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TLR2 and TLR4 expression after kidney ischemia and reperfusion injury in mice treated with FTY720

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ABSTRACT

Ischemia and reperfusion injury (IR) is an antigen independent inflammatory process that causes tissue damage. After IR, kidneys up-regulate leukocyte adhesion molecules and toll-like receptors (TLRs). Moreover, injured kidneys can also secrete factors (i.e. heat shock protein) which bind to TLRs and trigger intracellular events culminating with the increase in the gene expression of inflammatory cytokines.

FTY720 is an immunomodulatory compound and protects at least in part kidneys submitted to IR. The mechanisms associated with FTY720's beneficial effects on kidneys after IR remain elusive. We investigated whether FTY720 administration in mice submitted to kidney IR is associated with modulation of TLR2 and TLR4 expression. C57BL/6 mice submitted to 30 min of renal pedicles clamp were evaluated for serum parameters (creatinine, urea and nitric oxide), kidney histology, spleen and kidney infiltrating cells expression of TLR2 and TLR4, resident kidney cells expression of TLR2 and TLR4 and IL-6 protein expression in kidney.

FTY720-treated mice presented decrease in serum creatinine, urea and nitric oxide, diminished expression of TLR2 and TLR4 both in spleen and kidney infiltrating cells, and reduced kidney IL-6 protein expression in comparison with IR non-treated mice. However, acute tubular necrosis was present both in IR non-treated and IR + FTY720-treated groups. Also, FTY720 did not prevent TLR2 and TLR4 expression in kidney resident cells.

In conclusion, FTY720 can promote kidney function recovery after IR by reducing the inflammatory process. Further studies are needed in order to establish whether TLR2 and TLR4 down regulation should be therapeutically addressed as protective targets of renal function and structure after IR.

1. Introduction

The pathogenesis of kidney ischemia and reperfusion injury (IR) involves a complex interaction of parameters including renal hemodynamics, tubular injury and inflammatory responses. After IR, kidneys are not merely passive targets of the immune-mediated injury but in turn they up-regulate leukocyte adhesion molecules and toll-like receptors. Toll-like receptors (TLRs) are a family of transmembrane proteins that in addition to binding to a range of microbial products can also recognize endogenous ligands termed danger-associated molecular patterns (DAMPs). TLR2 and TLR4 bind to heat shock proteins (HSP), high mobility group box 1 (HMGB1) and breakdown products of fibronectin, heparan sulfate and hyaluronic acid. After TLR activation an intracellular cascade of events occurs resulting in the release of NF-κB from IκB, allowing NF-κB translocation from cytoplasm to nucleus where it mediates the increase in inflammatory cytokine gene expression leading to pro-inflammatory responses [1,2].

In the kidney TLR2 and TLR4 are constitutively expressed in both proximal and distal tubules, the thin limb of the loop of Henle and the collecting ducts with their up regulation in these sites after IR [3].

Rats submitted to 45 minutes of renal pedicles clamping and evaluated 24 h later presented an increase of the HSP70 protein expression on renal tubular cells with this expression peaking on day 3. An increase in the mRNA expression for TLR2 and TLR4 was also found on day 3. This result was confirmed by immunohistochemistry with TLR2 and TLR4 expressed in the injured proximal tubules and thick ascending limbs [4]. These findings suggest that renal tubular cells are the main source of HSP70 in IR and can activate toll-like receptors which are also present in this site.

Tubular epithelial cells (TECs) from TLR2$^{-/-}$ mice submitted to *in vitro* IR produced significantly less IL-6 than TECs from wild type mice. These results were confirmed using IR *in vivo* model (45 min of renal pedicles clamping) showing that C57BL/6 wild type mice produced more IL-6 than TLR2$^{-/-}$ mice [5].

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These findings provide further knowledge of the primary mechanisms associated with renal IR and are essential for the development of therapeutic strategies. As an example, Liu et al. showed that the administration of Eritoran (TLR4 inhibitor) to mice submitted to kidney IR decreased serum creatinine, attenuated renal tissue damage and diminished the expression of IL-6 mRNA [6].

Our group showed previously [7,8] that FTY720 treatment attenuates kidney damage and decreases kidney infiltrating cells in C57BL/6 mice submitted to IR. Therefore, our aim in the present study was to investigate whether the protective effects on kidney after IR due to FTY720 administration are associated with the modulation of TLR2 and TLR4 expression.

2. Materials and methods

Eight- to 10-week-old male C57BL/6 mice were anesthetized with a subcutaneous injection of a ketamine/xylazine (Vetbrands, São Paulo, Brazil) preparation.

Through a midline abdominal incision, the left and right renal vessels were bluntly dissected and occluded with a nontraumatic vascular clamp for 30 min. After both clamps were released, kidneys showed immediate restoration of renal blood flow excluding the possibility of a vascular thrombus. Finally, the abdominal incision was sutured and animals were returned to their cages for 24 h.

After this period and under anesthesia, 1 mL of blood was obtained from the vena cava to determine plasma creatinine (Cr), urea (UR), nitric oxide (NO) and lymphocytes (%).

Spleen was removed for flow cytometry evaluation of TL2 and TLR4.

Kidneys were removed either for histology, flow cytometry (TLR2 and TLR4), immunohistochemistry or Western blot evaluations.

2.1. FTY720 treatment

Immediately before renal pedicles clamping, 1 mg/kg of FTY720 (Novartis, Switzerland) was injected intravenously in the treated group (IR + FTY) whereas the non-treated group (IR) was submitted to ischemia-reperfusion only.

2.2. Renal parameters

Blood was collected and centrifuged at 1500 rpm for 5 min for serum collection. Serum creatinine concentration was assessed using a colorimetric assay (Creatinina, Labtest Diagnóstica/Brazil) based on Jaffe's reaction. Serum urea concentration was measured using an enzymatic colorimetric assay (Uréia CE, Labtest Diagnóstica/Brasil). Absorbance measurements were obtained at Genesys 5 (Spectronic/Denmark).

2.3. Nitric oxide measurement

Peripheral blood samples from C57BL/6 mice were centrifuged and stored at −80 °C until analysis. The assay is based on the enzymatic conversion of nitrate to nitrite by nitrate reductase. Nitrite was measured in blood serum samples by enzyme linked immunosorbent assay (ELISA). Samples (50 μl) were added to a 96 well plate with 50 μl of Griess solution and the absorbance was measured in a TP reader (Thermo Plate) in 550 nm with the obtained values adjusted to the standard curve. All samples were measured in triplicate.

2.4. Blood smear

In order to determine the differential (neutrophils, monocytes, lymphocytes) percentage of circulating leukocytes we collected blood (10 μL) from the vena cava and dropped it toward one end of a slide and with a cover slip it was smeared back over the slide. A Panotico kit (Laborclin, Paraná, Brazil) was used for staining and leukocytes were identified and counted under the microscope.

2.5. Cell suspensions

Kidneys and spleens were disrupted by a syringe plunge in phosphate buffered solution (PBS). The resulting cell suspension was forced through a 70 μm cell strainer mesh (BD Falcon, USA) and collected in a 15 ml Falcon tube. From this cell suspension 5 μl was added to 95 μl of Trypan blue for cell viability counting in Neubauer chamber under a microscope. It was possible to observe that the cell suspension was composed mostly of leukocytes with occasional appearance of cell debris. After cell counting they were adjusted for 1×10^6/ml.

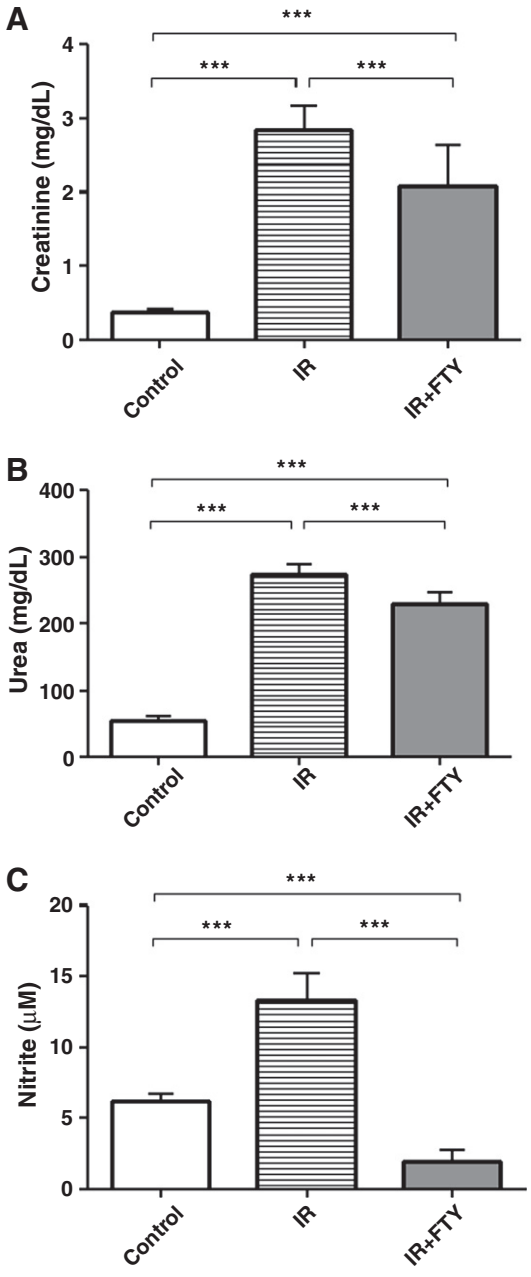

Fig. 1. Serum creatinine (A), serum urea (B) and serum nitrite (C) in Control, ischemia/reperfusion injury non-treated (IR) and FTY720-treated mice (IR + FTY). ***p < 0.0001.