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Beneficial Effect of Pentoxifylline on Hypoxia-Induced Cell Injury in Renal Proximal Tubular Cells

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Tumor necrosis factor-α (TNF-α) or its mRNA expression are increased in acute nephrosis of various types including ischemia/reperfusion injury. This study was undertaken to determine whether pentoxifylline (PTX), an inhibitor of TNF-α production, provides a protective effect against hypoxia-induced cell injury in rabbit renal cortical slices. To induce hypoxia-induced cell injury, renal cortical slices were exposed to 100% N_2 atmosphere. Control slices were exposed to 100% O_2 atmosphere. The cell injury was estimated by measuring lactate dehydrogenase (LDH) release and *p*-aminohippurate (PAH) uptake. Exposure of slices to hypoxia increased the LDH release in a time-dependent manner. However, when slices were exposed to hypoxia in the presence of PTX, the LDH release was decreased. The protective effect of PTX was dose-dependent over the concentrations of 0.05~1 mM. Hypoxia did not increase lipid peroxidation, whereas an organic hydroperoxide *t*-butylhydroperoxide (*t*BHP) resulted in a significant increase in lipid peroxidation. PTX did not affect *t*BHP-induced lipid peroxidation. Hypoxia decreased PAH uptake, which was significantly attenuated by PTX and glycine. *t*BHP-induced inhibition of PAH uptake was not altered by PTX, although it was prevented by antioxidant deferoxamine. The PAH uptake by slices in rabbits with ischemic acute renal failure was prevented by PTX pretreatment. These results suggest that PTX may exert a protective effect against hypoxia-induced cell injury and its effect may due to inhibition of the TNF-α production, but not by its antioxidant action.

Key Words: Hypoxia-induced cell injury, Renal cortical Slices, Pentoxifylline, LDH release, Rabbit

INTRODUCTION

Tumor necrosis factor-α (TNF-α) is a cytokine with pleiotropic actions that participates in inflammation and immunity. It has been reported that levels of TNF-α or its mRNA expression are increased in acute nephrosis induced by aminonucleoside (Diamond and Pesek, 1991) and adriamycin (Egido et al., 1993), inmmune glomerulonephritis (Tipping et al., 1991; Mulligan et al., 1993), diabetic nephropathy (Navarro et al., 1999), and endotoxin-induced acute renal failure (Baud et al., 1994). These studies suggest that TNF-α may be linked to the pathogenesis of diverse renal diseases. TNF-α production is increased in various cell types including the kidney subjected to ischemia/reperfusion *in vivo* (Colletti et al., 1990; Donnahoo et al., 1999; Kim et al., 2001) and hyoxia *in vitro* (Taylor et al., 1999). These data suggest that TNF-α may be an important mediator of ischemia/reperfusion injury.

Pentoxifylline (PTX) is a methylxanthine derivative with multiple hemorheologic properties, but the exact mechanisms of its pharmacology are not understood. Clinically, PTX has been used to treat intermittent claudication (Gillings, 1995). PTX has been known to decrease serum TNF-α levels (Staudinger et al., 1996; Loftis et al., 1997; Navarro et al., 1999) and TNF-α mRNA expression (Strieter et al., 1988). Therefore, PTX may exert the protective effect against ischemic cell injury. This study was undertaken to examine the effect of PTX on renal tubular injury induced by hypoxia in renal tubular cells using rabbit renal cortical slices.

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MATERIALS AND METHODS

1. Preparation of renal cortical slices

New Zealand white male rabbits weighing 1.5 to 2 kg

were killed and the kidneys were removed rapidly. The kidneys were perfused immediately through the renal artery with an ice-cold isotonic saline solution containing 140 mM NaCl, 10 mM KCl and 1.5 mM $CaCl_2$ to remove as much blood as possible. Thin slices (0.25~0.35 mm) of renal cortex were prepared using a Stadie-Riggs microtome and were stored in an ice-cold modified Cross-Taggart medium containing 130 mM NaCl, 10 mM KCl, 1.5 mM $CaCl_2$, 5 mM glucose and 20 mM Tris/HCl (pH 7.4).

2. Induction of hypoxia-induced cell injury

For hypoxia injury in renal tubules, slices were incubated in a modified Cross-Taggart medium for 60 min under a 100% N_2 atmosphere. Normoxic control slices were incubated under a 100% O_2 atmosphere for 60 min.

3. Measurement of cell injury in renal cortical slices

The injury in renal cortical slices was estimated by measuring the release of lactate dehydrogenase (LDH). After incubating, tissues were homogenized in 2 ml of distilled water and the homogenate was centrifuged at 2,000 g for 5 min. The pellet was discarded and the supernatant was saved. LDH activity was determined in the supernatant and incubation medium using a LDH assay kit (Iatron Lab., Japan). Protein content of tissue homogenate was measured by the method of Bradford (Bradford, 1976).

4. Lipid peroxidation measurement

Lipid peroxidation was estimated by measuring the renal cortical content of malondialdehyde (MDA) according to the method of Uchiyama and Mihara (Uchiyama and Mihara, 1978). Slices were homogenized in ice-cold 1.15% KCl (5% wt/vol). A 0.5 ml aliquot of homogenate was mixed with 3 ml of 1% phosphoric acid and 1 ml of 0.6% thiobarbituric acid. The mixture was heated for 45 min on a boiling water bath. After addition of 4 ml of n-butanol the contents were vigorously vortexed and centrifuged at 2,000 g for 20 min. The absorbance of the upper, organic layer was measured at 535 and 520 nm with a diode array spectrophotometer (Hewlett Packard, 8452A), and compared with freshly prepared malondialdehyde tetraethylacetal standards. MDA values were expressed as pmoles per mg protein. Protein was measured by the method of Bradford [13].

5. Uptake of *p*-aminohippurate (PAH) by renal cortical slices

PAH uptake was measured in slices subjected to normoxic and hypoxic conditions. Approximately 50 mg (wet wt.) of slices were transferred into a 20 ml beaker containing 4 ml of the modified Cross-Taggart medium, and incubated with 75 μM ^{14}C-PAH (Amersham, Alrington heights, IL). Incubation was carried out for 60 min in a Dubnoff metabolic shaker at 25℃ under a 100% oxygen atmosphere. Immediately after incubation, the slices were quickly removed from the beaker, blotted, weighed and solubilized in 1 N NaOH. Aliquots of the incubation medium and the solubilized tissue were pipetted into a scintillation vial containing scintillation cocktail and the radioactivity was determined using a liquid scintillation counter (Packard Tricarb 300C). PAH uptake by renal slices was expressed as the slice to medium (S/M) ratio: the concentration of the compound in the tissue (mole/g wet tissue) divided by that in the medium (mole/ml medium).

6. *In vivo* experimental protocol

New Zealand White male rabbits weighing 1.5~2.5 kg were used. Anesthesia was induced by intramuscular injection of a mixture of ketamine (15 mg/kg) and xylazine (2.5 mg/kg) and maintained by sustaining doses (ketamine 5 mg/kg and xylazine 1 mg/kg) at intervals of approximately 30 min. A ventral midline incision was made and both renal arteries were clamped with nontraumatic vascular clamps for 60 min. The incision was closed and the animal was allowed to recover. Hartman solution was infused at a rate of 0.5 ml/min into an ear vein throughout the entire experimental period. After completion of surgery and recovery from anesthesia, the rabbits were returned to their metabolic cages. After 24 hr of reflow, animals were sacrificed and the isolated kidneys were used for measurement of *p*-aminohippurate (PAH) uptake. The PTX-treatment groups recieved PTX (30 mg/kg, i.v.) 10 min before the reflow of blood. The control animals untreated with the drug received an equal volume of saline.

7. Statistical analysis

The data are expressed as mean ± SE and the difference between two groups was evaluated using Student's *t*-test. A probability level of 0.05 was used to establish significance.

RESULTS

Fig. 1. shows the effect of hypoxia on LDH release in renal cortical slices. LDH release was increased to 60 min of hypoxia exposure in a time-dependent manner. When slices were exposed to hypoxia for 60 min, the LDH release was increased from 2.41±1.15% of control to 20.56± 2.81%, indicating that hypoxia induced a significant cell death.

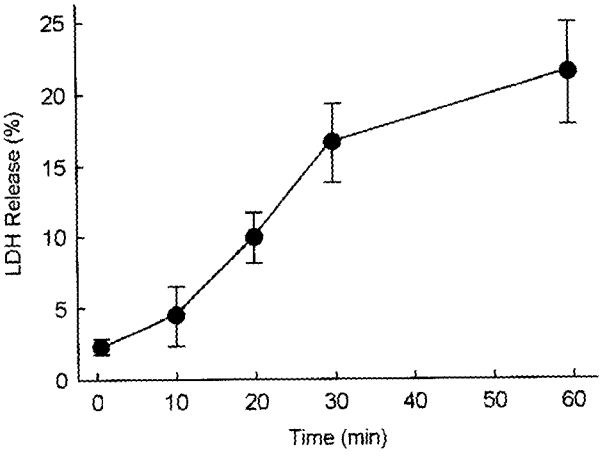

Fig. 1. Effect of hypoxia on the release of lactate dehydrogenase (LDH) release in rabbit renal cortical slices. Slices were incubated in a modified Cross-Taggart medium for various times at 37℃ under a 100% N_2 (hypoxia) or 100% O_2 atmosphere (control slices). Data are mean ± SE of four experiments.

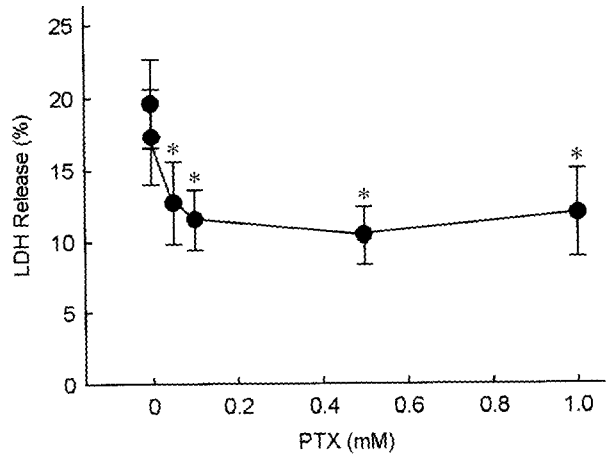

Fig. 2. Effect of pentoxifylline (PTX) on the release of lactate dehydrogenase (LDH) induced by hypoxia in renal cortical slices. Slices were incubated in a modified Cross-Taggart medium for 60 min under a 100% N_2 atmosphere in the presence of various concentrations of PTX. Data are mean ± SE of four experiments. *P<0.05 compared with the absence of PTX.

To evaluate if PTA exerts protective effect against the hypoxia-induced cell injury, slices were exposed to hypoxia in the presence or absence of various concentrations of PTX. As shown in Fig. 2, PTX at 0.05 mM significantly prevented the hypoxia-induced LDH release. The protective effect was increased to 0.1 mM and remained unchanged to 1 mM.

Since PTX has been reported to inhibit the generation of reactive oxygen species (Ciuffetti et al., 1991) and scavenge hydroxyl radicals (Franzini et al., 1993), the protective effect of PTX against the hypoxic injury may be attributed to an inhibition of lipid peroxidation. To test this possibility, changes in lipid peroxidation were examined in slices exposed to hypoxia in the presence of PTX. The results depicted in Fig. 3A indicated that hypoxia did not induce lipid peroxi-

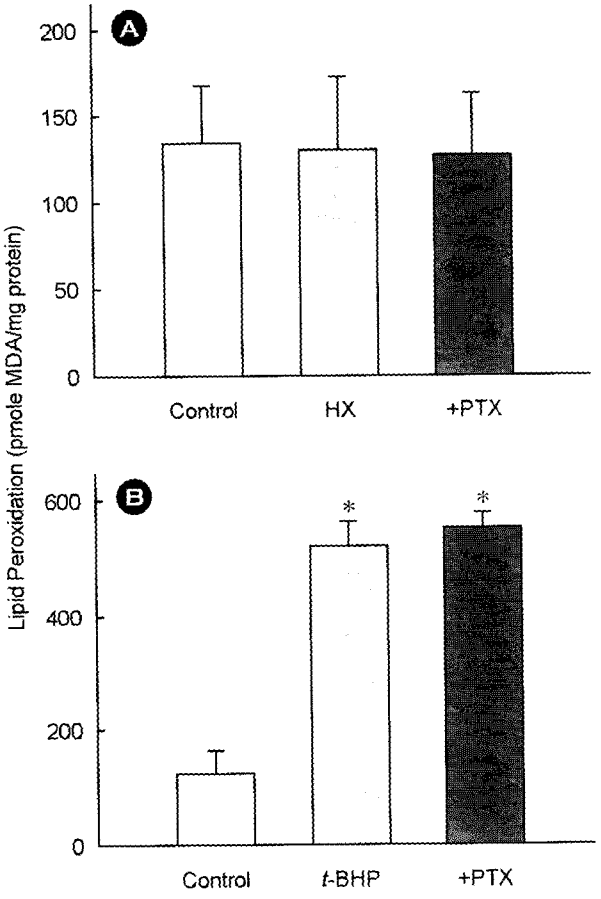

Fig. 3. Effect of pentoxifylline (PTX) on lipid peroxidation in slices exposed to hypoxia (HX, A) or *t*-butylhydroperoxide (*t*BHP). Slices were exposed to hypoxia for 60 min or 1 mM *t*BHP for 60 min under a 100% O_2 atmosphere in the presence of 0.5 mM PTX. Data are mean ± SE of five experiments. *P<0.05 compared with the control.

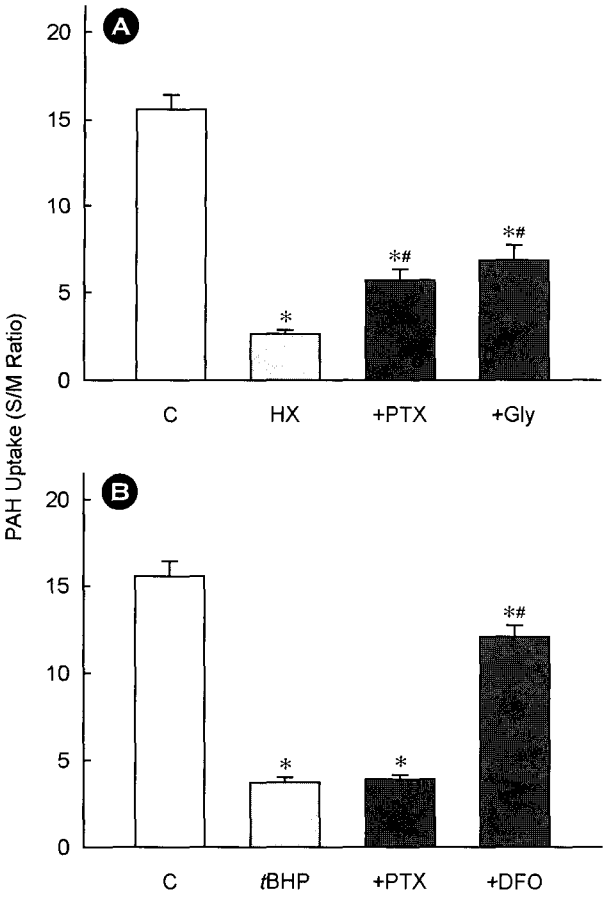

Fig. 4. Effect of pentoxifylline (PTX) on *p*-aminohippurate (PAH) uptake in slices exposed to hypoxia (HX, A) or *t*-butylhydroperoxide (tBHP). Slices were exposed to hypoxia for 60 min or 1 mM *t*BHP for 60 min under a 100% O_2 atmosphere in the presence of 0.5 mM PTX. The PAH uptake was then measured for 60 min. Glycine and deferoxamine (DFO) were employed as positive control preventing agent for hypoxia- and *t*BHP-induced inhibition of the uptake, respectively. Data are mean ± SE of five experiments. *P<0.05 compared with the control (C); #P<0.05 compared with HX or *t*BHP alone.

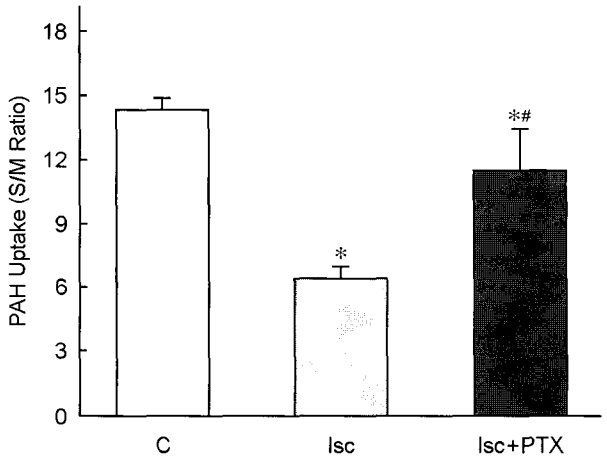

Fig. 5. Effect of pentoxifylline (PTX) on *p*-aminohippurate (PAH) uptake in slices of kidney exposed to ischemia *in vivo* (Isc). Ischemic acute renal failure in rabbits was induced by clamping renal arteries for 60 min. After 24 hr of reflow, animals were sacrificed and the isolated kidneys were used for measurement of PAH uptake. The PTX-treatment groups received PTX (30 mg/kg, i.v.) 10 min before the reflow of blood. The control animals untreated with the drug received an equal volume of saline. Data are mean ± SE of three animals. *P<0.05 compared with the control (C); #P<0.05 compared with Isc alone.

dation, suggesting that hypoxia induces cell injury through a mechanism independent of lipid peroxidation. To further verify if PTX exerts antioxidative effect in renal cortical slices, the effect of PTX on lipid peroxidation induced by an organic oxidant t-butylhydroperoxide (*t*BHP) was examined. As shown in Fig. 3B, *t*BHP produced a marked increase in lipid peroxidation and its effect was not affected by PTX. These results indicate that lipid peroxidation is not involved in hypoxic injury in renal cortical slices, and consequently the protective effect of PTX against the injury is not associated with an inhibition of lipid peroxidation.

PAH uptake by renal cortical slices has been used as a sensitive indicator in the assessment of renal tubular cell function (Hirsch, 1976; Kim and Kim, 1996). Therefore, the effect of PTX on hypoxia-induced alterations of PAH uptake was evaluated. Exposure of slices to hypoxia inhibited markedly PAH uptake from 15.49±0.86 in control to 2.57±0.23 S/M ratio, which was prevented by PTX (Fig. 4A). Glycine has been well known to protect hypoxic injury, although the underlying mechanism remains to be defined (Weinberg et al., 1991). As expected, hypoxia-induced reduction of PAH uptake was attenuated by addition of glycine (Fig. 4B).

In the last series of experiments, it was examined whether PTX treatment exerts protective effect against renal dysfunction induced by ischemic injury *in vivo*. As shown in Fig. 5, the PAH uptake was decreased by approximately 50% in kidney exposed to ischemia *in vivo* and its effect was prevented by PTX treatment.

DISCUSSION

The present study demonstrated that PTX provides a protective effect against the cell injury induced hypoxia *in vitro* and ischemia *in vivo*. PTX prevented the LDH release induced by hypoxia and attenuated reduced PAH uptake caused by hypoxia. Since the an increase in LDH release

indicates irreversible cell death and reduced PAH uptake means an impairment in renal function, these data suggest that PTX prevented both irreversible and function changes induced by hypoxia. These results are consistent with reported by Kim et al (Kim et al., 2001) in *in vivo* experiments. In those studies, PTX attenuated the ischemic acute renal failure and its effect was due to a suppression of the TNF-α production. Although, in the present study, it was not determined whether TNF-α production was increased in slices exposed to hypoxia, other studies have shown that TNF-α production is increased in various cell types including the kidney subjected to ischemia/reperfusion *in vivo* (Colletti et al., 1990; Donnahoo et al., 1999; Kim et al., 2001) and hyoxia *in vitro* (Taylor et al., 1999). Based on these data, it is suggested that the beneficial effect of PTX on hypoxia injury may be attributed to inhibition of TNF-α generation.

Since PTX has been shown to inhibit the generation of leukocyte-derived reactive oxygen species in humans subjected to exercise (Ciuffetti et al., 1991) and scavenge hydroxyl radicals in vitro (Franzini et al., 1993), the beneficial effect of PTX could be associated with its antioxidant action. However, the present study showed that lipid peroxidation was not increased by hypoxia, suggesting that hypoxia-induced cell injury was not attributed to lipid peroxidation resulting from reactive oxygen species generation. Similar results are reported by other investigators in kidney exposed to ischemia *in vivo* (Gamelin and Zager, 1988; Kim et al., 1998) and hypoxia *in vitro* (Borkan and Schwartz, 1989). To further ascertain if the protective effect of PTX was not due to its antioxidant action, the effect of PTX on changes in lipid peroxidation and the PAH uptake in slices exposed to *t*BHP, an organic hydroperoxide that has been known as a inducer of lipid peroxidation (Ross and Moldeus, 1993). The results showed the *t*BHP-induced lipid peroxidation was not altered by PTX and reduced PAH uptake caused by tBHP was also not changed. Taken together, it is unlikely that the protective effect of PTX against hypoxia-induced cell injury is associated with its antioxidant action.

The underlying mechanism of cell injury induced by hypoxia *in vitro* might be different from that of ischemia *in vivo* and thus PTX might be ineffective in cell injury induced by ischemia *in vivo*. However, reduced PAH uptake by ischemia *in vivo* was prevented by PTX pretreatment. These findings suggest that TNF-α production is involved in the renal cell injury caused by hypoxia *in vitro* and *in vivo*.

In conclusion, PTX prevented the LDH release and reduced PAH uptake induced by hypoxia. Inhibition of slices PAH uptake caused by ischemia *in vivo* was attenuated by PTX pretreatment. The beneficial effect of PTX was not attributed to its antioxidant action and thus may be due to a suppression of the TNF-α production.

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