

Inhibition of COX 1 and 2 prior to Renal Ischemia/Reperfusion Injury Decreases the Development of Fibrosis

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Ischemia and reperfusion injury (IRI) contributes to the development of chronic interstitial fibrosis/tubular atrophy in renal allograft patients. Cyclooxygenase (COX) 1 and 2 actively participate in acute ischemic injury by activating endothelial cells and inducing oxidative stress. Furthermore, blockade of COX 1 and 2 has been associated with organ improvement after ischemic damage. The aim of this study was to evaluate the role of COX 1 and 2 in the development of fibrosis by performing a COX 1 and 2 blockade immediately before IRI. We subjected C57Bl/6 male mice to 60 min of unilateral renal pedicle occlusion. Prior to surgery mice were either treated with indomethacin (IMT) at days -1 and 0 or were untreated. Blood and kidney samples were collected 6 wks after IRI. Kidney samples were analyzed by real-time reverse transcription-polymerase chain reaction for expression of transforming growth factor β (TGF- β), monocyte chemoattractant protein 1 (MCP-1), osteopontin (OPN), tumor necrosis factor α (TNF- α), interleukin (IL)-1 β , IL-10, heme oxygenase 1 (HO-1), vimentin, connective-tissue growth factor (CTGF), collagen I, and bone morphogenic protein 7 (BMP-7). To assess tissue fibrosis we performed morphometric analyses and Sirius red staining. We also performed immunohistochemical analysis of anti-actin smooth muscle. Renal function did not significantly differ between groups. Animals pretreated with IMT showed significantly less interstitial fibrosis than nontreated animals. Gene transcript analyses showed decreased expression of TGF- β , MCP-1, TNF- α , IL-1 β , vimentin, collagen I, CTGF, and IL-10 mRNA (all $P < 0.05$). Moreover, HO-1 mRNA was increased in animals pretreated with IMT ($P < 0.05$). Conversely, IMT treatment decreased osteopontin expression and enhanced BMP-7 expression, although these levels did not reach statistical significance when compared with control expression levels. The blockade of COX 1 and 2 resulted in less tissue fibrosis, which was associated with a decrease in proinflammatory cytokines and enhancement of the protective cellular response.

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INTRODUCTION

In renal ischemia and reperfusion injury (IRI), inflammation results in changes in the endothelial cell phenotype. These changes enhance leukocyte adhesion, entrapment, and transmigration, ultimately compromising the regional blood flow (1). In kidney transplantation, IRI is implicated as one of the potential contributors

to the development of chronic allograft nephropathy, now recognized as graft tubular atrophy/interstitial fibrosis, the main cause of graft loss after the first year of transplantation (2,3).

In the initial period of reperfusion, there is a rapid increase in proinflammatory cytokines, such as a tumor necrosis factor α (TNF- α) and interleukin (IL)-1 β ,

induced in part by infiltrating mononuclear and endothelial cells (4,5). There are also significant increases in the syntheses of leukotrienes and cyclooxygenase (COX) (3,6).

COX 1 and 2 can be detected in kidney tissue subjected to IRI, and recent data from our group and other investigators have shown that first these molecules were upregulated after IRI, and that their activation has a negative impact on organ function outcome (7,8). Indeed, COX blockade was associated with an improvement in renal function and a decrease in reactive oxygen species (9,10).

More importantly, the inflammation triggered by IRI is not restricted to the

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early days after the insult. Experimental data have demonstrated that neutrophil activation and CD4⁺ T-cell infiltration persisted 6 wks after the initial injury, culminating in tissue fibrosis (11,12). Normally, during the process of tubule-
interstitial remodeling, fibrosis results from an imbalance between the synthesis and the degradation of proteins of the extracellular matrix (13,14) with gradual loss of renal tubules and their function (15). Several cytokines participate in fibrogenesis, including transforming growth factor β (TGF- β), platelet-derived growth factor (PDGF), connective tissue growth factor (CTGF) (16,17), vimentin (18), collagen I (19–21), IL-1, fibroblast growth factor (FGF), osteopontin (OPN), endothelin, and TNF- α (22). Molecules such as heme oxygenase 1 (HO-1) and bone morphogenic protein-7 (BMP-7), however, are thought to balance the profibrotic effect of the former molecules.

In the current work, we hypothesized that COXs also contribute to this cascade of persistent inflammation. To address this question, we worked with an established model of unilateral IRI. Experimental animals were either pretreated with a COX inhibitor or not pretreated before being subjected to acute injury and followed for 6 wks after injury. We anticipated that the animals treated with the COX inhibitor would exhibit the lowest proinflammatory and profibrotic cytokine levels and have significantly less tissue fibrosis.

MATERIALS AND METHODS

Unilateral Renal IRI Model

Male C57Bl/6 mice, 10 wks of age (25–28 g), were purchased from the Animal Facilities Center from the Institute of Pharmacology at the Universidade Federal de São Paulo (São Paulo, Brazil). The mice were subjected to 60 min unilateral renal pedicle occlusion and were then monitored for 6 wks after ischemia. Animals were anesthetized with intraperitoneal ketamine and xylazine (100 mg/kg and 10 mg/kg, respectively; AgribRANDS do Brazil, São Paulo, Brazil).

After surgery, animals were housed in individual cages with free access to water and food. This project was approved by the Ethics Committee on Human and Animal Research of our institution.

Drug

Mice were treated with IMT (5 mg/kg/d) 1 d before and on the day of surgery (n = 8) or not treated (n = 8) (23,24). Sham-operated animals (n = 5) were used as controls.

Functional Analyses

Blood and kidney samples were collected 6 wks after unilateral IRI.

Assessment of Renal Function

Blood was collected for urea and serum creatinine measurements, and kidneys were harvested for histological and mRNA analyses at the end points. Urea was measured in duplicate by the Urease method and serum creatinine by Jaffe's method, using a Cobas Mira Plus auto-analyzer (Roche, Mannheim, Germany). The extent of fibrosis was determined by use of Masson Trichrome stain and performed by blinded morphometric assessment using a computer-assisted image system with an Olympus BX40F-3 microscope (Olympus Optical, Tokyo, Japan) and the software program Image Pro Plus for Windows 3.0 (Media Cybernetic, Silver Spring, MD, USA).

Gene Profile

Kidney samples were quickly frozen in liquid nitrogen. Total RNA was isolated from kidney tissues using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) methodology. RNA concentration was determined by spectrophotometer readings at an absorbance of 260 nm. First-strand cDNAs were synthesized using moloney murine leukemia virus reverse transcriptase (Promega, Madison, WI, USA). Reverse transcription–polymerase chain reaction (PCR) was performed using the SYBR Green real-time PCR assay (Applied Biosystems, Foster City, CA, USA) and specific primers for monocyte chemoattractant protein (MCP)-1 (sense:

5'-ACT GCA TCT GCC CTA AGG TCT TCA-3', antisense: 5'-AGA AGT GCT TGA GGT GGT TGT GGA-3'); *IL-1 β* (sense: 5'-CCT AAA GTA TGG GCT GGA CTG TTT-3', antisense: 5'-TAG AGA TTG AGC TGT CTG CTC ATT C-3'); *TGF- β* (sense: 5'-AAC TAT TGC TTC AGC TCC AGA GAG A-3', antisense: 5'-AGT TGG ATG GTA GCC CTT G-3'); and *OPN* (sense: 5'-GGC ATT GCC TCC TCC CTC-3', antisense: 5' CGA GGC TGT AAA GCT TCT CC-3'). We used a TaqMan real-time PCR assay for *TNF- α* (Mm00443258_m1), *IL-10* (Mm01288386_m1), *BMP-7* (Mm00432102_m1), *HO-1* (Mm00516007_m1) (a protective gene, *vimentin* (Mm01333430_m1), *CTGF* (Mm00515790_g1), and *collagen I* (Mm01302047_g1). *Hypoxanthine phosphoribosyltransferase (HPRT)* was used as a housekeeping gene. PCR data were presented as a ratio of genes to HPRT mRNA (sense: 5'-CTC ATG GAC TGA TTA TGG ACA GGA C-3' and antisense: 5'-GCA GGT CAG CAA AGA ACT TAT AGC C-3' and Mm03024075_m1) in arbitrary units (AU).

Immunohistochemical Analysis: Smooth Muscle Cell Actin Staining

Paraffin block sections (5 μ m) were placed on poly-L-lysine-coated slides. After the sections were dried in an oven for 1 h at 60°C, they were dewaxed in xylol for 15 min and hydrated in 3 alcohols (100% for 9 min, 95% for 4 min, and 70% for 2 min). Subsequently, sections were incubated for 10 min in 3% hydrogen peroxide to block endogenous peroxidase. For antigen retrieval, after being washed with phosphate-buffered saline (PBS), each section was unspecifically blocked. Sections were then incubated overnight at 4°C with anti-actin smooth muscle (α -SMA; Calbiochem, Schwalbach, Germany). After a new wash with PBS for 9 min, the sections were incubated in a wet chamber for 30 min with a secondary goat anti-mouse antibody (NCL-GAMP; NovoCastra, Norwell, MA, USA) and washed with PBS. Endogenous peroxidase activity

was detected by reacting fixed tissue sections with 3,3'-diaminobenzidine substrate plus 10% hydrogen peroxide for 10 min. After being washed with distilled water, the samples were stained with hematoxylin for 10 s. Dehydration was performed with 3 alcohols (70% for 1 min, 95% for 2 min, and 100% for 6 min) and with xylol for 9 min. All quantification was performed blinded. α -SMA staining was calculated as the percentage of renal cortex and medulla. We used computer image analysis (KS300, Zeiss system) to measure the percentage of area occupied by α -SMA staining per 4 mm² field at a magnification of 200 and calculate the mean percentage area for each biopsy (25).

Sirius red staining. Four micra formalin fixed slides were stained with Sirius red (F3BA CI 35782; Sigma, St. Louis, MO, USA) 0.1% in saturated picric acid for 60 min at room temperature. Slides were washed twice for 5 min in 0.5% glacial acetic acid and counterstained with Harris hematoxylin. Fibrosis was calculated as the percentage of renal cortex and medulla. We used computer image analysis (KS300, Zeiss system) to measure the percentage of area occupied by Sirius red staining per 4 mm² field at a magnification of 200 and calculate the mean percentage area calculated for each sample.

Statistics

Data were graphically displayed as mean and standard deviation (SD) or as median (histomorphometric analyses) owing to the Gaussian distribution of variables. Mann-Whitney *U* test, Student *t* test, and ANOVA on ranks tests were used to compare the data. PCR data were presented as a ratio of analyzed genes to HPRT mRNA, in arbitrary units (AU). Significant difference was considered when $P < 0.05$.

RESULTS

Long-term Renal Function Outcomes

Urea and creatinine levels were measured at 6 wks following IRI in animals treated with IMT or otherwise. In this

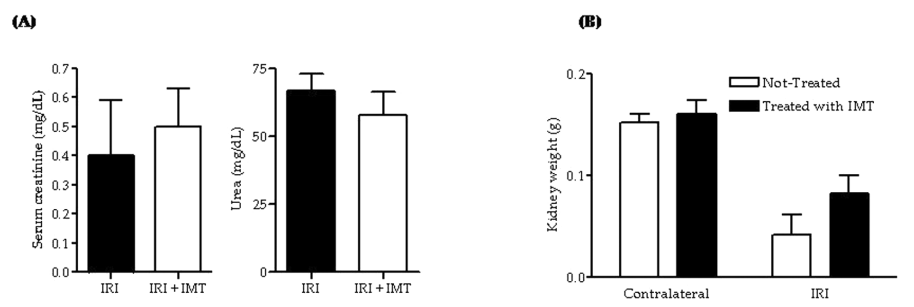


Figure 1. Renal function and renal fibrosis after unilateral IRI. (A) Blood urea and serum creatinine levels at 6 wks after 60 min of unilateral renal ischemia, in animals treated with IMT or not treated. There were no significant differences between groups ($P > 0.05$). (B) Kidney weights (in grams) at 6 wks after 60 min of unilateral renal ischemia, in animals treated with IMT or not treated. There were no significant differences between groups ($P > 0.05$).

model, renal function was not altered significantly and the previous treatment with IMT did not change these results (Figure 1A). The ischemic kidneys weighed significantly less than the contralateral nonischemic ones, but the IMT pretreatment did not change this relationship (Figure 1B). Therefore, we performed morphometric analyses to quantify tissue fibrosis at this time. Interestingly, animals treated with IMT prior to surgery had less fibrosis compared with nontreated animals (Figure 2). Sham-operated animals presented basal levels similar to normal non-manipulated animals (data not shown).

Cytokine Profiles

As previously seen in renal IRI, as early as 24 h after reperfusion there is strong amplification of proinflammatory cytokines such as IL-1 β , TNF- β , and interferon γ . Data

from our laboratory and other groups have demonstrated that the Th1 immune response is deleterious to renal tissue after IRI (26,27). Therefore, we investigated the profile of inflammatory cytokines in ischemic kidneys treated with IMT or otherwise. To compare these results, we also amplified the transcripts in the contralateral kidneys, as controls.

Ischemic kidneys presented a proinflammatory phenotype evidenced by the higher expression of MCP-1, IL-1 β , and TNF- α (Figure 3). Values for the expression of these cytokines were even higher when we compared them with values for the contralateral kidneys. Interestingly, animals pretreated with IMT showed a significant decrease in mRNA expression consistent with decreased cytokine levels, even in contralateral counterparts.

We also measured IL-10, an anti-inflammatory cytokine, and HO-1, con-

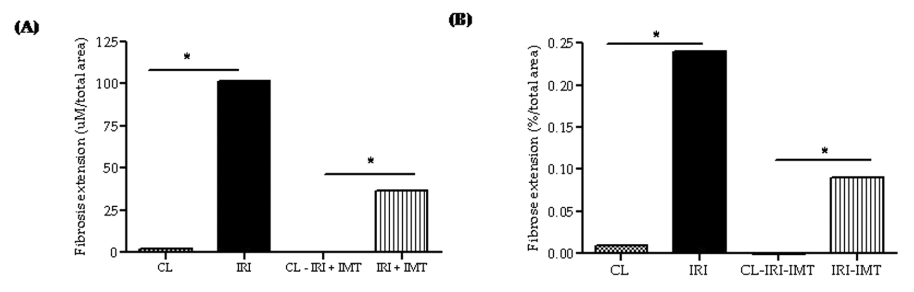


Figure 2. Morphometric analyses at 6 wks after unilateral IRI. (A) Histological analyses of kidneys (contralateral (CL) and ischemic kidneys) were performed at 6 wks after 60 min of unilateral renal ischemia in animals not treated or treated with IMT. * $P < 0.05$.

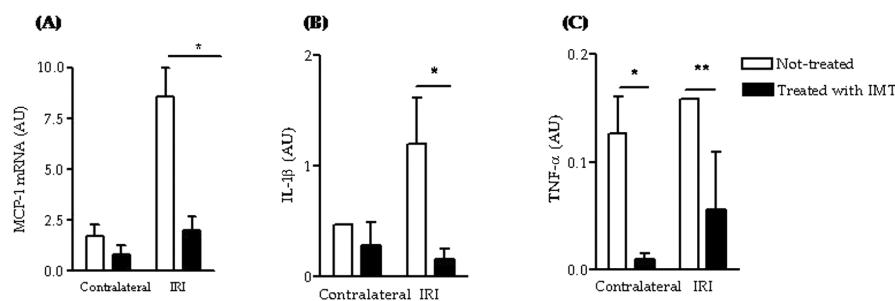


Figure 3. Proinflammatory cytokine profile at 6 wks after unilateral IRI. mRNA levels of MCP-1 (A, $*P < 0.05$), IL-1 β (B, $*P = 0.013$), and TNF- α (C, $*P = 0.029$ and $**P = 0.028$) in contralateral and ischemic kidneys in animals treated or not with IMT. mRNAs were standardized for HPRT mRNA and were expressed as AU.

considered a protective gene. IMT treatment decreased IL-10 expression compared with nontreatment (Figure 4A). Taking into consideration HO-1 expression, as previously described in acute renal injury, IMT treatment resulted in an impressive amount of HO-1 upregulation in this chronic model (Figure 4B).

Fibrotic Changes after IMT Treatment

Pro- and antifibrotic factor expression can change during the course of renal IRI. We therefore studied the expression of these molecules in mice at 6 wks after ischemia. We analyzed TGF- β , OPN, vimentin, CTGF, and collagen I. These molecules have previously been shown to be upregulated in fibrosis (28–31). In our model, we observed an increase of mRNA for TGF- β , OPN, vimentin, collagen I, and CTGF in ischemic kidneys

compared with contralateral kidneys. After IMT treatment, these increased levels were less evident, but reached statistical significance for expression of TGF- β , vimentin, collagen, and CTGF (Figure 5). The effect of IMT went beyond growth factor expression; compared with nontreated ischemic animals, treated animals showed increases in α -SMA staining (Figure 6A) and mirrored upregulation in BMP-7 expression, although these results were not statistically significant ($P > 0.05$) (Figure 5C). These quantitative immunohistochemical analyses of α -SMA-positive cells corroborated the effects of COX inhibition on renal tissue fibrosis seen in gene transcript analyses (Figure 6A). Furthermore, IMT-treated animals presented significantly less positive Sirius-red staining than nontreated animals (Figure 6B).

DISCUSSION

IRI is the foremost antigen-independent factor related to the development of chronic allograft nephropathy, the leading cause of graft loss after the first year of renal transplantation. The inflammation triggered by IRI, especially in the tubule-
interstitial compartment, results in fibrosis that is detectable in long-term follow-up (11). The development of alternative strategies to counteract the development of fibrotic tissue is essential for improving survival of patients who receive transplants from deceased individuals.

In this study, we aimed to demonstrate that blockade of the COX pathway could have a beneficial effect in preventing the development of fibrosis after IRI. Of note, we performed the COX blockade prior to acute renal injury. We have previously demonstrated that a single episode of severe unilateral warm ischemic injury to kidney is associated with significant long-term kidney inflammation and development of tissue fibrosis. These data are in accordance with the previously reported finding that a single ischemic episode can lead to maintenance of the inflammatory state, attributable mainly to neutrophils and CD4⁺ T-cell infiltration. We used a model of severe ischemia to mimic the long-term effects of ischemia seen in recipients of kidney transplants from deceased individuals (32). Lower degrees of ischemia are less likely to be associated with clinically significant long-term problems.

The extension of tissue damage did not alter renal function but did decrease organ size. The histological studies corroborated this finding. At this point, the IMT treatment had no effect on organ size but had a great impact on the development of fibrosis. Histomorphometric analyses showed that less fibrosis was seen in IMT-treated animals. These results indicate that early inflammation induced by ischemic stress leads to long-term fibrosis development, as previously demonstrated (11), and more importantly, the blockade of this early-onset inflammation is beneficial in halting this process.

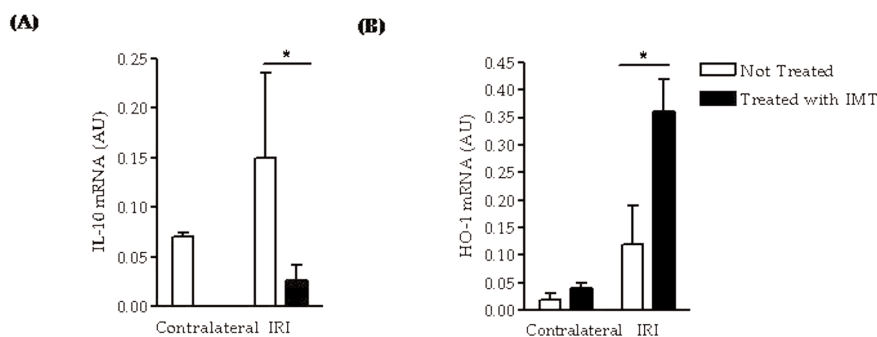


Figure 4. Anti-inflammatory profile at 6 wks after unilateral IRI. mRNA levels of IL-10 (A, $*P = 0.03$) and HO-1 (B, $*P < 0.05$) in contralateral and ischemic kidneys in animals treated or not with IMT. mRNA was standardized for HPRT mRNA and expressed as AU.

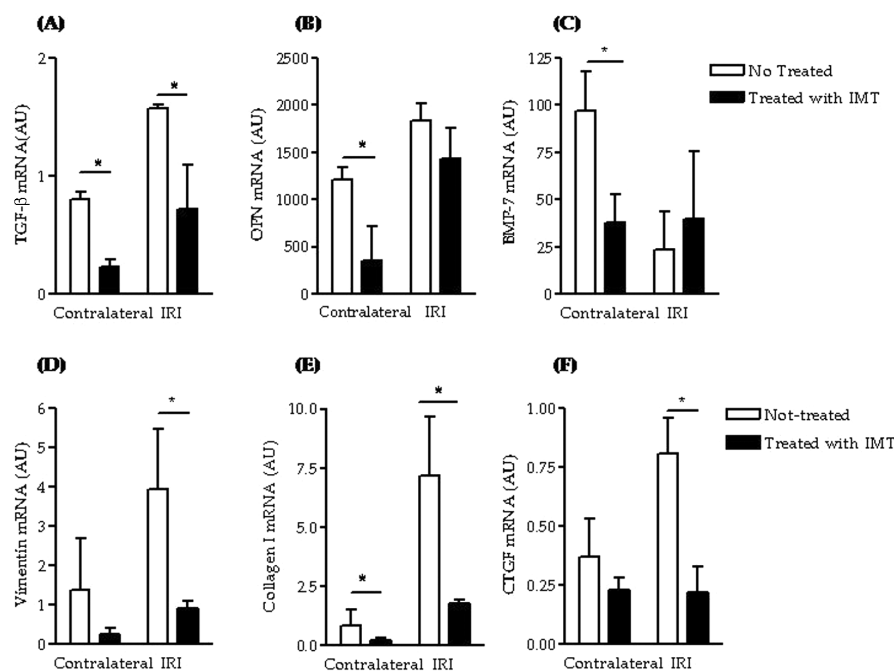


Figure 5. Pro- and antifibrotic profile at 6 wks after unilateral IRI. mRNA levels of TGF- β (A, $*P < 0.05$), OPN (B, $*P < 0.05$), BMP-7 (C, $*P < 0.05$), vimentin (D, $*P < 0.05$), collagen I (E, $*P < 0.05$), and CTGF (F, $*P < 0.05$) in contralateral and ischemic kidneys in animals treated or not with IMT. mRNA was standardized for HPRT mRNA and expressed as AU.

Because inflammation seems to be linked with fibrogenesis in our model, we opted to quantify a broad range of proinflammatory cytokines/growth factors and of BMP-7, HO-1, and IL-10, molecules that can counteract the actions of proinflammatory cytokines/growth factors.

It is well established that fibrogenesis results from an imbalance between growth factors and cytokines such as TGF- β , PDGF, CTGF, collagen I, OPN, FGF, IL-1 α and -1 β , and BMP-7 (16–21, 33–35). Proximal epithelial cells as well as infiltrating cells may produce these molecules, stimulated by the reactive oxygen species secreted by the injured cells (36,37). Vimentin has also been linked to fibrogenesis in the kidney (18) and to epithelial-to-mesenchymal transition (EMT) (38,39). These injured proximal tubular cells seem to release chemotactic factors, such as MCP-1, which promotes leukocyte attachment to tubular lumen and infiltration into the interstitium, amplifying this inflam-

atory cascade (40). Gloria and colleagues have demonstrated in an *in vitro* cell model of gaseous hypoxia that COX blockade decreased the expression of RANTES (regulated on activation normal T-cell expressed and secreted), another chemokine involved in leukocyte transmigration (41). Our data demonstrated that the animals pretreated with IMT presented less MCP-1 expression, a finding that supports this hypothesis.

Pretreatment with IMT significantly decreased the levels of proinflammatory cytokines, including IL-1 β and TNF- α . The production of IL-1 β by the glomerular cells is increased in several types of clinical and experimental glomerulopathy in which fibrosis occurs. IL-1 β stimulates the proliferation of fibroblasts and possibly the synthesis of extracellular matrix proteins, and it also has a chemotactic effect. Importantly, treatment with IL-1 β antagonists reduces fibrosis in rats with glomerulonephritis (42). Furthermore, Stanimirovic *et al.* demonstrated

that in cerebral IRI COX inhibition by IMT reduced neutrophil adhesion and completely inhibited IL-1 β mRNA expression (43). Thus, we can infer that the decreased extension of fibrosis may be partly attributable to decreased expression of these molecules owing to the action of IMT.

When we analyzed the profile of pro- and antifibrotic molecules, we observed that after 6 wks of reperfusion ischemic kidney tissue exhibited increased expression of profibrotic factors, namely TGF- β , vimentin, collagen I, CTGF, and OPN. The effect of IMT on TGF- β expression is important given the critical role of TGF- β in renal fibrosis. The EMT (44) may be attributable to TGF- β inducing myofibroblast transformation and increasing collagen synthesis. The present study demonstrated that COX inhibition can actually decrease TGF- β expression, a decrease associated with signs of decreased tissue fibrosis. Additional studies should be carried out to ascertain whether COX accelerates EMT. The expression of OPN, collagen I, and α -SMA were in accordance with that of TGF- β . α -SMA is expressed during EMT and is a marker of “activated” fibroblasts. Therefore, IMT could decrease the fibrosis by reducing these cells in the interstitium. The reduction in OPN expression indicates, beyond the reduction of fibrosis, lower macrophage infiltration, because OPN is considered one of the chemokines that attracts macrophages into the injured renal tissue (45). Macrophage infiltration correlates with the development of fibrosis (46–49). Finally, TGF- β signaling is regulated by BMP-7, also a member of the TGF- β family, through activation of Smad-6. This regulation can inhibit EMT (50).

Vimentin is a transitional filament protein mainly expressed in mesenchymal-derived cells. It can be reexpressed in renal tubular epithelial cells under many pathological conditions such as ischemia. Terzi and colleagues demonstrated that vimentin expression is up-regulated after renal ischemia in proximal tubular cells (51). CTGF seems

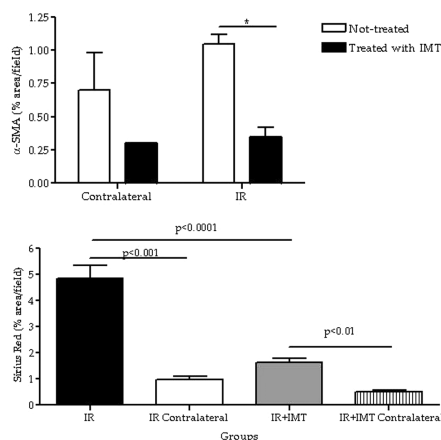


Figure 6. Immunohistochemical analyses of α -SMA and Sirius red staining. (A) Quantification of α -SMA-positive cells in medullar renal tissue 6 wks after 60 min of unilateral ischemia by immunohistochemical analysis (* $P < 0.05$). (B) Sirius red staining of kidneys (contralateral and ischemic kidneys) was performed 6 wks after animals were subjected to 60 min of unilateral renal ischemia and not treated or treated with IMT.

to be responsible for some TGF- β profibrotic activities (52). Nguyen and Goldschmeding have recently proposed that CTGF inhibition or BMP-7 induction could be a future therapy for renal fibrosis (52). Nguyen and Goldschmeding showed that BMP-7 expression was diminished in the ischemic kidneys compared with the contralateral kidneys and that treatment with IMT reversed BMP-7 expression, albeit without reaching statistical significance, and that IMT significantly decreased CTGF expression. These results could be promising because we have already observed that in animals treated with rapamycin and then subjected to unilateral ureteral obstruction, a model of renal fibrosis, the increased expression of BMP-7 was associated with less renal fibrosis (53). The blockade of CTGF has also been showed to be protective in reducing fibrosis in a unilateral ureteral obstruction model (54).

The action of proinflammatory cytokines could also be antagonized by favoring a Th2 immune response or by enhancing innate cellular protective gene machinery. We analyzed the expression

of IL-10, an anti-inflammatory cytokine, and HO-1, a protective gene. Some studies have shown that the increase of IL-10 expression diminishes intercellular adhesion molecule 1 and selectins (55,56), which are both responsible for neutrophil rolling in the endothelium, protecting the kidney from IRI. Furthermore, the inhibition of these molecules by chemical drugs or in knockout animals is associated with tissue protection (57). Consequently, we investigated whether treatment with IMT would have an influence on expression of HO-1 and IL-10. We observed an increase of HO-1 in the ischemic kidneys; however, this increase was visibly higher when the animals were treated with IMT. These data corroborate our observation that pretreatment with IMT in animals was associated with a sustained increase in HO-1 following an acute renal injury (3). Conversely, IL-10 expression was increased in ischemic kidneys but was reduced when the animals were pretreated with IMT. Although IL-10 and HO-1 can inhibit proinflammatory genes associated with activation of monocytes/macrophages, such as TNF- α , IL-1- β , and granulocyte-macrophage-colony-stimulating factor, in some specific models the anti-inflammatory effects of HO-1 seem to be independent of those of IL-10. Prostaglandin E2 can also have anti-inflammatory properties through induction of IL-10 (58,59), and the data expression found with IL-10 could be a reflection of an already protective response initiated by the organ after the initial injury. This response, however, is not strong enough to halt fibrosis. In this sense, the protection seen with IMT seems to be HO-1 dependent and IL-10 independent.

Recent data have established that IRI is an inflammatory syndrome and, more importantly, that the inflammatory response of IRI is persistent, culminating in the development of fibrosis. Renal fibrosis is the final pathway of many diseases, and no known treatment can halt or regress it. Our results demonstrated that COX 1 and 2 play an important role in the development of sustained inflamma-

tion. Their blockade prior to an acute injury could be beneficial in limiting the renal damage caused by fibrosis.

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DISCLOSURE

The authors have no conflicts of interest.

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