Effect of ischemic acute renal damage on the expression of COX-2 and oxidative stress-related elements in rat kidney

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Villanueva S, Céspedes C, González AA, Vio CP, Velarde V. Effect of ischemic acute renal damage on the expression of COX-2 and oxidative stress-related elements in rat kidney. Am J Physiol Renal Physiol 292: F1364–F1371, 2007. First published January 23, 2007; doi:10.1152/ajprenal.00344.2006.—Acute renal failure (ARF) is a clinical syndrome characterized by deterioration of renal function over a period of hours or days. The principal causes of ARF are ischemic and toxic insults that can induce tissue hypoxia. Transcriptional responses to hypoxia can be inflammatory or adaptive with the participation of the hypoxia-inducible factor 1α and the expression of specific genes related to oxidative stress. The production of peroxynitrites and protein nitrotyrosylation are sequela of oxidative stress. In several clinical and experimental conditions, inflammatory responses have been related to cyclooxygenase (COX)-2, suggesting that its activation might play an important role in the pathogenesis and progression of nephropathies such as ARF. In the kidney, renin and bradykinin participate on the regulation of COX-2 synthesis. With the hypothesis that in ARF there is an increase in the expression of agents involved in adaptive and inflammatory responses, the distribution pattern and abundance of COX-2, its regulators renin, kalikrein, bradykinin B2 receptor, and oxidative stress elements, heme oxygenase-1 (HO-1), erythropoietin (EPO), inducible nitric oxide synthase (iNOS), and nitrotyrosylated residues were studied by immunohistochemistry and immunoblot analysis in rat kidneys after bilateral ischemia. In kidneys with ARF, important initial damage was demonstrated by periodic acid-Schiff staining and by the induction of the damage markers α-smooth muscle actin and ED-1. Coincident with the major damage, an increase in the abundance of EPO, HO-1, and iNOS and an increase in renin and bradykinin B2 receptor were observed. Despite the B2 receptor induction, we observed an important decrease in COX-2 in the ischemic-reperfused kidney. These results suggest that COX-2 does not participate in inflammatory responses induced by hypoxia.

cyclooxygenase-2; acute renal failure

ACUTE RENAL FAILURE (ARF) is a clinical syndrome that is associated with high morbidity and mortality rates (30). ARF has an initiating phase, characterized by a reduced renal blood flow, that causes epithelial and vascular cell injury and a rapid decrease in glomerular filtration rate; the injury disrupts the ability of renal tubular epithelial cells and renal vascular endothelial cells to maintain normal renal function, initiating signaling cascades that contribute to inflammation and organ dysfunction (34). The initiating phase is immediately followed by an extension phase, which is characterized by the following two major events: persistent hypoxia and inflammatory response. During the extension phase, multiple interrelated processes exacerbate epithelial and endothelial cell injury and cell death, primarily in the corticomedullary region of the kidney (20). The maintenance phase is a period of injury stabilization during which corrective events facilitate cellular repair, division, and redifferentiation (34).

Acute tubular necrosis (ATN) with prerenal disease is the most common cause of ARF, accounting for two-thirds of intrinsic causes (8). The major cause of ATN is hypoxia induced by ischemia-reperfusion (I/R), which can be induced by clinical conditions, such as hemorrhagic shock or sepsis (18). Hypoxia could increase oxidative stress in acute renal I/R (24). Several data show that hydroxyl radical-like activities are generated from peroxynitrites. This latter compound is emerging as one of the important sequela of oxidative and nitrosative stresses. Peroxynitrite is produced by the reaction between nitric oxide (NO) and superoxide. In addition, transcriptional responses to hypoxia can be adaptive or inflammatory (36). Adaptive responses are controlled primarily through the nuclear accumulation of the heterodimeric hypoxia-inducible factor (HIF-1α) that regulates the expression of a number of adaptive genes coding for angiogenic, glycolytic, and other proteins, supporting tissue survival in hypoxia (6). These target genes, particularly erythropoietin (EPO) and heme oxygenase-1 (HO-1), are central to both local and systemic responses to hypoxia and to cellular responses to altered glucose, energy metabolism, and probably oxidative stress.

Westenfelder et al. (41) have demonstrated that the EPO receptor is expressed throughout the kidney, particularly in tubular epithelial cells, mesangial cells, and the glomerulus. In addition, it has been demonstrated that EPO stimulates endothelial cell mitogenesis and angiogenesis (3), which improve tissue oxygenation. This evidence supports a role for EPO in protecting the kidney against I/R injury.

On the other hand, HO-1 is part of the integrated response to oxidative stress. The expression of this protein increases in inflammatory cells and may be associated with the resolution phase of acute inflammation. In various models of oxidative tissue injuries, the induction of HO-1 confers protection from further damages by removing the prooxidant heme group or by virtue of the antioxidative, anti-inflammatory, and/or antiapoptotic actions of one or more of the products of heme metabolism, i.e., carbon monoxide, biliverdin, bilirubin, and iron by the HO reaction (13). However, the levels, together with the localization of EPO and HO-1, have not been evaluated in any I/R model.

The kidney produces several vasoactive substances that play important roles in the maintenance of renal blood flow and glomerular filtration. The renin-angiotensin system (RAS), with the production of ANG II, has important vasoconstrictor, antinatriuretic, and antidiuretic effects. On the other hand, the...
kallikrein-kinin system (KKS), with the production of bradykinin (BK), induces vasodilation, natriuresis, and diuresis. BK effects are mediated by prostaglandins derived from cyclooxygenases (COX). COX-1 is found in the glomerulus and afferent arteriole, whereas COX-2 is expressed in podocytes, thick ascending limb of the loop of Henle, macula densa, and afferent arteriole. Upregulation of COX-2 has been described in several clinical and experimental conditions characterized by inflammation, suggesting that activation of COX-2 might play an important role in the pathogenesis and progression of nephropathies, such as ARF. In addition, although pharmacological inhibition of COX-2 can protect from I/R (9), it can also lead to serious adverse events in kidney, including ARF and hyperkalemia (5). We hypothesize that in a model of ARF induced by I/R, which is characterized by a brief hypoxic condition, the levels of several vasoactive agents found in the kidney are altered because of a decrease in kallikrein levels even after several days of reperfusion. This would be associated with an increase in RAS and with a specific regulation of COX-2 and nitric oxide synthase (NOS). For this reason, we analyzed the relative abundance of COX-2, inducible nitric oxide synthase (iNOS), renin, kallikrein, and related elements of oxidative stress at different time periods after reperfusion.

**MATERIALS AND METHODS**

**Animals.** Adult male Sprague-Dawley rats (220–250 g, n = 7 for each I/R group; 24, 48, 72, and 96 h; n = 5 for each sham-operated group) were housed in a 12:12-h light-dark cycle. Control and treated animals were weighed at the time of initiation of bilateral ischemic injury and after completion of experiments. The animals had food and water ad libitum and were maintained at the university animal care facilities. All experimental procedures were in accordance with institutional and international standards for the humane care and use of laboratory animals (Animal Welfare Assurance Publication A5427-01, Office for Protection from Research Risks, Division of Animal Welfare, National Institutes of Health). In this study, the protocol for the use of animals was reviewed and approved by the institutional and independent ethical committee of the Pontificia Universidad Católica de Chile.

**Renal I/R injury.** An established model of renal I/R injury that resembles structural and functional consequences of renal ischemia, including apoptotic tubular epithelial cells was performed (4). Animals were anesthetized with ketamine-xylazine (25:2.5 mg/kg ip), and body temperature was maintained at 37°C. Both kidneys were exposed by a flank incision, and both renal arteries were occluded with a nontraumatic vascular clamp for 30 min. Next, clamps were removed, renal blood flow was reestablished, and both incisions were sutured. Rats were allowed to recover in a warm room with water and food ad libitum. Sham-operated rats were submitted to the same procedure without clamping.

![Fig. 1. Evidence of histological renal damage in hypoxic kidney induced by ischemia-reperfusion (I/R). Immunohistochemistry was performed in kidney samples obtained at 24 h after I/R (n = 7 for each I/R group). A clear induction of renal damage markers such as macrophages (ED-1; B) or α-smooth muscle actin (SMA; C) can be observed in the interstitial space from the inner and outer medulla. A: histological damage evaluated by periodic acid-Schiff (PAS) staining. Brush border, epithelial flattening, and mitosis are shown, indicating the renal damage induced by I/R. Kidneys from sham rats were also used to detect these markers (D–F); n = 5 for each control group. Scale bar = 100 µm. Arrows, localization of the markers.](http://ajprenal.physiology.org/)

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surgical procedure and conditions, without clamping the renal arteries. Rats were killed under anesthesia (ketamine-xylazine) 24, 48, 72, and 96 h after reperfusion; both kidneys were removed and processed for immunohistochemistry and Western blotting.

**Tissue processing and immunohistochemical analysis.** Tissue processing for immunohistochemical studies in paraplast-embedded sections was carried out according to methods previously described (40). Immunolocalization studies were performed using an indirect immunoperoxidase technique, as previously described (40). Briefly, tissue sections were dewaxed, rehydrated, rinsed in 0.05 M Tris-phosphate-saline buffer (pH 7.6), and incubated with the primary antibody overnight at 22°C. Afterward, sections were washed three times for 5 min each, followed by a 30-min incubation at 22°C with the corresponding secondary antibody and with the peroxidase-antiperoxidase (PAP) complex. Immunoreactive sites were revealed using 0.1% (wt/vol) 3,3′-diaminobenzidine and 0.03% (vol/vol) hydrogen peroxide solution.

**Antibodies and chemicals.** The following primary antibodies were used: rabbit polyclonal antibodies against renin, B2 receptor, and kallikrein were prepared as described previously (37). The monoclonal antibodies against macrophages (clone ED-1) were obtained from Biosource (Camarillo, CA), against α-smooth muscle actin (α-SMA; clone 1A4) from Sigma (St. Louis, MO), and against iNOS (catalog 39120) from Transduction Laboratories (Lexington, KY); the polyclonal antibodies against COX-2 (catalog 160126) were obtained from Cayman (Ann Arbor, MI), against EPO (catalog N-19) from Santa Cruz Biotechnology (Santa Cruz, CA), against HO-1 from StressGen Biotechnologies (Victoria, Canada), and against nitrotyrosine residues (PNT; clone 1A6) from Upstate (Charlottesville, VA).

Secondary antibodies and the corresponding PAP complexes were purchased from ICN Pharmaceuticals-Cappel (Aurora, OH). Triton X-100, 3,3′-diaminobenzidine, carrageenan, Tris-HCl, hydrogen peroxide, phosphate salts, and other chemicals were purchased from Sigma.

**Immunoblotting.** For immunoblotting analysis, inner/outer medulla kidney sections (~1 mm thick) were homogenized with an Ultra-Turrax homogenizer in buffer containing 0.05 M EDTA, 1× PBS, and protease inhibitor cocktail (Pierce, Rockford, IL). The protein concentration was determined through the Bradford method (Bio-Rad, Richmond, CA). Western blotting was performed as described by Harlow and Lane (11). Protein (60 μg) was mixed with an equal volume of SDS-PAGE sample buffer (100 mM Tris-HCl, pH 6.8, 200 mM dithiothreitol, 4% SDS, 0.2% bromphenol blue, and 20% glycerol) and boiled for 3 min. Proteins were separated on 12% SDS-polyacrylamide gels and transferred to nitrocellulose membranes. Blocking was carried out by incubation in blocking solution (8% nonfat dry milk in Tris-buffered saline-0.1% Tween) for 2 h at room temperature. After being blocked, membranes were probed with the corresponding antibody for 18 h at 4°C, washed with Tris-buffered saline-Tween, and incubated with the appropriate horseradish peroxidase-conjugated secondary antibody for 1 h at room temperature. Proteins were detected using enhanced chemiluminescence techniques (PerkinElmer, Life Sciences, Boston, MA).

The blots were scanned, and densitometric analysis was performed using the public domain National Institutes of Health (NIH) Image program version 1.61 (U.S. NIH, http://rsb.info.nih.gov/nih-image). The expression of tubulin was used to correct for variation in sample loading.

**Determination of tissue damage and immunohistochemical quantification.** Tissue damage was evaluated through periodic acid-Schiff (PAS) staining. Immunolocalization of ED-1 and interstitial α-SMA were used as markers of tissue damage.

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**Fig. 2.** Immunolocalization of oxidative stress-related elements in hypoxic kidney induced by I/R. Maximum staining intensity for erythropoietin (EPO; A), heme oxygenase (HO-1; B), inducible nitric oxide synthase (iNOS; C), and nitrotyrosine residues (PNT; D) was observed at 24 h after I/R (n = 7 for each I/R group). The staining for each marker was also performed at similar time periods in kidneys from sham-operated rats (D–G); n = 5 for each control group. The staining for HO-1 was localized in proximal tubules, INOS was localized in papilla and inner medulla ducts, and EPO and PNT were localized mainly in the cortex of the kidney. Scale bar = 100 μm. Arrows, localization of the markers.
The immunoreactive area in each image was determined by image analysis using Simple PCI software (Compix). Total immunostained (brown) cells were averaged and expressed as the mean absolute values or the mean percentage of stained cell area per field as previously described (38) with minor modifications.

**Statistical analysis.** Data from different groups were assessed with the parametric Student’s *t*-test when comparing two groups and ANOVA for multiple comparisons with Fisher’s post hoc test when comparing more than two groups. The significance level was *P* < 0.05.

**RESULTS**

**Determination of histological renal damage.** PAS staining renal sections after the induced damage showed clear alterations in kidney morphology consistent with ATN, such as brush borders and epithelial flattening (Fig. 1A). The following two indicators of renal damage were used after I/R: the presence of macrophages (ED-1) and interstitial α-SMA. Immunostaining in postischemic rats showed an increase of both markers in interstitial space (α-SMA: 9.1 ± 2.3 μm² in I/R rats vs. 2.7 ± 1.7 μm² in sham rats and ED-1: 42 ± 5 μm² in I/R rats vs. 10 ± 2 μm² in sham rats; *P* < 0.05; Fig. 1, B and C). No changes were observed in sham animals (Fig. 1, D–F).

Additionally, in PAS-stained slides from 24- to 48-h I/R kidneys, an important number of mitosis could be observed in proximal tubular cells (Fig. 1A) that was not observed in control rats (Fig. 1D).

**Increased levels of EPO, HO-1, and iNOS at 24 h after ischemia.** We previously demonstrated that HIF-1α level peaked at 24 h after I/R (38). Thus we wanted to analyze the expression pattern of the following three target genes of HIF-1α: EPO, HO-1 (32), and iNOS (24). In normal kidneys, immunostaining for iNOS was observed mainly in medullar structures (Fig. 2G), and HO-1 was observed in proximal tubules (Figs. 2F and 3, C and D), similar to what was reported previously (12, 28). In addition, kidneys from rats killed 24 h after I/R showed an increased staining for both proteins compared with their controls (for HO-1 sham: 11,306 ± 2,675 μm² vs. I/R: 15,667 ± 1,438 μm², *P* = not significant). Although the intensity was higher, the localization was similar to the one observed in normal kidneys (Fig. 2, B and C).

As can be observed in Fig. 4, A–C, the levels of the three proteins were maximal at 24 h, returning to control levels at 48 h (*n* = 4, *P* < 0.05). This increase was consistent with the increase in HIF-1α previously observed (38) and could be produced in response to this transcription factor.

**Immunostaining for EPO was not observed in normal kidneys (Fig. 2D); on the other hand, EPO immunostaining was present in discrete cells in kidneys from I/R animals after 24 h (Fig. 2A). The distribution was mainly peritubular close to proximal tubules and loops of Henle, identified by their immunoreactivity to GP330 and Tamm-Horsfall, respectively (Fig. 3, A, B, and D).

After reperfusion (24 h), iNOS was increased and returned to control levels at 48 h (Fig. 4A). EPO showed a similar behavior (Fig. 4B), returning to control levels at 72 h after reperfusion. Similarly, HO-1 was maximally increased at 24 h, returning to control levels at 48 h (Fig. 4C).

**Protein nitrosylation is increased in kidneys from I/R rats.** One of the consequences of I/R is the increase in oxidative stress. As mentioned before, iNOS levels were increased after...
24 h of I/R, so it was possible to expect an increase in the free radical peroxynitrite. This compound reacts with proteins to form nitrotyrosine residues. To determine the amount of peroxynitrite, we evaluated the presence of nitrotyrosilated proteins. As observed in Fig. 2D, nitrotyrosine reactivity was present in almost all tubular structures and was increased in kidneys from I/R animals after 24 h (14,792 ± 2,793 μm²; *P < 0.05) compared with control (7,025 ± 1,229 μm²; n = 5; Fig. 2H). In addition, when evaluated by Western blot, the pattern of nitrosylated proteins was different at 24 h after reperfusion compared with control, although the levels were not changed. In addition, protein nitrosylation was decreased at 48 h without returning to control levels, even at 96 h, suggesting an active degradation and resynthesis of proteins (Fig. 4D).

The levels of components of vasoactive systems are modified in I/R. For the kidney to have a proper function, the KKS and the RAS must be in balance (29). We evaluated the distribution and levels of certain components of these systems in kidneys after I/R (Figs. 5 and 6).

At 24 h after I/R, immunostained tissue sections showed a decreased amount of immunoreactive cells to COX-2, but the intensity was similar (Fig. 5, A and E). In addition, renin-immunoreactive cells were increased (Fig. 5, B and F) on the afferent arteriole, and kallikrein immunoreactivity was decreased not only in cell number but also in intensity (Fig. 5, C and G). Finally, BK B2 receptor immunoreactivity was increased in kidneys 24 h after I/R (1,299 ± 141 μm² vs. 749 ± 60 μm²; *P < 0.036; Fig. 5, D and H).

When analyzed by Western blot, we observed that 24 h after reperfusion renin was increased and returned to control levels at 48 h (P < 0.05; Fig. 6A). Interestingly, COX-2, which is expressed only in a few cells in control conditions, was decreased at 24 h (Fig. 6B) without returning to control levels, even at 96 h after reperfusion. Kallikrein was maintained relatively constant up to 72 h, where its levels decreased (Fig. 6C). Finally, the B2 receptor levels showed a biphasic behavior, slightly increasing at 24 h, returning to normal level at 48–72 h, but increasing again at 96 h (Fig. 6D).

**DISCUSSION**

The major findings of this study are that, although EPO, HO-1, and iNOS are increased 24 h after ischemia and return to basal 48 h later, correlating with the previously observed behavior for HIF-1α, components of the renin-angiotensin, the kallikrein-kinin, and the COX-prostaglandin systems have a differential regulation in the I/R model during the same time period.

I/R in rats is widely utilized to study ARF. ARF in rats is reversible, and the recovery response is characterized by the restoration of glomerular filtration rate and remodeling of the renal tubular system (4). However, there are persistent alterations in renal function postischemic injury that lead to a permanent compromise in urinary-concentrating ability associated with a reduction in renal medullary tonicity and permanent vascular damage. We studied the effects of ischemia after 24, 48, 72, and 94 h of reperfusion to follow how these persistent alterations are established. We used two methodological approaches, immunohistochemistry to evaluate the protein localization and Western blot to have a semiquantification of the levels of each protein.

We first analyzed proteins that are regulated by hypoxia, such as HO-1, EPO, and iNOS, followed by the evaluation of proteins that are involved in the regulation of urine concentration and pressure regulation such as kallikrein, renin, and COX-2.

Although three HO isoforms have been reported, only the inducible isoform, HO-1, and the constitutively expressed isoform, HO-2, have a bona fide heme oxygenase activity (5). Induction of HO-1 occurs as an adaptive and beneficial re-
sponse to several injury-signaling processes, and it has been implicated in many clinically relevant disease states, including acute renal injury. Increased HO-1 expression protects kidneys from oxidative injuries (22), rhabdomyolysis (23), cisplatin nephrotoxicity (2), ARF (33), and I/R-mediated tissue injury (26), probably through the generation of reaction products from heme degradation, iron, carbon monoxide, biliverdin, and in particular bilirubin, which exert important antioxidant, anti-

Fig. 5. Immunolocalization of vasoactive substances in hypoxic kidney induced by I/R. Staining for cyclooxygenase (COX)-2 (A), renin (B), kallikrein (C), and B2 receptor (D) was observed at 24 h after ischemia (n = 7 for each I/R group). The staining for each marker was also performed at similar time periods in kidneys from sham-operated rats (E–H; n = 5 for each control group. The expression of COX-2 and B2 was mainly observed in thick ascending limb, renin was observed in afferent arteriole, and kallikrein was observed in connecting tubule cells localized in the cortex of the kidney. Scale bar = 100 μm. Arrows, marker localization.

Fig. 6. Immunoblot for vasoactive proteins in ARF induced by I/R and vasoactive proteins renin (A), COX-2 (B), kallikrein (C), and B2 receptor (D) were analyzed in kidneys from sham animals and 24, 48, 72, and 96 h after 30-min ischemia. Tubulin was used as loading control; n = 4 for each group. *P < 0.05.
inflammatory, and cytoprotective functions (13). In our model, HO-1 expression after I/R correlates with HIF-1α activation (38). The hypothesis that HIF-1α can induce HO-1 expression is supported by results from Ockaili et al. (25), who observed an increase in HO-1 in rabbit hearts treated with HIF-1α activator, the prolyl hydroxylase inhibitor dimethylxalylglycine. In addition, in HIF-1α-deficient Hepa-1 cells, the hypoxia-induced increase in HO-1 was abolished (15), confirming the participation of HIF-1α on the induction of HO-1.

A hypoxia-responsive enhancer (HRE) has been identified in the 3′-flanking region of the Epo gene in a region required for EPO transcriptional activation (19). The HRE contains the consensus sequence for the binding of HIF-1α. It has been proposed that Epo has protective effects in retina and renal tubule cells. The mechanism proposed for this action involves the activation of Jak-2, Akt, and multiple targets with antiapoptotic effects (31). In our study, we have observed an increase in Epo immunoreactivity at 24 h by Western blot and immunocytochemistry, which is consistent with the increase in HIF-1α observed previously. This increase could be related to the molecular machinery elicited in the protection response to the I/R damage.

The promoter region of the iNOS gene also contains a sequence homology to the HRE. In murine macrophages, the induction of HIF-1α by the iron chelator desferrioxamine stimulates the expression of the mRNA for iNOS. iNOS has been postulated as a promoter of apoptosis in I/R (39). In addition, several in vivo and in vitro investigations have suggested that NO generated by iNOS contributes to renal I/R injury (7), possibly by the generation of peroxynitrite due to the reaction of NO with superoxide radical, and consequent protein tyrosine nitrosylation. An increased production of NO for prolonged periods of time in many pathological states is known to contribute to oxidative damage of critical cellular macromolecules, including proteins, which often show elevated levels of 3-nitrotyrosine (1, 14, 21, 27). The results shown by other authors in more aggressive models of I/R are consistent with our data and support the idea that lack of oxygen is not only inducing proteins that damage the kidney but also the induction of other proteins that protect the renal tissue. In this regard, we wanted to determine the effects of I/R on the expression of vasoactive systems of the kidney.

We previously reported that kallikrein was one of the genes downregulated 35 days after recovery from bilateral I/R injury (4). In the present study, we observed a decrease in kallikrein as early as 72 h. Kallikrein has been involved in the protection against I/R-induced myocardial injury (43). Kallikrein cleaves kininogen to produce BK, which binds to its receptor (B2), activating intracellular signaling. When BK concentration is increased, the receptor is downregulated by internalization followed by degradation. We speculate that the cyclic behavior of the BK receptor could be because of a decrease in BK produced by the decrease in kallikrein at longer periods, with the consequent upregulation of the receptor.

Matsuyama et al. (17) demonstrated that COX-2 was maximally expressed in a rat model at ~4 h after I/R injury, declining thereafter; but his observations were restricted to 24 h. In addition, it has been proposed that COX-1 and/or -2 blockade ameliorates the renal tissue damage triggered by I/R injury (9). Although we did not repeat the time points measured by Matsuyama et al., we can postulate that COX-2 is sensitive to tissue damage, so when tubules are well preserved, as happens in the first hours after reperfusion, COX-2 can be induced; but later, when a maximal tissue damage is observed, COX-2 decreases. A decrease in COX-2 activity with a concomitant increase in HO-1 activity has also been observed in a different model such as endothelial cells (10, 16, 42), suggesting that the heme-HO system can function as a cellular regulator of the expression of COX-2.

In summary, in kidneys with ARF, an important initial damage is observed at 24 h after I/R. Coincident with the major damage, there is an increase in EPO, HO-1, and iNOS. On the other hand, components of the renal vasoactive systems such as renin, kallikrein, BK B2 receptor, and COX-2 have dissimilar behaviors. Although renin was increased, the B2 receptor had a bifasical response, whereas kallikrein and COX-2 decreased in the I/R kidney, suggesting a differential regulation of these proteins during this type of damage, and could explain, in part, the increased propensity to damage later in life.

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