Protection From Unilateral Renal Ischemia/Reperfusion Induced Injury In The Non-Ischemic Distant Kidney With Fenoldopam

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Citation


Abstract

Objective: While NFκB signaling mediated inflammation and angiogenesis related transcription are important mediators of renal ischemia/reperfusion injury, its role in distant kidney injury after unilateral renal ischemia/reperfusion (UR-I/R) remains unknown. We therefore used a rat model to test the hypothesis that UR-I/R induces NF-κB- and angiogenesis-signaling mediated inflammation in distant kidney and, fenoldopam attenuates associated gene transcription.

Methods: 24 male Sprague-Dawley rats were divided into 4 groups (n=6 each): (1) sham operated, (2) sham operated with fenoldopam, (3) UR-I/R and, (4) UR-I/R with fenoldopam. Microarray analysis was performed using pathway specific NFκB signaling pathway and angiogenesis gene arrays. Gene expression was measured as % GAPDH, positive control.

Results: Of the 96 NFκB signaling pathway genes analyzed, 80 genes were induced and 3 were suppressed completely (≥ twofold change vs. control) in distant kidney after UR-I/R. Treatment with fenoldopam attenuated 76 of 80 genes UR-I/R induced genes. Similarly, UR-I/R induced 68 and downregulated another 18 angiogenesis related genes in the distant kidney. Fenoldopam attenuated 43 of 68 UR-I/R induced and 15 out of 18 UR-I/R suppressed genes.

Conclusion: These results constitute the initial demonstration that UR-I/R induces proinflammatory NFκB- and angiogenesis-signaling genes and fenoldopam may attenuate UR-I/R-induced inflammation in the distant kidney.

INTRODUCTION

Ischemic acute renal failure (ARF) remains a formidable clinical problem in perioperative and critically ill patients, that is associated with high morbidity and mortality. The pathophysiology of ARF is complex and multifactorial and includes persistent intra-renal vasoconstriction, hypoxic injury to microvascular endothelial cells and tubular epithelial cells and leukocyte-mediated cytotoxicity. Pathophysiologic mechanisms of ARF include intracellular damage with ATP depletion, and intracellular Ca$^{2+}$ accumulation. Cellular activation leads to reactive oxygen species generation, cytokine generation, neutrophil sequestration/activation, phospholipase activation, and membrane lipid alterations. Recently, we demonstrated that a period of unilateral ischemia followed by reperfusion induced the DNA binding activity of the transcription factor Nuclear factor kappa B (NFκB) and activation of proinflammatory NFκB signaling pathway genes, induced apoptosis and activated apoptosis-related signal transduction genes, and, modulated angiogenesis-related signal transduction. While these mediators are clearly involved in local tissue damage after ischemia/reperfusion (I/R), their role in remote organ injury has been less well defined. However, increasing evidence suggests that single organ ischemia can generate remote organ injury. Furthermore, Tnfα -dependent bilateral renal injury after unilateral renal ischemia/reperfusion (UR-I/R) has been demonstrated. Such signaling events may represent the earliest domino in the cascade of inflammatory events that culminate in contra lateral kidney after UR-I/R.
Although several experimental ameliorative strategies have been tested in I/R induced ARF, there is still a remarkable lack of definitive evidence supporting specific prophylactic therapies in any setting. Increasing evidence suggests that inhibition of inflammatory pathways, such as inhibition of redox-sensitive NF-κB signaling pathway is a beneficial strategy in I/R models. Recently we demonstrated that fenoldopam a short-acting, parenteral vasodilator, attenuate a number of signaling pathways including NF-κB signaling pathway, apoptosis signaling pathway and, angiogenesis signaling pathway. It is thus appropriate to investigate the effect of fenoldopam on UR-I/R-modulated genetic alterations in the distant non-ischemic kidney. We utilized a rat model of surgical ischemic renal failure to test the hypothesis that (1) UR-I/R modulate the proinflammatory NF-κB signaling and angiogenesis signaling in the non-ischemic distant kidney and, (2) fenoldopam attenuates UR-I/R-induced NF-κB and angiogenic gene transcription in the distant kidney.

MATERIALS AND METHODS

Animals and Instrumentation. All experiments were carried out in accordance with the guidelines laid down by the National Research Council and were approved by our institutional animal care and use committee. Twenty-four adult male Sprague-Dawley rats weighing 350–400 g each were acclimatized in standard animal quarters for at least 3 days prior to the study. Six rats were used for each group. In all animals, anesthesia was induced with an inhaled mixture of 3.5% isoflurane in oxygen. When the animal was sufficiently anesthetized (judged by loss of ear pinch reflex), a tracheotomy was performed, and the lungs were mechanically ventilated (Inspira ASV ventilator, Harvard Apparatus Inc., Holliston, MA, USA). Anesthesia was maintained using intraperitoneal urethane (50 mg. kg⁻¹). The inspired oxygen tension (FiO2) was maintained at 35% throughout the experiment. The carotid artery and jugular vein were cannulated (PE-20, Harvard Apparatus) to facilitate monitoring of arterial blood pressure and infusion of fluids. Muscle relaxation to permit mechanical ventilation (volume controlled, time cycled with tidal volume of 2 ml/100 g body weight and respiratory rate of 30 breaths per minute and zero PEEP, Inspira, Harvard apparatus) was achieved with intravenous boluses of pancuronium bromide (20 µg 100 g⁻¹) as required. An intravenous infusion of 0.9% saline was administered to prevent dehydration during the experiment (1 ml 100 g⁻¹ hr⁻¹). Core body temperature was maintained at 37°C by an automated rodent homeothermic blanket warming system (Harvard Apparatus).

EXPERIMENTAL PROTOCOL.

After instrumentation, and once the animal had reached a stable baseline, the randomization envelope was opened and the animal was assigned to one of the following four groups. Study infusions were begun immediately thereafter.

Saline sham group: After surgical preparation (including laparotomy), these rats (n≥6) underwent no further intervention. a 0.9% saline was infused via the left jugular vein throughout.

Saline UR-I/R group: These animals (n≥6) underwent laparotomy, after which the left renal artery was dissected free and then occluded with a microvascular clamp for 60 min. the clamp was then removed to allow reperfusion of the kidney for 6 h. a 0.9% saline was infused via the left jugular vein throughout.

Fenoldopam sham group: This group (n≥6) underwent the same surgical preparations (including laparotomy) as the saline sham group. fenoldopam 0.1µg.kg⁻¹.min⁻¹ (a dose designed to reproduce that used clinically in our ICU) was infused via the left jugular vein throughout.

Fenoldopam UR-I/R group: These animals (n=6) were infused with fenoldopam (0.1µg.kg⁻¹.min⁻¹) and underwent laparotomy followed by 60 min of left renal artery clamping and 6 h of reperfusion, as in the saline I/R group.

At the end of each experiment, the animals were euthanized by inhalation of 5% isoflurane and rapid exsanguination via the arterial line. At that time right kidneys were removed from the animal and snap frozen.

MICROARRAY ANALYSIS

Total cellular RNA from distant non-ischemic kidney tissues was isolated by phenol/chloroform extraction. Gene array expression analysis was performed as described earlier. In brief, single-stranded cDNA was synthesized from 2 µg of the group-wise pooled total RNA extract using MMLV reverse transcriptase and labeled with biotin-16-dUTP (Roche Applied Science, Branchburg, NJ, USA). Each membrane was prehybridized with hybridization solution supplemented with 100 µg ml⁻¹ of heat-inactivated salmon sperm DNA (Invitrogen Corporation, Carlsbad, CA, USA). The cDNA probes were denatured and then hybridized with nylon-based arrays spotted with cDNA fragments from 96 genes. We used mouse NF-κB signaling pathway gene array...
containing 96 genes representing seven functional groups: (a) Rel/NF-κB/UB family, (b) NF-κB-responsive genes, which include adhesion molecules, cytokines, and acute phase response proteins, (c) extracellular ligands, (d) transmembrane receptors, (e) adaptor proteins, (f) signal transduction kinases and (g) transcription factors and, mouse angiogenesis gene array containing 96 genes representing seven functional groups: (a) specific promoters and inhibitors, (b) growth factors and receptors, (c) cytokines and chemokines, (d) adhesion molecules, (e) matrix proteins, proteases and inhibitors, (f) transcriptional factors and, (g) other angiogenesis-related genes (Super Array Bioscience Corporation, Bethesda, MD). We started with these highly selective microchips instead of an all-encompassing gene array because the selected genes entail a well-characterized profile governing NFκB signal transduction-mediated inflammatory responses and angiogenesis, thereby facilitating interpretation of data, simplifying data acquisition and analysis, and avoiding the rationalization of genes not functionally characterized. Each array was also spotted with a negative control (pUC18) and four housekeeping genes: β-actin, GAPDH, cyclophilin and ribosomal protein L13a. The hybridized membrane was tagged with alkaline phosphatase–conjugated streptavidin and treated with CDP-Star and, exposed to radiographic film for 1 minute. The tetra spots from the autoradiograph were converted into a raw image file using a 1200 dpi scanner (Hewlett-Packard Company, Palo Alto, CA). The raw data were extracted from this image using ImageQuant array analysis software (Amersham Biosciences Piscataway, NJ). The gene sequences on the mouse GEArray Q series membranes were confirmed using the Basic Local Alignment Search Tool (BLAST), available in the National Center for Biotechnology Information (NCBI) website (http://www.ncbi.nlm.nih.gov/BLAST/), and the cross-species homology of each gene was investigated for both the mouse and rat genomes. GEArray analyzer 1.3 (Super Array, Inc.) was used for data analysis and to compare gene expression profiles. The relative abundance of a particular cDNA transcript was estimated by subtracting the background spot intensity (average of three blanks) from the values recorded for each spot. Because the overall signal of different arrays may fluctuate substantially, the spot intensity was then normalized to the signal derived from GAPDH, a positive housekeeping gene. The normalized data were then compared between the groups, and the relative expression level of each gene was expressed as a fold change.

When comparing each gene's signal intensity between arrays, we used a twofold or more increase in the experimental group compared with the control group to represent “stringent” criteria for upregulation and an increase of less than twofold to represent “less stringent” criteria. Classifying gene regulation criteria in this manner can provide an index of the reliability of the gene microarray data. Also, we recently validated the quality of these microarray expression levels using Reverse transcriptase-polymerase chain reaction (RT-PCR).

RESULTS

All 24 rats survived for at least the 6 hours after the reperfusion period (i.e. there was no operative mortality, and all animals required euthanasia in order to obtain kidney samples). In six cases (25%) an additional dose of urethane was required.

Gene array analysis: Oligonucleotide microarray analysis was performed using group wise pooled RNA. With both NFκB signaling pathway and angiogenesis signaling pathway gene chips, we observed intervention specific gene transcriptional response (Fig. 1A and 1B).
Figure 1: heat map: Color representation of relative expression levels (percent GAPDH, a positive control) of genes encoding (A) proinflammatory NFκB signal transduction pathway genes and (B) angiogenesis signaling pathway genes in the distant kidney of rats that underwent sham operation or unilateral renal ischemia/reperfusion in the presence or absence of fenoldopam.
NFκB SIGNALING PATHWAY

Expression of inflammatory molecules in distant kidney after UR-I/R: Of the 96 NFκB signaling pathway genes analyzed, UR-I/R induced 92 genes and suppressed 3 genes in the distant kidney. Expression of one gene, NFκB inhibitor-like 1 (NFκBIL-1) is not altered after UR-I/R. Eighty of the 92 upregulated genes including Rel/NF-κB/IκB family NFκB1, NFκB2, NFκbib, NFκbic, NFκbif, Rel, Rela, Relb; NFκB responsive adhesion molecules, Icam1, Icam2, Icam5, Sele, Sell, Selpl, Ncam1, Vcam1; NFκB responsive cytokines, Csf1, Csf2 Csf3, Ifn1, Ifn2, Il2, Il6, Il12, Il12r, Ifr1, Scy2a, Tnfβ, Lta; NFκB responsive acute phase response proteins C3, Orm1, Orm2, Saa1, Srf; extracellular ligands, Il1l, Il1l, Tnfsf11; transmembrane receptors, Il1r1, Il1r2, Il2rα, Ripk1, Thr1, Thr2, Thr3, Thr4, Thr5, Thr6, Thr7, Thr8, Thr9; adaptor proteins, Fadd, Myd88, Tnfaip3, Traf1, Traf2, Traf3, Traf4, Traf5, Traf6; signal transduction kinases, Chuk, Iκbkβ, Map3k1, Map3k14, Map3k2, Map3k5, Map3k7, Mapk14, Mapk9, Tab1, TBK1 and, transcription factors, Creb1, Elk1, Elk3, Max, Myc, Raf1 met stringent criteria for upregulation (at least a two-fold increase over control).

Attenuating effect of fenoldopam on UR-I/R modulated NFκB signaling pathway genes in distant kidney: Microarray analysis of the fenoldopam UR-I/R group compared that of saline UR-I/R group showed that, using less stringent criteria, of the 92 genes upregulated by UR-I/R injury, 88 genes were returned to the baseline level or less with fenoldopam. Using stringent criteria (≥ twofold) we observed that 76 of 80 upregulated genes were attenuated by fenoldopam. These include Rel/NF-κB/IκB family, NFκB1, NFκB2, NFκbib, NFκbic, NFκbif, Relb (Fig 2A); NFκB responsive adhesion molecules, Icam1, Icam2, Icam5, Sele, Sell, Selpl, Ncam1, Vcam1 (Fig 2B); NFκB responsive cytokines, Csf1, Csf2 Csf3, Ifn1, Ifn2, Il2, Il6, Il12, Il12r, Ifr1, Scy2a, Tnfβ, Lta (Fig 2C); NFκB responsive acute phase response proteins C3, Orm1, Orm2, Saa1 (Fig 2D); extracellular ligands, Il1l, Il1l, Tnfsf11 (Fig 2E); transmembrane receptors, Il1r1, Il2rα, Il2rβ, Ripk1, Thr1, Thr2, Thr3, Thr4, Thr5, Thr6, Thr7, Thr8, Thr9 (Fig. 2F); adaptor proteins, Fadd, Myd88, Tnfaip3, Traf1, Traf2, Traf3, Traf4, Traf5, Traf6 (Fig 2G); signal transduction kinases, Chuk, Iκbkβ, Map3k1, Map3k14, Map3k2, Map3k5, Map3k7, Mapk14, Mapk9, Tab1, TBK1 (Fig. 2H) and, transcription factors, Creb1, Elk1, Elk3, Max, Myc, Raf1 (Fig. 2I).

ANGIOGENESIS SIGNALING PATHWAY

Effect of UR-I/R on angiogenesis signaling genes in distant kidney: Of the 96 angiogenesis-related genes analyzed, we observed increased expression of 77 genes and suppression of 19 genes after UR-I/R injury in distant kidney. Sixty eight of 77 UR-I/R-induced genes including specific promoters and inhibitors, Agpt, Agpt2, Ang: growth factors and receptors, Efna2 Ephb4, Fgf1, Fgf16, Fgf2, Fgf6, Fgf7, Fgfr3, Fgfr4, Pdgfa, Pdgfb, Pdgfra, Pdgfrb, Pf4, Tgfa, Tgbf1, Tgbf2, Tgbf3, Tgbfr1, Tgbfr2, Ifgf, Pgf, Vegf, Vegfb, Cd36, Ctgf, Edg1, Egf, Egfr, I1f1, Grol, Tie1; cytokines and chemokines, Csf3, Ifnb, Ifng, Il10, Il12a, MDK, Nrp, Ptn, Rsn, Scya2, Sparc, Tnfβ; adhesion molecules, Cdh5, Iga5, Iga5, Igav, Igb3, Pecam, Tnc, Vcam1; matrix proteins, proteases and inhibitors, Adamt8, Colla1a1, Fn1, Mmp2, Mmp9, Plaur, Serpinb5, Serpine2, Thbs2, Thbs3, Thbs4; transcription factors, Erb2, Ets1, Idb1, Idb3, Madh1 and, other related genes Eng, F2, Mrsl, Nos3, Ptg1, Ptg1s2, Spp1 met stringent criteria for upregulation. Similarly, 18 of the 19 downregulated genes including Adamt1, Chga, Efna5, Efna2, Fgf4, Fgf5, Flk1, Flt1, Hgf, Hif1, Ifna1, Serpin1, Tek, Tgbfr3, Thbs1, Timp, Timp2 and Vegfc were completely suppressed (≥ 2 fold) after UR-I/R in the distant kidney.

Effect of fenoldopam on UR-I/R modulated angiogenesis related genes in distant kidney: In general, administration of low dose fenoldopam (0.1µg.kg⁻¹.min⁻¹) produced a blunting effect on the UR-I/R injury-modulated angiogenesis related gene expression. Forty eight of the 77 UR-I/R injury-
induced genes including specific promoters and inhibitors, Agpt (Fig. 3A); growth factors and receptors, Cd36, Ctgf, Edg1, Egfr, Fgf16, Fgf2, Fgfr3, Fgfr4, Figf, Igf1, Pdgfa, Pdgfb, Pdgfra, Pdgfrb, Pt4, Pgf, Tgfbl1, Tgfbr2, Tie1 (Fig. 3B); cytokines and chemokines, Csfr3, Ifng, Il12a, MDK, Nrp, Tnf (Fig. 3C); adhesion molecules, Cdh5, Itgav, Pecam, Tnc, Vcam1 (Fig. 3A); matrix proteins, proteases and inhibitors, Adamts8, Col18a1, Fn1, Mmp2, Mmp9, Plau, Serpinb5, Thbs2 (Fig. 3D); transcription factors, Idb3, Madh1 (Fig. 3C) and, other related genes Eng, Msr1 (Fig. 3A) met stringent criteria for upregulation. Similarly, 15 of 19 UR-I/R-suppressed genes showed enhanced expression with fenoldopam. This blunting effect was significant (brought to baseline level) for 4 genes, Efnb2, Flk1, Timp and Vegfc (Fig. 4).

**Figure 3**
Figure 3: Effect of fenoldopam on unilateral renal ischemia/reperfusion induced (>= 2 fold angiogenesis-related genes in distant kidney)

**DISCUSSION**
This study constitutes the initial demonstration of that UR-I/R induces proinflammatory NFκB- and angiogenesis-signaling in the non-ischemic distant kidney. We have previously shown that increased NFκB-DNA binding activity in response to renal I/R injury induces multiple pro-inflammatory molecules and mediate local cellular injury 18,19. Consistently, NF-κB a redox-sensitive transcription factor has been implicated in the pathophysiology of a number of inflammatory disorders including renal I/R injury 18,19,20. While NFκB signaling is clearly an important mediator of local I/R injury, its role in remote organ damage after UR-I/R, is just beginning to be elucidated. In this study, we demonstrated that UR-I/R activated the expression of 80 proinflammatory NFκB signaling molecules in the non-ischemic distant kidney. The induction of Rel/NFκB/IB family genes 18,19,21,22,23 after UR-I/R injury points to the general role of these genes in regulating the expression of cytokines, chemoattractant proteins, cell adhesion molecules, and other pro-inflammatory genes in the remote organ that subsequently lead to the cellular damage. Cytokines produced by renal tubular epithelial cells and infiltrated cells are critical factors in inflammatory processes of renal I/R injury. Induced expression of cytokines in remote organs including non-ischemic kidney 11 after renal I/R injury has been well documented. These served as positive controls for the system. The induction of adhesion molecules after UR-I/R is in response to cytokines after I/R injury. Recently, Kelly demonstrated induced expression of adhesion molecule in remote organ after I/R 12. We also observed
numerous UR-I/R injury-induced transmembrane receptors and adaptor proteins in the distant kidney. The induced TLRs expression during renal inflammation was found to be mediated by both IFN and TNF-α and is associated with a major increase of renal TLR protein expression. TLRs recruit an adapter protein, Myd88, to initiate a signaling pathway that is involved in the sequential activation of IRAKs, TRAFs, MAP3K14, and the IκB kinase complex. Activated IKKs lead to NFκB phosphorylation and degradation, resulting in NFκB activation. Li and his colleagues have previously suggested that the TLR-mediated Myd88-dependent NFκB signaling pathway may play a role in the inflammatory response to I/R injury. Consistently, in the present study, we observed the induction of all these molecules in the distant kidney which confirmed this concept of UR-I/R-injury induced cytokine mediated activation of NFκB signaling pathway.

A number of earlier studies demonstrated the protective effect of fenoldopam, a selective dopamine1-receptor (D1) agonist, in renal I/R injury, establishing the therapeutic potential of fenoldopam in this setting. The role of fenoldopam in multiple aspects of renal protection suggests that a complex interplay of transcriptional events underpin the renoprotective effect of fenoldopam in experimental I/R injury. Recently, we demonstrated that fenoldopam attenuates I/R injury modulated NFκB, apoptosis, and angiogenesis signaling pathway molecules. In the present study, fenoldopam significantly suppressed 76 of 80 UR-I/R-induced NFκB signaling molecules in the distant kidney. The reduced expression of these UR-I/R injury-induced proinflammatory NFκB family genes, cytokines, adaptor proteins, and MAP kinases in distant kidney after fenoldopam demonstrate the inhibitory effect of fenoldopam on UR-I/R-induced NFκB signaling molecules and subsequent mediated inflammation. Several studies have demonstrated that direct inhibition NFκB prevents I/R injury-induced tissue damage, and other renal diseases. Cao and colleagues (2004) demonstrated that inhibition of I/R injury-induced NFκB attenuated renal dysfunction, macrophage/microvascular infiltration, and tubular damage in the cortex and outer medullary stripe. Consistently, we found that the administration of low-dose fenoldopam, an established renoprotectant vasodilator, blocked UR-I/R injury-induced inflammatory genes.

In addition to the ischemic kidney induced cytokine mediated injury, UR-I/R will significantly increase the functional burden in the non-ischemic kidney. An important consequence of I/R is stimulation of angiogenesis, which in turn leads to vascular discontinuity, proliferation and migration of endothelial cells, and structural reorganization of the new vasculature. A complex interaction of biochemical and physiological processes, including imbalances in levels of angiogenic regulators, occurs during I/R. In the present study, we found that UR-I/R significantly induced 68 angiogenesis-related genes and downregulated another 18 in the distant kidney. Administration of fenoldopam attenuated 43 of UR-I/R injury-induced and 15 of UR-I/R-suppressed genes. Fenoldopam associated attenuation in the UR-I/R modulated angiogenesis-related genes might be highly beneficial in preventing the UR-I/R induced injury in distant kidney. For example, the ephrins, play a vital role in angiogenesis including primary network remodeling and tissue vascularization by the generation of new capillaries from existing vessels and, hence the enhanced expression of Ephb2 with fenoldopam administration observed in the present study suggests that it may be able to prevent UR-I/R-induced renal vasculature disruption in the distant kidney.

While it is tempting to speculate that these fenoldopam-mediated alterations in UR-I/R-induced gene expression might be beneficial to patients, if indeed these findings could be reproduced in the clinical setting, association is by no means proof of causation and we do not claim any such extrapolation here. Nevertheless, our data do provide an interesting platform on which to form future hypotheses about the role of vasodilator drugs in renal I/R injury. In the clinical setting it is rarely possible to administer an agent before an ischemic insult occurs to the kidney. The situation of descending thoracic aortic aneurysm (TAA) repair is just such a scenario however, and the high incidence of renal failure after this procedure shows that this is a serious, and presently unmet, medical need. Although it can never completely replicate the overall procedural insult, we feel that our model at least reproduces the temporal aspects of the renal ischemia induced by this operation, and the reason we gave the drug before the insult was because this would certainly be possible in the actual setting of TAA repair.

In conclusion, in the present study using pathway focused microarrays, we demonstrate that (a) UR-I/R significantly modulated the expression of both proinflammatory NFκB signaling pathway genes and angiogenesis related genes in the non-ischemic distant kidney and, (b) administration of
fenoldopam significantly attenuates UR-I/R-modulated genes. These data provide further evidence that UR-I/R causes inflammatory injury to the contralateral kidney and possibly to other remote organs. The degree of injury to the distant kidney may be insufficient to cause detectable functional impairment; nevertheless, it may make the distant kidney susceptible to further injury during states of additional physiological stress.

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