

Transient Hyperglycemia Affects the Extent of Ischemia-Reperfusion-induced Renal Injury in Rats

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Background: Chronic hyperglycemia is known to increase renal injury, particularly during ischemia-reperfusion episodes. The goal of this study was to examine whether transient hyperglycemia during or after renal ischemia-reperfusion increased renal dysfunction.

Methods: Male Lewis rats underwent sham operations or unilateral nephrectomies followed by contralateral renal ischemia-reperfusion. Hyperglycemic rats were given 25% dextrose to induce transient hyperglycemia lasting throughout the duration of ischemia (PI rats) or beginning 2 h after initiation of reperfusion (PR rats). Additional vehicle control rats received saline and underwent ischemia-reperfusion surgery as with PI and PR rats. Twenty-five minutes of mild renal ischemia followed by 24 h of reperfusion was induced by occluding the renal artery and vein.

Results: Terminal serum creatinine concentrations were significantly higher in the PI rats when compared with the PR or vehicle control rats. Histology demonstrated significantly increased necrosis in the PI rats relative to PR and control animals. Tissue analyses demonstrated significantly higher heat shock protein 70, heat shock protein 32, and cleaved caspase-3 protein levels in the PI rats. Oxidative stress generated through the xanthine pathway in the PI group was significantly increased compared with the oxidative stress in the PR and vehicle control rats. In contrast, vascular endothelial growth factor and erythropoietin were significantly decreased in the PI rats compared with the PR rats and controls.

Conclusions: Hyperglycemia that occurred during renal ischemia-reperfusion resulted in severe functional injury compared with normoglycemia or with hyperglycemia that occurred after reperfusion. Investigated molecular pathways are more profoundly affected by hyperglycemia that occurs before renal ischemia-reperfusion.

THE role of hyperglycemia in inducing renal dysfunction is incompletely understood. Several experimental studies have documented that renal ischemic injury in diabetic animals is worse than similar renal ischemic injury

in normoglycemic animals. This increased renal injury seems to be due to exaggerated oxidative stress and to increased inflammatory responses in the kidneys of hyperglycemic animals. However, these data were generated by investigations using experimental animals that had absolute insulin deficiency due to β -cell destruction.¹⁻³ Therefore, it is unclear whether acute hyperglycemia in nondiabetic animals would be equally injurious to renal function.

Multiple clinical studies have documented the adverse effects of chronic hyperglycemia on patient outcomes.^{4,5} However, adverse events associated with acute hyperglycemia are more difficult to prove. Acute hyperglycemia in patients who have sustained trauma, strokes, or myocardial ischemia or who have undergone cardiac surgery is associated with increased morbidity and mortality.⁵⁻⁹ These seemingly diverse events all involve ischemia-reperfusion, which may be the reason acute hyperglycemia is so detrimental to these patients because hyperglycemia seems to increase oxidative stress in ischemic organs.¹⁰⁻¹³ This hypothesis suggests that strict glucose control would only be of value in critically ill patients who had sustained ischemia-reperfusion. In fact, this hypothesis is supported by the finding that strict glucose control in all critically ill medical patients is not always beneficial, but can be harmful because of hypoglycemic events.^{9,14-19} What remains to be determined is the effect of acute hyperglycemia on renal ischemia-reperfusion injury, and whether the timing of the hyperglycemia relative to the ischemia-reperfusion event has an effect on renal injury.^{4,5,8} The goals in this study were to determine (1) the mechanism for increased renal ischemic injury in the presence of acute hyperglycemia and (2) whether acute hyperglycemia before or after ischemia-reperfusion is equally injurious to the kidney.

Materials and Methods

Animal Surgery (Production of Renal Ischemia and Unilateral Nephrectomy)

All animal experiments were conducted at the University of California at San Francisco; all experiments were approved by the University of California at San Francisco Committee on Animal Research. Animal care was in agreement with the National Institutes of Health guidelines for ethical research (National Institutes of Health publication 80-123, revised 1985). Inbred male Lewis rats (250–300 g body weight) were used for all experiments. Animals were anesthetized with isoflurane and

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kept normothermic during all procedures (approximately 37°C). Both kidneys were exposed through a midline incision. Subsequently, the right kidney was removed. After mobilization of the left kidney, the renal artery and vein were carefully dissected. Renal ischemia was induced by placing a nontraumatic microvascular clamp on both the renal artery and vein. After 25 min of ischemia, the clamp was removed, and reperfusion was confirmed by macroscopic appearance. Twenty-five minutes of renal ischemia causes only mild injury in rats. We chose this ischemia time to better delineate the impact of hyperglycemia.

Twenty-four hours after the operation, animals were killed, and blood and kidney samples were collected for further analysis.

Study Groups

In a preliminary study, we had a control group of animals that underwent sham surgery, which included exposure of the kidneys without nephrectomies and without induction of ischemia. These animals received either saline (normoglycemic sham, $n = 4$) or dextrose injections (hyperglycemic sham, $n = 4$) 30 min before what would have been the induction of ischemia.

Subsequently, we studied animals that were assigned to three experimental groups of renal ischemia-reperfusion:

1. *Vehicle control*: intraperitoneal injection 30 min before onset of ischemia (saline, $n = 4$)
2. *2.5 g/kg Dextrose*: intraperitoneal injection 30 min before ischemia (PI dextrose group, $n = 8$)
3. *2.5 g/kg Dextrose*: intraperitoneal injection 2 h after reperfusion (PR dextrose group, $n = 8$)

Glucose concentrations in blood were monitored at baseline, at 30 min after vehicle or dextrose injection, and at 24 h after reperfusion or sham operation. Before the experiment, the decision was made to include animals in the PI group only if they had a blood glucose concentration of greater than 200 mg/dl before the induction of ischemia. Blood samples obtained for glucose at baseline and at 24 h after reperfusion were also used for assessment of renal function.

Animal Recovery after Surgery

Animal recovery after the surgery or sham surgery was closely monitored. Animal behavior and appearance were assessed at 1, 3, and 6 h and at the time of euthanasia 24 h after surgery. Animals were observed for signs of poor clinical condition, which included lack of urine production, lethargy, ruffled fur, and guarding upon abdominal palpation. Upon euthanasia, all animals underwent necropsy and were examined for intraabdominal pathology, including hemorrhage, bowel obstruction, or other problems. Animals were included in the analysis only if they survived the 24-h experimental interval.

Renal Function

Renal function was assessed by serum creatinine (SCr) measurements. Blood samples were obtained twice for renal function: once from the tail vein before the ischemic injury (baseline sample) and once from the inferior vena cava during harvest 24 h after reperfusion. Blood samples (0.25 ml) were sent to a veterinary laboratory service (IDEXX Veterinary Services, Sacramento, CA) for determination of SCr.

Renal Morphology Assessments

A pathologist reviewed all histologic specimens without knowledge of the experimental group assignments. Semiquantitative assessments of histologic damage were performed: Kidney samples were cross-sectioned through their midportion, fixed in buffered formalin, and embedded in paraffin. For each kidney, two representative cross-sections containing the cortex and medulla were examined for tubular changes with an Axiolab light microscope (Carl Zeiss, Hamburg, Germany).

An ocular square ($20 \times 20 \mu\text{m}$; magnification $\times 400$) served as field of view. All tubules lying within a field of view were counted (cortex, 15 field of view; medulla, 30 field of view). The percentage of damaged tubules per field of view was used to measure histologic damage, typically appearing as tubular cell necrosis. The following light microscopic criteria for tubular cell necrosis were applied: nuclear pyknosis, karyorrhexis, and/or disruption of cell membranes. The severity of tubular damage was graded on a scale from 1 to 5. The different grades were defined as follows: grade 0 = no damage, grade 1 (mild damage) = less than 5% tubular necrosis, grade 2 (mild to moderate damage) = 5–25% tubular cell necrosis, grade 3 (moderate to severe damage) = 25–50% tubular cell necrosis, grade 4 (severe damage) = 50–75%, and grade 5 (severe to very severe damage) = greater than 75% tubular cell necrosis.²⁰

RNA Isolation and Quantitative Real-time Polymerase Chain Reaction

Total RNA was isolated from the frozen ischemic kidneys using RNeasy Mini Kit with on-column DNase digest according to manufacturer's instructions (QIAGEN, Valencia, CA). RNA yield and purity were determined on Smartspec 3000 (Biorad, Hercules, CA), and RNA integrity was confirmed by presence of intact 28S and 18S bands on 1% agarose gel. One microgram total RNA was converted to complementary DNA (cDNA) according to the manufacturer's protocol using 200U MMLV-Reverse Transcriptase with 250 ng of random primers (Invitrogen, Carlsbad, CA).

Gene expression for macrophage inflammatory protein 2 (MIP-2), interleukin 6, vascular endothelial growth factor (VEGF), and erythropoietin were assessed *via* quantitative real-time polymerase chain reaction (7300 Real-Time PCR System; Applied Biosystems, Foster City,

Table 1. Sequences of Quantitative Real-time Polymerase Chain Reaction Primers and TaqMan Probes

Gene	GenBank Accession No.	Sequence (5'→3')
β -actin	NM_031144	Forward CTG GCT CCT AGC ACC ATG AAG Reverse GAG CCA CCA ATC CAC ACA GA
Erythropoietin	NM_017001	Probe TCA AGA TCA TTG CTC CTC CTG AGC G Forward CGT CCC AGA TAC CAA AGT CAA CT Reverse CAG AGA CAG GCC TTG CCA AA
Interleukin 6	NM_012589	Probe TGG AAA AGA ATG AAG GTG GAA GAA CAG GCT Forward CGG AGG CTT AAT TAC ACA TGT TCT C Reverse AAG TGC ATC ATC GTT GTT CAT ACA
MIP-2	NM_053647	Probe TGA GAA AAG AGT TGT GCA ATG GCA ATT CTG Forward GAA GCC CCC TTG GTT CAG A Reverse GCC CAT GTT CTT CCT TTC CA
VEGF	NM_031836	Probe TCC AAA AGA TAC TGA ACA AAG GCA AGG CTA ACT Forward CGC AAG AAA TCC CGG TTT AA Reverse CAA ATG CTT TCT CCG CTC TGA Probe CCT GGA GCG TTC ACT GTG AGC CTT G

MIP-2 = macrophage inflammatory protein 2; VEGF = vascular endothelial growth factor.

CA). Table 1 shows primers and 5'-6-carboxyfluorescein/3' Black Hole Quencher (Biosearch Technologies, Novato, CA)-labeled TaqMan probes designed with Applied Biosystems Primer Express 2.0 software and synthesized by Integrated DNA Technologies (Coralville, IA). Each primer-probe combination was validated to within the accepted 95–105% efficiency. Fifty nanograms of reverse-transcribed cDNA was used as template along with 500 nM forward and reverse primers, 200 nM TaqMan probe, 200 nM dNTPs (Allstar Scientific, Sunnyvale, CA), 5.5 mM $MgCl_2$, and TaqMan buffer (University of California at San Francisco Mt. Zion Genome Core Facility, San Francisco, CA) for polymerase chain reaction amplification. Fluorescence was measured after each polymerase chain reaction cycle (initial one-time 10-min denaturation at 95°C, followed by 40 amplification cycles with 15-s denaturation at 95°C and 1-min annealing-extension at 60°C). Each sample was measured in triplicate, and all genes were normalized to the endogenous control β -actin. Reverse-transcriptase negative controls were run in duplicates and in parallel and were found to have no to negligible genomic expression contribution. Relative quantification of target genes was standardized to sham samples by the comparative $C(t)$ method using threshold cycle $C(t)$ values determined on ABI GeneAmp software (Applied Biosystems).

Protein Isolation and Western Blots

All steps for protein isolation were conducted at 4°C. Snap frozen kidney sections were homogenized in Tissue Protein Extraction Reagent (Pierce Biotechnology, Rockford, IL) containing 1 mM EDTA and 1:100 Protease Cocktail Inhibitor (Sigma, St. Louis, MO) and were centrifuged at 10,000g for 5 min. The supernatant was aliquoted, snap frozen, and stored at –80°C. Protein concentrations of kidney homogenates were measured by the Pierce BCA protein assay with bovine serum albumin as the standard. Fifty micrograms of kidney

homogenates was separated on a NOVEX-NuPAGE 10% Bis-Tris sodium dodecyl sulfate polyacrylamide gel electrophoresis gel (Invitrogen) and transferred to nitrocellulose membrane using the XCell SureLock system (Invitrogen). A mouse anti-heat shock protein 70 (HSP70) monoclonal antibody (SC-24), a goat anti-VEGF polyclonal antibody (SC-1386), a goat anti-erythropoietin polyclonal antibody (SC-1310), a rabbit anti-human p22^{phox} polyclonal antibody (SC-20781), a rabbit anti-human p47^{phox} polyclonal antibody (SC-14015), and a goat anti-actin antibody (SC-1616) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). In addition, a mouse anti-heat shock protein 32 (HSP32, or heme oxygenase 1) monoclonal antibody was purchased from Stressgen (Ann Arbor, MI), a mouse anti-nitrotyrosine antibody was purchased from Abcam Inc. (Cambridge, MA), and a rabbit anti-cleaved caspase-3 monoclonal antibody was purchased from Cell Signaling Technology (Danvers, MA). The membranes were incubated with a 1:100 or 1:1,000 dilution of the primary antibody followed by a 1:10,000-fold dilution of a secondary anti-mouse or anti-goat immunoglobulin G from Santa Cruz Biotechnology. Immunoreactive proteins were developed using Super-Signal West Dura (Pierce Biotechnology) and visualized on the FluorChem 5500 Imaging system from Alpha Innotech (San Leandro, CA). Band intensities were quantified *via* spot densitometry.

Enzyme-linked Immunosorbent Assay

Kits were purchased from Invitrogen to measure MIP-2 and interleukin-6 protein levels in the kidney according to the manufacturers' manuals.

Nicotinamide Adenine Dinucleotide (Phosphate) Oxidase and Nitrotyrosine Measurement

Protein abundance of nicotinamide adenine dinucleotide (phosphate) (NAD(P)H) oxidase subunits p22^{phox} and p47^{phox} and nitrotyrosine containing proteins were

measured by Western blot as described under Protein Isolation and Western Blots.

Allantoin Determination

Allantoin was determined by ^1H -nuclear magnetic resonance (NMR) analysis. Kidney tissues were extracted using 12% perchloric acid. Briefly, 0.2–0.8 g of frozen kidney tissue was homogenized in 4 ml perchloric acid using an electrical homogenizer and centrifuged for 20 min at 1,300g at 4°C. The supernatants were collected and the pellets were redissolved with 2 ml perchloric acid before being vortexed and centrifuged. The supernatants were combined, neutralized to pH 7 using potassium hydroxide, and centrifuged again to remove potassium perchlorate. The supernatants that contained water-soluble endogenous metabolites were then lyophilized overnight to remove water for NMR experiments. The water-soluble extracts were then dissolved in 0.45 ml deuterium oxide before ^1H -NMR. The pH of each extract inside of the 5-mm NMR tube was checked using a thin pH electrode. If necessary, deuterated chloride and sodium hydrogen were added to adjust the pH to 7.

After the second centrifugation, the pellets were redissolved in 4 ml ice-cold water, and a pH of 7 was adjusted using potassium hydroxide. The pellet suspensions that contained lipid fraction were then lyophilized overnight to remove water for NMR experiments. The lipid extracts were dissolved in 1 ml deuterated chloroform-methanol mixture (2:1, vol/vol) before ^1H -NMR.

All one- and two-dimensional ^1H -NMR spectra were obtained on a Bruker 500 MHz DRX spectrometer (Bruker Bisopin, Fremont, CA) using an inverse 5-mm TXI probe (tuned for proton detection) as previously described.²¹

Statistical Analysis

All data are presented as mean \pm SD. Normoglycemic and hyperglycemic sham groups were compared using a two-tailed unpaired *t* test (data not shown).

The three experimental groups that underwent ischemia-reperfusion were compared with the normoglycemic sham group using analysis of variance with *post hoc* Dunnett correction, with sham animals serving as controls. Data between the three experimental groups were compared using a two-tailed unpaired *t* test. *P* values less than 0.05 were recognized as being statistically significant.

Results

Normoglycemic and Hyperglycemic Sham Rats

Baseline blood glucose levels in all sham rats averaged 109.8 ± 13.4 mg/dl ($n = 8$). Blood glucose levels in sham animals treated with dextrose ($n = 4$) increased to 370.5 ± 40.9 mg/dl 30 min after dextrose administra-

tion. Animals underwent euthanasia 24 h later, and the blood glucose concentrations had returned to baseline in all four dextrose-treated sham animals.

Transient hyperglycemia did not result in creatinine changes in hyperglycemic sham animals when compared with normoglycemic sham animals. In addition, when tissue was examined for gene expression (table 1) or corresponding protein expression, profiles from the four transient hyperglycemic sham animals were not different from those of the four normoglycemic sham animals (data not shown). These results suggest that transient hyperglycemia in sham-operated animals did not have any discernible effect on gene and protein expression. All subsequent studies were performed in animals undergoing renal ischemia and reperfusion.

Ischemia-Reperfusion Experimental Groups

Blood Glucose Concentrations in Dextrose-treated Rats. The average baseline blood glucose concentration in the 20 experimental animals was 109.7 ± 15.5 mg/dl.

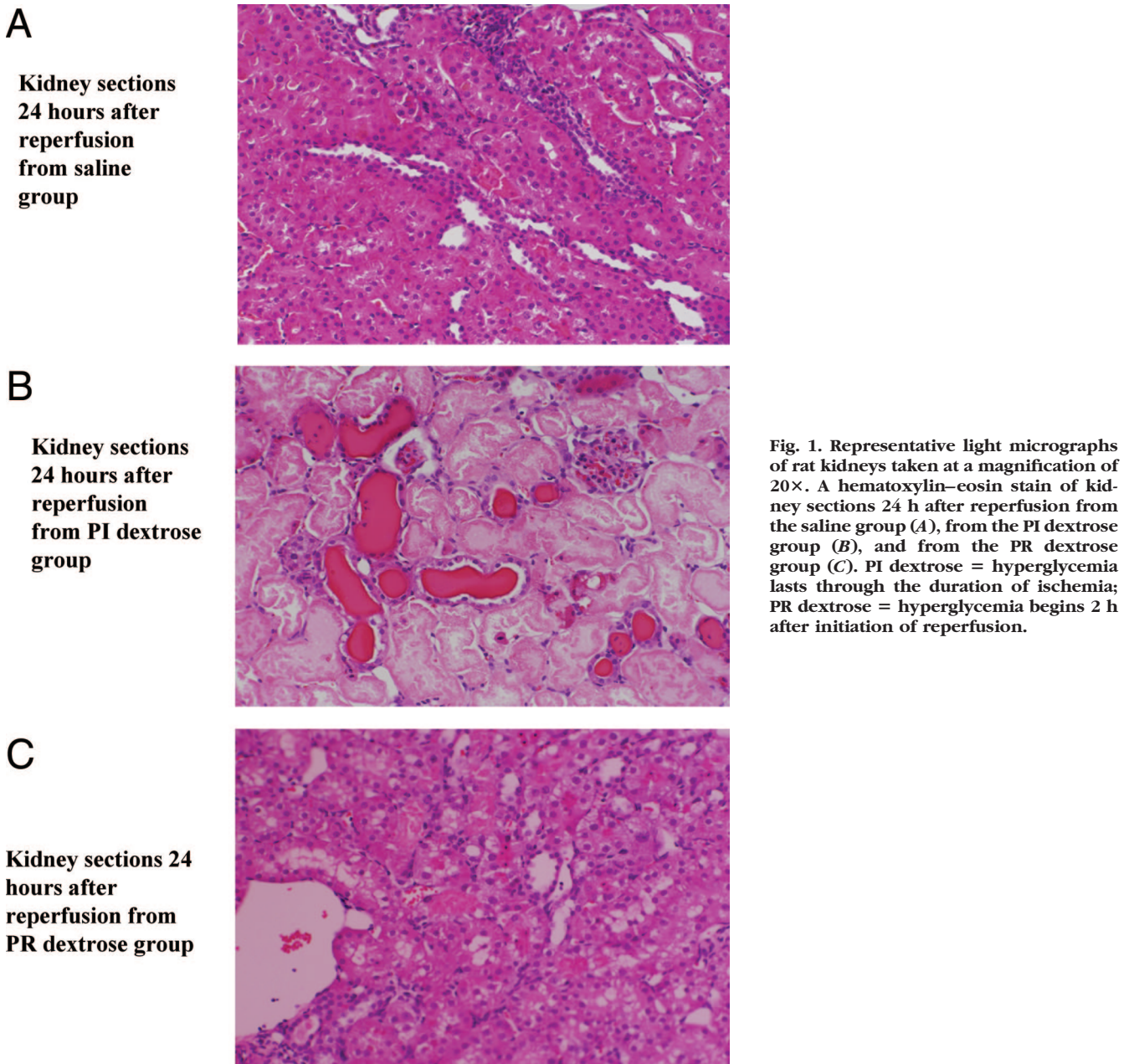
In the four vehicle control animals, the average blood glucose concentration was 163.0 ± 5.4 mg/dl at the time of ischemic onset. The average blood glucose concentration for the PI rats was 341 ± 111.3 mg/dl just before ischemia, which corresponded to 30 min after dextrose administration ($n = 8$).

The PR animals ($n = 8$) that received dextrose 2 h after reperfusion had an average preischemic blood glucose concentration of 160.8 ± 7.5 mg/dl, which was not statistically different from that of the vehicle control group. Blood glucose levels after injection of glucose increased to 471 ± 49 mg/dl 2.5 h after reperfusion.

All animals, regardless of sham or experimental group classification, were found to have similar average glucose concentrations after 24 h (total combined $n = 28$ for sham, PI, PR, and vehicle control rats). This result confirms the transient nature of the hyperglycemia in otherwise healthy animals with normal insulin secretion and resistance.

Serum Creatinine Levels at Baseline and 24 h after Ischemia-Reperfusion. Baseline SCr concentrations were 0.5 ± 0.1 mg/dl for all 28 animals. After 25 min of ischemia and 24 h of reperfusion, SCr concentration was significantly increased in the PI dextrose group (3.3 ± 2 mg/dl, $n = 8$) compared with the SCr concentrations in the PR dextrose and vehicle control groups (0.7 ± 0.2 and 0.8 ± 0.1 mg/dl, respectively, $n = 4$ in each group; $P < 0.05$). Four blood samples at the 24-h reperfusion time point from the PR group were lost during shipping, and creatinine could not be determined. All animals except for the sham animals, which did not undergo ischemia-reperfusion, had significantly increased terminal SCr concentrations compared with their respective baseline measurements ($P < 0.05$).

Renal Histology. Epithelial necrosis was significantly more extensive in the PI dextrose group (3.0 ± 0.8 , $n =$



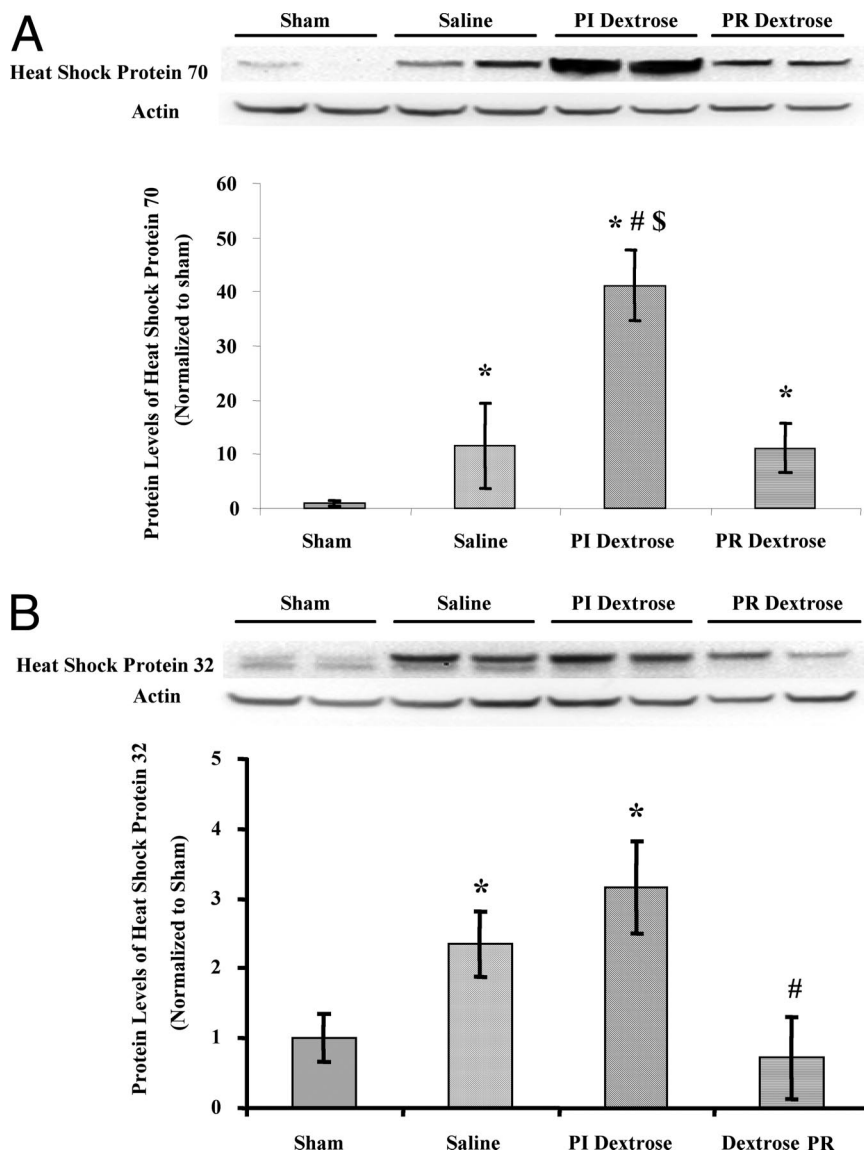
5) compared with the necrosis found in either the PR or vehicle control groups (1.0 ± 0 , $n = 4$ in each group; $P < 0.05$). There were only areas of unicellular and patchy necrosis without significant congestion in the kidneys taken from the vehicle control animals and from the PR animals. In contrast, there was significant congestion and prominent epithelial necrosis in the medullary and cortical areas of the kidneys from the PI animals (fig. 1).

Kidney Chemokine and Cytokine Expression. Macrophage inflammatory protein 2 and interleukin 6 are important chemokines and cytokines that have been found to mediate ischemia-reperfusion injury.²² After ischemia-reperfusion, MIP-2 messenger RNA (mRNA) expression increased in the kidneys from vehicle control animals compared with the kidneys from sham animals

($n = 4$ in each group; $P < 0.05$). In contrast, MIP-2 mRNA significantly increased in the kidneys from the PI dextrose group ($n = 6$) compared with the kidneys from the sham rats ($P < 0.05$).

Interleukin-6 mRNA expression increased in the kidneys from the vehicle control animals compared with the kidneys from the sham animals, whereas interleukin-6 mRNA expression in the PI rats significantly increased relative to the expression in sham kidneys ($n = 4$ in each group; $P < 0.05$). MIP-2 and interleukin-6 mRNA levels in the PR dextrose group were similar to those in the vehicle control group. However, there was no significant difference in MIP-2 and interleukin-6 protein levels between the sham and ischemic groups (data not shown).

Fig. 2. Heat shock proteins were induced by hyperglycemia. Representative immunoblots for heat shock protein 70 (HSP70; A) and heat shock protein 32 (HSP32; B) in kidneys after ischemia-reperfusion along with densitometric analysis. HSP70 and HSP32 proteins were measured by Western blot in 50 μ g of kidney homogenates. Densitometric values were normalized to actin and are expressed as a ratio of sham \pm SD, with $n = 4$ each in the sham and saline control groups and $n = 6$ each in the PI dextrose and PR dextrose groups. HSP70 and HSP32 were induced in the saline group after ischemia-reperfusion compared with sham but were dramatically induced in the PI dextrose group. * $P < 0.05$ versus sham. # $P < 0.05$ versus saline. \$ $P < 0.05$ versus PR dextrose group. HSP70 and HSP32 protein levels in the PR dextrose group were different than those in the saline group. PI dextrose = hyperglycemia lasts through the duration of ischemia; PR dextrose = hyperglycemia begins 2 h after initiation of reperfusion.



Heat Shock Protein Concentration in Kidneys.

The kidneys from either saline or dextrose-treated sham rats had barely detectable protein concentrations of HSP70 and HSP32 (the latter also known as heme oxygenase 1) 24 h after sham operation. HSP70 levels in the vehicle group increased 11-fold after ischemia-reperfusion relative to sham animals ($n = 4$ in each group; $P < 0.05$). The kidneys from the PI rats ($n = 6$) had significantly increased HSP70 protein concentrations compared with both the sham and vehicle control rats ($P < 0.05$; fig. 2A). In contrast, the HSP70 concentrations in the kidneys from the PR group ($n = 6$) were similar to the concentrations in the kidneys from the vehicle control animals.

Heat shock protein 32 concentrations increased more than twofold in the kidneys from the vehicle control animals compared with the sham animals ($n = 4$ in each group; $P < 0.05$). Renal HSP32 protein levels in the PI animals ($n = 6$) were threefold higher than in the sham

animals ($P < 0.05$; fig. 2B). Renal HSP32 protein concentrations were not significantly different between the PR dextrose animals ($n = 6$) and the sham animals. Interestingly, HSP32 concentrations in the kidneys from the PR animals were significantly lower than concentrations from the vehicle control animals ($P < 0.05$).

Hyperglycemia before and during Ischemia-Reperfusion Is Associated with Decreased VEGF and Erythropoietin Expression. Vascular endothelial growth factor mRNA and protein concentrations in the kidneys were not different in the vehicle control animals compared with the sham animals ($n = 4$ in each group). However, the PI group was found to have significantly decreased VEGF protein concentrations relative to the sham group ($n = 6$ in the PI group, $n = 4$ in the sham group; $P < 0.05$; fig. 3).

Erythropoietin mRNA and protein levels did not change significantly (albeit there was a trend) between vehicle control animals and sham animals ($n = 4$ in each

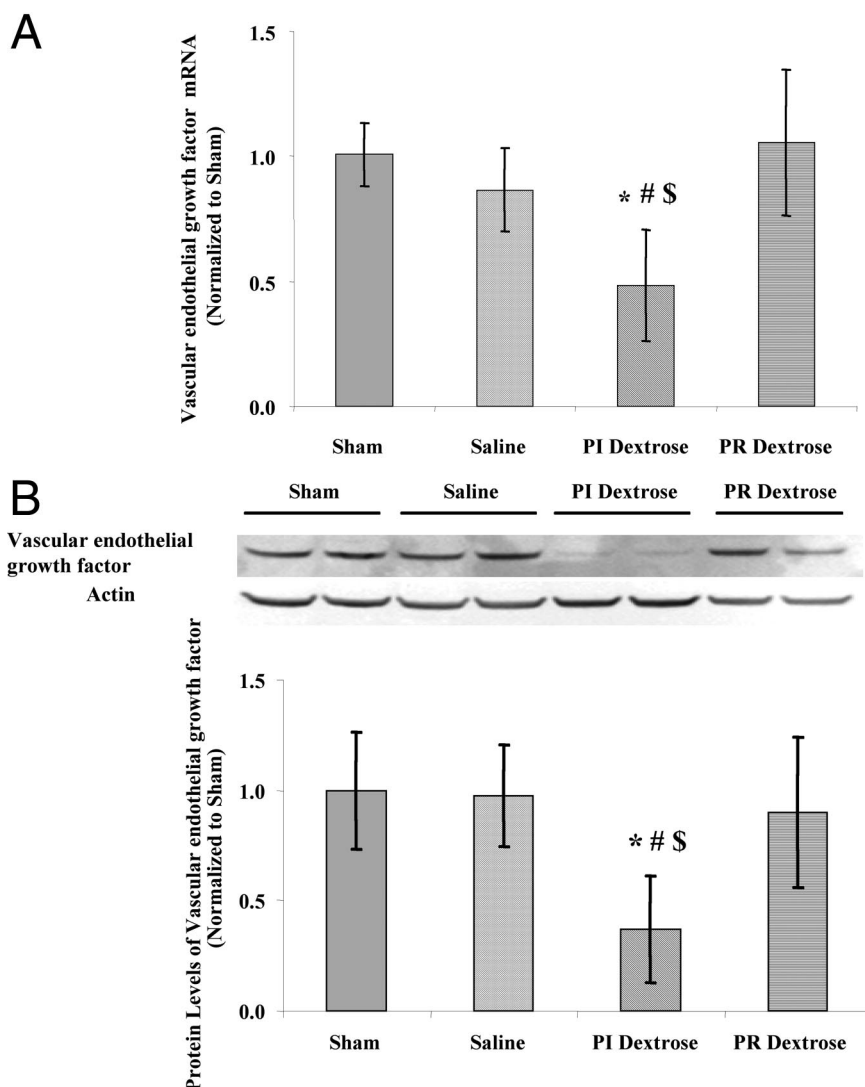


Fig. 3. Hyperglycemia down-regulated the expression of vascular endothelial growth factor (VEGF). **(A)** Quantitative messenger RNA (mRNA) expression of VEGF as assessed by TaqMan quantitative real-time polymerase chain reaction in renal tissue 24 h after reperfusion. Values were normalized to β -actin and are expressed as a ratio of sham \pm SD, with $n = 4$ in each group. VEGF mRNA levels did not change in the saline group after ischemia–reperfusion compared with sham but were significantly decreased in the PI dextrose group. **(B)** Representative immunoblot for VEGF in kidneys after ischemia–reperfusion along with densitometric analysis. VEGF protein was measured by Western blot in 50 μ g of kidney homogenates. Densitometric values were normalized to actin and are expressed as a ratio of sham \pm SD, with $n = 4$ each in the sham and saline control groups and $n = 6$ each in the PI dextrose and PR dextrose groups. VEGF protein levels did not change in the saline group after ischemia–reperfusion compared with sham but were significantly decreased in the PI dextrose group. * $P < 0.05$ versus sham. \$ $P < 0.05$ versus PR dextrose group. # $P < 0.05$ versus saline. VEGF expression in the PR dextrose group was similar to that in the saline group. PI dextrose = hyperglycemia lasts through the duration of ischemia; PR dextrose = hyperglycemia begins 2 h after initiation of reperfusion.

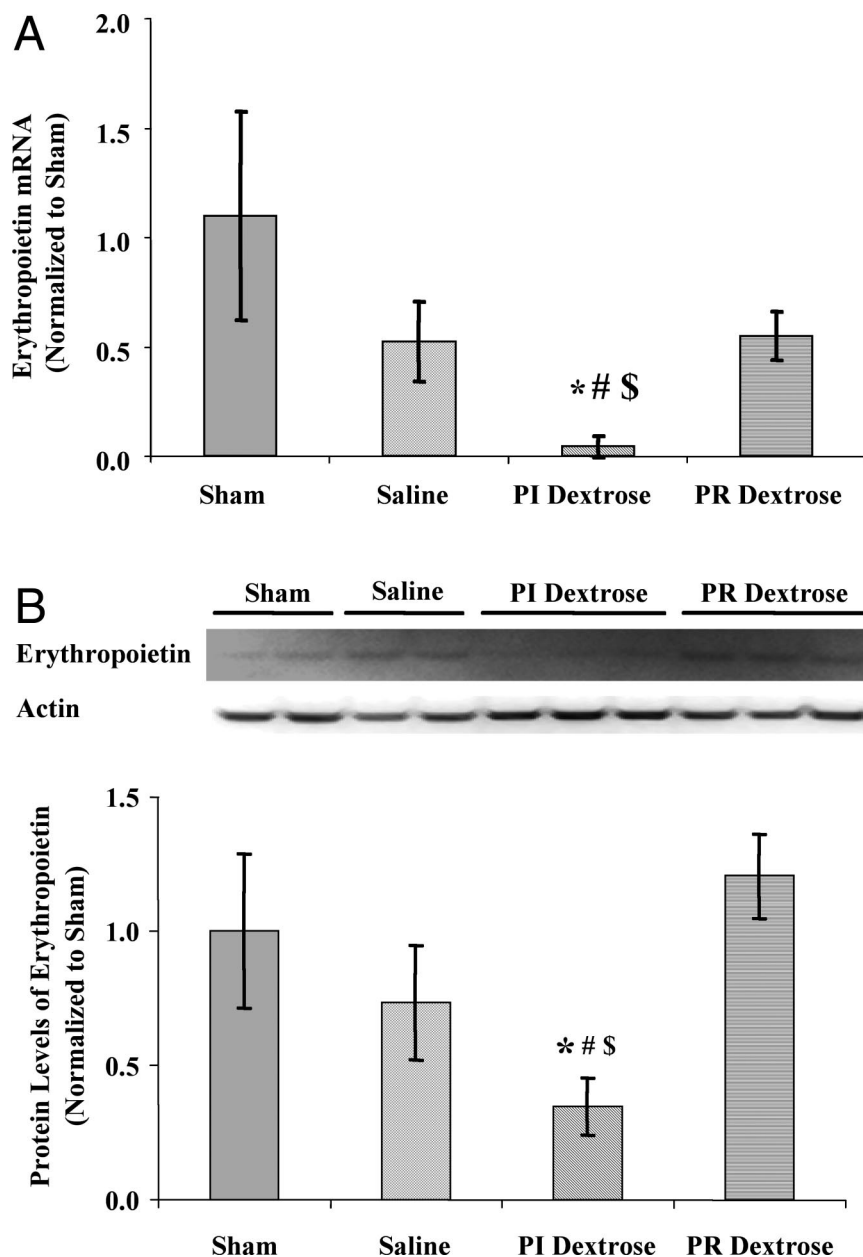
group). However, similar to the VEGF results, there was a significant decrease in renal erythropoietin mRNA and protein expression in the kidneys from the PI animals relative to sham animals ($n = 6$ in the PI group, $n = 4$ in the sham group; $P < 0.05$; fig. 4). VEGF and erythropoietin mRNA and protein levels in the kidneys from the PR animals ($n = 6$) were not different from those in the vehicle control animals ($n = 4$).

Hyperglycemia Induces Apoptosis. Cleaved caspase-3 protein levels in the kidneys were measured as a marker of apoptosis.^{23,24} Cleaved caspase-3 protein levels were not different in the kidneys between the vehicle control and the sham animals ($n = 4$ in each group). In contrast, the concentration of cleaved caspase-3 protein was significantly increased almost twofold in the kidneys from the PI animals when compared with the sham animals ($n = 6$ in the PI group, $n = 4$ in the sham group; $P < 0.05$; fig. 5). Similar to other results, cleaved caspase-3 protein levels in the kidneys from the PR animals were not different from those in the vehicle control animals.

Oxidative Stress Measurements. Other animal studies have provided evidence for up-regulation/activation of NAD(P)H oxidase in ischemia–reperfusion injury. We examined protein levels of NAD(P)H oxidase p22^{phox} and p47^{phox}. The protein expression of p22^{phox} increased 2.9-, 2.5-, and 2-fold, respectively, in the saline vehicle control, PI dextrose, and PR dextrose groups compared with sham animals ($n = 4$ each in the sham and saline groups, $n = 6$ each in the PI dextrose and PR dextrose groups; $P < 0.05$). However, there was no significant difference between the vehicle control and PI dextrose groups (fig. 6A). Expression of p47^{phox} was not different between groups (data not shown).

Up-regulation of the xanthine pathway has been demonstrated to be an important contributor to oxidative stress in transplantation, burn traumas, pulmonary endothelial cells exposed to tobacco smoke, and hypertensive and global ischemia models.²⁵ Xanthine oxidase is a molybdenum-requiring enzyme that uses molecular oxygen and produces hydrogen peroxide that in turn may be used for further reactions in the xanthine pathway in humans. In

Fig. 4. Hyperglycemia down-regulated the expression of erythropoietin. **(A)** Quantitative messenger RNA (mRNA) expression of erythropoietin as assessed by TaqMan quantitative real-time polymerase chain reaction in renal tissue 24 h after reperfusion. Values were normalized to β -actin and are expressed as a ratio of sham \pm SD, with $n = 4$ in each group. Erythropoietin mRNA levels did not change in the saline group after ischemia–reperfusion compared with sham but were decreased dramatically in the PI dextrose group. **(B)** Representative immunoblot for erythropoietin in kidneys after ischemia–reperfusion along with densitometric analysis. Erythropoietin protein was measured by Western blot in 50 μ g of kidney homogenates. Densitometric values were normalized to actin and are expressed as a ratio of sham \pm SD, with $n = 4$ in the sham and saline control groups and $n = 6$ each in the PI dextrose and PR dextrose groups. Erythropoietin protein levels slightly decreased in the saline group after ischemia–reperfusion compared with sham but were significantly decreased in the PI dextrose group. * $P < 0.05$ versus sham. # $P < 0.05$ versus saline. \$ $P < 0.05$ versus PR dextrose. Erythropoietin expression in the PR dextrose group was similar to that in the saline group. PI dextrose = hyperglycemia lasts through the duration of ischemia; PR dextrose = hyperglycemia begins 2 h after initiation of reperfusion.



mammals, allantoin is a metabolic end product of uric acid and, therefore, of xanthine metabolism and thus is metabolic marker of oxidative stress.²¹ Allantoin levels in the kidney were 0.14 ± 0.06 and 0.27 ± 0.16 μ mol/mg, respectively, in the sham and vehicle control animals ($n = 4$ in each group). Notably, the allantoin concentrations were significantly elevated in the kidneys taken from the PI animals compared with the concentrations in both the sham and vehicle control animals ($n = 7$ in the PI group, 1.29 ± 0.88 μ mol/mg; $P < 0.05$; fig. 6B). In contrast, the allantoin concentration in the kidneys taken from PR animals ($n = 5$, 0.29 ± 0.18 μ mol/mg) was similar to the concentration found in the kidneys from the vehicle control animals.

Nitrosative Stress. Four nitrotyrosine containing proteins with a size of 28, 38, 49, and 62 kD, respectively, were detected by Western blots performed on kidney homogenates

(fig. 7). Nitrotyrosine containing protein levels were not different in the kidney homogenates taken from the vehicle control and from the sham animals ($n = 4$ in each group). However, the concentrations of nitrotyrosine containing protein were significantly increased, almost sixfold, in the kidney homogenates from the PI animals when compared with sham or vehicle control animals ($n = 6$ in the PI group; $P < 0.05$; fig. 7). In contrast, the concentrations of the nitrotyrosine containing proteins in the kidney homogenates obtained from the PR animals ($n = 6$) were not different from the sham or vehicle control animals.

Discussion

Our most significant finding is that the presence of transient hyperglycemia initiated immediately before the

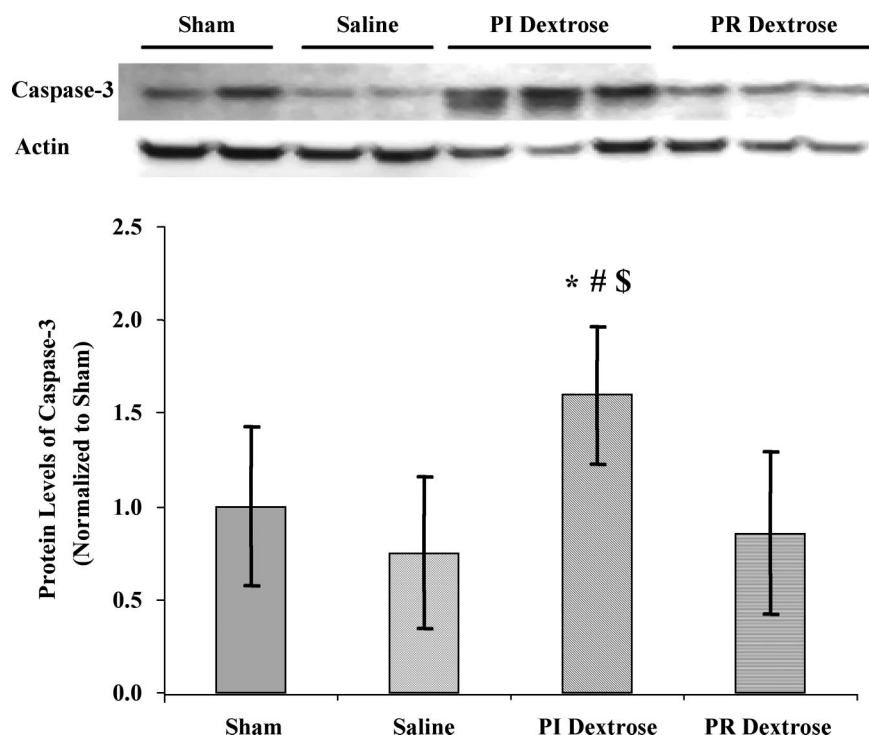


Fig. 5. Apoptosis was induced in hyperglycemia rats. Representative immunoblot for cleaved caspase-3 in kidneys after ischemia-reperfusion along with densitometric analysis. Cleaved caspase-3 protein levels were measured by Western blot in 50 μ g of kidney homogenates. Densitometric values were normalized to actin and are expressed as a ratio of sham \pm SD, with $n = 4$ each in the sham and saline control groups and $n = 5$ each in the PI dextrose and PR dextrose groups. Cleaved caspase-3 did not change in the saline group after ischemia-reperfusion compared with sham but was significantly increased in the PI dextrose group. * $P < 0.05$ versus sham. # $P < 0.05$ versus saline. \$ $P < 0.05$ versus PR dextrose. Cleaved caspase-3 expression in the PR dextrose group was similar to that in saline controls. PI dextrose = hyperglycemia lasts through the duration of ischemia; PR dextrose = hyperglycemia begins 2 h after initiation of reperfusion.

initiation and lasting throughout a period of renal ischemia-reperfusion significantly decreased kidney function and increased renal injury. These results are robust in that they represent multiple independent measurements, including histologic evidence of necrosis in the kidneys, molecular measurements on the kidneys taken from normal animals experiencing transient hyperglycemia just before and during ischemia. In contrast, we could not document that transient hyperglycemia affected renal function or caused renal injury when it occurred shortly after the initiation of reperfusion of the kidney.

Hence, our study suggests that timing of hyperglycemia in relation to the anticipated ischemic injury seems to be crucial for the observed increased injury. These experimental results have possible important implications for clinical care and suggest that glucose control before and during surgically induced ischemia-reperfusion may be important for the preservation of renal function as well as other organ function. In contrast, transient hyperglycemia, as frequently observed in the postoperative care unit, may be less harmful.

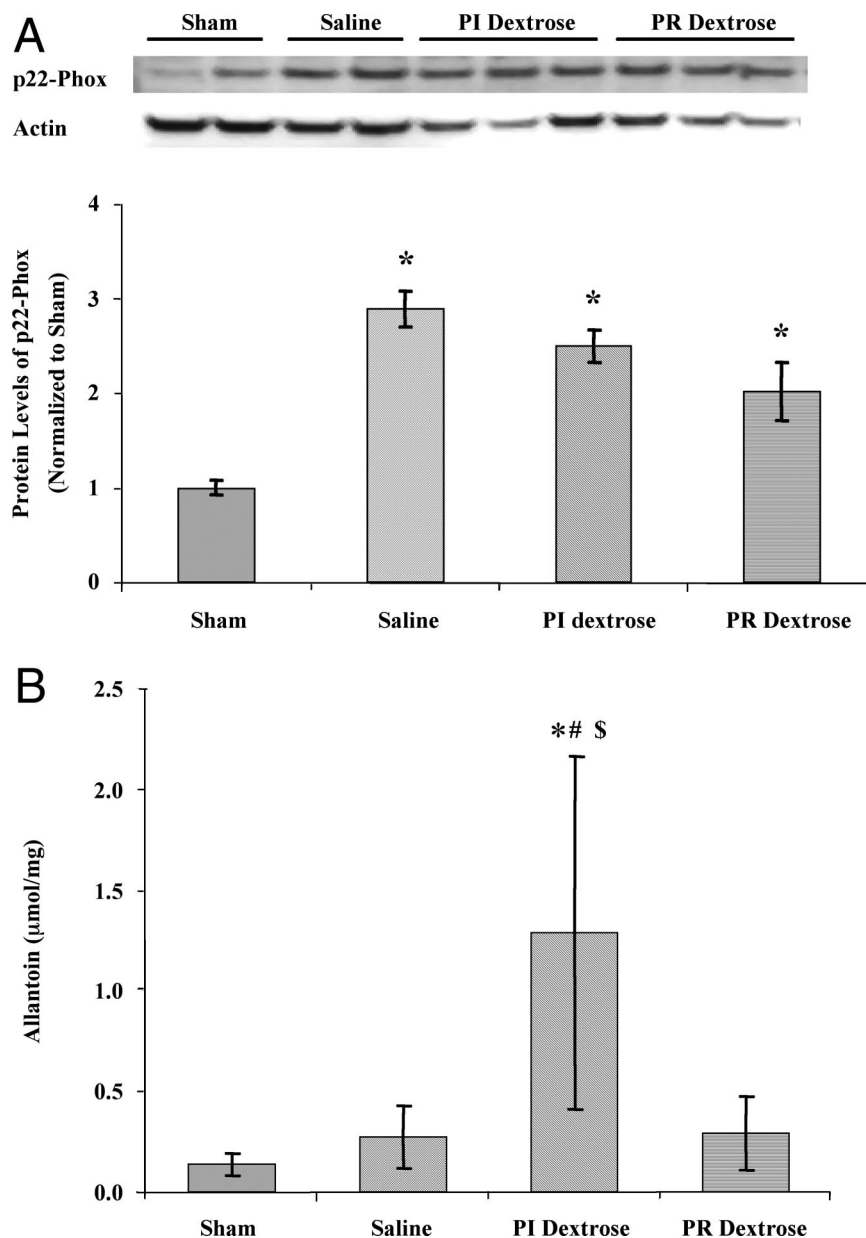
The glucose concentrations that were created by the dextrose administration averaged 341 mg/dl in the PI group of animals. These concentrations can be seen in perioperative patients, where strict glucose control is not a routine practice. The glucose concentrations observed in the vehicle control animals (average 163 mg/dl) were not associated with significant renal injury or loss of function. The elevation of glucose concentration in the PR group seems to be due to stress-induced hyperglycemia because it occurred even before dextrose was administered. These results suggest that there may

be a threshold at which elevated glucose concentrations become injurious to the ischemic kidney. More investigation is needed to determine which glucose concentration is safe for the preservation of organ function during surgically induced ischemia-reperfusion.

Unlike experiments in animals with chronic hyperglycemia, these experiments are unique in that the experimental animals had normal concentrations of insulin and normal insulin responses. This is documented by the return of blood glucose to normal concentrations in all the animals by the end of the experiments at 24 h. These results suggest that the hyperglycemia *per se* was the injurious agent. Similarly, in an analysis of critical care patients, a linear correlation between the degree of hyperglycemia and the risk of death persisted after correction for insulin dose and for the severity of illness scores.⁹ A reduction in blood glucose concentration seemed to be crucial for the prevention of morbidity, including acute renal failure, bacteremia, and anemia.¹⁷ Multivariate logistic regression analysis confirmed an independent role for blood glucose control in achieving most of the clinical benefits of intensive insulin therapy.^{26,27} Our results also suggest that insulin deficiency²⁸ is not the cause of the increased renal injury, which is similar to other recent findings.^{26,27}

Mechanisms for increased organ injury in chronic hyperglycemia have been attributed to an elevated baseline inflammatory state that increases oxidative stress, which is aggravated during physiologic stress.^{1,10,29-31} There have been few investigations of the effect of transient hyperglycemia in nondiabetic animals. Only two studies, published approximately 20 yr ago, used a similar ap-

Fig. 6. (A) p22^{phox} expression was measured by Western blots in 50 μ g of kidney homogenates. Densitometric values were normalized to actin and are expressed as a ratio of sham \pm SD, with $n = 4$ each in the sham and saline control groups and $n = 6$ each in the PI dextrose and PR dextrose groups. p22^{phox} was induced in the saline, PI dextrose, and PR dextrose groups after ischemia–reperfusion compared with sham, * $P < 0.05$. (B) Allantoin was measured in kidney tissues using ¹H-nuclear magnetic resonance analysis as described in the Materials and Methods. Values are expressed as mean \pm SD, with $n = 4$ each in the sham and saline control groups, $n = 7$ in the PI dextrose group, and $n = 5$ in the PR dextrose group. Allantoin concentration did not change in the saline group after ischemia–reperfusion compared with sham but increased dramatically in the PI dextrose group. * $P < 0.05$ versus sham. # $P < 0.05$ versus saline. \$ $P < 0.05$ versus PR dextrose. Allantoin concentration in the PR dextrose group was similar to that in saline controls. PI dextrose = hyperglycemia lasts through the duration of ischemia; PR dextrose = hyperglycemia begins 2 h after initiation of reperfusion.



proach to examine transient hyperglycemia and renal injury.^{28,32} Their investigations only evaluated histology and renal function. Detailed analysis of possible mechanisms of increased injury during transient hyperglycemia was not performed. Some of our results are similar to the findings in animals with chronic hyperglycemia (e.g., pronounced inflammatory response and increased oxidative stress), but other results are not, and this suggests that the duration of the transient hyperglycemia seems to affect not only organ function but also the vulnerability to ischemic injury.

The current results demonstrate that oxidative stress is increased in kidneys that are subject to ischemia during hyperglycemia. Oxidative stress can be documented by the production of reactive oxygen radicals, which can be produced *via* different pathways. These pathways in-

clude various enzymes such as lipoxygenases, peroxidases, NADPH oxidase, and xanthine oxidase. The prototypical NAD(P)H oxidase and its isotypes are significant source of reactive oxygen radicals in endothelial cells, vascular smooth muscle cells, and cellular constituents of the kidney.³³

Several experimental animal studies have provided evidence that up-regulation/activation of NAD(P)H oxidase and oxidative stress in the kidney promotes proinflammatory cytokines, chemokines, growth factors, and adhesion molecules, which then cause inflammation and fibrosis.³³ In the current study, only the NAD(P)H oxidase p22^{phox} was significantly up-regulated, whereas p47^{phox} was not different between groups. The observed up-regulation of p22^{phox} has been reported in other models of renal injury.^{34,35}

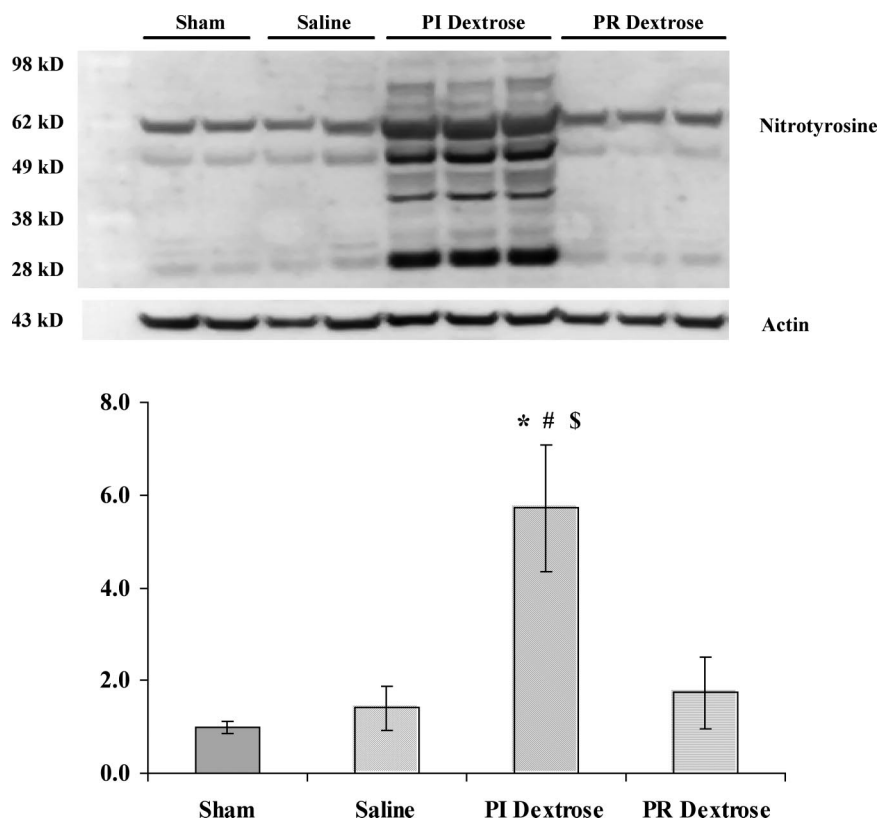


Fig. 7. Nitrotyrosine containing protein levels were measured by Western blot in 50 μ g of kidney homogenates. Densitometric values were normalized to actin and are expressed as a ratio of sham \pm SD, with $n = 4$ each in the sham and saline control groups and $n = 6$ each in the PI dextrose and PR dextrose groups. Nitrotyrosine containing protein levels did not change in the saline group after ischemia-reperfusion compared with sham but were significantly increased in the PI dextrose group. * $P < 0.05$ versus sham. # $P < 0.05$ versus saline. \$ $P < 0.05$ versus PR dextrose. Nitrotyrosine containing protein levels in the PR dextrose group were similar to those in saline controls. PI dextrose = hyperglycemia lasts through the duration of ischemia; PR dextrose = hyperglycemia begins 2 h after initiation of reperfusion.

In addition, 24 h after reperfusion, we were able to find increased allantoin, a marker of oxidative stress, after ischemia-reperfusion in all of the experimental groups. However, the levels of allantoin after reperfusion were significantly higher in the PI rats compared with all of the other animal groups. This result suggests that the xanthine pathway involving allantoin is differentially affected by hyperglycemia that occurs before and during renal ischemia-reperfusion. Increased concentrations of allantoin after reperfusion of the ischemic kidney indicate up-regulation of xanthine oxidase with increased degradation of xanthines and subsequent oxidative stress.²¹ Our results also suggest several important potential therapies. Identification of this metabolic pathway suggests that increasing xanthine concentration or blocking xanthine oxidase may be of benefit to prevent ischemia-reperfusion organ dysfunction in the setting of hyperglycemia. Blocking the xanthine oxidase pathway (e.g., allopurinol) may be an efficacious intervention in the setting of hyperglycemia and organ injury.

In the presence of oxidative stress, highly reactive nitric oxide species are formed, including nitrotyrosine. Nitrotyrosine is generated when superoxide anions react with nitric oxide, generating peroxynitrite, which in turn modifies tyrosine residues in signaling proteins. In this study, significantly higher nitrotyrosine concentrations were found in the PI group than in the other experimental group, indicating elevated nitrative stress in these animals. Therefore, the PI animals had both

increased oxidative and nitrative stress, and this combination is considered an important pathogenic mechanism for the induction of endothelial dysfunction.

Erythropoietin^{36,37} and VEGF³⁸⁻⁴⁰ have been suggested to modulate renal repair after injury and are known to be modulated after ischemia-reperfusion injury. Both of these protective factors were significantly down-regulated in the kidneys of the PI animals. VEGF is one of the most important survival factors for cells that are exposed to hypoxic injuries.^{39,40} It is a potent stimulator of angiogenesis and may help to restore the vascular supply to cells, thereby reducing hypoxia.⁴¹ Blunting of VEGF expression has been documented in animals with chronic hyperglycemia.³¹ This is the first demonstration of decreased expression of VEGF in the kidneys of acutely hyperglycemic animals.

Erythropoietin is another growth factor that is stimulated by hypoxia. Erythropoietin has been shown to be down-regulated during renal ischemia-reperfusion, and administration of erythropoietin has been shown to be highly protective during renal ischemia in several experimental models.^{36,37} These beneficial effects are believed to be mediated by antiapoptotic, antiinflammatory, and antioxidative properties of erythropoietin.⁴² Both VEGF and erythropoietin were significantly decreased in the PI rats 24 h after reperfusion, when compared with vehicle control animals. Consistent with these findings is the significantly elevated cleaved caspase-3 activity in the PI animals. Cleaved caspase-3 activity was not elevated

when rats were made hyperglycemic after reperfusion, again suggesting transient hyperglycemia after onset of ischemia-reperfusion does not increase renal injury. One hypothesis generated by these results is whether the exaggerated decrease in VEGF and erythropoietin expressions could be reversed by the administration of erythropoietin to decrease renal injury in transiently hyperglycemic rats exposed to ischemia-reperfusion.

Our results also suggested other therapies might not be beneficial. Increased renal expression of cytoprotective molecules, such as HSP70 or HSP32,⁴³⁻⁴⁵ are considered important cellular mechanisms of defense during physiologic stress. Interestingly, both HSP70 and the cytoprotective HSP32 were significantly increased in the kidneys from PI animals (fig. 2) compared with the levels in the PR and vehicle control animals. Our data do not support the notion that heat shock proteins are up-regulated in response to hyperglycemia alone, because hyperglycemia in the sham operated animals did not result in heat shock protein changes. Although a cause-effect relation cannot be established for certain, our data suggest that the timing of hyperglycemia in the context of surgical injury is important for the up-regulation of heat shock proteins. However, based on the increased HSP70 and HSP32 response during the transient hyperglycemia, it is unlikely that further increases in HSP32 expression would result in significant renal protection.

Finally, chronic hyperglycemia predisposes to an exaggerated inflammatory response.^{2,30,46} This is documented by polymorphonuclear neutrophil dysfunction (adhesion and influx), as shown by an increase in myeloperoxidase activity in different organs, including the kidney, and by elevated cytokines (e.g., interleukins) and chemokines, such as monocyte chemoattractants (MCP-1/CCL2)⁴⁶ and macrophage inflammatory protein 1 (MIP-2 α /CCL3).³⁰ Our study results suggest that even the very transient hyperglycemia we created resulted in an exaggerated inflammatory response (interleukin 6, MIP-2) as measured by mRNA. The magnitude of the inflammatory response seemed to be dependent on when the hyperglycemia occurred in relation to the ischemia-reperfusion. However, further confirmation on the protein level demonstrated no significant differences (albeit trends) between the experimental groups. One explanation for these results is that we only obtained samples at one time point and it is possible that increased protein expression was evident at a different time point.

One possible confounding variable in this study was the use of isoflurane as an anesthetic. Lee *et al.*⁴⁶ demonstrated that the use of isoflurane during renal ischemia reperfusion resulted in significant renal protection. In the current study, all animals, including sham rats, received isoflurane anesthesia. Renal function, gene expression, and protein concentrations were not different between the hyperglycemic sham rats and the normo-

glycemic sham rats. Therefore, it does not seem that the isoflurane affected our results.

In summary, transient hyperglycemia before and during ischemia-reperfusion in normal animals significantly increased renal injury. In contrast, hyperglycemia that occurred shortly after ischemia reperfusion was not associated with increased renal injury. These results suggest that glucose control must occur before ischemia-reperfusion to be effective. The concentration range for preischemic blood glucose that will not exacerbate ischemia-reperfusion injury has yet to be determined.

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