0041-1337/02/7408-1247/0
TRANSPLANTATION
Vol. 74, 1247–1251, No. 8, October 27, 2002
Printed in U.S.A.

RENAL PROTECTION BY BRIEF LIVER ISCHEMIA IN RATS[1]

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Background. **In this study, we evaluated the beneficial effect of brief ischemia and reperfusion, which was shown to have local effects on liver previously, on kidney as a remote organ in rats.**

Methods. **Male Wistar rats were divided into three groups: group I, sham; group II, renal ischemia for 45 min; and group III, 10 min of brief hepatic ischemia and 10 min of reperfusion after 45 min of renal ischemia. Biochemical determination, tumor necrosis factor (TNF)-α and tissue thiobarbituric acid-reactive substances (TBARS) levels, and histopathologic findings were evaluated at 45 min and 24 hr of reperfusion.**

Results. **Although blood urea nitrogen and creatinine levels were similar at 45 min in groups II and III, these levels were lower in group III at 24 hr. Creatine clearance values were higher and fraction excretion of sodium values were lower in group II than in group III at 24 hr. Lactate dehydrogenase levels of groups III and II were similarly elevated at 45 min, whereas group III values decreased more rapidly than those of group II at 24 hr. At 45 min of reperfusion, TNF-α and tissue TBARS levels were found lower in group III than in group II. Histopathologic parameters including congestion and tubular vacuolization, tubular cell detachment, and necrosis were significantly reduced in group III as compared with results of group II 45 min after ischemia. All histopathologic parameters were defined as statistically better in group II at 24 hr.**

Conclusions. **The beneficial effect of brief ischemia of liver on renal ischemia as a remote organ was confirmed by biochemical, histopathologic, and ultrastructural findings.**

In 1986, the term *ischemic preconditioning* (IP) was introduced by Murry et al. to describe the phenomenon wherein pretreatment with brief ischemia and a short period of reperfusion showed a beneficial effect on the size of infarctions caused by prolonged myocardial ischemia (*1*). Initially, IP was reported to have an effect only on myocardial tissue; more recently, it has been described in skeletal muscle (*2*), brain (*3*), liver (*4*), retina (*5*), spinal cord (*6*), and lung (*7*). Interestingly, there have been few studies of the effect of IP on renal ischemia, despite the fact that the kidney is a widely transplanted organ. Islam et al. claimed that IP had no effect on renal ischemic injury (*8*). Recently, however, Riera et al. and Toosy et al. have shown that the kidney is also protected by IP (*9*, *10*).

The mechanisms of IP related to its preventive effect are not fully understood and are under investigation. However, adenosine activation, bradykinin, prostaglandin, and nitric oxide secretion are the probable mediators concerning the protective role of IP (*11–15*). IP is a multifactorial process in which multiple signals, second messengers, and several effective mechanisms are interacting with each other.

Most studies of IP have focused on its capacity for limiting local ischemic damage. Przyklenk et al., however, showed that brief occlusion of the coronary artery could protect the outside coronary artery perfusion territory (*16*). Later, Gho et al. reported that brief ischemia in noncardiac tissue, such as the kidney and small intestine, has a protective effect on the myocardium (*17*).

Studies of the effects of IP on remote organs may help clarify the mechanisms of IP. Consequently, we undertook a study to determine whether a period of brief liver ischemia, which is known to be locally protective, will also protect the kidney.

MATERIALS AND METHODS

All experiments were performed in the Surgical Research Centre at Osmangazi University. The University Ethics Committee approved this study. Adult male Wistar rats weighing 250 to 300 g housed four to five to a cage were given standard rat chow with free access to water. All animals were fasted 12 hr prior to experimentation, and were allowed water ad libitum. The animals were anesthetized by intraperitoneal injection of ketamine (Ketalar, Parke-Davis, Turkey) and xylazine (Rompun, Bayer, Turkey). After the abdominal wall was cleansed with 10% povidone-iodine (Betadine, Seton, UK), the abdomen was opened by a midline incision. Rats were systemically heparinized (200 U/kg body weight intramuscularly); then, all rats had right nephrectomy and dissection of the left renal and hepatic pedicle.

The rats were divided into three groups. Group I (n=16) had a sham operation to assess the effect of laparotomy and dissection of the left renal and hepatic pedicle after right nephrectomy. In group II (n=24) and group III (n=24), renal ischemia was created by placing a microvascular clamp on the left renal pedicle for 45 min. In group III, IP was achieved by brief occlusion of the hepatic pedicle with a microvascular clamp for 10 min, followed by 10 min of reperfusion, before the 45 min of renal ischemia.

Blood samples and left kidneys were obtained after 45 min of reperfusion from half of the rats in each group. The remaining rats were followed for 24 hr in a metabolic cage. Samples from these animals were obtained under the same anesthesia protocol before they were killed. Left nephrectomy specimens were divided into three parts. One part was frozen in liquid nitrogen and stored at −70°C for determination of lipid peroxidation. The second part was

[1] This study was supported by a grant from the Osmangazi University Research Fund.

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Received 16 January 2002. Revision Requested 15 May 2002. Accepted 19 June 2002.

DOI: 10.1097/01.TP.0000032752.61372.36

stored in 10% buffered formaldehyde for light microscopy. The third part was processed as follows for ultrastructural studies.

Biochemical Analysis

Blood samples were immediately centrifuged at 5,000*g* for 5 min. Sodium (Na), blood urine nitrogen (BUN), serum creatinine (sCr), aspartate aminotransferase, alanine aminotransferase, lactate dehydrogenase (LDH), alkaline phosphatase, and gamma-glutamyl transferase levels in serum and Na and Cr in urine were determined with a Hithachi 911 automatic analyzer (Boehringer Mannheim, Germany). Creatine clearance (CrCl) and fraction excretion of Na (FENa) were calculated as follows:

$$CrCl\ (mL/min) = Urine\ Cr \times 24\ hr\ Urine\ volume/sCr \times 1{,}440$$

$$FENa(\%) = (Urine\ Na \times Plasma\ Cr/Plasma\ Na \times Urine\ Cr) \times 100$$

Tumor Necrosis Factor-α Assay

Serum samples were stored at −70°C until the assay was performed. The plasma concentration of tumor necrosis factor (TNF)-α was measured by enzyme-linked immunosorbent assay using a TNF test kit (R&D Systems, Quantikin M Murine, MN). According to the manufacturer, the detection limit of this assay was determined to be 5 pg/mL.

Measurement of Lipid Peroxidation

Tissue malondialdehyde levels were detected with the thiobarbituric acid-reactive substances (TBARS) method (*18*). Approximately 0.5 g of kidney tissue was taken and mixed with ice-cold 0.9% NaCl until the final volume reached 5 mL. This solution was then homogenized with a homogenizer (Janke & Kunker Ultra-Turrax T25, Germany) in +4°C. The supernatant was centrifuged at 15,000*g* for 15 min at +4°C in a cooled centrifuge. The reaction mixture contained 0.1 mL of sample, 0.2 mL of 8.1% sodium dodecyl sulfate, 1.5 mL of 20% acetic acid solution of various pH levels, and 1.5 mL of 0.8% aqueous solution of thiobarbituric acid. The pH of 20% acetic acid solution was adjusted with NaOH above pH 3.0, and in the pH range of 1.0 to 3.0, 20% acetic acid containing 0.27 M HCl was adjusted to the specified pH with NaOH. The mixture was finally made up to 4.0 mL with distilled water, and heated to 95°C for 60 min. After cooling with tap water, 1.0 mL of distilled water and 5.0 mL of the mixture of n-butanol and pyridine (15:1, v/v) were added and the mixture was shaken vigorously. After centrifugation at 4,000*g* for 10 min, the absorbance of the organic layer (upper layer) was measured at 532 nm.

Light Microscopy

Light microscopy studies were reviewed by a pathologist blinded to the protocol. Paraffin sections were stained with hematoxylin-eosin. Four sections were evaluated (10 random fields with ×20 magnification) for congestion, tubular vacuolization, intratubular cell detachment, and tubular cell necrosis using a semiquantitative scale adapted from Hauet et al. This scale is graded as follows: 0 to 4+ in which 0 denoted no abnormalities; 1+, changes affecting less than 25% of the sample; 2+, changes affecting 25% to 50% of the sample; 3+, changes affecting 50% to 75% of the sample; and 4+, changes affecting greater than 75% of the sample (*19*).

Electron Microscopy

Liver and renal tissues were fixed with 2% glutaraldehyde in phosphate buffer (pH 7.4) at +4°C for 2 hr. Specimen blocks were diced into 1-mm cubes. Samples were washed with Sorenson solution (SS) three to four times. The samples were then stored in fixative for 2 to 3 days before further processing. Specimens were postfixed with 2% osmium tetroxide for 90 min and washed with SS, dehydrated in graded alcohol, and embedded in Epon 812. Blocks were incubated at +60°C for 48 hr; 1-μm sections were stained with methylene blue and Azure II and evaluated under light microscopy. Ultrathin sections were cut on an ultramicrotome and contrasted with uranyl acetate and lead citrate. Stained sections were reviewed on a Zeus EM9-S2 (Orangeburg, SC) scanning electron microscope.

Statistical Analysis

Data are expressed as mean±SEM. Values of $P<0.05$ were considered statistically significant. Biochemical results and histologic findings were assessed by analysis of variance and the Dunnett post hoc test.

RESULTS

Effect of Ischemia

Ten minutes of hepatic ischemia and 10 min of reperfusion did not cause any difference compared to the sham group in liver tissue biochemically and histologically. BUN was significantly elevated in group II animals (renal ischemia-reperfusion [I/R]) compared with group I animals (sham operation) at all time points (immediately after I/R, 34±1.11 vs. 21±0.80 mg/dL, $P<0.001$; 24 hr after reperfusion, 113.8±6.59 vs. 21.4±0.82 mg/dL, $P<0.001$). Blood urea nitrogen values were also significantly elevated in group III animals compared with group I animals (sham operation) at all time points (immediately after I/R: 33±0.81 vs. 21±0.80 mg/dL, $P<0.001$; 24 hr after reperfusion: 71.3±4.43 vs. 21.4±0.82 mg/dL, $P<0.001$) (Fig. 1). There were no significant differences in sCr levels between group I versus groups II and III after 45 min of reperfusion, but sCr levels were elevated in group II and group III at 24 hr (Fig. 2). Furthermore, CrCl in group II and group III were significantly lower than in group I after 24 hr, and FENa values were significantly increased (Fig. 3). Lactate dehydrogenase levels were significantly higher in group II and group III than in group I after 45 min (1,713±197 for group II, 1,794±140 for group III vs. 918±59 U/dL) and 24 hr (1,518±39 for group II, 1,216±56 for group III vs. 950±53 U/dL, $P<0.001$). Although there were significant elevations in TNF-α level in group II and group III in relation to group I after 45 min of reperfusion, TNF-α levels in all groups declined to undetectable levels after 24 hr (Table 1). Thiobarbituric acid-reactive substances levels were significantly higher in group II, and group III versus group I; after 24 hr, TBARS levels declined in all groups but were still higher in group II and group III than in group I (Table 1).

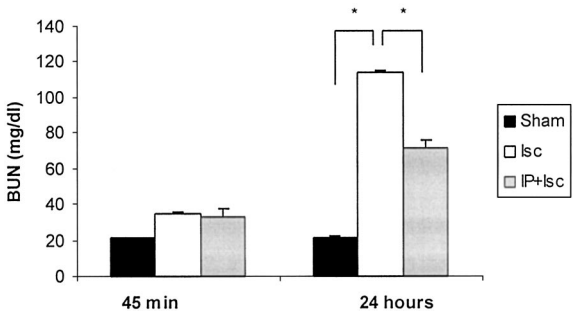

FIGURE 1. Changes in blood urea nitrogen levels in all groups. BUN levels were elevated in group II and group III, but the difference was statistically significant at 24 hr (*$P<0.001$).

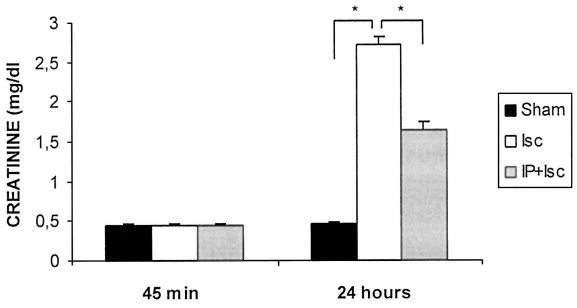

FIGURE 2. Changes in creatinine levels in all groups. There was no difference at 45 min, but at the end of 24 hr sCr levels were elevated much more in group II than in group III (*$P<0.001$).

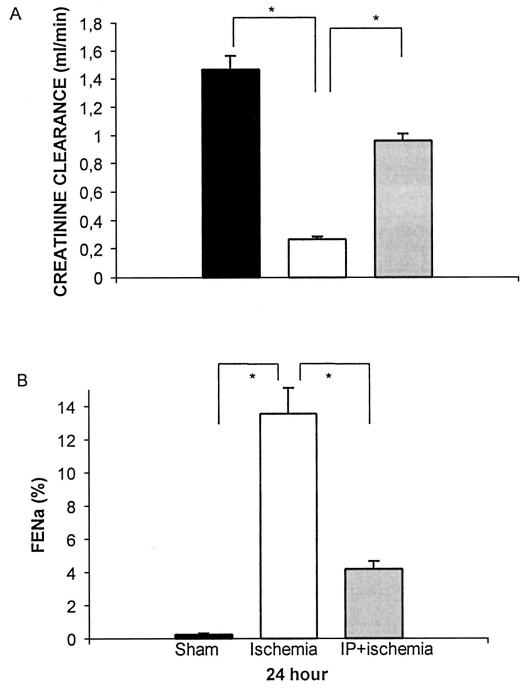

FIGURE 3. Creatinine clearance and FENa levels in all groups. Creatine clearance values of group II were less than those of group III and FENa values were higher in group II than in group III at 24 hr (*$P<0.001$).

On histopathologic analysis, group II showed evidence of congestion, tubular vacuolization and necrosis ($P<0.001$), and tubular cell detachment ($P<0.01$) at an early period of reperfusion (Table 1). Although histopathologic findings in group II deteriorated at 24 hr about the necrosis and tubular cell detachment, all findings were statistically different from those of group I. There was statistical significance about the early period and 24-hr levels of total sum of scores ($P<0.001$) (Table 1).

Under electron microscopy, the cytoplasmic architecture was disorganized and contained numerous autophagic lysosomes. The damaged cells showed intracristal swelling of mitochondria. The cytoplasm of damaged cells had accumulation vacuoles. Plasma membrane integrity was lost. Detachment of tubular cells from the basement membrane was evident. The paucity of basolateral interdigitating was examined. Detachment of intercellular junctions was evident in all tubular epithelium cells. At the apical surface of the tubular epithelium, microvilli were irregular and reduced in number (Fig. 4).

Effect of Brief Hepatic Ischemia on Renal Ischemia

The comparison of groups II and III revealed that BUN levels were similar 45 min after renal ischemia (34.8±1.11 vs. 33.0±0.81 mL/dL, ($P>0.05$); in contrast, BUN levels were elevated in group II and group III and the difference was statistically significant at 24 hr (113.8±6.59 vs. 71.3±4.43 mg/dL, $P<0.001$) (Fig. 1).

When the groups were compared in terms of the sCr levels, there was no difference at 45 min (0.45±0.023 vs. 0.45±0.019 mg/dL, $P>0.05$), but at the end of 24 hr sCr levels were elevated much more in group II than in group III (2.72±0.103 vs. 1.65±0.097 mg/dL, $P<0.01$). Creatine clearance values of group II were less than those of group III at 24 hr (0.268±0.022 vs. 0.960±0.050 mL/min, $P<0.001$). Fractional excretion of Na values of group III were also less than those of group II at 24 hr (4.18±0.48% vs. 13.53±1.56%, $P<0.001$) (Fig. 3).

Lactate dehydrogenase levels of groups III and II were similarly elevated at 45 min (1,794±140 vs. 1,713±171 U/dL, $P>0.05$), whereas group III LDH values decreased more rapidly than those of group II, and the difference was statistically significant (1,216±56 vs. 1,518±39 U/dL, $P<0.001$).

Tumor necrosis factor-α levels were found lower in group III than those of group II at 45 min of reperfusion (55.2±2.35 vs. 87.2±4.67, $P<0.001$) (Table 1). Tumor necrosis factor-α levels of both groups declined to undetectable levels after 24 hr.

Thiobarbituric acid-reactive substances levels of group III were significantly lower than in group II (2.00±0.076 vs. 4.58±0.174 μmol/g protein, $P<0.001$) at 45 min of reperfusion (Table 1), and despite the decrement at 24 hr, there was no significant difference between the groups (0.54±0.030 vs. 0.63±0.030 μmol/g protein, $P>0.05$) (Table 1).

On histopathologic analysis under light microscopy, congestion and tubular vacuolization ($P<0.001$) and tubular cell detachment ($P<0.01$) and necrosis ($P<0.001$) were significantly reduced in group III as compared with results of group II 45 min after ischemia (Table 1). Although congestion and tubular cell detachment were decreased in group III at 24 hr, vacuolization did not change and minimal focal necrosis was seen. Nevertheless, all histopathologic parameters were defined as statistically better in group III. The levels of total sum of scores at 45 min and 24 hr were statistically significant ($P<0.001$) (Table 1). Under electron microscopy, basolateral interdigitations and the mitochondria between them are examined. There was fewer vacuoles in cytoplasm. There was no detachment of tubular epithelium from the basement membrane (Fig. 5).

DISCUSSION

The locally protective effect of IP is well known (*20*). Recently, it has been suggested that IP also has a distant effect (*11*, *12*, *21*). In this study, we attempted to determine whether brief hepatic ischemia, known to be effective locally, has a remote effect on renal I/R injury.

Surprisingly, even though the kidney is a widely transplanted organ, only a few studies on the effect of IP on renal

TABLE 1. Serum TNF-α values and histopathologic evaluation, TBARS levels in kidney after 45 min and 24 hr of reperfusion

Groups	45 min of reperfusion			24 hr of reperfusion		
	I	II	III	I	II	III
Congestion	0.375±0.183	3.250±0.218	2.500±0.131	0.250±0.164	2.333±0.225	1.083±0.149
T. detachment	0.625±0.183	3.083±0.193	2.500±0.131	0.375±0.183	3.000±0.174	1.583±0.193
T. vacuolation	1.000±0.189	3.000±0.174	1.417±0.149	0.625±0.183	2.500±0.151	1.333±0.142
T. necrosis	0.000±0.000	0.833±0.112	0.000±0.000	0.000±0.000	2.500±0.151	1.083±0.083
Score	2.000±0.463	10.167±0.548	5.917±0.260[a]	1.250±0.366	10.333±0.513	5.083±0.468[a]
TNF-α	10.1±0.64	87.2±4.67	55.2±2.35[a]	5.1±0.52	5.8±0.43	5.4±0.54
TBARS	0.34±0.016	4.58±0.174	2.00±0.076[a]	0.25±0.020	0.63±0.030	0.54±0.030

[a] $P<0.001$ group II vs. group III.
T., tubular.

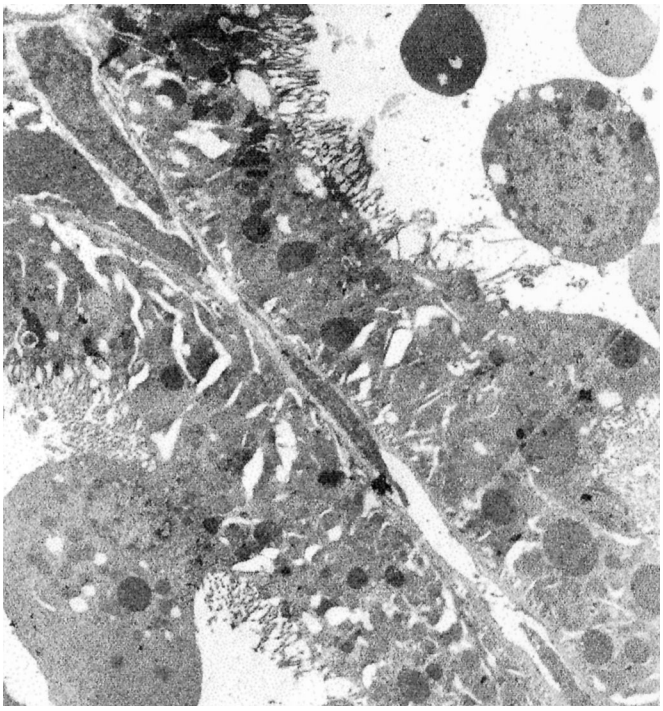

FIGURE 4. Group II showed numerous autophagic lysosomes and accumulated vacuoles in cytoplasm. Detachment of intercellular junctions was evident in all tubular epithelium cells. Microvilli were also irregular and reduced in number at the apical surface of the tubular epithelium (uranyl acetate, lead citrate; magnification × 7,500).

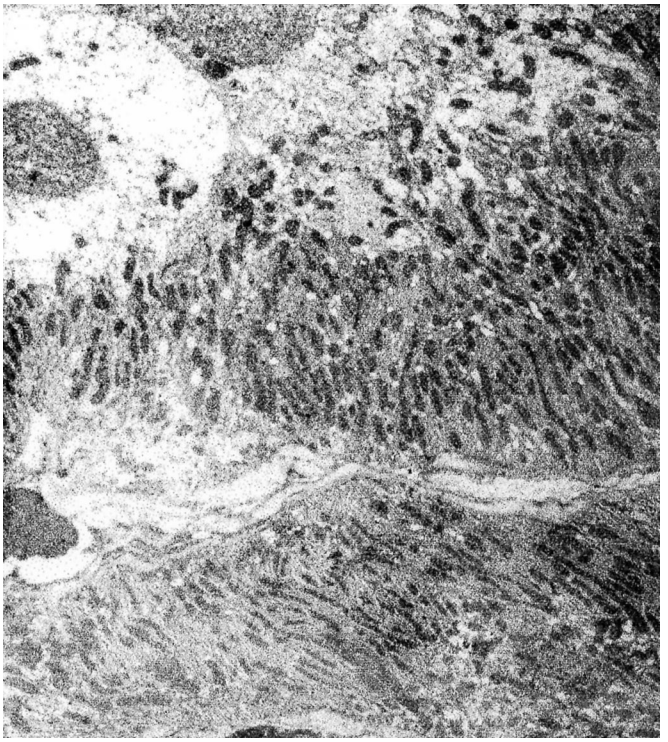

FIGURE 5. Group III showed fewer vacuoles in the cytoplasm. There was no detachment of tubular epithelium from the basement membrane (uranyl acetate, lead citrate; magnification × 7,500).

ischemia have been conducted. Most recently, Riera et al. stated that IP improves postischemic acute renal failure (*9*). They observed less renal ischemic injury after 15 min of ischemia and 10 min of reperfusion, as shown by lower elevations in sCr and more rapid normalization of Cr levels. The mechanisms of IP's remote organ effect has not been clarified clearly. However, what is known is that acute restoration of collateral blood flow, production of a special type of protective protein synthesis, inhibition of mitochondrial adenosine triphosphate, increase in paradoxical protection, or antioxidant production are not included in preventive mechanisms seen after IP (*20*, *22*).

There are conflicting results about the role of norepinephrine in IP. It was reported in a study that norepinephrine was inhibited after IP (*23*). However, IP was reported to decrease the injury caused by norepinephrine in another study (*24*). There are several reports claiming that the functional improvement of IP is abolished by lactate addition (*25*). Moreover, lactate, H^+, NH_3, and other harmful metabolites occurring during the ischemic period were shown to be washed with every reperfusion cycle (*26*). The stimulus arising after IP reaches the intracellular compartment with increasing strength with the help of several mediators serving to signal amplification. G protein and protein kinase C are two important mediators playing a role in IP (*27*).

In our study, in animals subjected to renal ischemia for 45 min or to renal ischemia plus hepatic IP, we found similar levels of BUN and sCr immediately after operation. Yet, in the animals in the hepatic IP group, BUN and sCR elevations were significantly lower at 24 hr after IP. In addition, CrCl was higher and FENa was lower in the hepatic IP group after

24 hr. Lactate dehydrogenase levels followed a similar pattern in both groups, declining to lower levels in the hepatic IP group at 24 hr after operation.

Finally, lipid peroxidation in response to I/R injury, as measured by TBARS**,** was elevated less in group III than in group II after 45 min of reperfusion, but diminished more rapidly after 24 hr. Tumor necrosis factor-α, a proinflammatory agent, is elevated acutely after ischemic events. It is responsible not only for local effects but also remote organ effects (*28*, *29*). For example, Peralta et al. reported that TNF-α elevation was responsible for pulmonary damage after hepatic I/R (*21*). Recent studies have shown that IP prevents TNF-α elevation after I/R (*30*). We also, in our present study, showed that TNF-α levels after 45 min of ischemia were lower in animals treated with IP than in animals not so treated. Our finding that TNF-α levels were decreased by brief IP confirms that TNF-α is one of the factors responsible for the remote effect of IP.

The lesser extent of histopathologic injury in kidneys of IP-treated animals was predictive of better renal function in this group after 24 hr. In fact, histopathologic findings at 24 hr were nearly normal in IP-treated animals. Gho et al. reported the beneficial remote effects of brief renal and small intestinal ischemia on myocardial tissue (*17*) and showed that intestinal IP had a neuronal pathway. Assuming that IP affects different target organs by means of different pathways can be a reasonable explanation for the remote effect of IP on kidney by means of different pathways as well.

CONCLUSION

Our study shows that brief hepatic I/R has a beneficial remote effect on the kidney. This finding was confirmed by biochemical, histopathologic, and ultrastructural studies. We suggest that treatment of cadaveric livers with IP prior to procurement would protect kidneys as well.

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