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Role of Phospholipase A_2 in Oxidant-induced Alteration in Phosphate Transport in Primary Cultured Rabbit Renal Proximal Tubule Cells

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The present study was undertaken to examine the role of phospholipase A_2 (PLA_2) in oxidant-induced inhibition of phosphate transport in primary cultured rabbit renal proximal tubule cells. Uptakes of phosphate and glucose were dose-dependently inhibited by an oxidant *t*-butylhydroperoxide (*t*BHP), and the significant inhibition appeared at 0.025 mM of *t*BHP, whereas *t*BHP-induced alterations in lipid peroxidation and cell viability were seen at 0.5 mM. *t*BHP stimulated arachidonic acid (AA) release in a dose-dependent fashion. A PLA_2 inhibitor mepacrine prevented *t*BHP-induced AA release, but it did not alter the inhibition of phosphate uptake and the decrease in cell viability induced by *t*BHP. *t*BHP-induced inhibition of phosphate transport was not affected by a PKC inhibitor, staurosporine. *t*BHP at 0.1 mM did not produce the inhibition of Na^+-K^+-ATPase activity in microsomal fraction, although it significantly inhibited at 1.0 mM. These results suggest that *t*BHP can inhibit phosphate uptake through a mechanism independent of PLA_2 activation, irreversible cell injury, and lipid peroxidation in primary cultured rabbit renal proximal tubular cells.

Key Words: Phosphate uptake, Oxidant, PLA_2, Proximal tubular cells

INTRODUCTION

Reactive oxygen species (ROS) have been suggested to play a role in pathogenesis of several different renal diseases including glomerulonephritis, ischemic reperfusion injury, and toxic nephritis (Rehan et al, 1984; Baud & Ardaillou, 1986; Andreoli & McAteer, 1990; Paller & Neumann, 1991). ROS can affect membrane structure resulting in an increased permeability (Arstila et al, 1972; Chance et al, 1979) and alterations in the function of essential proteins such as Na^+-K^+-ATPase (Kako et al, 1988) and membrane transport systems (Andreoli et al, 1993). Although ROS may attack a variety of cellular targets (Farber et al, 1990), an important mechanism by which oxidants cause cell death is the generation of lipid peroxidation in renal proximal tubules (Schnellmann, 1988; Salahudeen, 1995; Sheridan et al, 1996). However, it is uncertain that oxidant-induced alterations in membrane transport systems are associated with cell death. In the previous study, we observed that oxidant-induced inhibition of *p*-aminohippurate (PAH) uptake and Na^+-K^+-ATPase activity were not related to irreversible lethal cell injury (estimated by lactate dehydrogenase release) in rabbit renal cortical tissues (Kim & Kim, 1996).

Oxidants have been shown to increase the synthesis of arachidonic acid (AA) metabolites in vascular endothelial cells (Ager & Gordon, 1984; Harlan & Callahan, 1984; Whorton et al, 1985) and kidney cells (Baud et al, 1981). The augmented synthesis is believed to be secondary to an increase in phospholipase A_2 (PLA_2) activity (Boyer et al, 1995; Natarajan, 1995). Furthermore, Chakraborti et al (1989 & 1993) have demonstrated that an oxidant *t*-butylhydroperoxide (*t*BHP) induces a direct activation of PLA_2 enzyme in endothelial cells. Thus, PLA_2 activation has been proposed to be a critical mediator in the injury that occurs in response to ROS (Sapirstein et al, 1996). Oxidants affect alterations in membrane trans-

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port function in renal proximal tubular cells (Andreoli et al, 1993) and endothelial cells (Elliott & Schilling, 1992; Elliott & Koliwad, 1995). However, whether such changes are attributed to PLA_2 activation is not yet determined. PLA_2 has been known to affect fluid reabsorption mediated by receptors in renal proximal tubule (Li et al, 1994). Also, oxidants modulate sodium transport via generation of PGE_2 resulting from PLA_2 activation in gerbil middle ear epithelium (Herman et al, 1995), suggesting that PLA_2 activation might be responsible for oxidant-induced alterations of phosphate uptake in renal proximal tubules.

In this study, therefore, we determined whether alterations in membrane transport function induced by oxidants are associated with cell death or PLA_2 activation in renal proximal tubular cells. We examined effect of an oxidant *t*BHP on phosphate uptake in primary cultured rabbit proximal tubular cells.

METHODS

Primary culture of rabbit renal proximal tubular cells

Proximal tubules were isolated by the method of Brendel & Meeanz (1975) and prepared for cultures as described by Chung et al (1982) with some modifications. In brief, adult male New Zealand white rabbits were sacrificed by cervical dislocation. The kidneys were immediately removed, cleaned of fat and debris, and washed with sterile antibiotic-supplemented medium. The kidneys were perfused with phosphate buffer saline (pH 7.4) through the renal artery and subsequently with Dulbecco's modified Eagle's medium/Ham's F12 (DMEM/F12, Sigma Co., St. Louis, MO) containing 0.5% (wt/vol.) iron oxide until the kidneys turned grey-black in color. The cortex was removed and homogenized with 4 strokes of a sterile glass homogenizer. The homogenate was passed through a series of sterile nylon mesh sieves (254 and 85 micron: TETCO, Inc., Depew, NY). Tubules and glomeruli retained on the 85-micron sieve were suspended in a tube containing DMEM/F12 medium and magnetic stirring bar. Glomeruli containing iron oxide were attracted to the magnetic stirring bar. The stirring bar was removed from the solution. The isolated proximal tubules were briefly incubated in DMEM/F12 medium containing 80 μg/ml collagenase A and 0.025% soybean trypsin inhibitor. The dissociated tubules were then washed by centrifugation, resuspended in DMEM/F12 medium, and transferred into tissue culture plates. Proximal tubule cells were grown on 24-well tissue culture plates in DMEM/F12 medium supplemented with bovine insulin (5 ug/ml), human transferrin (5 ug/ml) and hydrocortisone (5×10^{-8} M). The cultures were maintained in a humidified 95% air/5% CO_2 incubator at 37°C. Culture medium was changed every 48 or 72 hr and 24 hr before the beginning of each experiment. All experiments started 7 days after plating when a confluent monolayer culture was achieved. For induction of oxidant stress, cells were treated with *t*BHP for 60 min at 37°C.

Transport experiments

Uptake of phosphate and glucose was determined in cell monolayers grown on 24-well plate after 60 min of exposure to *t*BHP in Hanks' balanced salt solution (HBSS). Following oxidant stress, the cells were washed once with HBSS and incubated in the uptake medium containing 0.5 mM ^{32}Phosphate or 0.05 mM ^{14}C-α-methylglucoside (α-MG). The composition of the uptake medium was 137 mM NaCl, 5.4 mM KCl, 2.8 mM $CaCl_2$, 1.2 mM $MgSO_4$, and 10 mM HEPES (pH 7.4). Phosphate and glucose uptake was measured for 30 and 60 min at 37°C, respectively. At the end of incubation, uptake was stopped by drawing off the medium, and the monolayers were washed three times with ice-cold HBSS. The cells were solubilized in 1 ml of 0.5% Triton X-100, and an aliquot of the solubilized cells was transferred into scintillation vials to determine radioactivity in a liquid scintillation counter (Packard, Tricarb 2100TR). Phosphate and glucose uptake was normalized to cell protein and expressed as nmoles per mg cell protein per hour.

Cell viability

The viability in cultured cells was determined by a trypan blue exclusion assay, since this method was a more sensitive indicator of cell death than LDH release in cultured cells, as demonstrated by Aleo et al (Aleo et al, 1991). Following oxidant stress, the cells were harvested using 0.025% trypsin, incubated with 4% trypan blue solution, and were counted using a hemocytometer under light microscopy. Cells failing to exclude the dye were considered nonviable, and the data were expressed as a percentage of nonviable cells.

Lipid peroxidation assay

The grown cells in the 6-well dishes were exposed to the various concentrations of *t*BHP in HBSS (4 ml/well) for 60 min. Cells were lysed by addition of 0.1% sodium deoxycholate. Lipid peroxidation was estimated by measuring the tissue content of malondialdehyde (MDA) according to the method of Uchiyama and Mihara (1978). Briefly, cell lysates (4 ml) were mixed with 0.5 ml of 10% phosphoric acid and 1 ml of 0.6% thiobarbituric acid aqueous solution. 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 diode array spectrophotometer (Hewlett Packard, 8452A), and was compared to results obtained using freshly prepared malondialdehyde tetraethylacetal standards. MDA values were expressed pmoles per mg protein. Protein was measured by the method of Bradford (1976).

Arachidonic acid (AA) release

Confluent cells were incubated with [^{3}H]AA (2 μCi/ml) in DMEM/F12 for 20 hour. After incubation, cells were gently washed three times with HBSS (pH 7.4) to remove unbound [^{3}H]AA. To measure *t*BHP-induced AA release, cells were exposed to various concentrations of *t*BHP in HBSS (pH 7.4) for 60 min at 37°C in 95% air/5% CO_2. The medium was removed and centrifuged, and the radioactivity in 400 μl of supernatant was measured in a liquid scintillation counter (Packard Tricarb A2100TR). Cells were solubilized with 0.1% deoxycholate and the radioactivity in 400 μl of cell lysate was measured. The amount of [^{3}H]AA released into the medium was expressed as a percent of total (cell-associated plus released).

Na^+-K^+-ATPase activity measurement

The Na^+-K^+-ATPase activity was measured in the microsomal fraction prepared from cultured cell. For the preparation of microsomal fraction, cells were grown to confluence in 100 mm dish, scraped from the dish in 10 mM mannitol and 2 mM Tris/HCl (pH 7.1) at 4°C, and briefly sonicated. Then, the cell lysate was centrifuged for 2 min at 2,000 g to remove unbroken cells and the supernatant was saved, centrifuged for 12 min at 15,000 g. Pale-pink layer on top of pellet was removed and resuspended in 10 mM mannitol and 2 mM Tris/HCl (pH 7.1). Microsomal fraction was treated with *t*BHP for 60 min at 37°C and Na^+-K^+-ATPase was measured.

The ATPase activity was determined by measuring inorganic phosphate (Pi) released by ATP hydrolysis during incubation of microsome with an appropriate medium containing 3 mM ATP (Sigma) as the substrate. The total ATPase activity was determined in the presence of 100 mM Na^+, 20 mM K^+, 3 mM Mg, 2 mM EDTA, and 40 mM imidazole (pH 7.4). The Mg^{2+}-ATPase activity was determined in the absence of K^+ and in the presence of 1 mM ouabain. The difference between the total and the Mg^{2+}-ATPase activities was taken as a measure of the Na^+-K^+-ATPase activity. At the end of a 10-min incubation, the reaction was terminated by the addition of ice-cold 6% perchloric acid. The mixture was then centrifuged at 3,500 g, and Pi in the supernatant fraction was determined by the method of Fiske and SubbaRow (1925).

Statistical analysis

Results were expressed as mean$\pm$SE. Statistical differences between groups were evaluated using Student's *t*-test. Differences were considered significant at $p<0.05$.

RESULTS

Effect of tBHP on phosphate and α-AMG uptake

Fig. 1 depicts time course of phosphate and glucose uptake in *t*BHP-treated and untreated cells. In *t*BHP-untreated cells, phosphate and α-MG increased to 60 and 120 min, respectively, as function of incubation time. When cells were treated with 0.05 and 0.5 mM *t*BHP, the uptakes of phosphate and α-AMG were significantly reduced.

In order to quantify the inhibition potency of *t*BHP, phosphate uptake was measured for 30 min in cells treated with various concentrations of *t*BHP. As shown in Fig 2, *t*BHP inhibited phosphate and α-AMG uptake in a dose-dependent manner and the significant inhibition was present at a concentration as low as 0.025 mM.

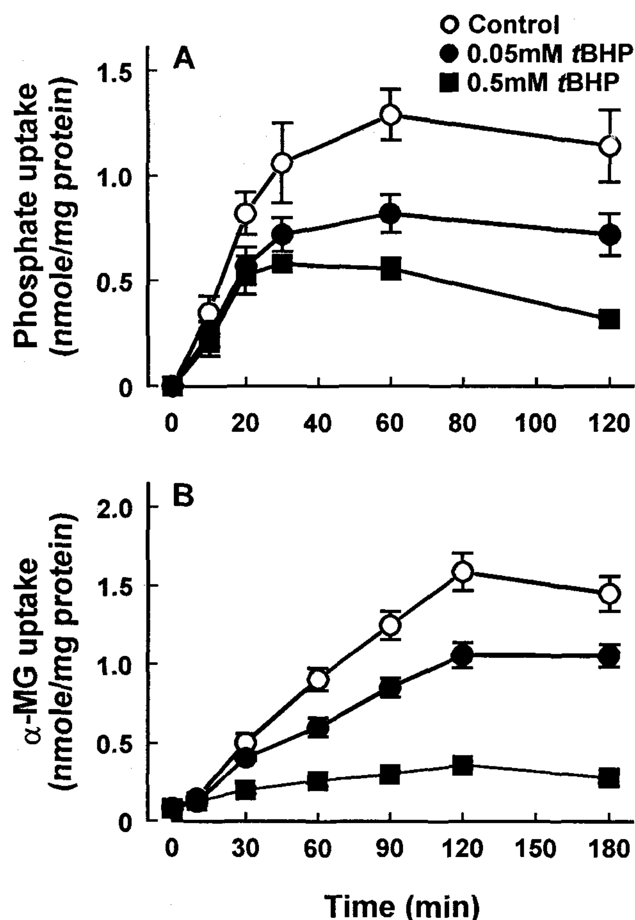

Fig. 1. Time course of phosphate (A) and α-methylglucoside (α-MG, B) uptakes in the primary cultured renal proximal tubular cells. Cells were exposed to 0, 0.05 and 0.5 mM *t*BHP for various times at 37°C, and the uptakes were measured. Data are mean±SE of four experiments. *P<0.05 compared with cells exposed to 0 mM *t*BHP.

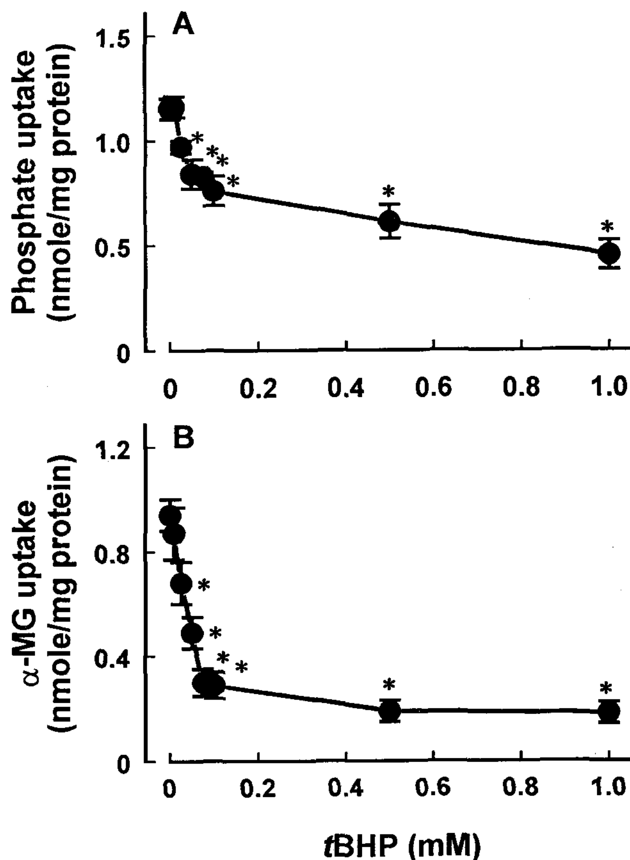

Fig. 2. Dose-dependency of *t*BHP effect on the uptakes of phosphate (A) and α-methylglucoside (α-MG, B) in the primary cultured renal proximal tubular cells. Cells were exposed to various concentrations of *t*BHP for 60 min at 37°C, and the uptakes were measured for 30 min. Data are mean±SE of five experiments. *P<0.05 compared with cells exposed to 0 mM *t*BHP.

Effect of tBHP on cell viability, lipid peroxidation and AA release

In order to determine if *t*BHP-induced inhibition of phosphate uptake was attributed to cell death, effect of *t*BHP on cell viability was measured. As shown in Fig. 3A, *t*BHP caused loss of cell viability in a dose-dependent manner. However, a significant loss occurred at 0.5 mM *t*BHP, a concentration higher than that required for significant inhibition of phosphate uptake. These results indicate that *t*BHP-induced inhibition of phosphate uptake may be not due to irreversible cell injury.

It has been reported that lipid peroxidation plays an important role in oxidant-induced cell injury in renal epithelial cells (Schnellmann, 1988; Salahudeen, 1995; Sheridan et al, 1996). In order to determine if lipid peroxidation contributes to *t*BHP-induced inhibition of phosphate uptake, effect of *t*BHP on lipid peroxidation was examined. As shown in Fig. 3B, *t*BHP induced a significant increase in lipid peroxidation at 0.5 mM, which is identical to the concentration required to cause cell death but higher than the concentration required to inhibit phosphate uptake. These results suggest that cell death is resulted from the peroxidation of membrane lipids, and the inhibition of phosphate uptake can occur without lipid peroxidation.

To confirm that functional PLA_2 activity is increased by oxidants in primary cultured proximal tubular cells, $[^3H]$AA release was measured in pre-

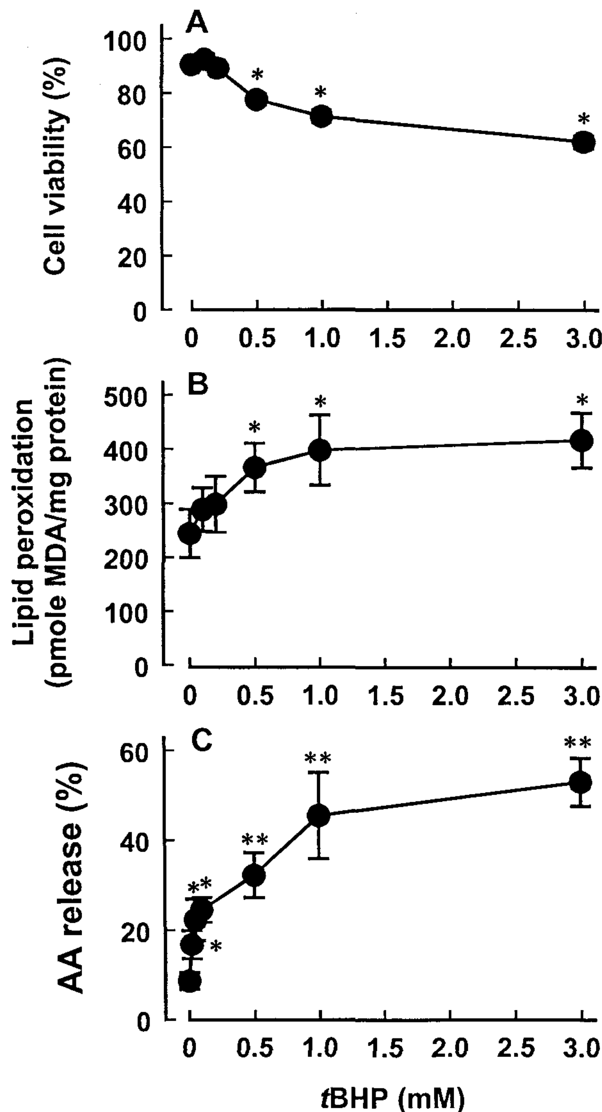

Fig. 3. Effect of *t*BHP on cell viability (A), lipid peroxidation (B), and arachidonic acid (AA) release (C) in primary cultured renal proximal tubular cells. Cells were exposed to various concentrations of *t*BHP for 60 min at 37°C. The viability was measured with trypan blue uptake. Cells failing to exclude the dye were considered nonviable. Viability were expressed as a percent of total (viable plus nonviable). Lipid peroxidation was estimated by measuring MDA. For AA release measurement, cells were prelabelled with [^{3}H]AA for 20 h, washed, and exposed to various concentrations of *t*BHP for 60 min. Aliquots of medium were removed and analyzed for [^{3}H]AA radioactivity. Data are mean±SE of four experiments. *P<0.05 compared with cells exposed to to 0 mM *t*BHP.

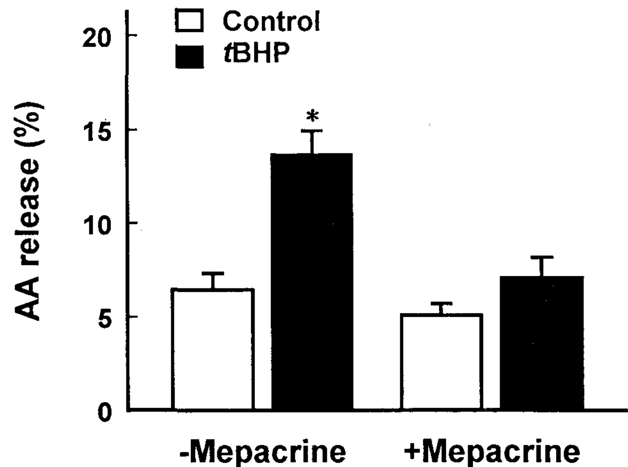

Fig. 4. Effect of PLA2 inhibitor on oxidant-induced arachidonic acid (AA) release in the primary cultured renal proximal tubule cells. Cells were prelabelled with [^{3}H]AA for 20 hr, washed, and exposed to 0.05 mM *t*BHP in the presence or absence of 0.1 mM mepacrine for 60 min. Aliquots of medium were removed and analyzed for [^{3}H]-AA radioactivity. Data are mean±SE of four experiments. *P<0.05 compared with the control.

labeled cells exposed to *t*BHP (Fig. 3C). *t*BHP caused an increase in AA release in a dose-dependent manner over concentration range of 0.02~3.0 mM and the significant increase occurred at 0.02 mM concentration. Therefore, the minimum concentration of *t*BHP for significant AA release was almost identical to that for inhibition of phosphate uptake, but was lower than that required for cell death.

Effect of PLA2 inhibitor mepacrine

In order to determine if *t*BHP-induced AA release resulted from PLA2 activation, effect of mepacrine, a PLA2 inhibitor, was examined. As shown in Fig. 4, 0.1 mM mepacrine completely inhibited AA release induced by 0.05 mM *t*BHP. However, the inhibitor did not affect the inhibition of phosphate uptake and cell death induced by *t*BHP (Figs. 5 and 6).

Role of protein kinase C (PKC) in tBHP-induced inhibition of phosphate uptake

Phosphate transport in renal proximal tubular cells is inhibited by PKC (Friedlander et al, 1996) which has been known to be activated by oxidants such as H_2O_2 and *t*BHP in hepatocytes (von Ruecker et al,

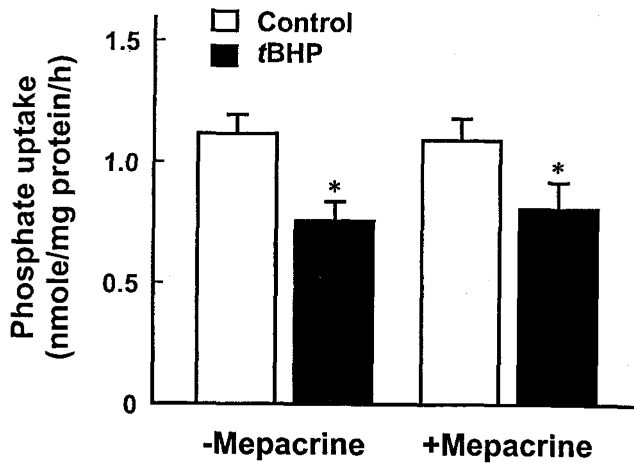

Fig. 5. Effect of PLA_2 inhibitor on oxidant-induced inhibition in phosphate transport in primary cultured renal proximal tubular cells. Cells were exposed to 0.05 mM *t*BHP in the presence or absence of 0.1 mM mepacrine for 60 min, and the uptake was measured. Data are mean ±SE of four experiments. *P<0.05 compared with the control.

Table 1. Effects of staurosporine on inhibition of phosphate uptake induced by protein kinase C activator and *t*BHP in primary cultured renal proximal tubule cells

Treatments	Phosphate uptake (nmole/mg protein/hr)
Control	1.162±0.067
Staurosporine	1.170±0.018
PMA	0.878±0.031*
PMA + Staurosporine	1.101±0.110
*t*BHP	0.771±0.092*
*t*BHP + Staurosporine	0.793±0.079*

Cells were pretreated with staurosporine (1 μM) for 3 h, followed by treatment of phorbol 12-myristate 13-acetate (PMA, 1 μM) or *t*BHP (0.05 mM) for 60 min in the presence or absence of 10 nM staurosporine, and the phosphate uptake was measured. Data are mean±SE of five determinations. *p<0.05 compared with the control.

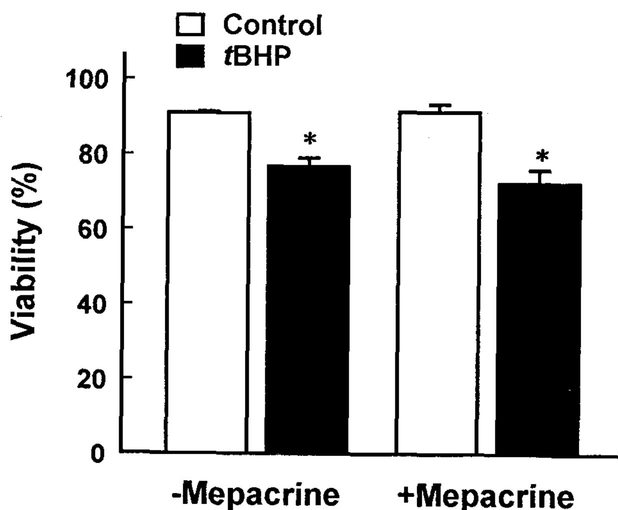

Fig. 6. Effect of PLA_2 inhibitor on oxidant-induced decrease of cell viability in primary cultured renal proximal tubular cells. Cells were exposed to 0.5 mM *t*BHP in the presence or absence of 0.1 mM mepacrine for 60 min at 37°C, and the viability was measured with trypan blue uptake. Cells failing to exclude the dye were considered nonviable. Viability were expressed a percent of total (viable plus nonviable). Data are mean±SE of four experiments. *P<0.05 compared with the control.

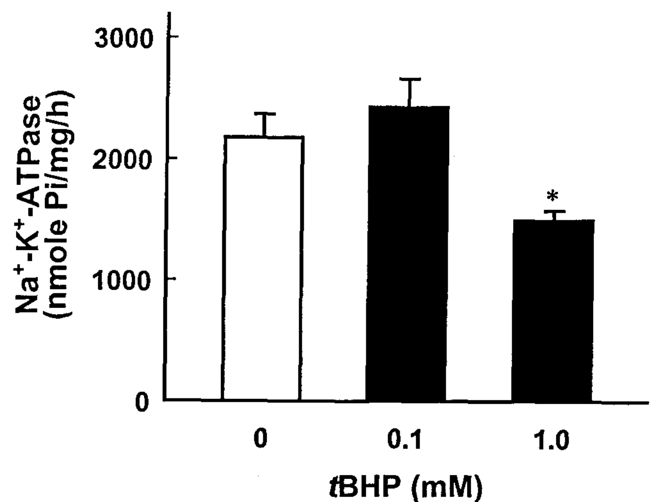

Fig. 7. Effect of *t*BHP on Na^+-K^+-ATPase activity in microsomal fraction prepared from primary cultured renal proximal tubular cells. Microsomal fraction was exposed to 0.1 and 1.0 mM *t*BHP for 60 min, and the enzyme activity was measured. Data are mean±SE of four experiments. *P<0.05 compared with 0 *t*BHP.

1989) and vascular endothelial cells (Taher et al, 1993). Thus, *t*BHP could inhibit the phosphate uptake through the PKC activation. To test the possibility, effect of a PKC inhibitor staurosporine was examined. A PKC activator, phorbol 12-myristate 13-acetate (PMA), at 1 μM inhibited the phosphate uptake. Staurosporine (10 nM), a PKC inhibitor, prevented the inhibition induced by PMA, but it did not alter *t*BHP-induced inhibition (Table 1).

Effect of tBHP on Na^+-K^+-ATPase activity

In the last series of experiments, we determined whether *t*BHP inhibits Na^+-K^+-ATPase activity at concentrations which cause a significant reduction in phosphate uptake. In microsomal fraction prepared from primary cultured cells, *t*BHP produced a significant inhibition of the enzyme activity at 1.0 mM but not 0.1 mM (Fig. 7).

DISCUSSION

Oxidants have been reported to affect membrane transport function in various cell types (Elliott & Schilling, 1992; Andreoli et al, 1993; Elliott & Koliwad, 1995; Herman et al, 1995). Since oxidants such as H_2O_2 and *t*BHP have been reported to induce renal cell injuries by generation of lipid peroxidation (Schnellmann, 1988; Salahudeen, 1995; Sheridan et al, 1996), oxidant-induced alterations in membrane transport function may be due to irreversible cell injury resulting from lipid peroxidation. Lipid peroxidation has been reported to decrease glucose transport in intestinal brush border membranes (Meddings et al, 1990; Jourd'heuil et al, 1993). However, in the present study, the oxidant *t*BHP significantly inhibits phosphate uptake at concentrations lower than those required for induction of cell death and lipid peroxidation (Figs. 2 and 3), indicating that *t*BHP inhibits phosphate uptake without inducing irreversible cell injury.

Eicosanoids have been suggested to play a role in pathophysiological state (Bonventre & Nemenoff, 1991) and to mediate the agonist-induced inhibition of tubular reabsorption (Satoh et al, 1993). AA and eicosanoid metabolites play critical roles in the initiation or modulation of a broad spectrum of physiological responses and certain abnormal processes in mammalian cells (Smith, 1989; Bonventre & Nemenoff, 1991). AA release can be catalyzed by PLA_2 and is believed to be the limiting step in the biosynthesis of eicosanoids in response to stimulation by receptors such as G protein-coupled receptors (Mukherjee et al, 1994). Since oxidants activate directly or indirectly PLA_2 and thereby exert various biological effects (Chakraborti et al, 1989; Schnellmann et al, 1994; Natarajan, 1995), oxidants may inhibit membrane transport function in renal proximal tubules. In fact, it has been demonstrated that effects of oxidants on sodium transport in gerbil middle ear epithelium are attributed to generation of PGE_2, which is believed to be due to PLA_2 activation (Herman et al, 1995). In the present study, we examined the role of PLA_2 in *t*BHP-induced inhibition of phosphate uptake using a PLA_2 inhibitor mepacrine. *t*BHP increased AA release at concentrations similar to those for inhibition of phosphate uptake (Fig. 3C), which was completely inhibited by 0.1 mM mepacrine (Fig. 4). However, reduction in phosphate uptake and cell viability was not altered by mepacrine (Figs. 5 and 6). These results suggest that PLA_2 may not contribute to inhibition of phosphate uptake and cell death induced by *t*BHP.

In hepatocytes (von Ruecker et al, 1989) and vascular endothelial cells (Taher et al, 1993), oxidants such as H_2O_2 and *t*BHP activate the PKC enzyme which inhibits phosphate transport in renal proximal tubular cells (Friedlander et al, 1996). Therefore, oxidants may inhibit phosphate uptake through PKC activation. However, the present study clearly showed that *t*BHP-induced inhibition of phosphate uptake is not prevented by a PKC inhibitor staurosporine (Table 1), indicating that *t*BHP effect is not mediated by the PKC activation in renal proximal tubules.

Since phosphate is taken up by Na^+-phosphate cotransport system in the renal proximal tubular cells, maintenance of intracellular Na^+ gradient is necessary to drive phosphate transport. The Na^+ gradient across plasma membrane is generated by the active Na^+ pump in the basolateral membrane. Thus, *t*BHP-induced inhibition of phosphate obtained from the present study could be a consequence of decrease in the Na^+-K^+-ATPase activity in the basolateral membrane. However, the present study showed that *t*BHP-induced reduction of phosphate uptake does not result from the inhibition of Na^+-K^+-ATPase activity, although it may be, in part, due to the inhibition of the enzyme activity at higher concentrations (Fig. 7). Andreoli et al (1993) reported in LLC-PK_1 cells that inhibition of Na^+-K^+-ATPase activity by 0.5 mM H_2O_2 mediates the inhibition of glucose and phosphate transports.

An increase in intracellular Ca^{2+} may play a role in mediating oxidant-induced inhibition of phosphate uptake. Oxidants increase intracellular Ca^{2+} in renal cells (Kuroda et al, 1995) and renal phosphate transport is modulated by intracellular Ca^{2+} (Dousa, 1996). In vascular endothelial cells, Ca^{2+}-signaling mechanisms have been proposed to be possible mechanisms

of oxidant-mediated cell dysfunction (Schilling & Elliott, 1992; Natarajan, 1995). Since oxidants at noncytotoxic levels act as chemical messengers (Saran & Bors, 1989), Ca^{2+}-dependent and/or independent signaling mechanisms may be responsible for oxidant-induced inhibition of phosphate uptake in renal proximal tubular cells.

In conclusion, a PLA_2 inhibitor mepacrine reduced *t*BHP-induced AA release, but it did not alter the inhibition of phosphate uptake and cell injury induced by *t*BHP. The oxidant caused irreversible cell injury and lipid peroxidation at concentrations higher than those required to inhibit phosphate uptake. These results suggest that the inhibition of phosphate uptake induced by lower concentrations of *t*BHP at lower concentrations may be due to other actions rather than PLA_2 activation, irreversible cell, and lipid peroxidation.

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