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Contribution of γ glutamyl transpeptidase to oxidative damage of ischemic rat kidney

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Contribution of γ glutamyl transpeptidase to oxidative damage of ischemic rat kidney.

Background. A variety of mechanisms have been considered in the pathogenesis of the cell damage occurring in the kidney that is undergoing transient ischemia. However, little information is available about the role of oxidative stress in building up the tissue injury in the hypoxic organ during short-term ischemia.

Methods. After a standard brief period (25 min) of unilateral kidney ischemia in rats, pretreated or not with acivicin (60 μmol/L/kg i.v.), tissue samples from both ischemic and not ischemic kidneys were obtained to measure malondialdehyde (MDA) and glutathione (GSH) content, γ glutamyl transpeptidase (GGT) activity by spectrophotometry, localization and intensity of enzyme activity, and tissue damage by histochemistry.

Results. GGT activity was found to be increased in both cortical and medullar zones of the ischemic kidneys, where the GSH level was only slightly decreased and the MDA level, in contrast, was markedly increased; in parallel, the cytosolic volume of the proximal tubular (PT) cells showed a significant increment. The animal pretreatment with acivicin, a specific inhibitor of GGT, besides preventing the up-regulation of the enzyme during ischemia, afforded good protection against the observed changes of MDA and GSH tissue levels, as well as of tubular cell volume.

Conclusions. Ex vivo data supporting a net pro-oxidant effect of up-regulated GGT during short-term ischemia of rat kidney have been obtained. The enzyme stimulation appears to contribute to the renal morphological damage exerted by a brief hypoxic condition at the level of PT cells. The actual impact on kidney function by GGT-dependent oxidative damage during transient ischemia and the potential protective action of GGT inhibitors require subsequent investigation.

The mechanisms responsible for tubular damage and cell death following acute renal ischemia are still not fully understood. It is accepted that a loss of membrane-selective permeability and the collapse of the ionic gradients through cell membrane, as well as the abnormal activation of phospholipases and proteases caused by impaired calcium homeostasis induced by adenosine 5′-triphosphate (ATP) depletion, play the main roles in the genesis of the biochemical and morphological alterations observed in the proximal tubules during prolonged renal ischemia [1–9]. However, because these changes do not account for the whole cell damage, other pathogenetic processes must contribute. Despite the very low oxygen concentrations that can be reached in ischemic conditions, free radical-mediated reactions appear to take place in the ischemic organ and lead to oxidative damage, or at least this may be assumed from the increased lipid peroxidation and/or antioxidant consumption observed in such conditions [10–12].

γ Glutamyl transpeptidase (GGT; EC 2. 3. 2. 2.) is a glycoprotein attached to the external surface of various cell types and in which the physiological function in the glutathione (GSH) metabolism is to recover cysteine from extracellular GSH (and GSH derivatives) [13]. High enzyme activity has been observed in cells that exhibit intense secretory and absorption functions, such as epithelial cells of the proximal tubular (PT) cells, jejunum, biliary tract, choroid plexus, thyroid follicles, and canalicular pole of hepatocytes [14, 15].

In the kidney, the primary site of GGT activity is the outer surface of the microvillus membrane (brush border) in the PT [14, 15]. Microdissection of rat nephron showed that proximal straight tubules (S3 segment in the inner cortex, medullary rays of the cortex, and the outer medulla) exhibit about twice as much GGT activity as proximal convoluted tubules (S1-S2 segments in the cortex) [15]. In addition, it has been reported that some enzyme is also present on the basolateral membrane of the PT, and it was claimed that the transpeptidase activity on the basolateral membrane plays a role in the clearance of plasma GSH [16, 17].

Key words: cell damage, oxidative stress, renal ischemia, transplantation, hypoxia.

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Because GGT favors the reconstitution of intracellular GSH, it could be considered as a member of the antioxidant defense. On this basis, the expression of high GGT levels in tumors has been interpreted as one of the factors involved in the resistance of transformed cells to the oxidant damage produced by chemical or radiation therapies [18–22]. However, recent studies have shown that GGT itself plays a marked pro-oxidant role under certain conditions. The cysteinyl-glycine resulting from the GGT-mediated cleavage of GSH rapidly reduces ferric iron, thus triggering iron redox cycling, which stimulates the production of hydroxyl radicals and enhances membrane lipid peroxidation [23–26].

Our experiments were aimed to assess the role of GGT in the pathogenesis of ischemic tubular cell damage as a promoter of oxidative stress and particularly of lipid peroxidation. A rat model of unilateral renal ischemia was set up, and the degree of tubular cell damage and lipid peroxidation was evaluated. The activity of GGT *in vivo* was inhibited by pretreating the animal with acivicin, a glutamine analogue that irreversibly inactivates GGT without affecting its synthesis and degradation [27].

METHODS

Animals

Male Wistar rats (Harlan-Nossan, Correzzana, MI, Italy) of 250 to 280 g were used. All experiments were conducted according to the ethical guidelines of the Animal Welfare Committee of the University of Turin (Turin, Italy). The rats had free access to standard chow diet and water. The animals were randomly divided in two experimental groups. One group received acivicin at a dose of 60 μmol/L/kg. Acivicin was dissolved in 0.9% sterile NaCl solution (wt/vol) and injected into the tail vein two hours before the application of renal ischemia. The other group of control rats received an equal volume of saline solution.

Renal ischemia

Rats were anesthetized with sodium pentobarbital given intraperitoneally (50 mg/kg) and placed under a warm lamp to maintain the body temperature. An abdominal incision was made, and perirenal fat, which supplies small arteries to the surface of the kidneys, was stripped off. The vascular pedicle of the left kidney was then occluded with a nontraumatic vascular clamp for 25 minutes. At the end of this short ischemic period, not followed by organ reperfusion, both kidneys were directly removed for morphological, histochemical, and biochemical determinations. The right kidney was used as a sham-operated, internal control.

Morphological and histochemical studies

Two slices, cross-sections of approximately 0.2 cm thickness, were cut from the middle portion of each control and ischemic kidneys. One slice was placed in 4% formaldehyde, 50 mmol phosphate buffer (pH 7.4) overnight, dehydrated in graded alcohols, and embedded in paraffin. Tissue sections of 5 μm were stained with periodic acid-Schiff (PAS) reagent. The sections were subjected to a point-counting technique. Ten randomly chosen areas from the inner cortex and the outer medulla were quantitatively analyzed using a 551-point grid. The grid points falling on the cytosol (C points) or the nucleus (N points) of the PT were counted. The degree of cell swelling was assessed by the ratio of C points/N points, which reflects the cytosolic volume/nuclear volume ratio.

The second kidney slice was placed under liquid nitrogen and stored at −80°C until use for histochemical determination of GGT activity. The latter was performed according to the procedure described by Rutenburg et al with minor modifications [28]. Briefly, 10 μm cryosections were air dried at room temperature and then fixed in cool acetone for 10 minutes. The slides were incubated at room temperature for 5 to 15 minutes in 0.1 mol/L Tris-HCl buffer, pH 7.0, containing 0.4 mmol/L γ glutamyl-4-methoxy-2-naphthylamide, 3.0 mmol/L glycylglycine, and a diazonium salt (Fast Blue B). The resulting azo dye was then chelated with $CuSO_4$ to yield an insoluble orange-brown dye at the site of enzyme activity. Slides were counterstained with hemalum reagent solution, and the intensity of the reaction was evaluated in 10 nonconsecutive fields randomly chosen by a blinded observer using an arbitrary scale as follows: 0 point, negative reaction; 1 point, slight positive reaction; 2 points, strong positive reaction (the maximum score was 20 points).

Biochemical determinations

To this purpose, homogenates of the cortical and outer medullar zones of each kidney were made at 10% (wt/vol) in 0.1 mol/L Tris-HCl buffer, pH 7.4, and malondialdehyde (MDA) steady-state levels, GSH concentration, and GGT activity were determined. MDA was measured according to the method described by Poli et al with minor modifications [29]. Thiobarbituric acid 0.67% (wt/vol) was added to aliquots of the homogenates, previously precipitated with 10% trichloroacetic acid (wt/vol). Then the mixture was centrifuged, and the supernatant was heated for 15 minutes in a boiling water bath. After cooling, 2N KOH was added to neutralize the mixture, and the absorbence was measured at 543 nm. The results were expressed as nmol of MDA/mg protein by using $E = 17$ mmol/L^{-1}cm^{-1}. The GSH concentration was measured by the method of Sedlak and Lindsay in which 5,5′-dithiobis-(2-nitrobenzoic acid), reacting with sulfydryl groups, yields 2-nitro-5 mercaptobenzoic acid with maximal absorbence at 412 nm [30].

The hypothetical antioxidant capacity of acivicin was tested in an *in vitro* standard model, that is, a rat liver microsomal suspension (20 mg protein/mL). Aliquots of

such suspension were incubated in 0.1 mol/L Tris/KCl buffer, pH 7.4, at 37°C for 40 minutes with or without acivicin (5 μmol/L), in the presence or absence of the pro-oxidant systems cumene hydroperoxide 0.1 mol/L (CUOOH) or ferrous sulfate 5 μmol/L/ascorbate 500 μmol/L ($FeSO_4$/Asc). Then the MDA content was determined as previously described and was expressed as nmol MDA/mg of protein.

The enzymatic activity of GGT in the homogenates was measured in 0.1 mol/L Tris-HCl buffer, pH 8.0, following the method of Tate and Meister [13]. Aliquots were incubated at 37°C in the presence of 5 mmol/L L-gamma-glutamyl-*p*-nitroanilide and 20 mmol/L glycylglycine as γ-glutamyl acceptor. The rate of *p*-nitroanilide release was determined spectrophotometrically at 410 nm using $E = 8.8$ mmol/L^{-1}cm^{-1}. One unit of GGT activity is defined as the amount of enzyme releasing 1 μmol of *p*-nitroanilide per minute at 37°C. The specific activity of the enzyme was expressed as units/mg protein.

The protein content of tissue homogenates was assayed by the Bradford's spectrophotometric method [31].

Statistical analysis

All values were expressed as means ± SE of more than three different experiments. For data group comparison, the unpaired two-tailed Student's *t*-test was used.

Chemicals

Acivicin, γ glutamyl-4-methoxy-2-naphthylamide, glycylglycine, Fast Blu B, L-γ glutamyl-*p*-nitroanilide and 5, 5′-dithiobis-(2-nitrobenzoic acid) were obtained from Sigma (St. Louis, MO, USA). All other reagents were of analytical grade.

RESULTS

Renal ischemia and γ glutamyl transpeptidase activity: Effect of acivicin

After 25 minutes of unilateral ischemia, which is a commonly analyzed period of ischemia because it is consistently associated with reversible damage of S1-S2 cells and death of S3 cells [3, 4], GGT activity increased by about twofold in both cortical and medullar zones of the kidney. Rat pretreatment with acivicin fully prevented such increases of GGT total activity in the kidneys undergoing ischemic condition, but it also markedly inhibited the enzyme activity of both cortical and medullar zones of control kidneys (Fig. 1 A, B).

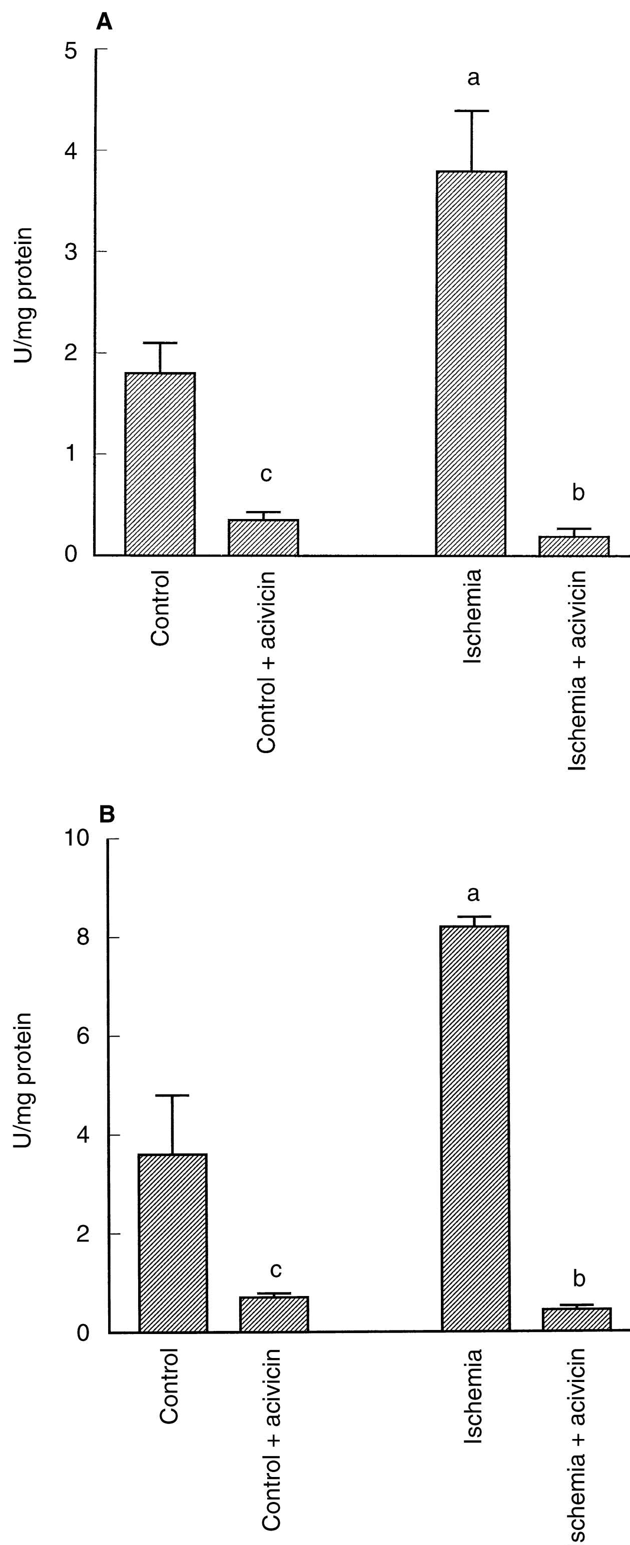
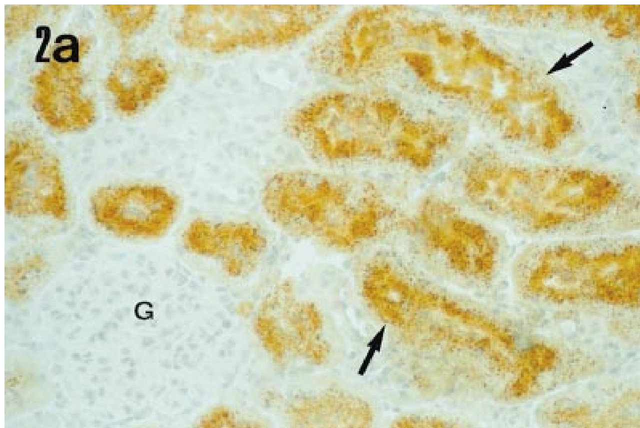


Fig. 1. Effects of 25 minutes of ischemia on the γ glutamyl transpeptidase (GGT) activity of rat kidney. (*A*) Cortex. (*B*) Outer medulla. Values are means ± SE from five rats per experimental group. [a]Different from control ($P < 0.05$); [b]different from ischemia ($P < 0.01$); [c]different from control ($P < 0.05$).

Histochemical determination of γ glutamyl transpeptidase activity in control and ischemic kidneys of untreated and acivicin-treated rats

The histochemical assays of GGT activity in the inner cortex and outer medulla zones of kidneys from control



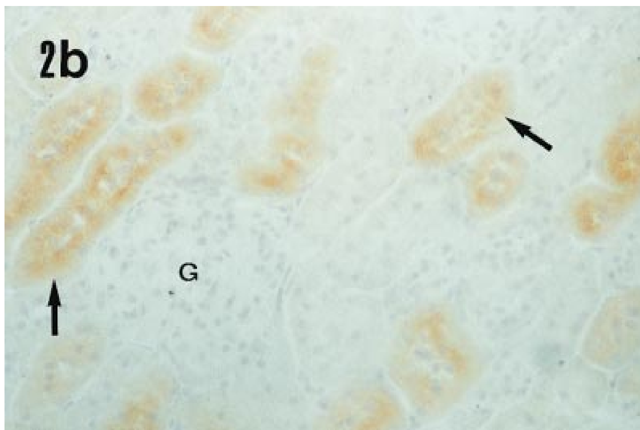
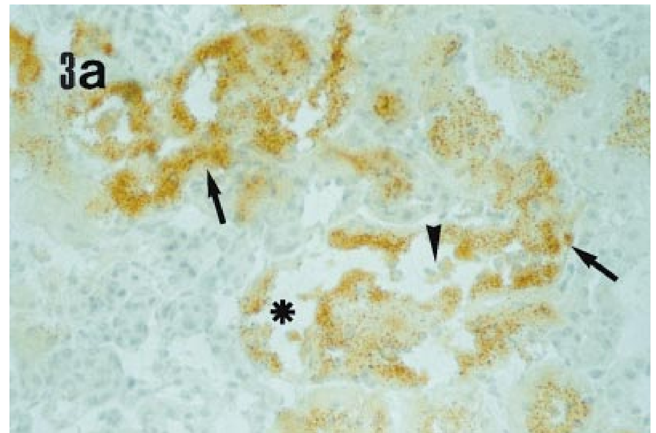


Fig. 2. Histochemistry of GGT activity in kidneys from control and acivicin-pretreated rats. (*a*) Renal inner cortex of a control rat. (*b*) Renal inner cortex of a rat pretreated with acivicin. G, glomeruli; arrows, proximal tubules.



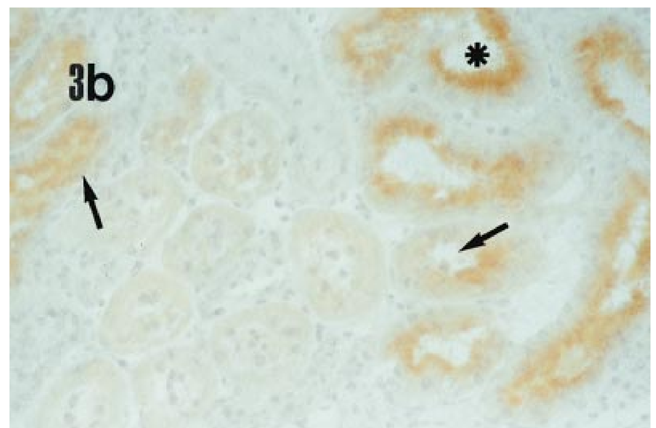
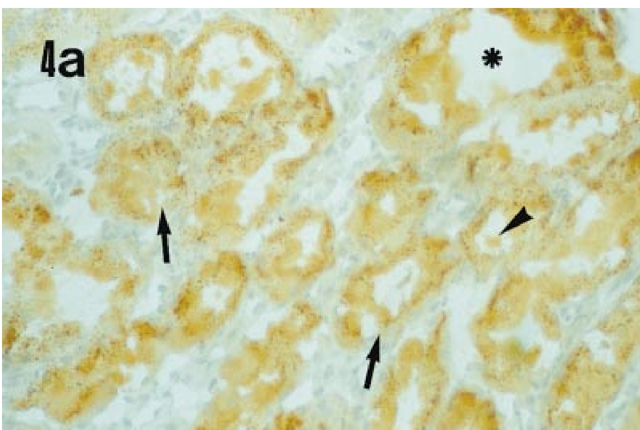


Fig. 3. Histochemistry of GGT activity in the inner cortex of ischemic rat kidneys in the presence or absence of acivicin pretreatment. (*a*) Inner cortex of ischemic kidney from acivicin-untreated animal. (*b*) Inner cortex of ischemic kidney from acivicin-treated rat. Arrows, proximal tubules; arrowheads, desquamated tubular cells; asterisks, dilated proximal tubules.



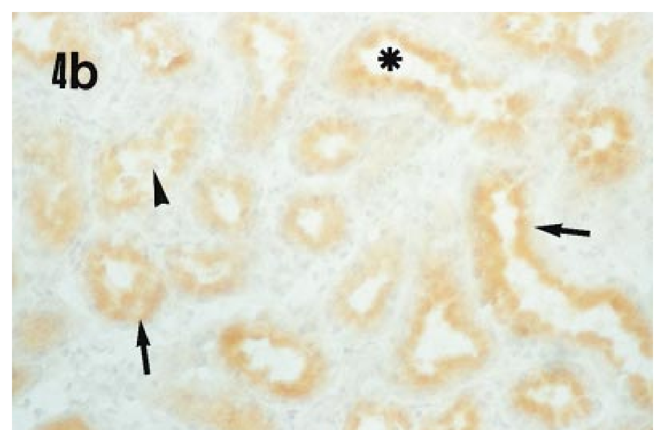


Fig. 4. Histochemistry of GGT activity in the outer medulla of ischemic rat kidneys in the presence or absence of acivicin treatment. (*a*) Outer medulla of ischemic kidney from control rat. (*b*) Outer medulla of ischemic kidney from acivicin-treated rat. Arrows, proximal tubules; arrowheads, desquamated tubular cells; asterisks, dilated proximal tubules.

animals showed the PT with an intense and homogeneous reaction (score, 17 ± 3). The treatment with acivicin strongly inhibited enzyme activity; only a few tubules exhibited a slight positive reaction in feel agreement with the biochemical method (score, 7 ± 1; different from control, $P \leq 0.001$; Fig. 2 a, b). Renal ischemia did not modify the intensity of the histochemical reaction of GGT in cortex (score, 16 ± 4; Fig. 3a) and medulla (score, 18 ± 2; Fig. 4a). However, tubular dilation, cell swelling, and desquamation were clearly evident in both kidney zones (Figs. 3a and 4a). In contrast, acivicin-treated rats had ischemic kidneys that showed only mild cytological and architectural alterations of PT, as well as a slight histochemical positive reaction for GGT activity, both in the cortex (score, 6 ± 2) and the medulla (7 ± 3; Figs. 3b and 4b).

Renal ischemia and tissue lipid peroxidation: Effect of acivicin

After 25 minutes of unilateral ischemia, a marked increase of MDA levels was observed in the cortex (about 2.9-fold) and medulla (about 1.7-fold) of ischemic kidneys (Fig. 5). The increase in MDA, a major end-product of cellular lipid peroxidation, indicates a marked oxidative stress and an accelerated rate of lipid peroxidation reactions in ischemic cortex and medulla. Acivicin pretreatment totally prevented the ischemia-induced increase in MDA and showed, in addition, some lowering effect on endogenous MDA content (Fig. 5). With regard to GSH tissue levels, acivicin pretreatment significantly increased control values and well counteracted the really modest lowering effect exerted by mild ischemia on the tripeptide content (Fig. 6).

Tubular cell swelling after kidney ischemia: Effect of acivicin

Renal ischemia produced a marked tubular cell swelling that was about 60% in the inner cortex and 87% in the outer medulla, as evaluated by the morphometric ratio: cytosolic volume/nuclear volume (Table 1). Acivicin treatment prevented the ischemia-induced cell swelling by 60 and 95% in the cortex and medulla, respectively (Table 1). The GGT inhibitor *per se* did not exert changes on cellular volume.

Lack of effect of acivicin on microsomal lipid peroxidation

To exclude that the reported effect of acivicin was not dependent on an antioxidative action of the compound, the latter was tested on a classic in vitro pro-oxidant model employing isolated liver microsomes. The addition of acivicin to this system did not affect the extent of the lipid peroxidation measured at all (Table 2).

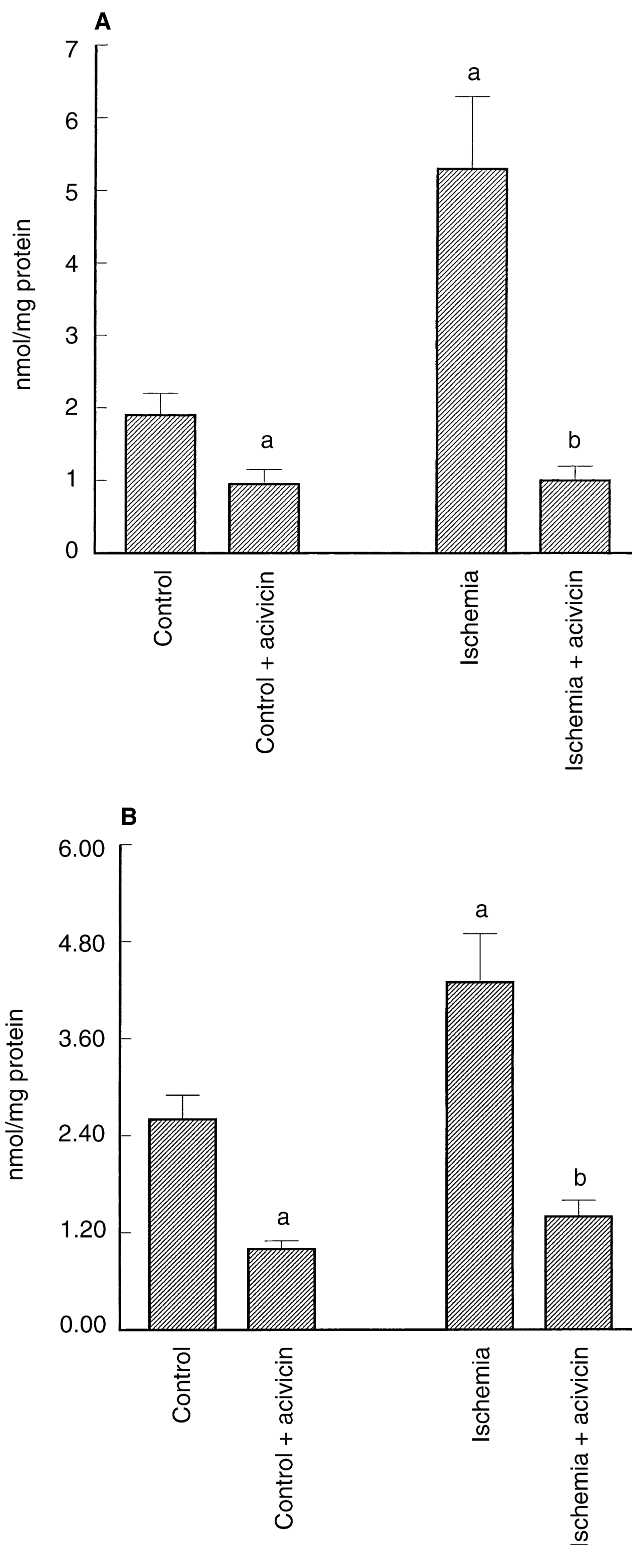


Fig. 5. Effect of 25 minutes of ischemia on malondialdehyde (MDA) levels in rat kidney. (*A*) Cortex. (*B*) Medulla. Values are means ± SE from five animals per experimental group. [a]Different from control ($P < 0.05$); [b]different from ischemia + acivicin ($P < 0.001$).

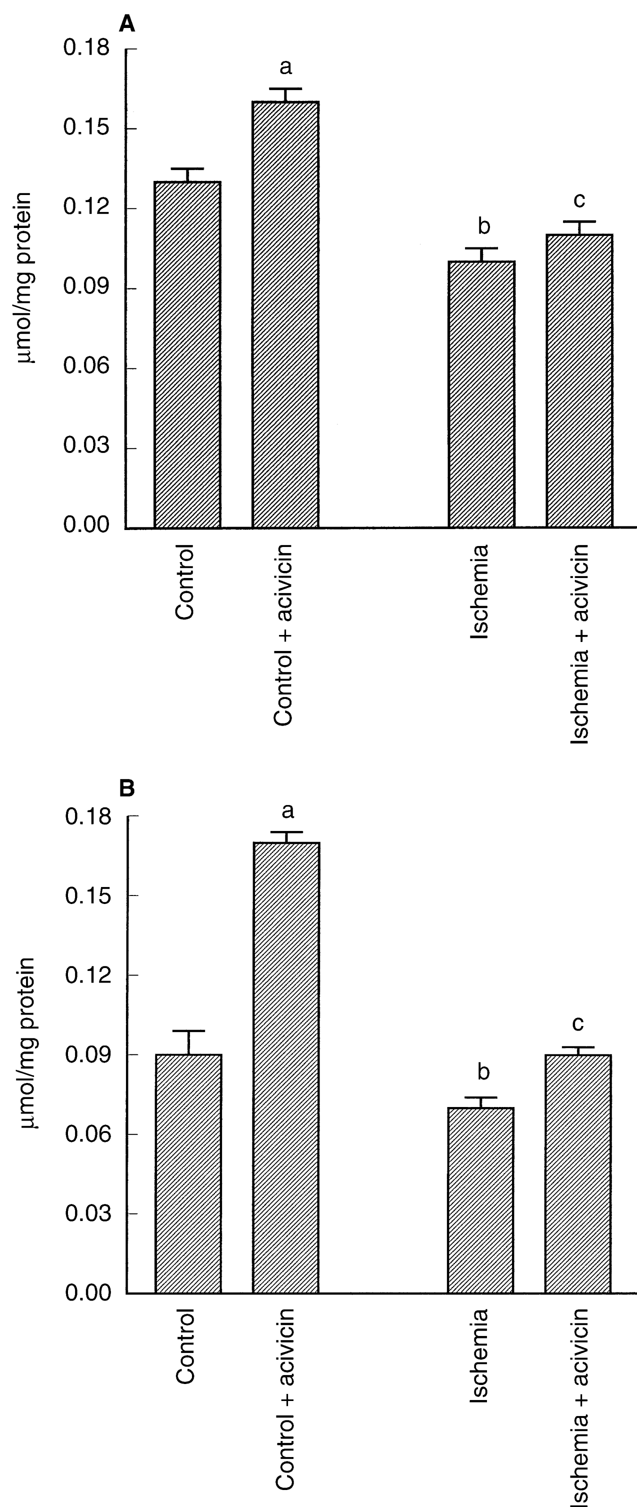


Fig. 6. Effect of 25 minutes of ischemia on GSH content of rat kidney. (*A*) Cortex. (*B*) Medulla. Values are means ± SE from five animals per experimental group. [a]Different from control ($P < 0.001$); [b]different from control ($P < 0.01$); [c]different from ischemia ($P < 0.03$).

Table 1. Cytosolic volume of tubular cells after kidney ischemia plus or minus acivicin pretreatment

	Cytosolic/nuclear volume ratio	
Exp. group	Inner cortex	Outer medulla
Ischemia	8.0 ± 0.5[a,b]	9.0 ± 0.8[a,b]
+ acivicin	6.2 ± 0.4[c]	5.0 ± 0.2
Control	5.0 ± 0.3	4.8 ± 0.2
+ acivicin	5.3 ± 0.2	4.5 ± 0.3

Values are means ± SE of twenty determinations per experimental group (4 fields each rat and 5 rats each group).
[a] Different from control ($P < 0.001$)
[b] Different from ischemia + acivicin (cortex, $P < 0.05$ and medulla, $P < 0.001$)
[c] Different from control + acivicin ($P < 0.05$)

Table 2. Effect of acivicin on microsomal lipid peroxidation

Experimental group	MDA level *nmol/mg protein*
Control	0.5 ± 0.1
Acivicin	0.6 ± 0.1
CUOOH	4.0 ± 0.2
CUOOH + acivicin	4.0 ± 0.1
$FeSO_4$/ascorbate	19.1 ± 0.1
$FeSO_4$/ascorbate + acivicin	18.1 ± 1.0

See **Methods** section for details on microsomal incubation and concentration of pro-oxidants. Values are means ± SE of at least three determinations per experimental group.

DISCUSSION

Application of a short-term ischemia led to extensive cell swelling and morphological injury of rat kidney at the level of S3 cells of proximal tubules. Together with the renal damage, two other events resulted from the application of a mild ischemia: (*a*) a marked increase of GGT activity and (*b*) a net stimulation of membrane lipid oxidative breakdown, indirectly revealed by increased MDA tissue levels as well as by decreased tissue concentration of GSH. The animal pretreatment with a single dose of acivicin, the most reliable GGT inhibitor [27], able to prevent fully the effect of ischemia on the enzyme activity, allowed protection of the ischemic organ against lipid peroxidation enhancement and morphological alterations. These findings indicate that the up-regulation of GGT activity contributes to the ischemic damage of the PT through a pro-oxidant effect.

With respect to the mechanisms behind the observed increase of renal GGT activity during short-term ischemia, post-translational changes appear most likely. It is known that kidney GGT is normally sequestered in the form of a propeptide within the microsomal membrane system, before its insertion into the brush border membrane. The relatively late cleavage of the transpeptidase precursor in the post-translational processing of the enzyme may therefore serve to prevent degradation of GSH in the cytoplasm [32]. It has been claimed that the catalytic function of GGT is influenced by its inner core

structure, which determines the number of branches of the complex oligosaccharide chains and that the saccharide moiety influences the substrate affinity and the activation energy of GGT [33]. Short-term ischemia could exert changes in the oligosaccharide chains by this way modifying enzyme conformation and activity. The only slight decrease of renal GSH observed after the application of transient ischemia (Fig. 6) indirectly indicates that the enzyme hyperactivation occurred mainly, if not exclusively, at the cellular outer surface.

Concerning the growing body of evidence that points to GGT activity itself as a source of free radicals, Stark, Zeiger, and Pagano showed that the GGT-dependent metabolism of GSH leads to generation of thiyl radicals and other oxidant species responsible for cytotoxicity, mutagenesis, and lipid peroxidation [23, 24]. The proposed mechanism was the reduction of Fe^{3+} to Fe^{2+} by cysteinylglycine resulting from the GGT-mediated cleavage of GSH; then the redox cycling of iron is able to initiate a lipid peroxidation chain reaction [34, 35]. The actual occurrence of this series of chemical reactions was conclusively proved by Pompella et al, who showed that rat hepatocytes and human hepatoma cells, in the presence of substrates for GGT activity and chelated iron, are able to initiate a nonlethal membrane lipid peroxidation process [25, 26].

It is noteworthy that the initiation of membrane lipid oxidative breakdown may also take place under low O_2 tension. Thiyl radicals originated from Fe^{2+}-induced thiol oxidation may directly react with double bonds of polyunsaturated fatty acids, yielding a carbon centered radical able to propagate a radical chain reaction [35, 36]. Furthermore, data are available in the literature on the rate of oxygen-dependent free radical reactions occurring at very low oxygen tensions (83% of the maximal rate at a pO_2 of approximately 6 mm Hg and 50% at a pO_2 of approximately 1 mm Hg) [37]. Indeed, in the acute *in vivo* model employed in this investigation, short ischemia of the kidney also led to increased MDA steady-state levels in both cortical and medullar zones (Fig. 5), clearly dependent on stimulation of GGT activity. In agreement with these results, cold preservation of isolated rabbit proximal tubules appears to induce cell injury, with increased lipid peroxidation (in terms of MDA) and release of lactate dehydrogenase by an iron-dependent and nitric oxide-independent free radical mechanism [38].

It has been reported that renal levels of GSH decrease during ischemia may be due to catabolism of cysteine [39, 40]. Our data on acivicin-induced prevention or even an increase of GSH in ischemic and control rat kidneys (Fig. 6) suggest a major pathway of GSH consumption through GGT activation. Furthermore, it cannot be excluded that the observed increase of membrane lipid peroxidation would in part contribute to lower the renal content of the tripeptide.

In relationship to the tissue injury that a short period of warm ischemia is able to exert on the kidney, independent from that triggered by organ reperfusion, the data presented here appear to favor the hypothesis that GGT-driven oxidative mechanisms, in particular lipid peroxidation, could contribute to a certain degree to morphological changes of tubular cells. To evaluate if tissue alterations caused by ischemia-induced oxidative stress would result in a significant impairment of renal function, further *in vivo* studies that would consider and compare different ischemic terms than different degrees of renal hypoxia are necessary.

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