Distant Effects of Experimental Renal Ischemia/Reperfusion Injury

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Abstract. Acute renal failure results in significant morbidity and mortality, yet renal failure is not the usual cause of death in the clinical situation. We have previously reported systemic increases in the inflammatory mediators tumor necrosis factor-α (TNF-α) and interleukin-1 (IL-1) after renal ischemia in the mouse. In the present study, an animal model of bilateral renal ischemia was used to test the hypothesis that cytokines released with renal ischemia have effects on other organ systems. Increased levels of immunoreactive TNF-α and IL-1 and intercellular adhesion molecule-1 mRNA were found in the heart after renal ischemia in the rat. This was accompanied by increases in myeloperoxidase activity, an index of tissue leukocyte infiltration, in the heart as well as the liver and lung. Functional changes in the heart 48 h after renal ischemia included increases in left ventricular end diastolic diameter, left ventricular end systolic diameter, and decreased fractional shortening by echocardiography. Evidence of apoptosis of cardiac cells was also found 48 h after an abbreviated period of renal ischemia insufficient to induce azotemia but not bilateral nephrectomy (which resulted in significant renal failure), suggesting that renal ischemia but not uremia is necessary for the apoptosis observed. It was also found that blocking the action of TNF-α limited cardiac apoptosis. Renal ischemia results in distant effects and the alterations observed in the heart may be important in the morbidity and mortality observed clinically.

The mortality of acute renal failure (ARF) remains unacceptably high and has not improved significantly in more than 40 yr (1). One of the most common causes of death in ARF patients is cardiac failure (2,3). Many agents have recently been shown to provide marked protection from ARF in animals (1). In contrast to studies in animals, synthetic atrial natriuretic peptide did not result in improved survival or dialysis free survival in a randomized trial of 504 patients with ARF (4). In this study, cardiac abnormalities were associated with an increased risk of mortality and provision of dialysis (relative risk [RR], 1.51 to 3.14) (5). A randomized trial of insulin-like growth factor-1 (IGF-1) in ARF was terminated due to lack of efficacy after an interim analysis of 72 patients (6). The authors of this study conclude, "Recombinant human insulin-like growth factor-1 does not accelerate the recovery of renal function in acute renal failure patients with substantial comorbidity." A recent study found increases in the number of neutrophils in rat kidneys after ischemia and IGF-1 (*versus* ischemia alone) (7), suggesting that increased neutrophil accumulation might be responsible for the increases in mortality seen in critically ill patients treated with some growth factors (8).

Although myocardial depressant factors have been postulated, the pathophysiology of cardiac dysfunction in ARF remains unclear (9). Leukocytes have long been thought to be important in cardiac dysfunction after ischemia (10), and agents that block leukocyte function or localization protect against injury (11–13). An elevated white cell (WBC) count markedly increases the risk of acute myocardial infarction in humans (14), and removing WBC from cardioplegia solution improves myocardial function in stressed piglet hearts (15).

Leukocyte localization to sites of injury or inflammation is largely mediated by adhesion receptors (16). The initial rolling of leukocytes on activated endothelium is mediated by members of the selectin family. This allows exposure of WBC to inflammatory mediators such as interleukin-1 (IL-1) and tumor necrosis factor-α (TNF-α) and activation of the rolling leukocytes. Adhesion receptors, including intercellular adhesion molecule-1 (ICAM-1), on endothelial cells interact with their counterreceptors on leukocytes, resulting in immobilization of leukocytes on the endothelium and diapedesis of WBC across the vessel wall. ICAM-1 expression is increased by IL-1 and TNF-α *in vitro* (16). A significant body of data demonstrates the detrimental effects of these cytokines on the heart. The negative effects include left ventricular dysfunction and apoptosis of both cardiac myocytes and endothelial cells (17).

We have previously reported increases in the systemic levels of IL-1 and TNF-α after renal ischemia in an animal model (18). The purpose of the present study was to investigate the effect of these systemic increases in inflammatory mediators on the heart.

Materials and Methods

Animal Protocols

All experiments were conducted in conformity with the "Guiding Principles for Research Involving Animals and Human Beings." Male

Sprague-Dawley rats (180 to 240 g; Harlan Laboratories, Indianapolis, IN) were anesthetized with intraperitoneal pentobarbital (50 mg/kg) and placed on a homeothermic table to maintain core body temperature at approximately 37°C. Both renal pedicles were occluded for 30 min with microaneurysm clamps as described (19). To assess the effect of blocking the TNF-α action, polyclonal anti-TNF-α antibody (1 mg/kg; Endogen, Inc, Woburn, MA) was administered intravenously to some animals at the time of ischemia. Sham surgery consisted of an identical surgical procedure with the exception of application of microaneurysm clamps. In some experiments, mouse antibody against intercellular adhesion molecule-1 (ICAM-1) (20) or vehicle (0.9% NaCl) was administered intravenously at the time of ischemia. To determine whether renal failure was necessary for the changes observed, in another series of experiments, different periods of ischemia (15, 30, and 45 min) were employed. Bilateral nephrectomy (which produced renal failure without ischemia) was performed by ligating both renal arteries and veins through a midline incision after insuring adequate anesthesia. Both kidneys were removed, and the incision was closed in two layers. Blood was obtained via tail vein for determination of hematocrit as well as urea nitrogen and creatinine (by standard urease and picric acid reactions).

TNF-α Levels

TNF-α in sera obtained before surgery or 1, 2, 6, 24, or 48 h after ischemia or sham surgery was determined by immunoassay using reagents available from R & D Systems (Minneapolis, MN) according to the manufacturer's protocol (18).

Fluorescence Microscopy

A Zeiss confocal microscope (LSM 510) equipped with UV, Argon, and Helium lasers was used. Pieces from the *in situ* fixed hearts were preserved in 20% sucrose before 10-μm frozen sections were obtained. Sections were stained with rabbit anti-rat IL-1 or anti-rat TNF-α polyclonal antibody (Endogen, Inc., Woburn, MA) followed by FITC-conjugated goat anti-rabbit antibody (Amersham Pharmacia Biotech, Piscataway, NJ) and then FITC-conjugated donkey anti-goat antibody (Amersham) and DAPI (a nuclear stain, Molecular Probes, Eugene, OR). Control sections were stained with secondary and tertiary antibodies only (21).

Northern Analysis

Renal RNA was isolated using TRI reagent (Molecular Research Center, Cincinnati, OH) according to the manufacturer's protocol (22). Blots were probed with ^{32}P-labeled ICAM-1 (23) as described previously (18). Membranes were exposed for 24 to 72 h and read by PhosphorImager (Molecular Dynamics, Sunnyvale, CA).

Myeloperoxidase (MPO) Activity

MPO activity, used as an indicator of leukocyte infiltration, was measured as described previously (19). Briefly, aliquots of supernatants of tissue homogenates were analyzed using *o*-dianisidine dihydrochloride and H_2O_2. MPO activity was normalized to protein content and expressed as percent increase over animals subjected to sham surgery.

Echocardiographic Analysis

Rats were lightly anesthetized with pentobarbital (30 mg/kg). M-mode echocardiograms were obtained from the short axis view of the left ventricle at the tip of the mitral valve leaflets using an Interspec-ATL Apogee X200 ultrasound machine (Ambler, PA) with a dynam-ically focused 9-mHz annular array transducer. Tracings were interpreted without knowledge of the experimental group. Left ventricular (LV) end diastolic dimension (EDD) and anterior (interventricular septal; AWT) and posterior wall thicknesses (PWT) were measured by the leading edge method at the time of maximum diastolic dimension. LV end systolic dimension (ESD) was determined at the time of the maximum anterior motion of the posterior wall.

TUNEL Reaction

Forty-eight hours after surgery, hearts were perfusion fixed with 4% paraformaldehyde, sectioned at 4 microns, and the terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) reaction (Promega, Madison, WI) was performed according to the manufacturer's protocol. All nuclei were counterstained with propidium iodine or DAPI. TUNEL-positive nuclei were quantified as percent of total nuclei in coded sections. The negative control was incubated without enzyme (terminal deoxynucleotidyl transferase), and the positive control was treated with DNAse before the TUNEL reaction. To assess the specificity of the TUNEL reaction in this model, nuclear morphology after DAPI staining was examined in a subset of the TUNEL-positive nuclei (21).

DNA Electrophoresis

Given the experimental difficulty of identifying apoptosis in tissue sections (24), DNA electrophoresis was also used to document apoptosis. Cardiac DNA was extracted using TRI reagent according to the manufacturer's protocol. Electrophoresis was carried out using a 1% agarose gel. DNA was visualized by staining with ethidium bromide.

Sphygmomanometry

Systolic BP was measured by tail cuff using an IITC (Woodland Hills, CA) amplifier before anesthesia, after anesthesia but before surgery, during surgery, and 6 and 24 h after renal ischemia or sham surgery.

Statistical Analyses

Data are expressed as means $\pm$ 1 SE. ANOVA was used to determine if differences among mean values reached statistical significance. *t* test was used for comparisons between groups. Tukey test was used to correct for multiple comparisons. The null hypothesis was rejected at $P < 0.05$.

Results

Effect of Renal Ischemia on Systemic TNF-α Levels

We have previously demonstrated increases in systemic IL-1 and TNF-α after experimental renal ischemia/reperfusion (18). IL-1 was increased 1 and 2 h after ischemia and returned to basal levels by 4 h. In the present study, we further define the increases in systemic TNF-α. Before ischemia and after sham surgery, TNF-α was undetectable in the serum. TNF-α increased by 1 h post-ischemia, increased further at 2 h and remained elevated compared with levels after sham surgery for 24 h, but levels were not significantly different from the sham surgery group 48 h post-ischemia (Figure 1).

Effect of Renal Ischemia on Cardiac IL-1 and TNF-α

Induction of immunoreactive IL-1 and TNF-α was seen 6, 24, and 48 h after renal ischemia (30 min; Figure 2), both
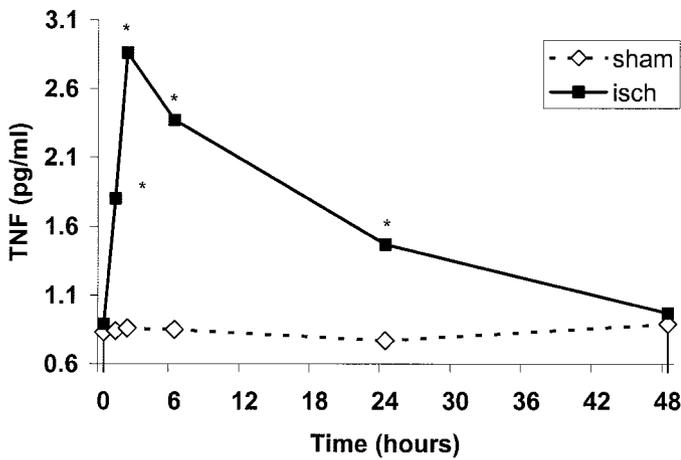
Figure 1. Effect of renal ischemia on systemic tumor necrosis factor–α (TNF-α) levels. TNF-α was measured in sera obtained before surgery and 1, 2, 6, 24, and 48 h after sham surgery or bilateral renal ischemia. Data are expressed as means $\pm$ 1 SEM. $P < 0.05$ *versus* sham surgery.

proximal to blood vessels and in multinucleated cells. No staining was apparent after sham surgery.

Effect of Renal Ischemia on Cardiac ICAM-1 mRNA Level

Increases in ICAM-1 mRNA were seen 6 h after renal ischemia (30 min). ICAM-1 mRNA increased further by 24 h and remained elevated at 48 h (Figure 3). Mean density at 24 and 48 h post-ischemia was significantly greater than after sham surgery ($P < 0.05$; Figure 3B).

Effect of Renal Ischemia on Cardiac Myeloperoxidase Activity

Increases in MPO activity, an index of leukocyte infiltration/ activation (25), were seen in kidney tissue as well as in the heart, liver, and lung 6 h after renal ischemia (30 min; Figure 4A). The differences reached statistical significance ($P < 0.05$) in the heart, liver, and kidney. Administration of anti-ICAM-1
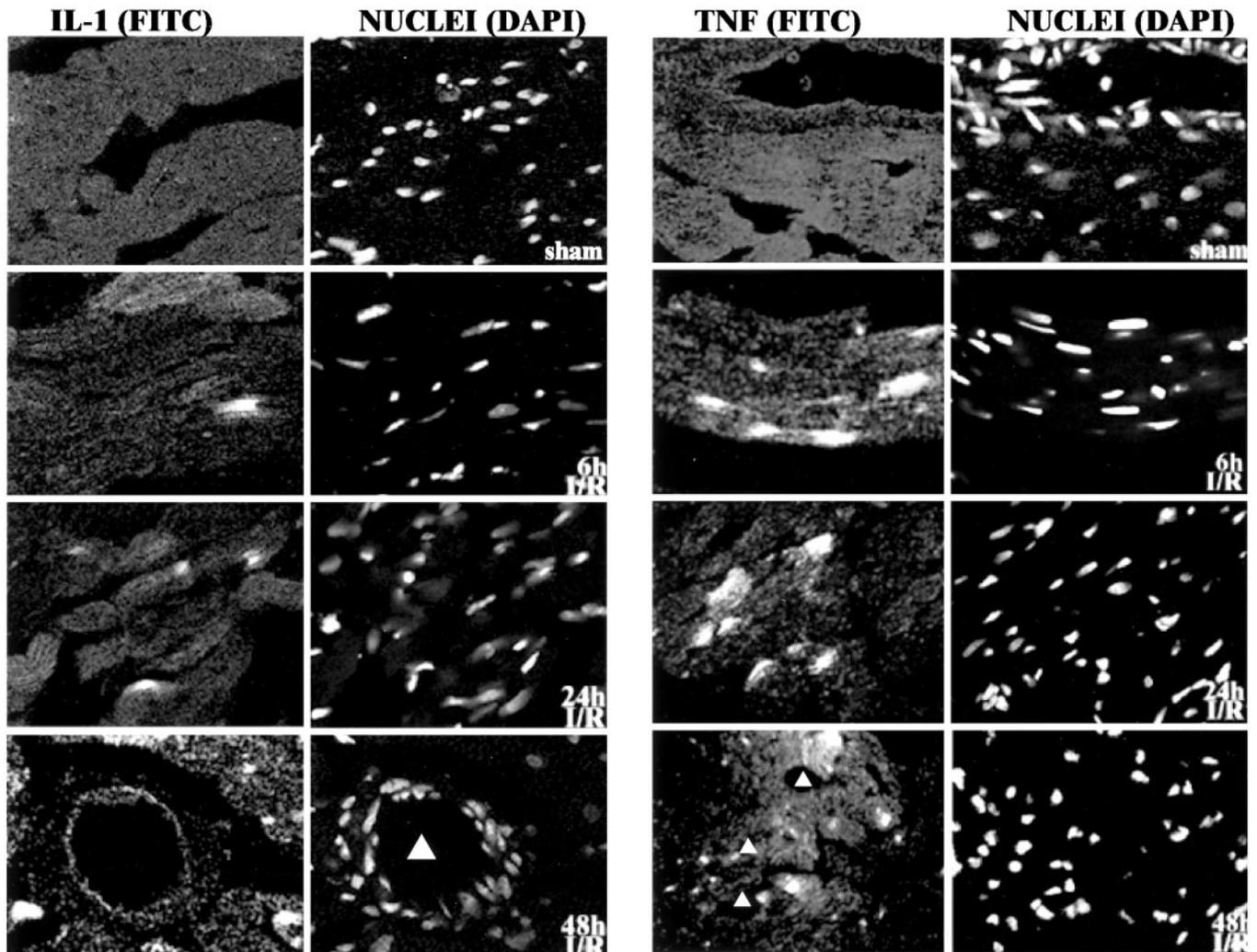

Figure 2. Effect of renal ischemia on cardiac interleukin-1 (IL-1) and TNF-α. Sections of hearts removed 6, 24, or 48 h after renal ischemia (30 min) were stained with polyclonal anti-IL-1 (left) or anti-TNF-α (right) and then FITC-conjugated secondary and tertiary antibodies. Sections were counterstained with the nuclear stain DAPI. Representative sections after ischemia and reperfusion (I/R) or sham surgery are shown. Arrowheads indicate vessel lumina.
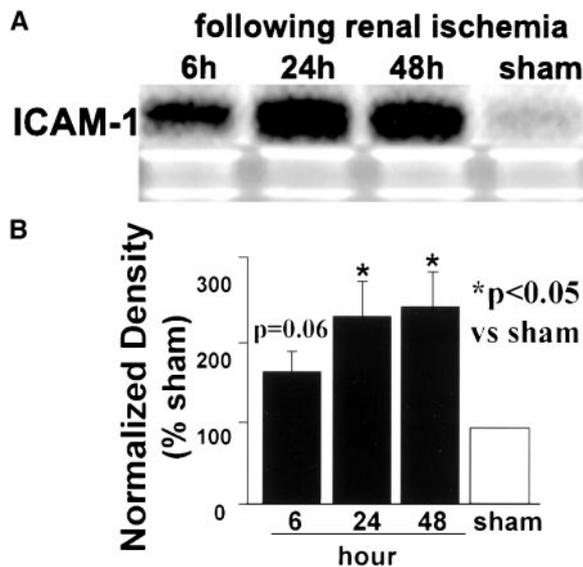
Figure 3. Effect of renal ischemia on cardiac ICAM-1 mRNA level. A representative blot is presented in panel A. Mean density of the band corresponding to ICAM-1 mRNA ($n = 3$) was significantly greater after renal ischemia (30 min) than after sham surgery at 24 and 48 h after ischemia (B). *$P < 0.05$ *versus* sham surgery.

antibody at the time of ischemia prevented the increase in cardiac MPO activity seen after renal ischemia (Figure 4B).

Effect of Renal Ischemia on Cardiac Function

To assess the effect of these changes in cardiac function, echocardiograms were obtained 48 h after 30 min of renal ischemia or sham surgery. Representative M-mode tracings are presented in Figure 5. Left ventricular dilatation is apparent after ischemia (lower panel). Quantification of echocardiographic parameters (Table 1) revealed increases in left ventricular end diastolic and end systolic diameter, relaxation time, decreased fractional shortening, and no significant change in anterior or posterior wall thickness.

Effect of Renal Ischemia on Apoptosis in the Heart

Apoptosis was evident in the heart by the TUNEL reaction 48 h after renal ischemia (30 min; Figure 6A). Significantly more TUNEL-positive nuclei were seen in sections of heart harvested after renal ischemia than after sham surgery (Figure 6B). DNA fragmentation (laddering) characteristic of apoptosis was also evident by electrophoresis (Figure 6C). A short period of renal ischemia (15 min) insufficient to induce azotemia resulted in cardiac apoptosis, whereas bilateral nephrectomy (which resulted in an 18-fold increase in mean serum creatinine; $P < 0.01$) was not accompanied by an increase in apoptosis in the heart (Figure 7). In addition, hearts from animals with cisplatin-mediated renal failure showed an increase in TUNEL-positive cells in the heart ($0.4 \pm 0.1\%$ *versus* $0.05 \pm 0.05\%$; $P < 0.05$). The number of TUNEL-positive nuclei was also decreased in hearts from the group treated with anti-TNF-α antibody at the time of renal ischemia when compared with hearts from the group subjected to isch-

emia only (Figure 8). Examination of the DAPI channel alone (21) in 20 TUNEL-positive nuclei showed nuclear condensation and/or fragmentation consistent with apoptosis (data not shown).

Systolic BP

No significant difference in systolic BP between the ischemia and sham groups was evident at baseline, after anesthesia, during surgery, or 6 or 24 h after surgery (Table 2). Mean hematocrit ($46 \pm 3\%$) in the ischemia group was no different from that in the sham surgery group ($44 \pm 2\%$) 48 h after ischemia. There was also no significance difference in weight between the ischemia and sham surgery groups before surgery or 24 or 48 h after surgery.

Discussion

Cardiac failure is the most common cause of death in uremic patients (26). In most studies of acute renal failure, the mortality rate remains $>50\%$, although renal failure itself is not usually the cause of death (1). Several investigators have demonstrated that congestive heart failure is associated with increased mortality in patients with acute renal failure (2,3,27). In one study of patients with acute renal failure, the adjusted odds ratio of death for cardiac failure (7.7) was greater than that for respiratory failure (3.6), hepatic failure (6.3), neurologic failure (3.0), massive transfusion (5.3), or age >60 (3.7) (28). Cardiac dysfunction was also felt to be a factor in the synthetic atrial natriuretic peptide (5) and insulin-like growth factor (6) trials.

Cytokines are thought to be important in the pathophysiology of congestive heart failure (29,30). Overexpression of TNF-α in the heart results in severely impaired cardiac function, myocyte apoptosis, and leukocyte infiltration of the heart (31). In the isolated rat heart, perfusion with TNF-α results in a decrease in left ventricular developed pressure (LVDP) and coronary vasoconstriction (32). TNF-α–mediated apoptosis is one of the proposed mechanisms for its association with cardiac dysfunction (31). Increased plasma TNF-α has been found in human CHF (33). However, trials with the TNF-α receptor antagonist etanercept or anti–TNF-α antibody (infliximab) in human CHF did not improve death rate or hospitalization for CHF (34). Chronic treatment with IL-1 in the dog results in sustained myocardial dysfunction, WBC infiltration of the heart, and increased cardiac myeloperoxidase activity. Myocardial dysfunction can be prevented by blocking adhesion receptors (35).

In vitro, cytokines such as TNF-α and IL-1 upregulate adhesion receptors such as ICAM-1 on endothelia. Adhesion receptors mediate the localization of WBC to sites of injury or inflammation. The interaction of adhesion receptors, including ICAM-1, on endothelial cells with their counter-receptors on leukocytes facilitates the immobilization of leukocytes on the endothelium, WBC exposure to cytokines, and activation and diapedesis of WBC across the vessel wall (16).

In this study, we found increases in immunoreactive IL-1 and TNF-α and increases in ICAM-1 mRNA levels in the heart after renal ischemia. These increases were accompanied by
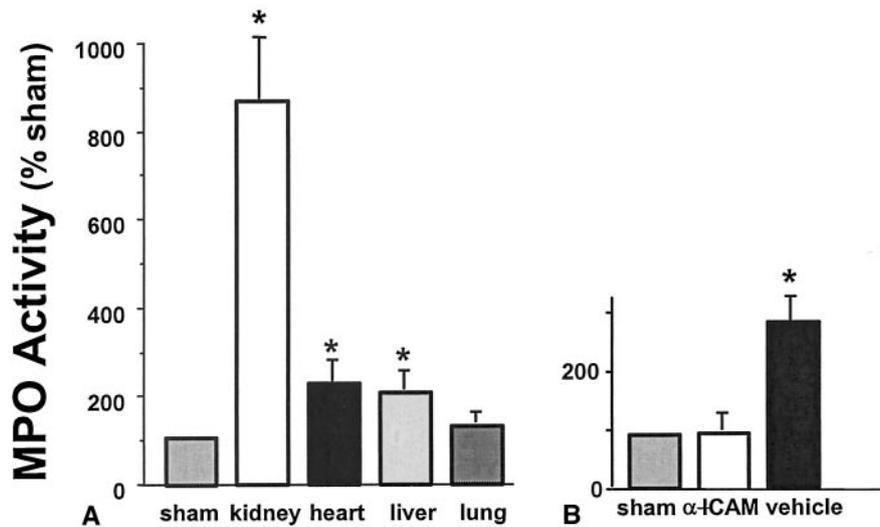
Figure 4. Effect of renal ischemia on myeloperoxidase (MPO) activity in distant organs. MPO activity was determined as an index of leukocyte infiltration. Mean activity $\pm$ 1 SEM in the heart, liver, lung, and kidney after renal ischemia (30 min) is presented in panel A. Activity in the kidney, heart, and liver is significantly greater ($P < 0.05$) than that after sham surgery. Increases in cardiac MPO activity post-ischemia could be prevented by the administration of anti-ICAM-1 antibody at the time of ischemia (B). *$P < 0.05$ *versus* sham surgery; $n = 3$ for each mean.
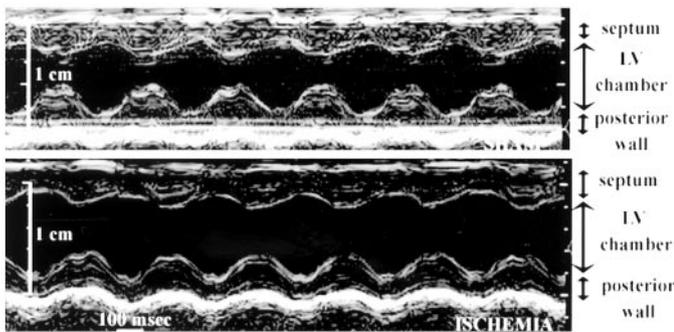

Figure 5. Effect of renal ischemia on cardiac function. Representative M-mode echocardiographic tracings 48 h after sham surgery (top panel) or renal ischemia (30 min; bottom panel) are presented. Mean values of echocardiographic parameters are presented in Table 1. LV, left ventricle.

Table 1. Echocardiographic parameters after renal ischemia

	Sham ($n = 8$)	Post-Ischemia ($n = 8$)
LV end diastolic diameter[a] (mm)	6.13 ± 0.02	6.28 ± 0.05[b]
LV end systolic diameter (mm)	3.17 ± 0.07	3.29 ± 0.05[b]
Relaxation time (msec)	190 ± 12	239 ± 2[c]
Fractional shortening (%)	48.04 ± 1.05	44.10 ± 1.45[b]
Anterior wall thickness (mm)	0.43 ± 0.06	0.44 ± 0.06
Posterior wall thickness (mm)	0.44 ± 0.06	0.44 ± 0.07

[a] LV, left ventricular.
[b] $P < 0.05$.
[c] $P < 0.01$.

increased cardiac MPO activity, consistent with leukocyte infiltration and activation in the heart. The increases in MPO activity could be prevented by administration of anti-ICAM-1 antibody at the time of ischemia. It is possible that anti-ICAM-1 prevents leukocyte infiltration of the heart after renal ischemia. We have previously demonstrated that treatment with anti-ICAM-1 antibody at the time of renal ischemia results in preservation of renal function (19). Thus, an alternate explanation for the lack of increase in cardiac MPO after renal ischemia and anti-ICAM-1 antibody is the lack of renal failure in this setting.

We assessed the functional significance of the changes observed in the heart using echocardiography. Echocardiography can be used to assess the risk of death in patients with essential hypertension (36). We thus used echocardiography to evaluate cardiac function in rats with renal failure and found evidence of

impaired cardiac function 48 h after renal ischemia. At this time, there was no difference in systolic BP, hematocrit, or body weight between the sham surgery and renal ischemia groups.

Loss of cardiomyocytes is also believed to be critical in the pathogenesis of congestive heart failure. Transgenic models have shown that apoptosis alone can result in heart failure (37). The increase in cardiac apoptosis we observed after renal ischemia may account for the decrease in cardiac function post-ischemia. Although renal failure can result in hemodynamic changes, the controls used were subjected to the same anesthetic and sham surgery, and no differences in systolic BP were observed. Renal ischemia, but not uremia, was critical in the induction of apoptosis in the heart, because TUNEL-positive cells were found after 15 min of renal ischemia (which did not produce renal failure), but not bilateral nephrectomy (which resulted in significant renal failure). Nephrectomy produces renal failure without inflammation. In contrast, cisplatin-
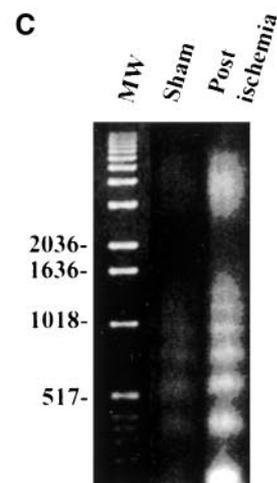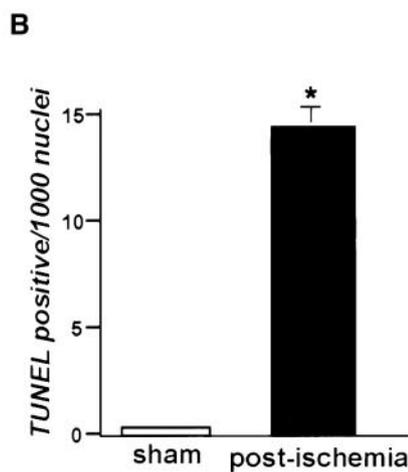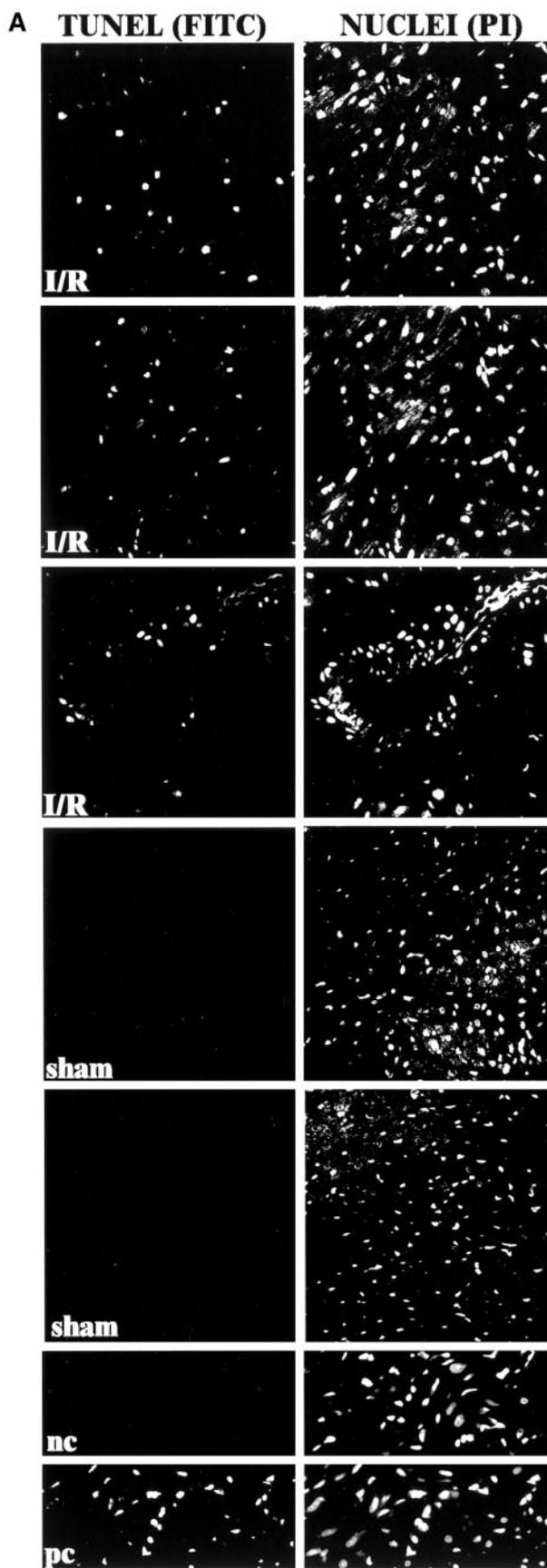
Figure 6. Effect of renal ischemia on apoptosis in the heart. The terminal deoxynucleotidyl transferase-mediated nick-end labeling (TUNEL) reaction was used to identify apoptotic nuclei. All nuclei were counterstained with propidium iodine (PI). Representative sections 10 mm from the apex of hearts removed 48 h after renal ischemia (30 min) are presented in panel A. Quantification of TUNEL-positive nuclei in 120 sections from four animals is presented in panel B. Electrophoresis of DNA extracted from rat hearts 48 h after renal ischemia or sham surgery is presented in panel C. *P < 0.01 *versus* sham surgery; I/R, ischemia/reperfusion; nc, negative control; pc, positive control.
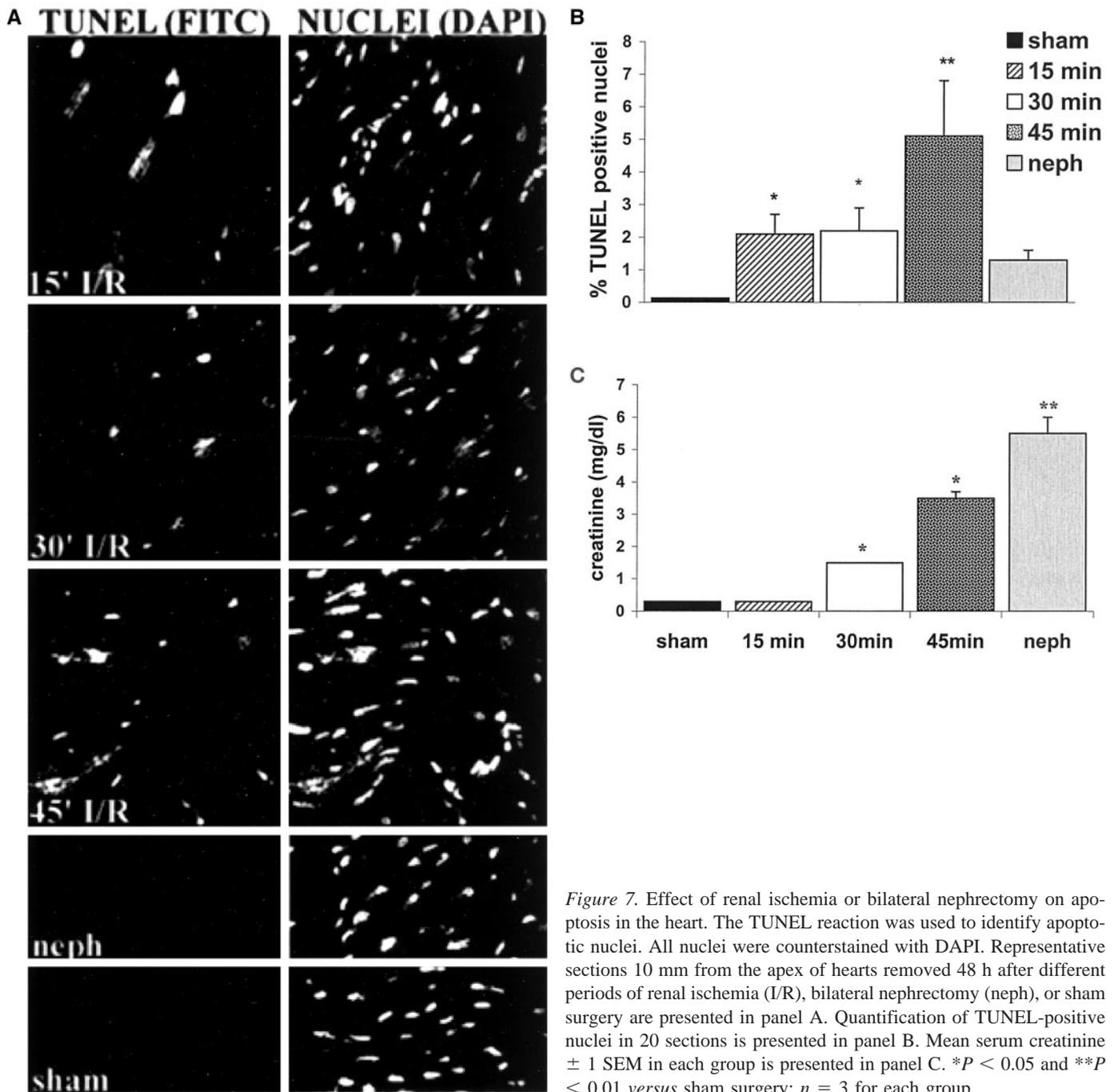
Figure 7. Effect of renal ischemia or bilateral nephrectomy on apoptosis in the heart. The TUNEL reaction was used to identify apoptotic nuclei. All nuclei were counterstained with DAPI. Representative sections 10 mm from the apex of hearts removed 48 h after different periods of renal ischemia (I/R), bilateral nephrectomy (neph), or sham surgery are presented in panel A. Quantification of TUNEL-positive nuclei in 20 sections is presented in panel B. Mean serum creatinine ± 1 SEM in each group is presented in panel C. *$P < 0.05$ and **$P < 0.01$ *versus* sham surgery; $n = 3$ for each group.

mediated renal injury, which may have an inflammatory component (38), increases the number of TUNEL-positive cells in the heart.

To our knowledge, this is the first demonstration of alterations in cardiac function after experimental renal ischemia. Decreases in cardiac index and stroke volume have been observed after intestinal ischemia/reperfusion in the rat (39). Injury to the contralateral kidney has been demonstrated with unilateral renal ischemia (40). Alterations in phagocyte function have also been demonstrated after renal injury (41). Ischemia/reperfusion injury in several models (for example, intestine [42], liver [43], muscle [44], and contralateral lung [(45)]

result in lung injury, although pulmonary dysfunction was not found after renal ischemia/reperfusion (46). Renal dysfunction has also been demonstrated after ischemic injury in other organ systems (47). In many of these systems, dysfunction in other organ systems was associated with increased systemic levels of cytokines after ischemic injury (39,43,47,48). Experimental renal ischemia has also been shown to stimulate the production of granulocyte-colony stimulating factor by the kidney and IL-10 by the liver (49).

In summary, this study demonstrates multiple alterations in the heart after renal ischemia. The changes include increases in mRNA levels of the adhesion receptor intercellular adhesion
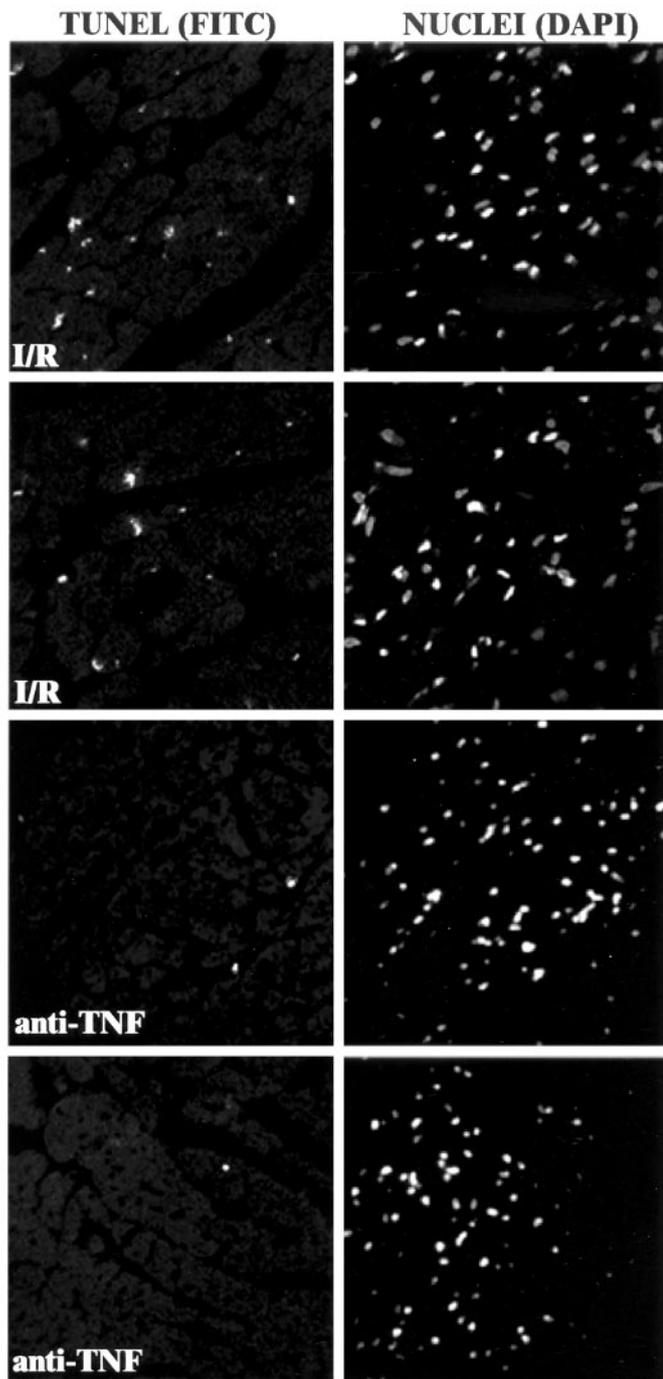
Figure 8. Effect of blocking TNF-α action on cardiac apoptosis after renal ischemia. The TUNEL reaction was used to identify apoptotic nuclei. All nuclei were counterstained with DAPI. Representative sections 10 mm from the apex of hearts removed 48 h after renal ischemia with (lower panels) or without (upper panels) the administration of anti-TNF-α antibody are shown.

molecule-1, myeloperoxidase activity, and presence of apoptosis. This is accompanied by left ventricular dilation and decreased fractional shortening. If similar changes occur in human acute renal failure, the decreased cardiac function may be an important determinant of the unacceptably high mortality in acute renal failure.

Table 2. Systolic BP (mmHg) in sham surgery and renal ischemia groups

	Sham Surgery ($n = 3$)	Renal Ischemia ($n = 3$)	P
Before anesthesia	112 ± 3.5	113 ± 5.7	0.93
After anesthesia	100 ± 1.1	99 ± 2.4	0.82
During surgery	99 ± 2.4	103 ± 1.8	0.26
6 h after surgery	105 ± 8.7	99 ± 4.1	0.58
24 h after surgery	105 ± 4.4	104 ± 5.8	0.27

Acknowledgments

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