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Prevention of Renal Ischemia–Reperfusion-Induced Injury in Rats by Picroliv

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ABSTRACT. Picroliv is a potent antioxidant extracted from the roots and rhizome of *Picrorhiza kurrooa*. It has been shown to impart significant hepatoprotective activities, partly by modulation of free radical-induced lipid peroxidation. Lipid peroxidation and reactive oxygen species are associated with tissue injury in post-ischemic acute renal failure. The efficacy of picroliv was assessed in an *in vivo* model of renal ischemia–reperfusion injury (IRI) in rats at a dose of 12 mg/kg orally for 7 days. The animals were killed at various times after reperfusion. Increased lipid peroxidation and apoptotic cell number reflected the oxidative damage following renal IRI. Picroliv-pretreated rats exhibited lower lipid peroxidation, improved antioxidant status, and reduced apoptosis, indicating better viability of renal cells. Immunohistochemical studies revealed that picroliv pretreatment attenuated the expression of intercellular adhesion molecule-1 in the glomerular region. These results suggested that picroliv pretreatment protects rat kidneys from IRI, perhaps by modulation of free radical damage and adhesion molecules. BIOCHEM PHARMACOL **59**;10:1315–1322, 2000. © 2000 Elsevier Science Inc.

KEY WORDS. picroliv; renal; ischemia–reperfusion injury; free radicals; apoptosis

Renal ischemia is associated mostly with acute renal failure. Moreover, transplantation of any organ necessarily involves an unavoidable period of ischemia. Clinical and experimental studies have provided evidence that ischemic cell injury is mediated by ROS¶ [1, 2]. ROS *per se* have also been shown to compromise renal function, depress glomerular filtration, impair glomerular sieving function [3–5], and induce apoptosis in renal cells [6]. Antioxidants improve organ functions [7] and attenuate apoptosis [8, 9]. IRI in the renal vascular bed rapidly results in cellular injury associated with lipid peroxidation [1, 10]. Lipid peroxidation causes production of conjugated dienes and secondary products, among which MDA and 4-HNE are used widely as markers of oxidative stress, as they are stable and easily measurable. Cellular defense against free radical injury is provided by enzymatic (catalase, superoxide dismutases, and glutathione peroxidase) and nonenzymatic (GSH, α-tocopherol, vitamin C, and urate) free radical scavenging systems present in the cell. As lipid peroxidation is the main pathway for tissue radical damage irrespective of the source of free radicals, blocking of this pathway appears to be an attractive strategy to protect the kidney from ROS-mediated damage. Recent overwhelming attention to plant products and alternative medicine has encouraged plant chemists, pharmacologists, biochemists, and molecular biologists to combine their efforts in a search for natural agents that can limit free radical-mediated injuries during and following ischemia–reperfusion, for better therapeutic management of IRI. Prolonged ischemia–reperfusion triggers migration of neutrophils into ischemic tissue areas. Overexpression of adhesion molecules and recruitment of leukocytes during IRI have also been implicated as important mediators of renal ischemia [11]. Novel anti-adhesion therapy for treatment of IRI-mediated renal failure has been suggested recently [12].

Picroliv is a natural plant product extracted from the roots and rhizome of *Picrorhiza kurrooa*. It is a mixture of two iridoid glycosides, picroside-1 and kutkoside (1:1.5, w/w), and has been shown to impart significant hepatoprotective activities by modulation of free radical-induced lipid peroxidation in *in vitro* systems [13]. We have reported previously its ability to enhance the expression of certain hypoxia-inducible factors at the mRNA level in human umbilical vein endothelial cells and glioma cell lines following hypoxia and hypoxia–reoxygenation [14]. The present study was designed to confirm the

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¶ *Abbreviations*: ROS, reactive oxygen species; IRI, ischemia–reperfusion injury; MDA, malondialdehyde; 4-HNE, 4-hydroxynonenal; GPx, glutathione peroxidase; GR, glutathione reductase; SOD, superoxide dismutase; ICAM-1, intercellular adhesion molecule-1, adhesion molecule CD-54; TUNEL, terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling; and TdT, terminal deoxynucleotidyl transferase.

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cytoprotective role of picroliv and to assess its potential to be developed as a therapeutic agent against IRI, in an *in vivo* setting, using a renal IRI model in rats. In the present study, we evaluated the effect of picroliv pretreatment on lipid peroxidation, GSH, GPx, GR, SOD, total NO release, adhesion molecules, and apoptosis following renal IRI. The results imply that picroliv protects renal tissue from IRI-induced damage, perhaps by modulating lipid peroxidation, apoptosis, and adhesion molecules.

MATERIALS AND METHODS

Induction of IRI

IRI was induced in the left kidney of adult male Sprague–Dawley rats (250–325 g; Charles River Laboratories) as described elsewhere [15]. Briefly, 24 animals were divided randomly into two main groups. One of the groups (picroliv-treated; N = 12) was pretreated orally with 12 mg/kg of picroliv once daily for 7 days, while the other (vehicle-treated; N = 12) was administered the same volume of water. Experiments were repeated with consistent results. Rats were anesthetized by Pentothal (50 mg/kg, i.p.; Abbott Laboratories). Autoclave-sterilized surgical instruments were used for the procedure. A laparotomy was performed with a vertical midline incision, and the left renal artery was exposed by blunt dissection. A hemostatic micro clamp was applied on the renal artery of the left kidney for 60 min to create complete renal ischemia. The right kidney was left intact to serve as a control. The clamp was removed later to allow restoration of blood flow to the kidney, for various times of reperfusion. Animals were killed following 5-, 60-, 120-, and 240-min initiation of reperfusion. After the animals were killed, both right and left kidneys were dissected out and split into two equal halves vertically. At random, one half was fixed in 10% buffered formalin for paraffin-embedded sections, and the other was stored at −70° for biochemical assays.

Biochemical Parameters

MEASUREMENT OF FREE RADICAL-MEDIATED LIPID PEROXIDATION. Frozen kidneys were homogenized in ice-cold 20 mM Tris–HCl buffer (pH 7.4) to prepare 10% (w/v) homogenates. Homogenates were centrifuged at 3000 *g* for 10 min at 4° to remove cell debris. Free radical-mediated lipid peroxidation was assessed in tissue homogenates in terms of micromolar MDA per milligram of tissue protein using a colorimetric biochemical kit (Calbiochem-Novabiochem Corp.), following the manufacturer's protocol. MDA contents were calculated using a standard MDA curve plotted using the standard samples provided with the kit.

MEASUREMENT OF GSH, GPX, AND GR. GSH was estimated colorimetrically using a glutathione assay kit procured from the Calbiochem-Novabiochem Corp. Briefly, 5% tissue homogenates were prepared in ice-cold 5% metaphosphoric acid and centrifuged at 3000 *g* for 10 min at 4° to remove cell debris. One hundred microliters of supernatant was used for GSH estimations, to which 800 μL of 200 mM potassium phosphate buffer, pH 7.8 (containing 0.2 mM diethylene triamine pentaacetic acid and 0.025% LUBROL) was added. Fifty microliters of chromogenic agent in 0.2 N HCl was added to this mixture to form thioesters. Further, 50 μL of 30% NaOH was added for β-elimination in alkaline conditions, which transforms the substitution product obtained with GSH into a chromophoric thione, which was read at 400 nm.

GPx and GR activities were assayed using biochemical kits obtained from Oxis International Inc. Briefly, for GR activity, 10% tissue homogenates prepared in ice-cold 50 mM Tris–HCl, pH 7.5, containing 1 mM EDTA were centrifuged to remove cell debris. Four hundred microliters of GSSG was added to 200 μL of diluted samples and incubated at 25°. Four hundred microliters of NADPH was added to this reaction mixture, and reaction kinetics were followed at 25° for 6 min at 340 nm, spectrophotometrically. Each sample was assayed against its own blank containing potassium phosphate buffer, pH 7.5, to replace GSSG. GR activity is based on the oxidation of NADPH to $NADP^+$ catalyzed by GR. For GPx activity, 5 mM EDTA and 1 mM mercaptoethanol were added to the homogenizing buffer. A reaction mixture containing 350 μL of assay buffer, 350 μL of NADPH reagent, and 70 μL of diluted sample was incubated at 25°. Three hundred fifty microliters of enzyme substrate (0.007% *tert*-butyl hydroperoxide) was added to initiate the reaction, which was estimated by following the oxidation of NADPH to $NADP^+$ for 3 min at 340 nm to monitor GPx activity, spectrophotometrically.

ESTIMATION OF TOTAL NO LEVELS. Tissue homogenates prepared for MDA levels were filtered through 10,000 MW cutoff microcentrifuge filters (NanoSpin plus). Filtrates were used for the measurement of total NO levels, as its stable form, nitrite. Nitrite assay was carried out following the protocol suggested for biochemical kits procured from Assay Designs Inc. Briefly, samples were incubated with reaction buffer, reduced β-NADH, and diluted nitrate reductase, provided in the kit, for 30 min at 37°, to allow conversion of sample total nitrate into nitrite. Griess reagent, also provided in the kit, then was added to the reaction mixtures and allowed to stand at room temperature for 10 min. Finally, samples were read at 570 nm for their total nitrite contents, which were calculated using a sodium nitrate standard.

Protein estimation in all samples was carried out using a bicinchoninic acid protein assay kit obtained from Pierce.

Immunohistochemical Studies

Immunostaining for adhesion molecule CD-54 (ICAM-1) and the free radical scavenging enzyme Cu/Zn SOD was performed using rat monoclonal anti-ICAM-1 (PharMingen) and human polyclonal anti-Cu/Zn SOD (Calbiochem-Novabiochem Corp.), respectively, by an indirect avidin–biotin–immunoperoxidase technique (Quick Universal Kit, Vector Laboratories) as described by the manufacturer's protocol. Briefly, tissue sections on slides were deparaffinized, hydrated, and treated with 3% hydrogen peroxide

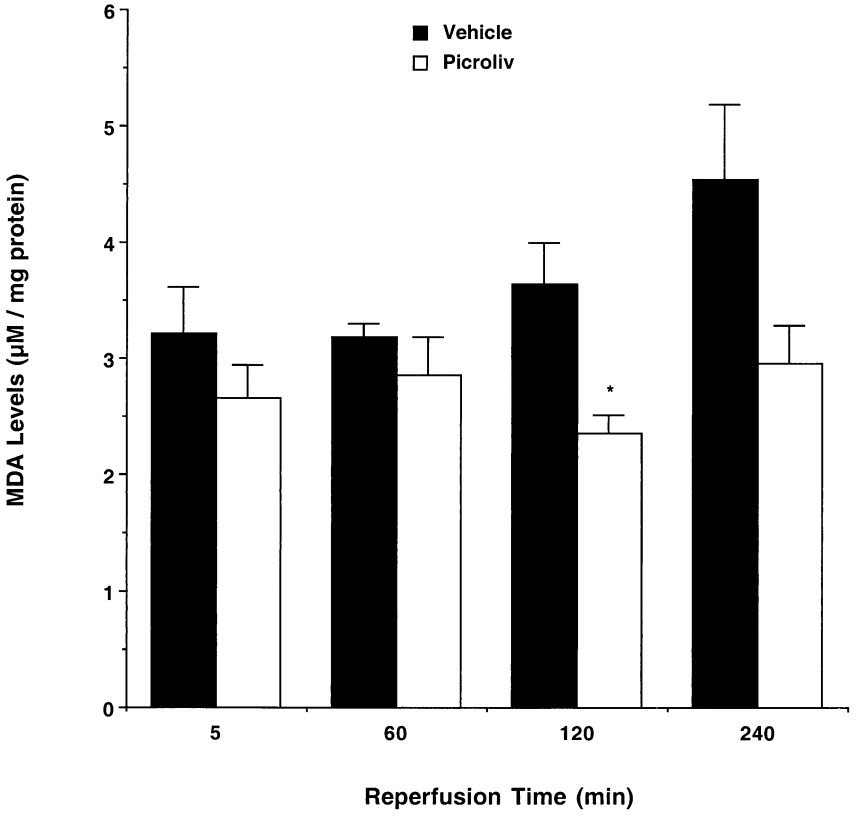

FIG. 1. Alterations in lipid peroxidation levels by picroliv following IRI. MDA levels were measured in 10% homogenates of kidney samples from rats subjected to 60 min of ischemia and various times of reperfusion. Solid bars represent MDA levels following IRI of vehicle-treated rat kidney samples, and open bars represent MDA levels following IRI of picroliv-pretreated rats. Key: (*) $P < 0.05$ as compared with vehicle-treated rat kidney, at 60 min of ischemia and 120 min of reperfusion.

in methanol for 10 min at room temperature to inactivate endogenous peroxidase. Blocking serum, provided in the kit, was used to block nonspecific staining. Then sections were incubated with respective antibody for 1 hr at room temperature in a humidified chamber. Slides were washed with PBS after each incubation with antibody/kit reagents. The respective biotinylated secondary antibody IgG (H+L) was applied to sections for 15 min at room temperature, followed by avidin–biotin–peroxidase complex for 15 min. Diaminobenzidine was used as a substrate for peroxidase; slides were incubated in the dark for 5–8 min, and sections were counterstained with Gill's hematoxylin (Vector Laboratories). Specificity of test antibody was ascertained by incubating sections from each set with normal serum IgG separately.

Apoptotic Studies

The extent of cell death, apoptosis, was determined by the TUNEL technique using an Apoptag kit (Oncor Laboratory) and following the manufacturer's instructions. The TUNEL method is based on the specific binding of TdT to the 3′-OH end of DNA, and ensuing synthesis of a polydeoxynucleotide polymer. Briefly, sections were digested using proteinase K, and the endogenous peroxidase activity was blocked using 2% hydrogen peroxide in PBS. These slides then were placed in equilibration buffer and incubated with a working strength of TdT. The reaction was terminated after 30 min using stop/wash buffer, provided with the kit. The apoptotic nuclei were visualized by direct immunoperoxidase detection of digoxigenin-labeled DNA in test sections.

Statistics

Unless otherwise indicated, data are presented as means ± SEM of 5–6 animals. Comparisons of means were performed using Student's *t*-test, which was contained within the StatView II program (Abacus Concepts, Inc.). A *P* value of less than 0.05 was accepted as statistically significant.

RESULTS

Modulation of MDA Levels by Picroliv

The degree of free radical damage following IRI was assessed by lipid peroxidation, which was measured as MDA levels. MDA levels were expressed as micromolar per milligram of protein of tissue homogenates. Picroliv treatment, *per se*, had no effect on the basal MDA levels, as there was no change in the MDA levels in non-IRI picroliv-treated rats (3.000 ± 0.431 μM/mg protein) compared with their controls (2.889 ± 0.615 μM/mg protein). There was an increase in the MDA levels following 60 min of ischemia and 5, 60, 120, and 240 min of reperfusion as compared with right kidneys from vehicle-treated rats at the same time points. MDA levels gradually increased with increasing time of reperfusion and were maximal at 240 min of reperfusion. As compared with vehicle-treated rat kidney, following 60 min of ischemia and 120 min of reperfusion, picroliv pretreatment resulted in a significant reduction ($P < 0.05$) at 120 min post-reperfusion in the free radical-mediated lipid peroxidation, as indicated by a decrease in the MDA levels (Fig. 1).

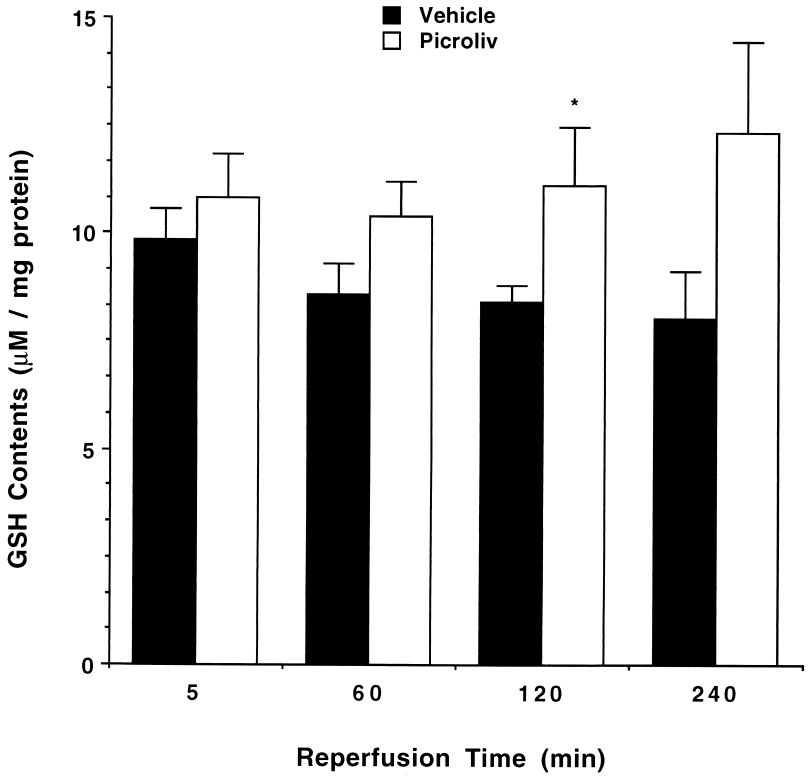

FIG. 2. Modulation of GSH levels by picroliv following IRI. GSH levels were measured in kidney sample homogenates following 60 min of ischemia and different reperfusion times. Solid bars represent GSH values obtained from vehicle-treated rats, and open bars represent values from picroliv-treated rats. Key: (*) $P < 0.05$ as compared with corresponding vehicle-treated group, at 60 min of ischemia and 120 min of reperfusion.

Effect of Picroliv on GSH, GPx, and GR (Glutathione Redox Cycle)

Biochemical studies revealed that in vehicle-treated rats, the levels of GSH were decreased following IRI. The non-IRI right kidneys had a GSH level of 11.174 ± 0.550 μM/mg protein, whereas in kidneys subjected to 60 min of ischemia and 240 min of reperfusion, the GSH levels decreased to 8.017 ± 1.094 μM/mg protein. Furthermore, in non-IRI kidneys, picroliv treatment by itself exhibited a tendency for a slight increase in the GSH levels (12.711 ± 0.808 μM/mg protein) in comparison with the vehicle-treated controls (11.174 ± 0.550 μM/mg protein).

In kidneys undergoing IRI, there was a decrease in GSH content, following different times of reperfusion (Fig. 2, solid bars). However, the attenuated GSH levels were found to be improved in kidney samples obtained from picroliv-pretreated rats ($P < 0.05$) following IRI (Fig. 2, open bars).

To have a complete picture of the glutathione redox cycle, enzyme kinetic studies were performed to assess the activities of GR, the enzyme responsible for replenishing the decreased GSH pool, and GPx, the enzyme responsible for scavenging hydrogen peroxides and lipid peroxides. We observed that following IRI there was no significant change in the GR activity with or without picroliv (Table 1).

GPx activity was decreased to 3.868 ± 0.362 from 4.281 ± 0.470 U/min/mg protein following 5 min of reperfusion, to 3.629 ± 0.262 from 4.322 ± 0.482 U/min/mg protein following 60 min of reperfusion, to 3.361 ± 0.430 from 4.726 ± 0.714 U/min/mg protein following 120 min of reperfusion, and to 4.083 ± 0.715 from 5.000 ± 0.670 U/min/mg protein following 240 min of reperfusion, which was 10, 16, 29, and 18%, respectively, as compared with right kidneys with no IRI. Picroliv treatment resulted in an increase of GPx activity by 47.6% (4.863 ± 0.067 from 3.293 ± 0.240 U/min/mg protein), 13.3% (4.874 ± 0.485 from 4.301 ± 0.107 U/min/mg protein), 30.9% (4.887 ± 0.683 from 3.732 ± 0.320 U/min/mg protein) and 12.63% (4.281 ± 0.282 from 3.801 ± 0.463 U/min/mg protein) at 5, 60, 120, and 240 min of reperfusion as compared with right kidneys with no ischemia and no reperfusion. Observations after 60 min of ischemia and 5, 60, 120, and 240 min of reperfusion in left kidneys clearly suggest that picroliv treatment did enhance the GPx activity as compared with vehicle-treated kidneys following IRI (Table 2). Furthermore, the increase in GPx activity was statistically significant ($P < 0.05$) following 5, 60, and 120 min of reperfusion (Table 2).

TABLE 1. Alterations in activity of glutathione reductase (GR) in kidney tissue samples obtained from rats with or without picroliv undergoing 60 min of ischemia and various time of reperfusion

Reperfusion time (min)	GR (× 10^{-3} U/min/mg protein)	
	Vehicle treated	Picroliv treated
5	1146.94 ± 260.84	952.62 ± 92.88
60	882.60 ± 182.31	1180.40 ± 35.56
120	1091.64 ± 72.95	1009.77 ± 52.71
240	1097.47 ± 45.84	1177.21 ± 80.68

Values are means ± SEM, N = 5–6. Following IRI, there was no significant change in the GR activity with picroliv pretreatment.

TABLE 2. Modulation of glutathione peroxidase (GPx) activity in kidney samples from rats with or without picroliv undergoing 60 min of ischemia and various time of reperfusion

Reperfusion time (min)	GPx U/min/mg protein)	
	Vehicle treated	Picroliv treated
5	3.868 ± 0.362	4.863 ± 0.067*
60	3.629 ± 0.262	4.874 ± 0.485*
120	3.361 ± 0.430	4.887 ± 0.683*
240	4.083 ± 0.715	4.281 ± 0.282

GPx activities were assayed in kidney homogenates by studying reaction kinetics at 25° and by following the oxidation of NADPH to $NADP^+$. Values are means ± SEM, N = 5–6.

*$P < 0.05$ compared with vehicle-treated kidneys following IRI at respective time points.

Alteration in NO Release

Biochemical estimations for total nitrite contents were performed to assess the NO production in kidneys obtained from vehicle and picroliv-treated rats with or without IRI. Total nitrite contents in vehicle-treated rats tended to increase with reperfusion time with a maximum at 120 min (1.390 ± 0.231 nmol/mg protein). We observed that at 60 min of ischemia and 5, 60, and 120 min of reperfusion, the total nitrite contents were 0.948 ± 0.178, 1.189 ± 0.187, and 1.390 ± 0.231 nmol/mg protein. Picroliv modulated the total NO release in our studies and showed a trend to attenuate the release of NO, which was measured in terms of nitrite. Picroliv attenuated the nitrite contents to 0.877 ± 0.104, 0.766 ± 0.181, and 1.098 ± 0.156 nmol/mg protein following 5, 60, and 120 min of reperfusion, which was 7.5, 35.6, and 21%, respectively.

Effect of Picroliv on SOD

Immunohistochemical studies revealed that picroliv treatment by itself results in noticeable expression of SOD protein in the tubular and glomerular region, as seen by the brown diaminobenzidine staining, thereby suggesting an enhanced presence of the enzyme or better antioxidant status (Fig. 3A). Furthermore, picroliv appeared to be able to maintain augmented SOD expression even after 60 min of ischemia followed by 120 min of reperfusion (Fig. 3B).

Effect of Picroliv on ICAM-1 Expression Following Renal IRI

Immunohistochemical studies of paraffin-embedded sections showed that following IRI the expression of ICAM-1 was up-regulated mainly in the glomerular region, which was best seen in sections obtained from rats that were subjected to 60 min of ischemia and 120 min of reperfusion. Amazingly, the expression of the adhesion molecule was found to be diminished almost completely in kidney sections from picroliv-pretreated groups (Fig. 4B) following IRI. Our results indicate that picroliv treatment down-regulates the expression of adhesion molecules.

Modulation of Apoptosis by Picroliv

The *in situ* end labeling technique, which labels the large number of DNA fragments generated within apoptotic nuclei, was used on renal sections obtained from animals fed with or without picroliv during IRI to study the effect of picroliv on apoptosis. There was an increase in apoptotic injury to renal epithelial cells with increasing time of

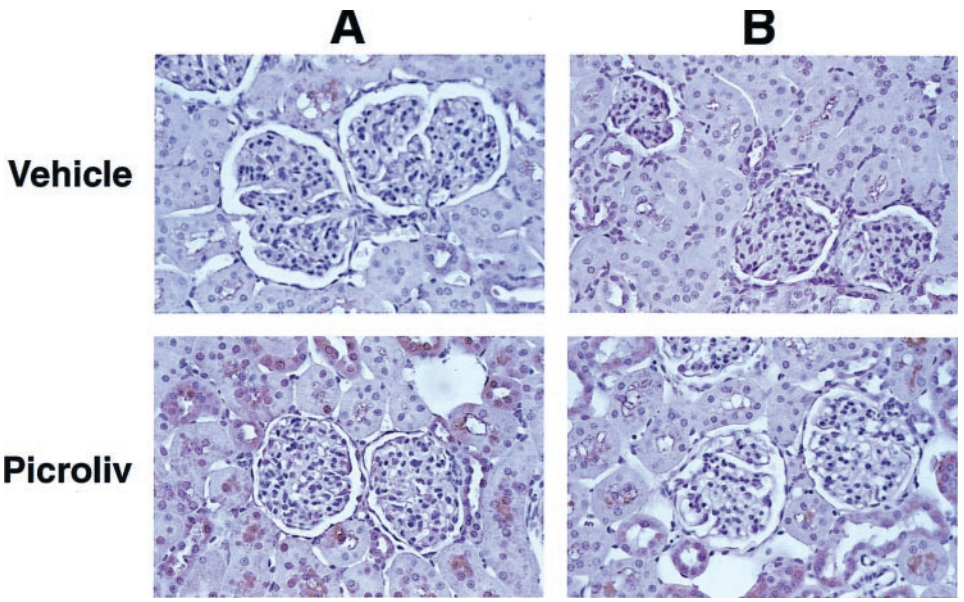

FIG. 3. Immunohistochemical staining of superoxide dismutase (SOD) in kidney sections. Immunohistochemistry was performed on kidney sections from vehicle-treated and picroliv-treated groups using a Quick Universal Kit and polyclonal anti-Cu/Zn SOD. (A) Sections from vehicle- and picroliv-treated rats not subjected to IRI. (B) Sections from kidneys that were subjected to IRI at 40x magnification.

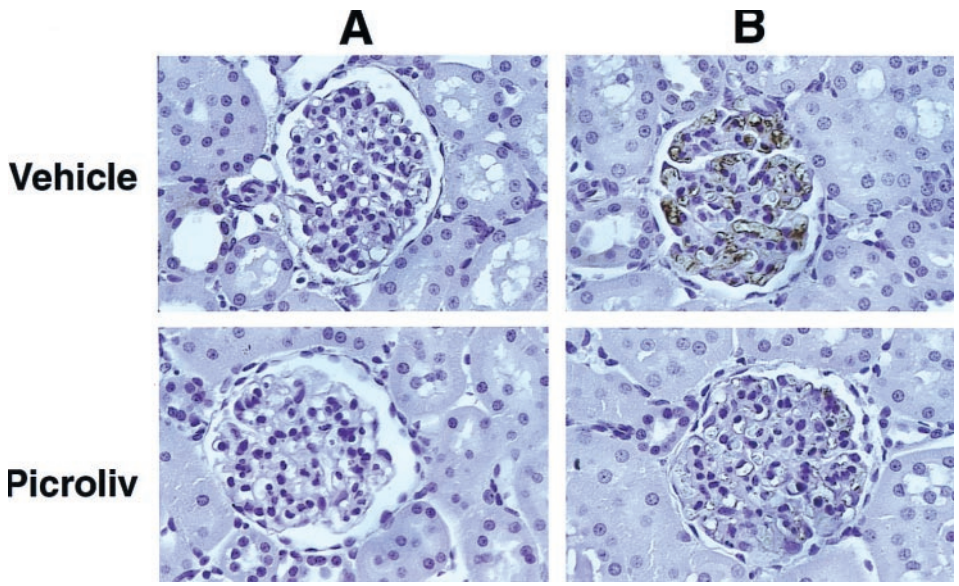

FIG. 4. Expression of ICAM-1 in kidney sections. Anti-ICAM-1 monoclonal antibody was used for immunohistochemical staining of kidney sections from vehicle-treated and picroliv-treated groups. (A) Sections from vehicle- and picroliv-treated rats not subjected to IRI. (B) Sections from kidneys that were subjected to IRI at 60x magnification.

reperfusion, which was seen to be maximal after 60 min of ischemia and 240 min of reperfusion. Picroliv-fed animals showed a significantly lesser number of apoptotic positive nuclei within the glomeruli and tubular epithelial cells as compared with vehicle-treated controls (Fig. 5).

DISCUSSION

A great deal of effort has been directed toward searching for compounds that can be used for better management of the clinical consequences arising from renal ischemia–reperfusion, without much success. The results obtained in the present investigation suggest that picroliv has an overall protective effect against kidney ischemia/reperfusion injury in a rat model. We observed that picroliv pretreatment offered protection against free radical-mediated lipid peroxidation, apoptosis, and overexpression of adhesion molecules following IRI.

Free radicals have been shown to play a major role in IRI [1, 2]. ROS collectively are instrumental in impairing overall renal function [3–5] and in inducing apoptosis in renal cells [6]. Antioxidant therapy has been well documented to help in the improvement of organ functions [7] and to prevent apoptosis [8, 9]. The protection provided by free radical scavengers against ROS produced during IRI supports the hypothesis that free radical species are involved in the cellular pathogenesis of IRI [16, 17].

We assessed the potential of picroliv by studying its effect on lipid peroxidation, which was measured in terms of MDA, a stable metabolite of the free radical-mediated lipid peroxidation cascade. MDA levels increased with increasing time of reperfusion following renal IRI. Picroliv reversed the increase of MDA levels to a considerable extent, thereby confirming its antioxidant role in IRI. We also studied the effect of picroliv on yet another cellular bio-antioxidant, GSH, which is known to be depleted following an ischemic insult [18]. Similarly, in our studies, GSH was decreased with increasing reperfusion time following IRI. Picroliv-pretreated rats exhibited higher GSH contents than their respective controls, indicating that

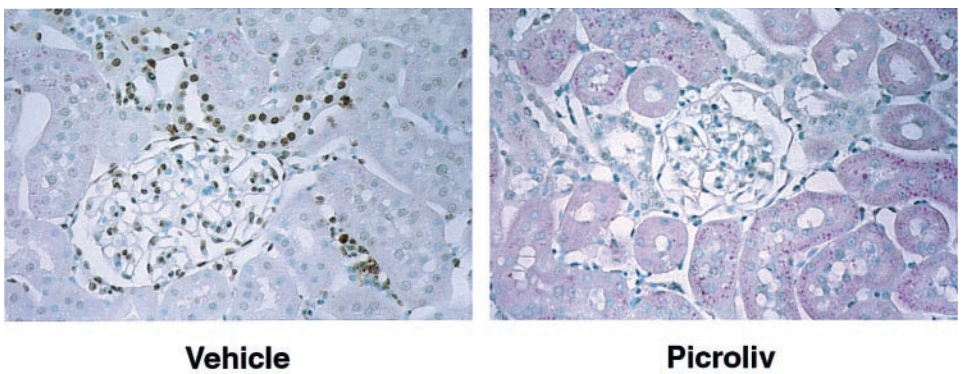

FIG. 5. Representative picture of TUNEL staining in renal sections. Apoptotic nuclei were determined, using an Apoptag kit in kidney sections from vehicle- and picroliv-treated rats following 60 min of ischemia and 240 min of reperfusion as seen at 60x magnification.

picroliv helped in replenishing the GSH pool; however, picroliv-mediated GSH replenishment was not as impressive as expected. Hence, we conducted studies to look into the activity of an important enzyme, GR, responsible for maintaining cellular GSH pools. We observed that picroliv-treated tissues had no significant increase in GR activity. Hence, moderate elevation of GSH may be the result of unaffected GR activity by picroliv. IRI resulted in a decrease in GPx activity, probably because of overwhelming production of lipid peroxides following an ischemia–reperfusion insult. Picroliv tended to enhance the GPx activity that was lowered due to IRI. As GPx helps in limiting the buildup of lipid peroxides [19], our observations indicate that picroliv-mediated attenuation of MDA may be due partly to increased GPx activity. We also observed a decrease in the GPx activity in non-IRI kidneys, although it was not statistically significant. More importantly, our immunohistochemical studies showed that SOD levels increased following picroliv treatment. Although, after 60 min of ischemia and 120 min of reperfusion, SOD staining was reduced significantly as compared with the picroliv control, it was still maintained at a noticeably elevated level from the vehicle control. It has been reported that total SOD is down-regulated following renal IRI [20]. It is also well known that SOD enzyme is the first line of defense against free radical generation, and, hence, any decrease in SOD renders the tissue susceptible to oxidant injury. Superoxide radicals are formed following respiratory bursts and ischemic, hypoxic, or IRI conditions and lead to formation of other oxyradicals, collectively known as ROS [21]. Use of SOD [22], SOD mimics [23], transgenic mice [24], and other such preparations [25] have been shown to exhibit significant protection against ROS-mediated injuries. We believe that the elevated SOD levels induced by picroliv may help in reducing superoxide radicals following IRI and, hence, reducing free radical-mediated lipid peroxidation.

IRI-induced damages are also brought about by NO, which is also a free radical [26]. Although the role of NO has been controversial ever since it was discovered, there are ample reports to indicate that NO contributes further to hypoxia and reoxygenation injury [27]. Peroxynitrite radical is formed by the interaction of NO and superoxide radical and has been reported to be much more damaging than either of them alone [28, 29]. NO and ROS are also known to induce apoptosis [30, 31]. In our IRI model, we found that the NO levels increased with reperfusion time. Similar observations were made by Weight *et al.* [32]. Picroliv treatment reversed the elevated NO levels resulting from IRI. Our observations are in accord with that of Sharma *et al.* [33], who have shown that antioxidant(s) attenuates the expression of constitutive neuronal NO synthase (nNOS), which is involved in the pathophysiology of brain injury during heat stress. As free radicals and NO induce apoptosis, we were interested in studying the effect of picroliv on apoptosis following IRI. We observed that in comparison to vehicle-treated controls, there was a significant reduction in the number of apoptotic nuclei in kidney sections from picroliv-pretreated animals. We believe that the reduction in number of apoptotic nuclei was due to better antioxidant status as evidenced by enhanced GSH content, increased GPx enzyme activity, better SOD staining, and decreased NO levels.

Finally, we looked into yet another important factor, the expression of adhesion molecules. Overexpression of adhesion molecules has been implicated as the critical mediator in renal ischemia [11]. Our observations reveal that picroliv down-regulates the overexpression of ICAM-1 in glomerular regions. We also studied the expression of CD-18 (integrin β2 chain), another important adhesion molecule, in our study and observed that picroliv treatment, as with ICAM-1, decreased the IRI-induced expression of CD-18 (data not presented). These observations with picroliv in regard to adhesion molecules suggest that picroliv treatment prevents overexpression of adhesion molecules, and hence prevents neutrophil recruitment to ischemic areas. This, in turn, may protect tissue from the possible consequences of activated neutrophils following IRI. Furthermore, we have also observed that picroliv prevents neutrophil infiltration in liver tissue following IRI in a liver IRI model (unpublished observation). Over-expression of adhesion molecules can be suppressed by administration of exogenous NO or NO donors [34]. However, picroliv might mediate regulation of ICAM-1 and CD-18 independently of NO, since our studies did not involve use of any exogenous NO or NO donor.

To summarize, our investigations demonstrated that picroliv pretreatment has a protective effect on lipid peroxidation, GSH, GPx, total NO release, adhesion molecules, and apoptosis following renal IRI. Thus, picroliv has the potential for being developed as a therapeutic agent for the better management of renal IRI.

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