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Antioxidative effects of exogenous nitric oxide versus antioxidant vitamins on renal ischemia reperfusion injury

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Abstract The objectives of this study were to compare the protective influence of exogenous nitric oxide on renal ischemia reperfusion (I/R) injury with that of the antioxidant vitamins C and E. Sprague-Dawley rats were divided into three groups ($n = 12$ per group). Normal saline solution was given in group 1, a vitamin C (200 mg/kg/d) plus vitamin E (100 mg/kg/d) combination in group 2 for 3 days before operating and Na-nitroprusside (5 mg/kg/d) in group 3 before reperfusion. The left kidneys were exposed to warm ischemia for 40 min followed by reperfusion for 90 min. The right kidneys were used as internal controls. After both kidneys were removed, histopathological examinations were performed, and oxidative and antioxidative parameters were measured. In the postischemic reperfused rat kidneys, the renal lipid peroxidation level was significantly lower, and the renal GSH level higher in the group given Na-nitroprusside compared with groups 1 and 2. Renal specific xanthine oxidase activity was also significantly lower in the group treated with Na-nitroprusside than in the groups given vitamins or saline. There was a significant, negative correlation between lipid peroxidation and reduced glutathione levels. Our results suggest that the exogenous nitric oxide (Na-nitroprusside) inhibits xanthine oxidase, and has more apparent preventive features for renal I/R injury than the antioxidant vitamins C + E.

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Introduction

Free oxygen radical generation is an important mechanism of cellular injury in ischemic and reperfused tissues [2]. Although several mechanisms have been hypothesized to be involved in the generation of free oxygen radicals (FOR), xanthine oxidase (XO) has been shown to be a central mechanism in a variety of postischemic cells and tissues [11].

During ischemia there is massive breakdown of the adenine nucleotide pool due to the low energy status of the tissue. Adenosine is converted to inosine and then to hypoxanthine, which accumulates in abundance.

In reperfused tissues, XO in the presence of its substrates hypoxanthine or xanthine reduces molecular oxygen to the superoxide radical (O_2^-) and hydrogen peroxide, which can further react to form the more reactive hydroxyl radical (OH*). The O_2^- and OH* radicals produced by the enzyme can then in turn react with cellular proteins and membranes inducing cellular injury [20]. The increase in XO activity has been proposed to play a key role in triggering postischemic free radical generation. It has been demonstrated that the enzyme and its substrates are present and give rise to a burst of free radical generation upon postischemic reperfusion.

Several drugs have been used to prevent ischemia reperfusion (I/R) injury including allopurinol, superoxide dismutase, coenzyme Q, antioxidant vitamins and N-acetylsistein [8, 22]. The main target of these drugs is the inhibition of XO, which is the main source of the superoxide radical, or the removal of this radical.

It has been noticed that nitric oxide (NO), a small molecule with multiple physiological functions, plays an important role in modulating tissue injury and renal blood flow in the healthy kidneys as well as several pathologic kidney conditions. The role of NO in I/R

injury is controversial [21]. Exogenous NO has a beneficial effect in renal I/R injury, while endogenous NO does not appear to be an important contributor to renal I/R injury. The infiltration of neutrophils (migration) was decreased in animals pretreated with the NO donor Na-nitroprusside [13]. Recently, in vitro studies have shown that nitric oxide and peroxynitrite at high concentrations regulate XO activity [12].

In this study, the lipid peroxidation level was measured to determine oxidative damage, xanthine oxidase specific activity to evaluate superoxide radical production as well as renal reduced glutathione level, glutathione peroxidase and superoxide dismutase specific activities to determine the antioxidative capacity in renal tissue. We examined whether exogenous nitric oxide has an in vivo inhibitory effect on XO activity in the I/R injury of the kidney, and whether it has a protective effect on oxidative injury. We also compared exogenous nitric oxide with the strongly antioxidant vitamins C and E in terms of antioxidative effect.

Materials and methods

A total of 36 male Sprague-Dawley rats, weighing 250–300 g were used. Animals were divided into three groups, each of which contained 12 rats. Group 1 was the control group in which saline solution was given intraperitoneally. In group 2, vitamin C (200 mg/kg/d) plus vitamin E (100 mg/kg/d) were given intraperitoneally for 3 days before operating. In group 3, Na-nitroprusside (5 mg/kg) was given before reperfusion.

The animals were subjected to left renal warm ischemia for 40 min, and reperfusion for 90 min. Briefly, under intraperitoneal sodium pentobarbital anesthesia (60 mg/kg body weight) and through a midline incision, the abdominal contents were displaced to the right side. The left renal artery and vein were dissected and the perirenal fat was preserved. The vascular pedicule was temporarily ligated with 2–0 silk before the abdominal contents were replaced and the incision was covered with a moistened polyethylene sheet.

At the end of the ischemic period, the abdominal cavity was reentered, the ligature was removed and reperfusion was supplied. Na-nitropusside (5 mg/kg) was given at the beginning of the reperfusion [13]. At the 90th min of reperfusion both kidneys were removed.

The kidney tissue was divided into four parts. The first part was used for the determination of lipid peroxidation, the second part was used for reduced glutathione, the third part was used for the measurements of enzyme activities. The last part was used for histopathological examination. Superoxide dismutase (SOD), glutathione peroxidase (GSHPx), XO enzyme activities, reduced glutathione (GSH) and thiobarbituric acid reactive products (TBAR) of lipid peroxidation were determined in the kidney tissues.

The lipid peroxidation level of the kidney tissue was measured as malondialdehyde (MDA) which is the end product of lipid peroxidation, and reacts with thiobarbituric acid as a thiobarbituric acid reactive substance (TBAR). Kidney TBAR level was determined at 4°C by homogenization of the tissues in potassium phosphate buffer (0.4 ml buffer per 100 mg tissue) 0.02 mol/l, pH 7.4 containing butylated hydroxytoluene (0.5 mg per 100 ml buffer) to prevent further lipid oxidation. A total of 0.7 ml of this homogenate was treated with 0.7 ml TCA, centrifuged and 1 ml of the supernatant was then added to 0.5 ml thiobarbituric acid . After 30 min at 70°C the cuvettes were centrifuged and colour development was determined spectrophotometrically at 532 nm. Values were calculated on a reference scale plotted with different dilutions of standard malondialdehyde [4].

For the estimation of reduced glutathione (GSH), the kidney tissue was homogenized in 0.2 M citrate, 5 mM EDTA pH 5. The homogenate was deproteinized by the addition of a reagent consisting of 120 g NaCl, 6.68 g metaphosphoric acid and 0.8 g EDTA dissolved in 400 ml distilled water. After centrifugation at 3,000 rpm for 20 min 0.5 ml of supernatant was added to 2 ml 0.3 M $Na_2HPO_4.2H_2O$ solution. SH groups were assayed by the method of Ellman [5].

The enzyme analyses of kidney tissues were performed on the supernatant fractions of the tissue homogenates. The tissues were homogenized in 150 mmol/l ice-cold KCl to make a 10% homogenate, using a glass Teflon homogenizer. Then the samples were sonicated in ice ten times for 5 s. The homogenates were centrifuged at 12,500 *g* for 30 min at 3°C and the supernatant fractions were obtained.

Superoxide dismutase activity was determined with Ransod (Randox, Ireland), a commercial superoxide dismutase activity measurement kit, based on the capacity of superoxide dismutase to inhibit the reduction of phenyltetrazolium chloride by XO [15].

Glutathione peroxidase activity was measured with Ransel, a commercial glutathione peroxidase activity measurement kit (Randox, Ireland) based on the modified method of Paglia and Valentine using cumene hydroperoxide as a substrate [16]. Superoxide dismutase and glutathione peroxidase assays were performed on a Hitachi 911 (Boehringer, Mannheim, Germany), an automated chemistry analyser.

XO activity was measured by monitoring the absorbance change at 292 nm (for uric acid production) in the presence of 50 μM xanthine and 50 mM potassium phosphate buffer with 0.1 mM EDTA pH 7.4 [19].

The protein content of the supernatant fraction was determined according to the method of Lowry et al [14]. Results were expressed in U/mg protein.

Histopathological examinations of the samples were performed according to the criteria of Jablonski et al. [10]. Briefly, the injury was graded from one (minimal damage) to four (maximal damage) : grade 1: mitoses and necrosis of individual cells; grade 2, necrosis of all cells in adjacent proximal convoluted tubules with survival of surrounding tubules; grade 3, necrosis confined to the distal third of the inner cortex; and grade 4, necrosis affecting all three segments of the proximal convoluted tubule. The histological damage of the kidney samples was blindly evaluated by the pathologist (M.B.).

The biochemical data were normally distributed, so parametric statistics were selected. Data were expressed as the mean ± SD throughout the study. Analyses of variance (ANOVA), Student's *t* test and correlation analyses were performed. The histological grades were analysed according to the Kruskal-Wallis test by using the SPSS statistical package (SPSS for Windows, Release 7.5, SPSS, USA). Significance was defined as $P < 0.05$.

Results

The oxidative and antioxidative parameters both for reperfused kidney tissues and those tissues which were not reperfused are given in Table 1. Lipid peroxidation levels, an indicator of oxidative stress, were found to be higher in the reperfused kidneys (left) of all groups than those in the control (right) counterpart. Reduced glutathione levels, the most important intracellular antioxidant, were lower in all treated kidneys compared to their control counterparts.

The XO activity of ischemic kidneys of the group treated with Na-nitroprusside was lower than those of the other groups. Superoxide dismutase and glutathione peroxidase activities were not statistically different in all groups. A significant negative correlation was found between lipid peroxidation and reduced glutathione levels ($r = -0.43$, $P = 0.03$).

Table 1. Oxidative and antioxidative parameters in reperfused and control kidneys. Values are mean ± SD. Left: ischemia-reperfused kidney, right: non ischemia-reperfused kidney, group 1: control group, group 2: the group treated with vitamin C + E, group 3: the group treated with Na-nitroprusside, MDA: malondialdehyde (indices lipid peroxidation), GSH: reduced glutathione, SOD: superoxide dismutase, GSHPx: glutathione peroxidase

	Group 1		Group 2		Group 3	
	Right	Left	Right	Left	Right	Left
MDA, nmol/g wet tissue	84.78 ± 12.31	131.82 ± 17.19[a]	82.26 ± 12.43	103.84 ± 15.72[ab]	83.01 ± 13.58	91.47 ± 14.63[abc]
GSH, μmol/g wet tissue	4.53 ± 0.43	2.05 ± 0.25[a]	4.45 ± 0.42	2.98 ± 0.29[ab]	4.46 ± 0.47	3.50 ± 0.32[abc]
Xanthine oxidase, mU/mg protein	16.82 ± 1.68	17.02 ± 1.87	16.30 ± 1.66	16.88 ± 1.74	16.52 ± 1.82	10.15 ± 1.93[abc]
SOD, U/mg protein	2.94 ± 0.19	2.73 ± 0.18	2.95 ± 0.20	2.82 ± 0.18	2.91 ± 0.18	2.81 ± 0.21
GSHPx, U/mg protein	13.21 ± 1.27	14.09 ± 1.41	13.27 ± 1.38	13.88 ± 1.39	13.44 ± 1.43	13.09 ± 1.36

[a] $p < 0.05$ vs counterpart kidney
[b] $p < 0.05$ vs reperfused kidney of group 1
[c] $p < 0.05$ vs reperfused kidney of group 2

Histological evaluation revealed that, the median value of the damage grade of the Na-nitroprusside group was lower than that of the control group ($P = 0.01$). However, the median value of the grade of vitamin C + E group was not statistically different from those of both other groups. Typical samples of damage relating to the groups were shown in Fig. 1.

Discussion

FOR have very important roles in I/R injury. They are derived from two main sources during I/R injury. The most important of these is XO [11]. During ischemia, intracellular ATP is consumed and metabolised to uric acid by XO, releasing a superoxide anion and hydrogen peroxide. The other source of FOR is neutrophils. Ischemia leads to the infiltration of neutrophils into the tissues, and activated neutrophils release FOR by myeloperoxidase and NADPH oxidase [7, 13].

Exogenous NO has been shown to prevent migration of neutrophils in the I/R of the kidneys [7, 13]. Under normal conditions, a superoxide anion reacts with NO, producing a peroxynitrite radical which is a potent oxidant [1]. Therefore, it is expected that exogenous NO causes oxidative injury in the I/R of tissues. On the contrary exogenous NO has been shown to prevent oxidative injury. This contradiction has not yet been clearly explained.

Recently, Lee et al. [12] demonstrated in an in vitro study that NO, at high concentration, combines with the element molybdenum which is a cofactor of XO and that this combination inhibits XO. According to our knowledge there is no report on the effect of exogenous nitric oxide on xanthine oxidase activity in renal I/R injury. In this study we demonstrated that NO inhibits xanthine oxidase activity in I/R injury of the kidney.

We also investigated the effect of exogenous NO on the antioxidative enzymes SOD and GSHPx, and found that NO had no significant effect on them.

There are various antioxidative mechanisms against oxidative injury in the tissues. The most important endogenous antioxidant among them is reduced glutathione. This tripeptide endogenous antioxidant is present in especially high concentrations in kidney cells. A reduction in GSH levels occurs in the kidney after a period of ischemia followed by reperfusion. The GSH redox cycle catalyzed by an endogenous antioxidative enzyme glutathione peroxidase (GSHPx) reduces hydrogen peroxide (H_2O_2), thus breaking the chain reaction leading production from the superoxide radical to the highly reactive hydroxl radical. Additionally, GSH scavenges superoxide radicals and protects protein thiol groups from oxidation. GSH also plays a major role in restoring other free radical scavengers and antioxidants such as vitamin E and ascorbic acid to their reduced state [17, 18]. Following decreases in the level of GSH , oxidative stress increases and thereafter cell damage occurs. In our study, we determined a significant negative correlation between reduced glutathione and lipid peroxidation levels in reperfused kidneys. In the third group, given Na-nitroprusside, kidney GSH level was significantly higher than those of control and vitamin C + E groups.

Oxidative injury to cells is evaluated by measuring the lipid peroxidation level [3]. To determine this in the kidney during I/R, we measured malondialdehyde (MDA) concentration, which is the end product of lipid peroxidation and gives a colored complex with thiobarbituric acid (TBA) [4]. As seen in Table 1, the lipid peroxidation level of the group given exogenous nitric oxide was lower than in the C + E and control groups.

In the third group, the lipid peroxidation level was lower and the reduced glutathione level higher than that of the second group. Accordig to our results, exogenous nitric oxide is a more potent protective agent against oxidative injury than the antioxidant C + E vitamin combination.

XO activity, which is the main source of the superoxide radical, was lower in the third group than in the other groups. An inhibitor effect of antioxidant vitamins on XO activity was not observed.

On the other hand, these antioxidative protective effects of nitroprusside were also compatible with the histopathological findings showing the lower grade of

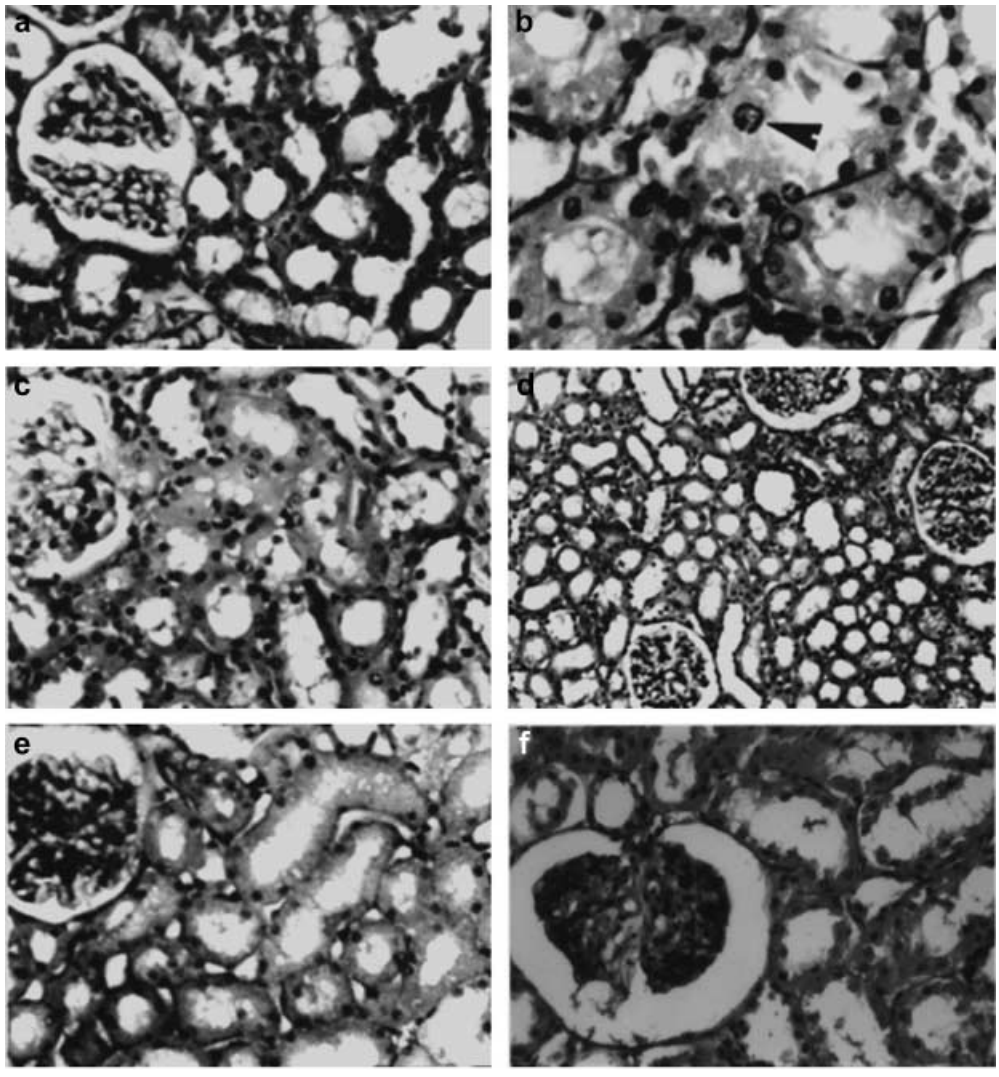

Fig. 1a–f. Histological samples of kidneys. A grade 1 histological sample showing individual cell necrosis (**a**, HE×200) and mitosis (**b**, *arrow*, HE×400) from the group treated with Na-nitroprusside. Grade 2 and 3 damage figures obtained from the group treated with vitamin C + E, demonstrating more necrosis in adjacent proximal convoluted tubules (**c**, HE×200), and necrosis reaching to distal part of the proximal tubule (**d**, HE×100), respectively. Also shown are a massive necrosis sample in all segments of the proximal convoluted tubule conforming to grade 4 damage (**e**, HE×200) of the control group, and the normal histology of the kidney (**f**, HE×200)

damage in the Na-nitroprusside group in our study. Although vitamin C + E had some antioxidative effects on the reperfusion injury, a statistically significant, positive effect could not be demonstrated in the histopathological evaluations.

While antioxidant vitamins remove only FOR and allopurinol inhibits only XO activity, exogenous nitric oxide removes superoxide radicals, inhibits XO and NADPH oxidase activities, and also reduces neutrophil infiltration which are the main superoxide radical sources [6, 7, 8, 9, 12, 13]. Thus it has more effective, multifaced antioxidative features.

In conclusion, exogenous nitric oxide inhibits XO, and has more apparent preventive effects on renal I/R injury than the antioxidant vitamins C + E.

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