 24] and summarized here, frozen kidney sections were homogenized in liquid nitrogen and then resuspended in TRIzol LS Reagent (Invitrogen, Carlsbad, CA USA) for RNA extraction (GE Healthcare Life Sciences, BW, Germany) according to the manufacturer's instructions. Two hundred nanograms of RNA was used for the synthesis of cDNA (RT-PCR) from the High-Capacity RNA-to-cDNA kit (Applied Biosystems, Foster City, CA, USA) and Mastercycler thermocycler (Eppendorf, Hamburg, GER), according to the product protocol. The cDNA (25 ng) obtained was used for the analysis of gene expression through real-time polymerase chain reaction (PCR) (StepOnePlus Real Time PCR System, Applied Biosystems). The following FAM-labeled probes obtained in the TaqMan Gene Expression Assays format (Applied Biosystems) were used: nephrin (*Nphs1*), Mm01176615_g1; kidney injury molecule-1, Kim-1 (*Havcr1*), Mm00506686_m1; antigen Ki67 (*Mki67*), Mm01278617_m1; interleukin 1 beta (*Il1b*), Mm00434228_m1; tumor necrosis factor alpha (*Tnf*), Mm00443258_m1; monocyte chemoattractant protein-1, MCP1 (*Ccl2*), Mm00441242_m1; nuclear factor kappa B 1 (*Nfkb1*), Mm00476361_m1; alpha smooth muscle actin, αSMA (*Acta2*), Mm00725412_s1; transforming growth factor beta 2 (*Tgfb2*), Mm00436955_m1; collagen type I (*Col1a1*), Mm00801666_g1; collagen type III (*Col3a1*), Mm01254476_m1; collagen type IV (*Col4a1*), Mm01210125_m1; and glyceraldehyde-3-phosphate dehydrogenase (*Gapdh*), Mm99999915_g1 (reference gene). The real-time PCR reactions were performed in duplicate and the data were analyzed by the comparative cycle threshold ($2^{\Delta\Delta Ct}$) method. The results were normalized to *Gapdh* expression and are shown as the fold change relative to the control group.

Western blotting analysis

Immunoblotting analysis was performed on 30-μg protein aliquots resolved by 10–15% SDS-PAGE. Then, the protein samples were transferred to polyvinylidene fluoride (PVDF) membranes (GE Healthcare Life Sciences). The membranes were incubated with primary antibodies against autophagic markers [rabbit anti-AMPKα (1:1000, Cell Signaling, Danvers, MA, USA [25]), phospho-AMPKα (1:1000, Cell Signaling [26]) rabbit anti-Beclin-1 (1:1000, Cell Signaling [27]), rabbit anti-LC3 I and II (1:1000, Cell Signaling [28]), rabbit anti-SQSTM1/p62 (1:1000, Cell Signaling [29])] and mouse anti-β-actin (1:8000, Abcam, Cambridge, MA,

UK) and then incubated with conjugated secondary antibodies. Then, the membranes were exposed to the reagent for immunodetection with ECLTM luminescence (Amersham, GE) and subsequently to a photodocumenter (Amersham Imager 600, GE). The intensity of the bands was assessed by optical densitometry using ImageJ software (National Institute of Health). The protein expression was quantified relative to the expression of the endogenous control β-actin, and the results are presented as protein expression relative to the control group.

Statistical analysis

The data were analyzed by two-way ANOVA for the effect of diabetes (STZ), renal IR, and the interaction between these two factors (STZ/IR). When the interaction was statistically significant, the Bonferroni post-hoc test was performed for multiple comparisons. When analysis between two groups was necessary, a t test was performed. The statistical analysis of the data was performed by GraphPad Prism 8 (GraphPad Software, Inc., San Diego, CA, USA). Values of $p < 0.05$ were considered statistically significant, and the results are presented as the mean ± S.D.

Results

Initial body weight and blood glucose levels

Before surgery, all the animals were weighed, and the blood glucose levels were evaluated. STZ treatment did not change the body weight of the animals compared to the control treatment [(g) Control, 23.2 ± 1.2 (n = 16) *vs* STZ, 22.4 ± 1.5 (n = 14)]; however, STZ induced an increase in the blood glucose levels compared to the control treatment [(mg/dL) Control, 143.6 ± 19.2 (n = 16) *vs* STZ, 325.8 ± 39.7 (n = 14), $p < 0.0001$]. This result confirms the effectiveness of the drug.

Metabolic parameters and kidney function

As shown in Table 1, we observed an effect of renal IR in terms of weight loss and an increase in the kidney weight/body weight ratio. On the other hand, renal IR did not affect the plasma glucose levels, but as expected, the STZ or STZ/IR groups maintained increased blood glucose levels.

Table 1
Physiological parameters

Two-way ANOVA					
Parameters	**Sham (8)**	**IR (8)**	**STZ (7)**	**STZ/IR (5–8)**	**STZ IR STZ/IR**
Final body weight, g	22.2 ± 1.0	19.0 ± 1.0	21.4 ± 1.7	18.7 ± 0.9	ns p < 0.0001 ns
Body weight gain, g	-1.6 ± 0.5	-3.6 ± 0.7	-1.0 ± 0.5	-3.7 ± 1.1	ns p < 0.0001 ns
Final blood glucose, mg/dL	98.5 ± 13.0	92.5 ± 25.4	251.8 ± 90.7	273.4 ± 139.2	p < 0.0001 ns ns
Kidney weight /body weight, mg/g	6.24 ± 0.45	8.80 ± 0.98	6.47 ± 0.44	8.78 ± 0.71	ns p < 0.0001 ns
Parameters	**Sham (4–8)**	**IR (4–7)**	**STZ (4–7)**	**STZ/IR (3–6)**	**STZ IR STZ/IR**
Plasma Creatinine, mg/dL	0.203 ± 0.068	0.939 ± 0.582*	0.179 ± 0.078	1.839 ± 0.709$^{\#\#\#\#\&}$	p < 0.5 p < 0.0001 p < 0.05
Plasma Urea, mg/dL	41.49 ± 2.82	283.95 ± 142.90	35.29 ± 4.01	323.61 ± 153.91	ns p < 0.0001 ns
Urinary albumin, Alb/Cr ratio	0.155 ± 0.114	1.569 ± 0.951*	0.247 ± 0.106	3.159 ± 0.742$^{\#\#\#\&}$	p < 0.5 p < 0.0001 p < 0.05
Parameter	**Sham (8)**	**IR (8)**	**STZ (7)**	**STZ/IR (7)**	**STZ IR STZ/IR**
Glomerular area, μm^2	6932 ± 585	7012 ± 593	7576 ± 616	7737 ± 744	p < 0.01 ns ns

Values are mean ± S.D, number of animals (n) per group is indicated in parenthesis. Data was analyzed by Two-way ANOVA for diabetes (STZ), renal IR or interaction between STZ/IR. When interaction was significant, Bonferroni post-test was performed: $^{*}p < 0.05$ *versus* sham group; $^{\#\#\#}p < 0.001$, $^{\#\#\#\#}p < 0.0001$ *versus* STZ group. $^{\&}p < 0.05$ *versus* renal IR group. IR: ischemia/reperfusion; ns: not significant.

In nondiabetic and STZ-treated mice, renal IR resulted in increased plasma creatinine levels, urea levels, and albuminuria compared to the sham and STZ-treated mice. STZ treatment did not change these parameters compared to the sham treatment. However, when the STZ-treated group was subjected to renal IR, the plasma creatinine levels and albuminuria were robustly increased compared with those in the nondiabetic renal IR group (Table 1, **Fig. 1A, B, C and Supplementary Fig. 1C**).

Glomerular and tubular injury

We started the glomerular analysis by assessing the glomerular area, and we did not observe a significant difference in the studied groups. (Table 1). Next, we evaluated nephrin mRNA expression. As shown in

Fig. 1D, in the nondiabetic and STZ-treated mice, renal IR resulted in a significant decrease in nephrin mRNA expression compared to that in the sham and STZ groups. STZ treatment did not change nephrin mRNA expression compared to the sham treatment, but renal IR in the STZ-treated mice resulted in a significant decrease in this parameter compared to renal IR in the nondiabetic mice. The average values are shown in Table 2.

Table 2

Genes and proteins expression

Parameters Two-way ANOVA					
mRNA expression (fold change)	**Sham (7–8)**	**IR (7–8)**	**STZ (7)**	**STZ/IR (5–7)**	**STZ IR STZ/IR**
Nephrin	1.00 ± 0.10	0.70 ± 0.09****	0.96 ± 0.10	0.48 ± 0.10$^{\#\#\#\#\&\&}$	p < 0.01 p < 0.0001 p < 0.05
Kim-1	1.09 ± 0.53	284.70 ± 78.70****	3.90 ± 2.54	659.18 ± 205.13$^{\#\#\#\#\&\&\&\&}$	p < 0.0001 p < 0.0001 p < 0.0001
Ki-67	1.04 ± 0.31	20.91 ± 9.65****	2.49 ± 1.00	1.55 ± 1.27$^{\&\&\&\&}$	p < 0.0001 p < 0.0001 p < 0.0001
IL-1β	1.08 ± 0.46	5.58 ± 1.44***	1.90 ± 0.71	11.89 ± 3.71$^{\#\#\#\#\&\&\&\&}$	p < 0.0001 p < 0.0001 p < 0.001
TNF-α	1.06 ± 0.38	5.62 ± 1.74	2.30 ± 0.60	9.63 ± 6.25	p < 0.05 p < 0.0001 ns
MCP-1	1.08 ± 0.42	15.94 ± 7.00	3.84 ± 1.47	18.97 ± 21.48	ns p < 0.001 ns
NFκB	1.01 ± 0.15	1.72 ± 0.47	1.07 ± 0.13	2.13 ± 0.80	ns p < 0.0001 ns
TGF-β2	1.02 ± 0.24	1.72 ± 0.79	1.34 ± 0.53	1.95 ± 0.63	ns p < 0.01 ns
α-SMA	1.04 ± 0.32	4.48 ± 1.79*	0.99 ± 0.32	8.94 ± 4.50$^{\#\#\#\#\&\&}$	p < 0.05 p < 0.0001 p < 0.05
Collagen I	1.03 ± 0.31	15.90 ± 3.28****	1.49 ± 0.36	5.77 ± 5.44$^{\&\&\&\&}$	p < 0.001 p < 0.0001 p < 0.0001
Collagen III	1.07 ± 0.45	23.43 ± 7.16****	1.43 ± 0.91	12.22 ± 6.16$^{\#\#\&\&}$	p < 0.01 p < 0.0001 p < 0.01
Collagen IV	1.01 ± 0.21	2.92 ± 0.77	1.24 ± 0.23	3.20 ± 1.09	ns p < 0.0001 ns
Protein/ β-actin expression	**Sham (4–6)**	**IR (4–5)**	**STZ (3–6)**	**STZ/IR (4–6)**	**STZ IR STZ/IR**

Values are mean ± S.D, number of animals (n) per group is indicated in parenthesis. Data was analyzed by Two-way ANOVA for diabetes (STZ), renal IR or interaction between STZ/IR. When interaction was significant, Bonferroni post-test was performed: *p < 0.05, ***p < 0.001, ****p < 0.0001 *versus* sham group. $^{\#}$p < 0.05, $^{\#\#}$p < 0.01, $^{\#\#\#\#}$p < 0.0001 *versus* STZ group. $^{\&\&}$p < 0.01, $^{\&\&\&\&}$p < 0.0001 *versus* renal IR group. IR: ischemia/reperfusion; ns: not significant.

Parameters Two-way ANOVA					
AMPKα	1.00 ± 0.12	0.91 ± 0.25	0.91 ± 0.17	0.92 ± 0.21	ns ns ns
SQSTm1/p62	0.93 ± 0.16	1.47 ± 0.22	1.35 ± 0.29	1.54 ± 0.32	ns p < 0.05 ns
pAMPKα	1.00 ± 0.10	0.76 ± 0.10	1.16 ± 0.11	0.90 ± 0.15	p < 0.05 p < 0.001 ns
Beclin-1	1.00 ± 0.12	0.92 ± 0.11	0.92 ± 0.13	0.98 ± 0.06	ns ns ns
LC3 I	1.00 ± 0.12	0.92 ± 0.05	0.75 ± 0.10*	0.95 ± 0.02#	p < 0.05 ns p < 0.01
LC3 II	1.00 ± 0.16	0.97 ± 0.24	0.66 ± 0.04	1.20 ± 0.27#	ns p < 0.05 p < 0.05

Values are mean ± S.D, number of animals (n) per group is indicated in parenthesis. Data was analyzed by Two-way ANOVA for diabetes (STZ), renal IR or interaction between STZ/IR. When interaction was significant, Bonferroni post-test was performed: *p < 0.05, ***p < 0.001, ****p < 0.0001 *versus* sham group. #p < 0.05, ##p < 0.01, ####p < 0.0001 *versus* STZ group. &&p < 0.01, &&&&p < 0.0001 *versus* renal IR group. IR: ischemia/reperfusion; ns: not significant.

To assess tubular injury, 4-µm hematoxylin and eosin-stained kidney slices were evaluated, and the results indicated that kidney morphology was similar between the sham and STZ-treated mice. However, there was prominent formation of intratubular casts (indicated by arrows) and infiltration of interstitial immune cells (indicated by asterisks) in the groups subjected to renal IR (Fig. 2A). Renal IR resulted in increased Kim-1 mRNA expression, and STZ treatment did not change this parameter compared to the sham treatment. However, in the STZ-treated group, a robust increase in Kim-1 gene expression was observed after renal IR compared to that observed in the nondiabetic renal IR group (Fig. 2B). The renal IR group exhibited increased Ki-67 mRNA expression compared to the sham group. STZ treatment did not change this parameter compared to the sham treatment. However, in the STZ-treated mice subjected to renal IR, this response was completely abolished (Fig. 2C). The average values are shown in Table 2.

Autophagy

Considering that autophagy is a conserved cellular recycling process, we evaluated the main autophagic components in our experimental models, focusing on AMPKα, beclin-1, LC3 I and II, and SQSTM1/p62 protein expression. As shown in **Fig. 3 (A – F and Supplementary Fig. 3A)**, our results demonstrate that STZ treatment and renal IR changed pAMPK expression, but an interaction between these factors was not observed. In addition, total beclin 1 protein expression was similar among all the studied groups, and an effect of renal IR in increasing SQSTM1/p62 protein expression was observed. We also evaluated the expression of the LC3 proteins. Our results demonstrated that renal IR did not change the total protein expression of either LC3 I or LC3 II when compared to the sham operation. In contrast, STZ treatment only induced a decrease in LC3 I protein expression and did not statistically alter LC3 II protein expression

compared to the sham treatment. When renal IR occurred after STZ treatment, these parameters were similar to those observed in the sham group. The average values are show in Table 2.

Inflammation

Next, we investigated the gene expression of proinflammatory factors. As demonstrated in **Fig. 4A**, in the nondiabetic and STZ-treated mice, renal IR resulted in a significant increase in IL-1β mRNA expression compared to that in the sham and STZ-treated mice. STZ treatment did not alter the expression of this gene compared to the sham treatment. However, renal IR in the STZ-treated mice resulted in an amplification of IL-1β mRNA expression compared to that in the nondiabetic mice subjected to renal IR. On the other hand, we observed independent effects of STZ treatment and renal IR on increasing TNF-α mRNA expression and an effect of renal IR alone on increasing MCP-1 and NFκB mRNA expression (Fig. 4B **– D**). The average values are shown in Table 2.

Fibrosis

Considering that inflammatory processes are associated with fibrosis, we investigated the mRNA expression of two profibrotic components: TGF-β2 and α-SMA. As shown in **Fig. 5A and B**, the renal IR group exhibited a significant increase in TGF-β2 and α-SMA mRNA expression compared to the sham group. STZ treatment did not change either parameter compared to the sham treatment. However, renal IR intensified the increase in α-SMA mRNA expression in the STZ-treated mice compared to that observed the nondiabetic mice subjected to renal IR (Fig. 5B).

We also evaluated the mRNA expression of collagens I, III and IV. Figure 5C **and D** demonstrate an interaction between renal IR and STZ treatment on increasing collagen I and III mRNA expression. Renal IR resulted in increased collagen I and III mRNA expression compared to the sham and STZ treatments. In contrast, when renal IR was combined with STZ treatment, the increase in the mRNA expression of both collagens was less robust than that in the nondiabetic mice subjected to renal IR. In addition, we observed an independent effect of renal IR on increasing collagen IV mRNA expression (Fig. 5E). The averages values are shown in Table 2.

Discussion

Studies have reported that DKD aggravates renal IR-induced AKI [17, 30]. However, there is still a lack of studies that show an association of the early stages of diabetes with IR-induced AKI. By using STZ-induced diabetes and acute renal IR models, we observed that early diabetes may also aggravate IR-induced AKI by exacerbating glomerular and tubular injury and pro-inflammatory and pro-fibrotic mRNA expression. These conditions may be associated with a maladaptive repair process.

In our study, only renal IR-induced AKI resulted in a significant decrease in final body weight gain, probably due to difficulty eating food after surgery and increased kidney weight. These results confirmed the severity of IR in kidney tissue. For the other groups, most of the metabolic parameters remained unchanged, except for the hyperglycemia observed in the diabetic groups, as expected.

We also observed a significant decline in kidney function in the mice subjected to renal IR since, under this condition, the animals showed increased creatinine and urea plasma levels and albuminuria. Moreover, in the STZ-treated group, renal IR resulted in largely increased plasma creatinine levels and albuminuria compared to those in the nondiabetic renal IR group. The plasma creatinine level is an important marker of the glomerular filtration rate (GFR), although it can also be associated with other factors, such as tubular secretion rate and urine volume [31, 32]. Glomerular injury in both the renal IR and STZ/IR groups was confirmed by a significant decrease in nephrin mRNA expression. Nephrin is a protein related to the podocitary slit diaphragms (SDs) [33]; the loss of nephrin is closely related to podocyte injury and consequent changes in ultrafiltration barrier function, resulting in proteinuria [34], which is the major risk factor for progressive kidney injury. Taken together, our results indicate that early diabetes aggravates the loss of kidney function and glomerular injury in renal IR-induced AKI.

Since albuminuria may be associated with both glomerular and tubular injury, we extended our observations to the renal tubules. Microscopic analysis revealed strong tubular cast formation in the groups subjected to renal IR. Tubular and urinary casts are classified as hyaline, granular, fatty, and leukocyte casts [35]. Hyaline casts are the most common and are mainly formed by the Tamm-Horsfall (THP)-glycoprotein produced by the epithelial cells of the thick ascending limb (TAL) of Henle's loop [35, 36]. However, other casts can also be formed via the inclusion or adhesion of different elements to the hyaline base. In addition, granular casts can be organized by aggregates of plasma proteins, particularly IgG and IgG light chains [37]. In general, most casts appear in patients with acute tubular necrosis [38]. Consistent with these findings, our results suggest that the pronounced formation of casts in the renal IR and renal IR-diabetic groups is mainly related to tubular injury and that these casts seem to be assembled with tubular albumin. To confirm tubular injury, we investigated Kim-1 and Ki-67 gene expression. Kim-1 is a transmembrane glycoprotein that is undetectable in the healthy kidney, but its expression is highly upregulated on the apical surface of proximal tubular epithelial cells (PTECs) during AKI, where it acts as a marker of cell differentiation [39, 40]. Kim-1 expression on the apical surface of PTECs allows them to recognize and involve not only apoptotic but also necrotic material during AKI [41], and Ki-67 is widely described as a marker of cell proliferation [42]. In agreement with these findings, our results indicate severe PTEC injury, since Kim-1 and Ki67 gene expression appeared to be significantly increased in the nondiabetic renal IR group, suggesting that after insult, there is a predisposition of cell differentiation and proliferation, which are essential for the subsequent tissue repair. Interestingly, in the STZ/IR group, Kim-1 gene expression was robustly increased, and in contrast, diabetes impaired Ki-67 gene expression, suggesting that hyperglycemia promotes a negative response to tubular cell proliferation, resulting in maladaptive tissue repair. Together, these results indicate that diabetes may hinders renal tubular tissue repair after renal IR.

Next, we evaluated autophagy, since it is a dynamic process activated in response to various cellular stress conditions, including hypoxia, oxidative stress, nutrient deprivation and organelle damage [43]. The autophagic flux involves phagophore nucleation and elongation into an autophagosome, which engulfs intracellular damaged components and then fuses with the lysosome for subsequent hydrolysis [44]. Energy stress triggers autophagy in mammalian cells by activating the energy sensor AMP-activated

protein kinase (AMPK), which induces autophagy by directly phosphorylating the uncoordinated51like protein kinase (ULK1/ATG1) complex at serine residues Ser317, Ser555, and Ser777 [45, 46]. Moreover, AMPK phosphorylates Beclin1 at Ser91/94 [47] and mediates the inactivation of mammalian target of rapamycin (mTOR), a suppressor of the autophagic flux [48]. The ULK1/AGT complex and beclin-1 initiate phagophore nucleation [43]. The elongation of the phagophore involves multiple protein complexes, including the AGT12 system and LC3-II, the latter a protein specifically related to autophagosome formation. Simultaneously, cytosolic LC3 is cleaved to form LC3-I, which is conjugated to phosphatidylethanolamine (PE) by ATG7 and ATG3, facilitating the conversion of cytosolic LC3-I to membrane-bound LC3-II, which in turn maintains autophagosome formation [49]. The membrane protein LC3-II can interact with p62/SQSTM1, also referred to as sequestosome 1, which mediates the autophagic transport of ubiquitinated substrates, such as misfolded proteins for degradation in autolysosomes [50]. Thus, p62 seems to be critical in the autophagic cascade since inhibition of autophagy is often accompanied by upregulation of p62 expression. In the healthy kidney, autophagy acts as a quality control system for cellular metabolism and organelle homeostasis. Under pathological conditions, such as renal IR-induced AKI, autophagy is activated but seems to be unable to provide protection from cellular stress [51]; however, the mechanisms are unknown. In this context, we evaluated the same proteins involved in the autophagic flux in the injured kidneys of nondiabetic and diabetic animals with renal IR-induced AKI. Our results revealed that there was an effect of renal IR on decreasing phosphorylated AMPK and maintenance of beclin 1 and LC3-I or LC3-II protein expression. We also found a renal IR effect on increasing p62 protein expression, which indicates a deficiency in the autophagic response. Together, these results complement the above observations regarding Kim-1 and Ki-67 and are consistent with the absence of autophagy during renal IR-induced AKI. Interestingly, early diabetes did not change the IR-induced effects on the protein expression of autophagic components, maintaining the inability of kidney tissue repair after renal IR insult.

It is known that the autophagy pathway may prevent tissue inflammation through its role in apoptotic cell clearance [52]. Thus, it is possible that the loss of autophagic machinery can potentiate the inflammatory response in many tissues. Consistent with these findings, we observed that renal IR resulted in increased gene expression of IL-1β, TNF-α, MCP-1, and NFκB compared to the sham surgery, and only IL-1β gene expression was exacerbated in the early diabetic group subjected to renal IR. Together, these results may suggest a relevance of autophagy in preventing exacerbated inflammation in renal IR-induced AKI, highlighting the contribution of early diabetes in enhancing IL-1β gene expression compared to that observed in the nondiabetic mice subjected to renal IR. Interestingly, the contribution of IL-1β has been reported not only in proximal tubular injury but also in fibrosis development [53].

Considering the relationship between inflammation and fibrosis [54], we next investigated the gene expression of profibrotic components such, as TGF-β2, a key transcription factor associated with tubulointerstitial and glomerular fibrosis [55], and α-SMA, a commonly used marker of myofibroblasts [56]. Our results revealed that renal IR in both nondiabetic and diabetic mice resulted in increased TGF-β2 and α-SMA gene expression compared to that in sham and STZ-treated mice, suggesting a fibrotic mRNA expression response. Furthermore, early diabetes amplified the α-SMA gene expression. These results are

consistent with other studies that reported increased profibrotic mRNA expression during AKI [23] and myofibroblast recruitment, extracellular matrix deposition and subsequent tubulointerstitial fibrosis in DKD [6]. Here, we highlight that early diabetes aggravated the profibrotic mRNA expression induced by renal IR.

In addition to the inflammatory state, TGF-β2 plays a central role in the glomerular and tubulointerstitial pathobiology of kidney disease since it promotes monocyte recruitment and macrophage differentiation (53). TGF-β2 contributes to a decrease in nephrin protein synthesis and glomerular extracellular matrix (ECM) accumulation by stimulating mesangial cells to produce type I, III and IV collagens and by inhibiting matrix degradation (20, 37, 38). In accordance with these findings, our results revealed that in the renal IR group, collagen I and III gene expression was significantly increased. However, compared with nondiabetic mice subjected to renal IR, mice with early diabetes subjected to renal IR exhibited moderate collagen I and III gene expression. Interestingly, it is known that matrix metalloproteinases (MMPs), such as MMP-2 and MMP-9, are upregulated in diabetic kidneys (57). MMPs are proteases involved in the turnover of extracellular matrix and degradation of bioactive proteins, including collagen (19). Thus, it is possible that both MMPs may contribute to the suppression of collagen I and III gene expression induced by renal IR. In contrast, renal IR resulted in an increase in collagen IV gene expression, suggesting a compensatory mechanism of GBM against the possible loss of its components. Collagen IV is the main component of GBM, and its function is to provide structure and support for other cell types, such as podocytes [57].

In conclusion, our results suggest that early diabetes aggravates renal IR-induced AKI mainly by exacerbating glomerular and tubular injury and proinflammatory and profibrotic responses. We believe our findings will contribute to the understanding of the molecular mechanisms associated with renal IR-induced AKI in early diabetic conditions and may provide relevant information to prevent adverse outcomes in T1DM patients affected by renal IR.

Declarations

Acknowledgement

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Statement of Ethics

All of the experimental protocols were conducted in accordance with the ethical standards approved by the Institutional Animal Care and Use Committee of the University of Sao Paulo (Protocol no. 12/2017*).*

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Disclosures

No conflicts of interest, financial or otherwise, are declared by the authors.

Author contributions

M.C.P designed and performed all experiments, analyzed the data, wrote and reviewed the manuscript; V.G.C. helped with animal treatment and surgical procedures. J.M.C.P helped with immunoblotting experiments and M.O.S. supported and supervised the study and contributed with the writing and review of the manuscript. All authors approved the final manuscript.

Data availability statement

All data generated or analyzed during this study are included in this published article.

ARRIVE statement

The authors confirms that the study was carried out in compliance with the ARRIVE guidelines.

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Figures

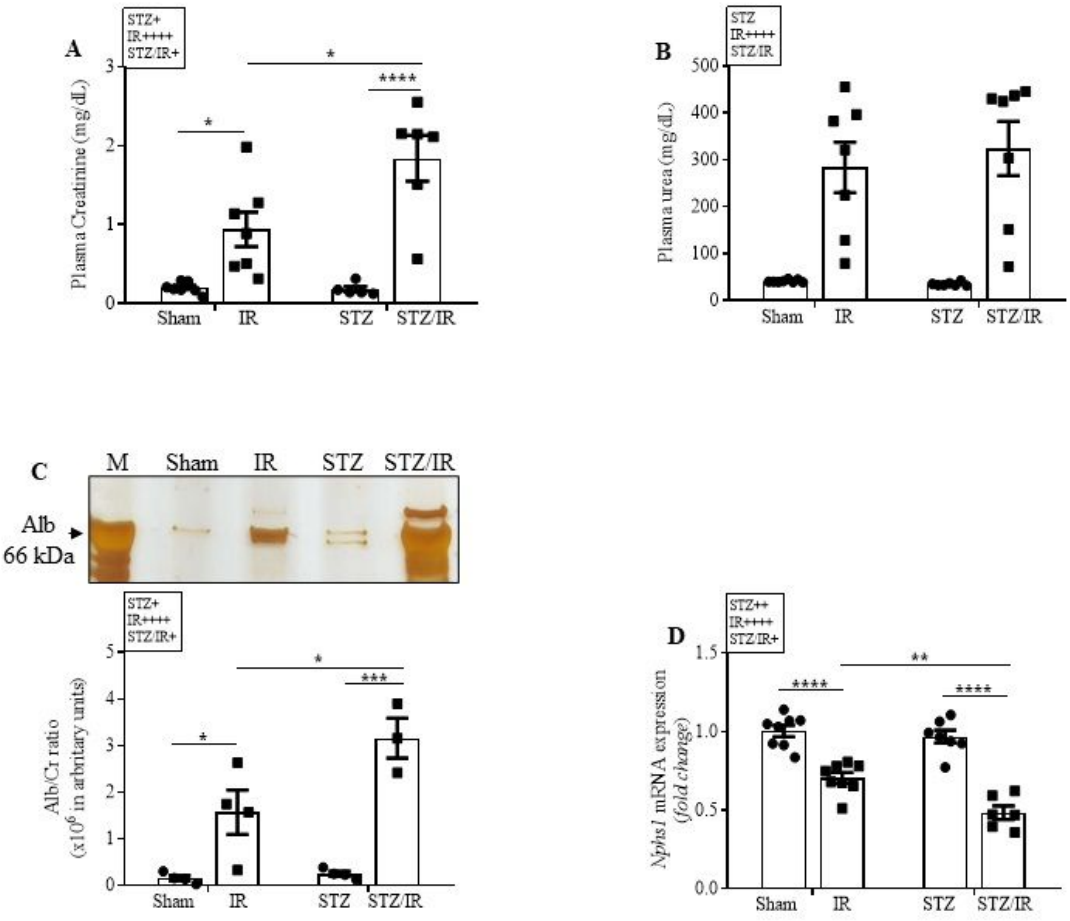

Figure 1

Effects of renal ischemia/reperfusion in control and STZ-treated mice on plasma levels of creatinine (A), urea (B), albuminuria (C), and mRNA expression of nephrin (D). +p<0.05, ++p<0.01, ++++p<0.0001 for diabetes (STZ), renal IR, and interaction between these two factors (STZ/IR), as indicated by two-way ANOVA. When the interaction was significant, the Bonferroni post-hoc test was performed: *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001. The values are the mean ± S.D. (n = 3-8). The samples were obtained from the same experiment. Full-length gel is presented in Supplementary Figure 1. IR: ischemia/reperfusion; Alb: albumin; M: marker; Cr: creatinine; Nphs1: nephrin.

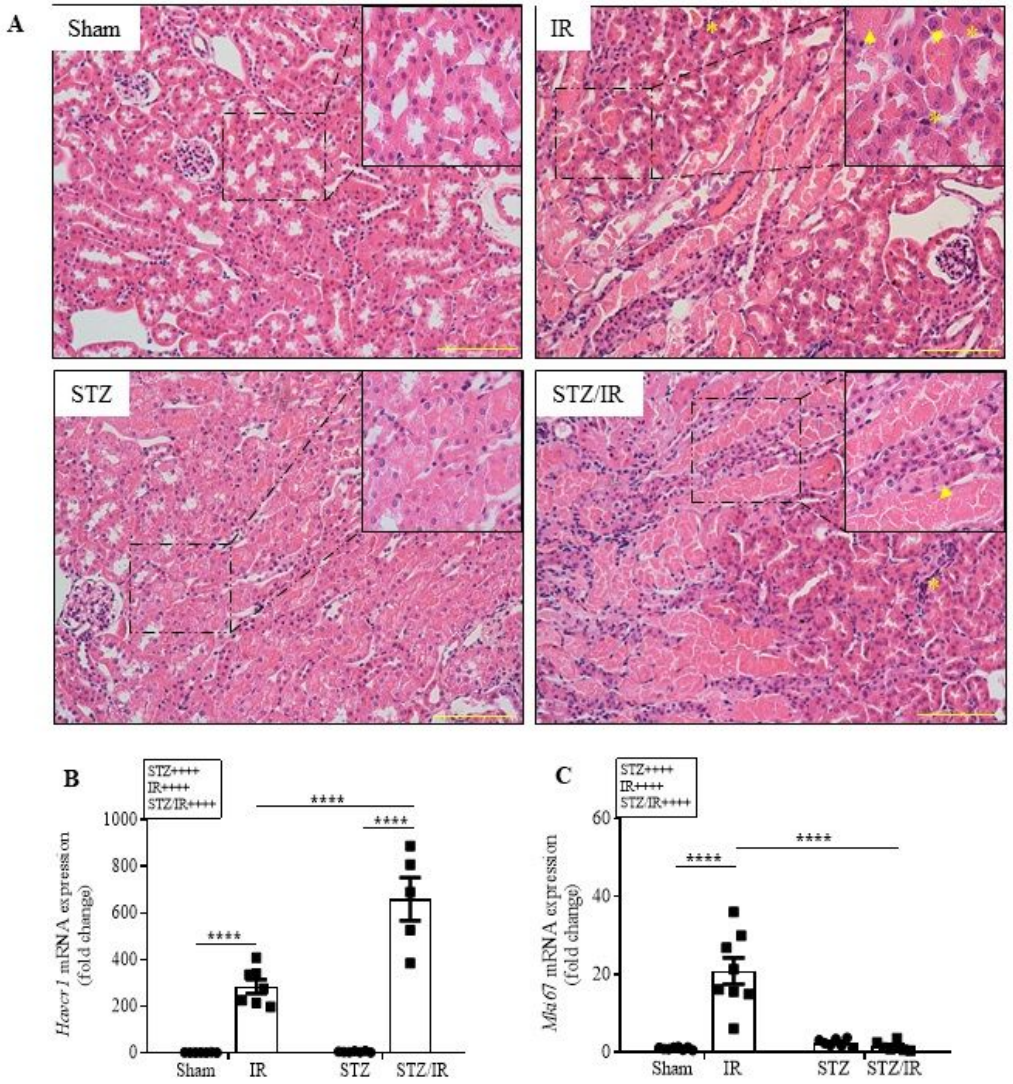

Figure 2

Effect of renal ischemia/reperfusion in control and STZ-treated mice on tubular injury (A), Kim-1 mRNA expression (B), and Ki67 mRNA expression (C). ++++p<0.0001 for diabetes (STZ), renal IR, and interaction between these two factors (STZ/IR), as indicated by two-way ANOVA. When the interaction was significant, the Bonferroni posttest was performed: ****p<0.0001. The arrows indicate the formation of intratubular cylinders, and the asterisks indicate interstitial infiltration. The values are the mean ± SD (n = 5-8). IR: ischemia/reperfusion; STZ, streptozotocin; Havcr1: Kim-1; Mki67: Ki-67. Bars, 100 µm.

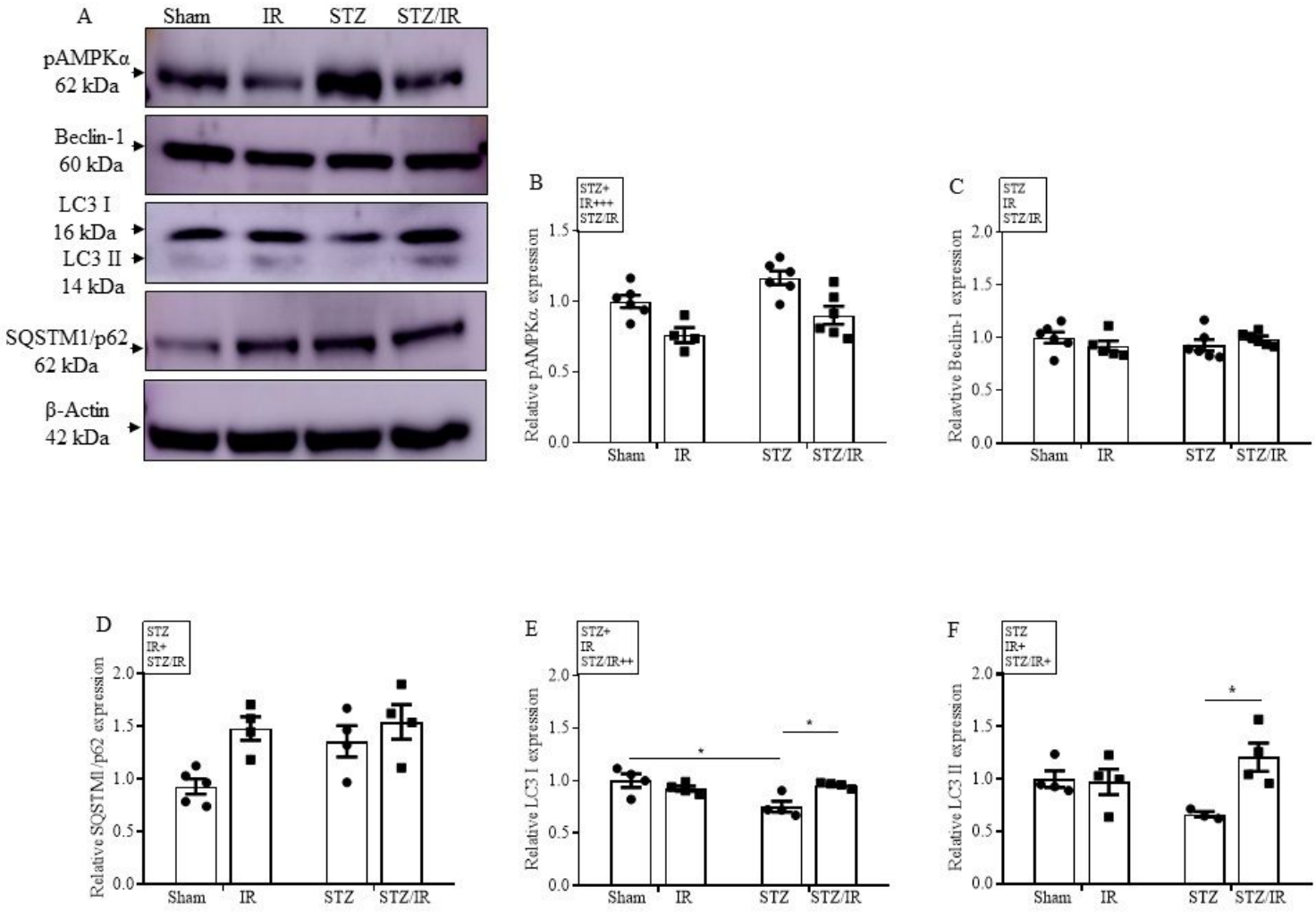

Figure 3

Effect of renal ischemia/reperfusion in control and STZ-treated mice on the protein expression (A) of phosphorylated AMPKα (B), beclin-1 (C), SQSTM1/p62 (D), LC3 I (E), and LC3 II (F). +p<0.05, ++p<0.01, +++p<0.001 for diabetes (STZ), renal IR, and interaction between these two factors (STZ/IR), as indicated by two-way ANOVA. When the interaction was significant, the Bonferroni post-hoc test was performed: *p<0.05. The values are the mean ± SD (n = 3-6). The samples were obtained from the same experiment and the blots were processed in parallel. Full-length blots are presented in Supplementary Figure 3 (A). IR: ischemia/reperfusion. β-actin: positive control.

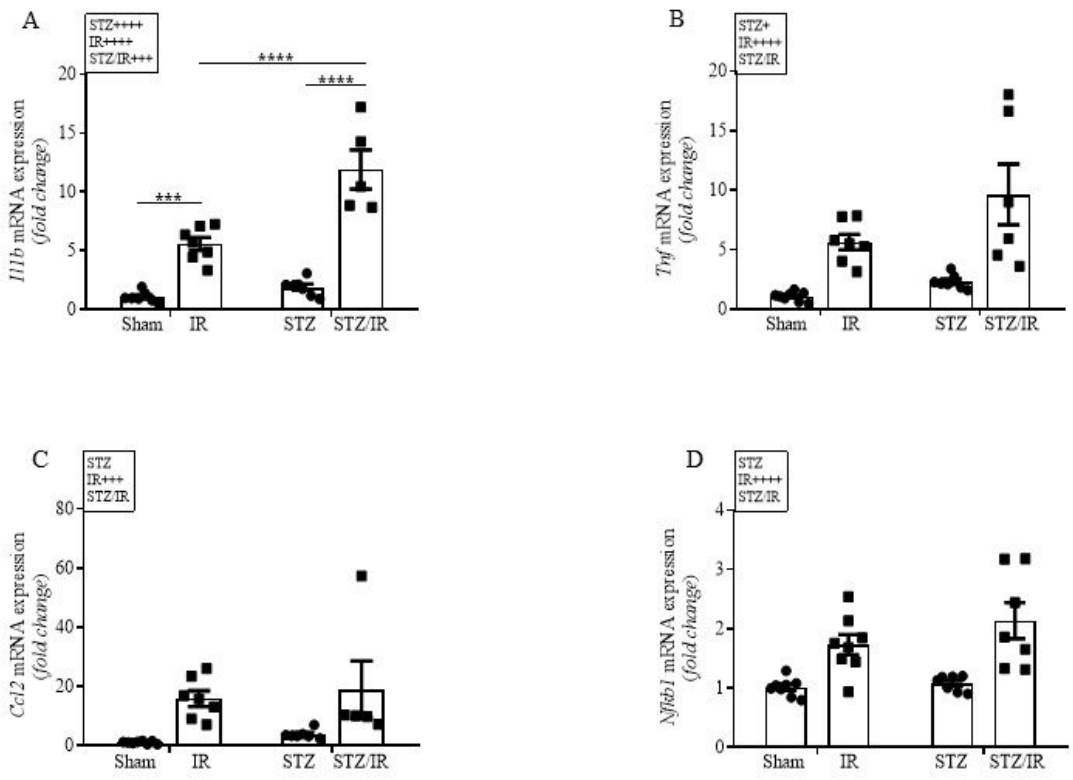

Figure 4

Effect of renal ischemia/reperfusion in control and STZ-treated mice on IL-1β (A), TNF-α (B), MCP-1 (C), and NFκB (D) mRNA expression. +p<0.05, +++p<0.001, ++++p<0.0001 for diabetes (STZ), renal IR, and interaction between these two factors (STZ/IR), as indicated by two-way ANOVA. When the interaction was significant, the Bonferroni post-hoc test was performed: ***p<0.001, ****p<0.0001. The values are the mean ± SD (n = 5-8). Il1b: interleukin 1 beta; Tnf: tumor necrosis factor alpha; Ccl2: monocyte chemoattractant protein-1; Nfkb1: nuclear factor kappa B 1; IR: ischemia/reperfusion.

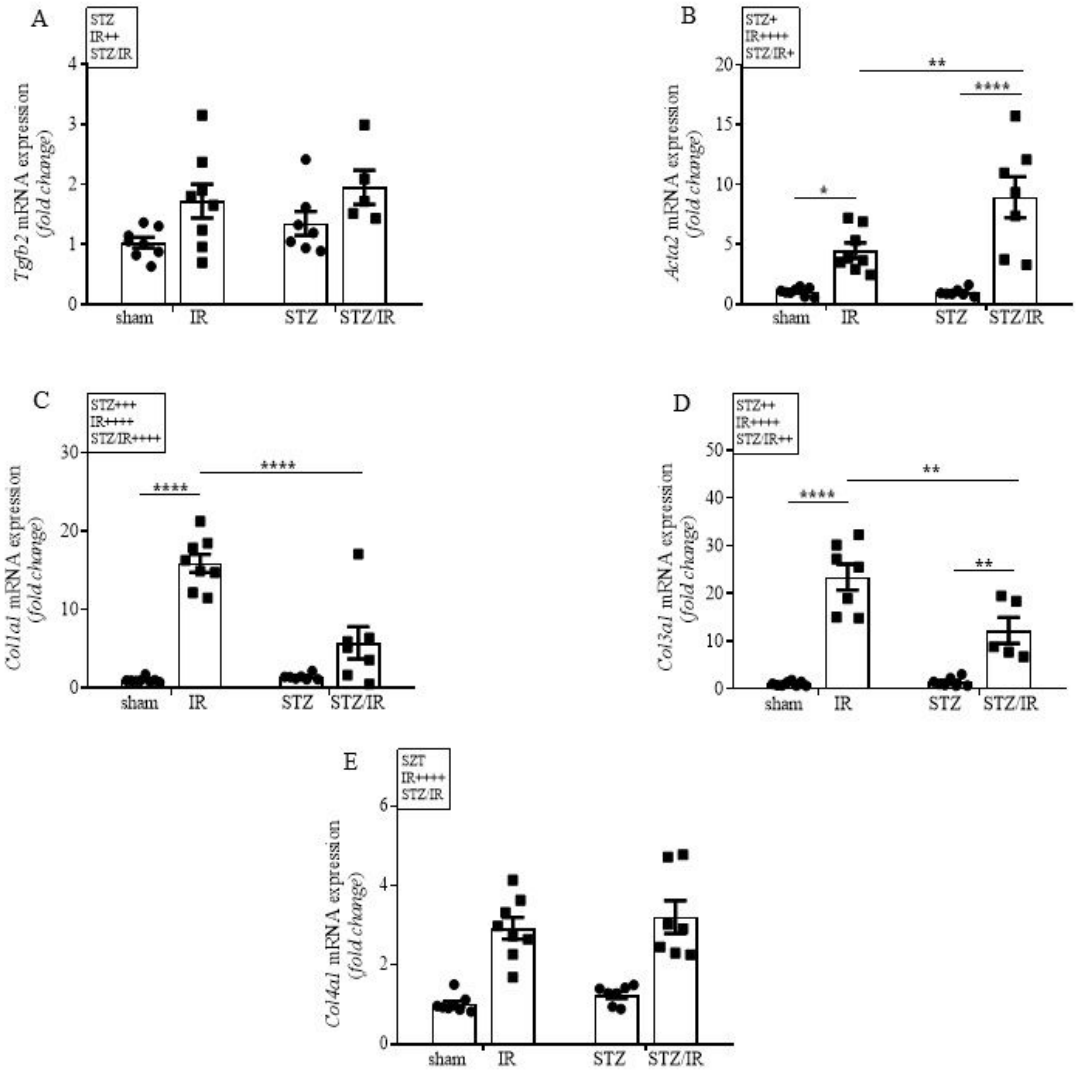

Figure 5

Effect of renal ischemia/reperfusion in control and STZ-treated mice on TGF-β2 (A), α-SMA (B), collagen I (C), collagen III (D), and collagen IV (E) mRNA expression. +p<0.05, ++p<0.01, +++p<0.001, ++++p<0.0001 for diabetes (STZ), renal IR, and interaction between these two factors (STZ/IR), as indicated by two-way ANOVA. When the interaction was significant, the Bonferroni post-hoc test was performed: *p<0.05, **p<0.01, ****p<0.0001. The values are the mean ± SD (n = 5-8). Tgfb2: transforming growth factor beta 2; Acta2: alpha smooth muscle actin; Col1a1: collagen type I; Col3a1: collagen type III; Col4a1: collagen type IV; IR: ischemia/reperfusion.

Supplementary Files

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