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Role of Phospholipase A_2 in Hypoxia-Induced Renal Cell Injury

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The present study was designed to assess the roles of PLA_2 activation and arachidonic acid (AA) metabolites in hypoxia-induced renal cell injury. Hypoxia increased LDH release in a dose-dependent manner in rabbit renal cortical slices, and this increase was significant after 20-min hypoxia. The hypoxia-induced LDH release was prevented by amino acids, glycine and alanine, and extracellular acidosis (pH 6.0). Buffering intracellular Ca^{2+} by a chelator, but not omission of Ca^{2+} in the medium produced a significant reduction in hypoxia-induced LDH release. The effect of hypoxia was blocked by PLA_2 inhibitors, mepacrine, butacaine, and dibucaine. A similar effect was observed by a 85-kD $cPLA_2$ inhibitor $AACOCF_3$. AA increased hypoxia-induced LDH release, and albumin, a fatty acid absorbent, prevented the LDH release, suggesting that free fatty acids are involved in hypoxia-induced cell injury. These results suggest that PLA_2 activation and its metabolic products play important roles in pathogenesis of hypoxia-induced cell injury in rabbit renal cortical slices.

Key Words: Oxidant, PLA_2, LDH release, Hypoxia, Rabbit kidney

INTRODUCTION

The underlying mechanisms of cell injury induced by ischemia or hypoxia remain obscure. Accumulation of unesterified fatty acids, especially arachidonic acid (AA), has been observed during ischemic or hypoxic injury in liver, heart, and kidney (Chien et al, 1978; Corr et al, 1984; Matthys et al, 1984; Humes et al, 1989; Bunnachak et al, 1994). This accumulation of free fatty acids has been attributed to the degradation of membrane phospholipids which is believed to be secondary to phospholipase A_2 (PLA_2) activation (Nguyen et al, 1988; Bonventre & Nemenoff, 1991; Portilla et al, 1994). Phospholipids provide the major structural framework of cell membranes (Singer & Nicolson, 1972) and participate in the regulation of membrane enzyme activity, permeability, and hormone activation (Rubalcava & Rodbell, 1973; Dahl & Hokin, 1974; Cullis et al, 1980; Green et al, 1980). Thus, phospholipids play important roles in the maintenance of membrane function and cell viability. The activation of intracellular PLA_2 has been suggested to be an important mediator of ischemic or hypoxic tubular cell injury (Nakamura et al, 1991; Weinberg, 1991; Bonventre, 1993; Portilla et al, 1994; Choi et al, 1995). Indeed, PLA_2 enzyme activity has been demonstrated to increase in ischemic and hypoxic proximal tubules (Nakamura et al, 1991; Portilla et al, 1992). However, the role of PLA_2 activation in hypoxic cell injury in renal proximal tubules remains controversial (Weinberg, 1991; Bunnachak et al, 1994; Portilla et al, 1994).

This study was undertaken to determine whether PLA_2 activation play any roles in hypoxia-induced renal cell injury employing inhibitors of PLA_2 enzyme in rabbit renal cortical slices and primary cultured proximal tubules.

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METHODS

Preparation of renal cortical slices

Male New Zealand white rabbits weighing 1.5∼2 kg were sacrificed and the kidneys were rapidly removed. The kidneys were immediately perfused through the renal artery with an ice-cold isotonic solution containing 140 mM NaCl, 10 mM KCl and 1.5 mM $CaCl_2$ to remove as much blood as possible. Thin (0.4∼0.5 mm thick) slices 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).

Experimental protocols

Renal cortical slices were incubated in a modified Cross-Taggart medium under a 100% O_2 atmosphere (control) and under a 100% N_2 atmosphere (hypoxia). Unless stated otherwise, slices were incubated for 30 min at 37°C. Following incubation, slices were treated for the measurement of LDH release.

Isolation and culture of proxiaml 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 (1.5~2.0 kg) were sacrificed by cervical dislocation. The kidney was immediately removed, cleaned of fat and debris, and washed with sterile antibiotic-supplemented medium. The kidney was perfused with antibiotic-supplemented medium through the renal artery to remove as much blood as possible, and then perfused with a 0.5% (wt/vol) iron oxide suspension until the kidney turned a greyish-black in colour. The cortex was removed, and homogenized with 4 strokes of a Dounce homogenizer (Wheaton type A). The resulting homogenate was passed through a series of sterile nylon mesh sieves (254 and 85 micron: TETCO, Inc., Depew, NY) and washed with antibiotic-supplemented medium. The proximal tubules and glomeruli retained on the 85 micron sieve were suspended in a centrifuge tube containing antibiotic-supplemented medium and magnetic stirring bar. Glomeruli containing iron oxide were attracted to the magnetic stirring bar, and the procedure was repeated until a purified preparation of isolated proximal tubules was obtained.

The proximal tubules were briefly incubated in DMEM/F12 medium containing 80 ug/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 onset of each experiment. All experiments started 7 day after plating when a confluent monolayer culture was achieved.

Measurement of LDH release

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).

Arachidonic acid (AA) release

After cells were labelled with 0.1 μCi [^{3}H] AA/ml per well for 20 hr in a 24 well plate, they were washed twice with Hanks balanced salts solution (HBSS) and incubated in the same solution at 37°C for 15 min for the temperature equilibration. Cells were exposed to the medium containing to 5 mM KCN/0.1 mM iodoacetic acid in the presence or absence of 250 M butacaine or 20 M $AACOCF_3$ for 2 hr at 37°C. The medium was removed and centrifuged, and the radioactivity in the supernatant was measured by a liquid scintillation spectrophotometer. Cells left attached to the plate were scraped off with 0.2% Triton X-100 and also counted for radioactivity. The amount of [^{3}H] AA released into the medium was expressed as a percent of the total (cell-associated plus released).

Chemicals

Mepacrine, butacaine, dibucaine, Quin-2/AM, and bovine serum albumin were purchased from Sigma Chemical (St. Louis, MO). N,N'-diphenyl-p-phenylenediamine (DPPD) was purchased from Aldrich Chemical (Milwaukee WI). $AACOCF_3$ from Calbiochem (San Diego, CA). All other chemicals were of the highest commercial grade available.

Statistical analysis

The data are expressed as the mean ± SE. Comparison between two groups was made by unpaired *t* test. Multiple group comparison was done using one-way analysis of variance followed by Tukey *post hoc* test. The difference with $p < 0.05$ were considered statistically significant.

RESULTS

Hypoxia-induced LDH release in renal cortical slices

Fig. 1. shows the effect of hypoxia on LDH release in renal cortical slices. During 60-min incubation, the LDH release from hypoxic tissues increased gradually with time, whereas that from control tissues remained unchanged. The amount of LDH release after 20-min incubation was significantly greater in hypoxic tissues than in controls. Thus, in subsequent experiments, 30-min hypoxia was employed in evaluating the effects of various agents on hypoxic cell injury.

Fig. 2. depicts the effects of amino acids on hypoxia-induced LDH release. The addition of 5 mM glycine or alanine completely prevented hypoxia-induced LDH release. The protective effect of these amino acids against hypoxic cell injury in proximal tubules has also been demonstrated by other investigators (Weinberg, 1991; Wetzels et al, 1993).

Extracellular acidosis has been reported to protect against the cell injury induced by hypoxia or toxic agents in renal tubular cells and hepatocytes (Bonventre & Cheung, 1985; Nieminen et al, 1990; Rodeheaver & Schnellmann, 1993). In accordance with this, the extracellular acidosis (pH 6.0) significantly reduced the hypoxia-induced LDH release in the present study (Fig. 3).

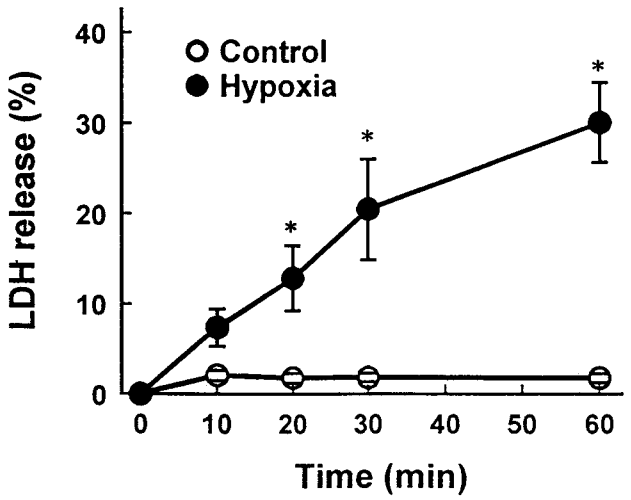

Fig. 1. Time course of hypoxia-induced LDH release in renal cortical slices. Slices were incubated for indicated time period at 37°C under 100% O_2 (Control) or 100% N_2 atmospheres (Hypoxia) and then LDH release was measured. Data are mean±SE of four experiments. *p< 0.05 compared with the respective control.

Effects of Ca^{2+} on hypoxia-induced LDH release in renal cortical slices

In an attempt to evaluate the effects of extracellular

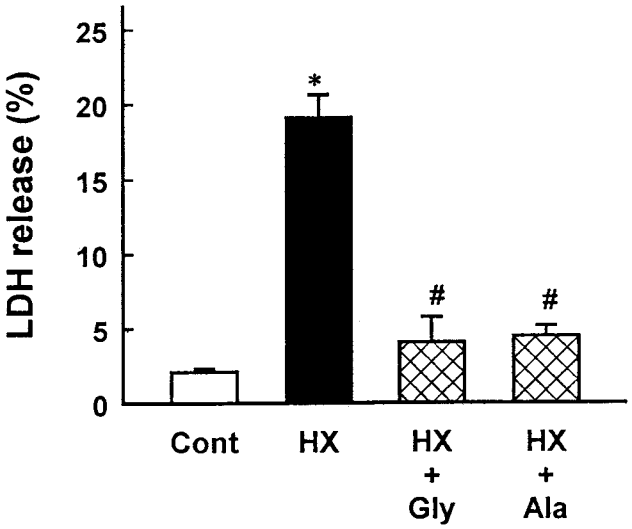

Fig. 2. Effects of glycine and alanine on hypoxia-induced LDH release in renal cortical slices. Slices were incubated for 30 min at 37°C under 100% O_2 (Cont) or 100% N_2 (HX) atmospheres in the presence or absence of 5 mM glycine (Gly) or alanine (Ala), and then LDH release was measured. Data are mean±SE of seven experiments. *p<0.05 compared with the control. #p< 0.05 compared with hypoxia alone.

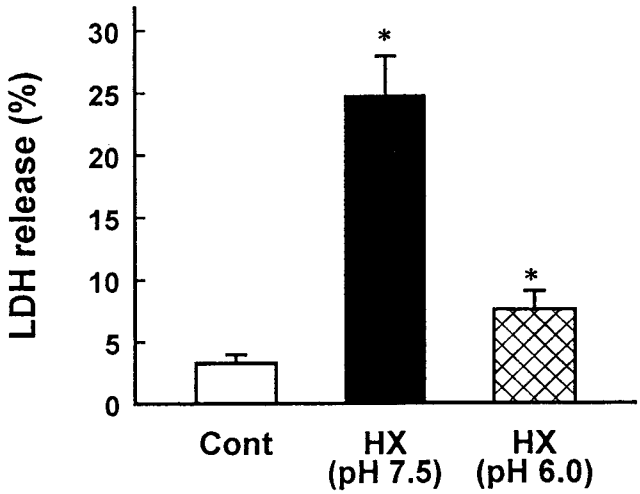

Fig. 3. Effect of extracellular acidosis on hypoxia-induced LDH release in renal cortical slices. Slices were incubated for 30 min at 37°C under 100% O_2 (Cont) or 100% N_2 (HX) atmospheres in the medium with pH 7.5 or 6.0, and then LDH release was measured. Data are mean±SE of seven experiments. *p<0.05 compared with the control. #p<0.05 compared with hypoxia alone.

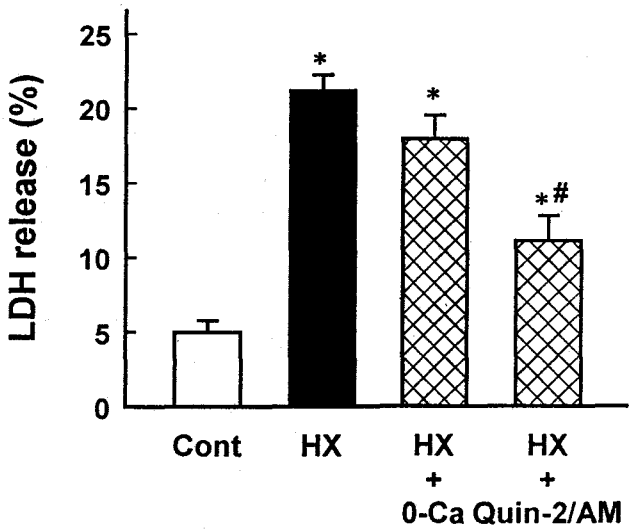

Fig. 4. Effects of Ca^{2+}-free medium and intracellular Ca^{2+} chelation by quin-2/AM on hypoxia-induced LDH release in renal cortical slices. Slices were incubated for 30 min at 37°C under 100% O_2 (Cont) or 100% N_2 (HX) atmospheres in Ca-free medium (0-Ca) or in the medium containing 50 μM quin-2/AM, and then LDH release was measured. Data are mean±SE of five experiments. *p< 0.05 compared with the control. #p<0.05 compared with hypoxia alone.

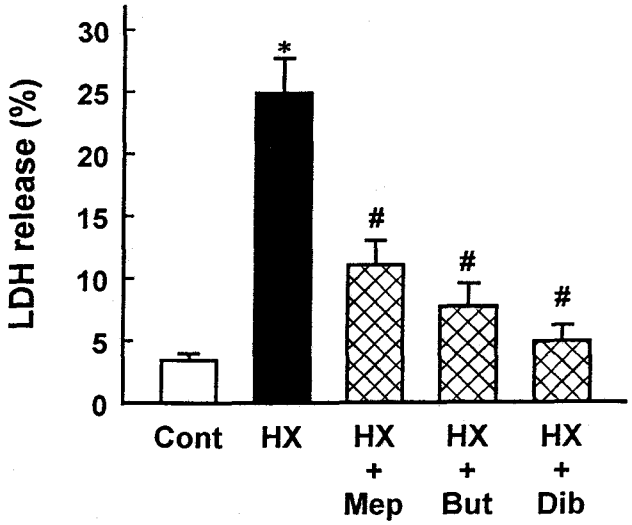

Fig. 5. Effect of PLA_2 inhibitors on hypoxia-induced LDH release in renal cortical slices. Slices were incubated for 30 min at 37°C under 100% O_2 (Cont) or 100% N_2 (HX) atmospheres in the presence or absence of 0.25 mM mepacrine (Mep), butacaine (But), or dibucaine (Dib), and then LDH release was measured. Data are mean±SE of five experiments. *p<0.01 compared with the control. #p<0.05 compared with hypoxia alone.

Ca^{2+} and buffering intracellular Ca^{2+} on hypoxia-induced cell injury, slices were incubated in Ca^{2+}-free medium or in the medium containing 50 μM quin-2/AM, an intracellular Ca^{2+} chelators, which is known to protect against the oxidant-induced cell injury (McConkey et al, 1990; Ueda & Shah, 1992; Kim & Kim, 1996). As shown in Fig. 4. the hypoxia-induced LDH release was not prevented by removing extracellular Ca^{2+}, but significantly reduced by quin-2/AM.

Effects of PLA_2 inhibitors on hypoxia-induced LDH release in renal cortical slices

In order to determine if PLA_2 inhibition prevents hypoxia-induced cell injury, we studies effects of PLA_2 inhibitors such as mepacrine, dibucaine, and butacaine (Billah et al, 1981; Harrison et al, 1991; Sheridan et al, 1993) on LDH release. As shown in Fig. 5. these drugs significantly reduced the hypoxia-induced LDH release at 0.25 mM. Protective effect of butacaine was dependent on concentration and was maximal at 0.5 mM (Fig. 6. When slices were treated with these agents under 100% oxygen atmospheres, the LDH release was not altered.

In the next series of experiments, we examined the effect of $AACOCF_3$, a trifluoromethyl ketone analogue

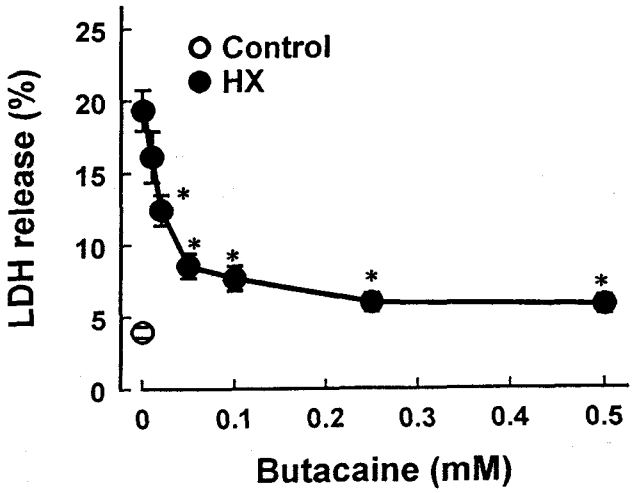

Fig. 6. Dose dependency of butacaine effect on hypoxia-induced LDH release in renal cortical slices. Slices were incubated for 30 min at 37°C under 100% O_2 (Control) or 100% N_2 (HX) atmospheres in the presence or absence of various concentrations of butacaine, and then LDH release was measured. Data are mean±SE of six experiments. *<0.05, compared with hypoxia without butacaine.

of arachidonyl acid which inhibits the 85-kD $cPLA_2$ but not the 14-kD PLA_2 (Street et al, 1993), on LDH release (Huang et al, 1994). As shown in Fig. 7. the LDH release increased from 5.60±1.45% to 33.10±

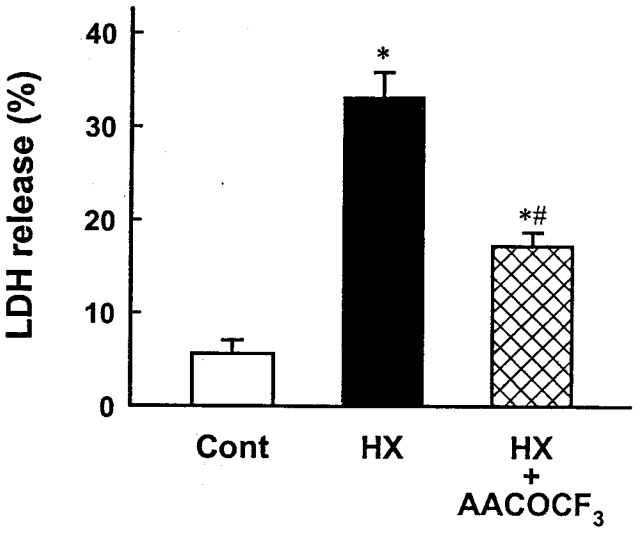

Fig. 7. Effect of $AACOCF_3$ on hypoxia-induced LDH release in renal cortical slices. Slices were incubated for 30 min at 37°C under 100% O_2 (Cont) or 100% N_2 (HX) atmospheres in the presence or absence of 20 μM $AACOCF_3$, and then LDH release was measured. Data are mean ± SE of four experiments. *p < 0.05 compared with the control. #p < 0.05 compared with hypoxia alone.

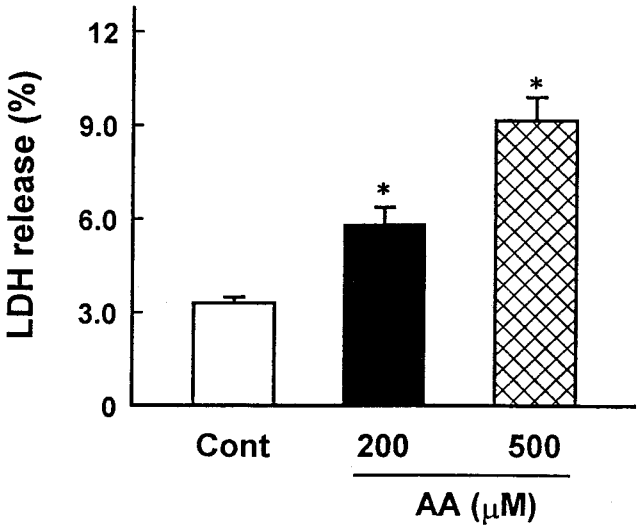

Fig. 8. Effect of arachidonic acid (AA) on LDH release in renal cortical slices. Slices were incubated for 30 min at 37°C at a 100% O_2 atmosphere in the presence or absence of 200 and 500 μM AA, and then LDH release was measured. Data are mean ± SE of seven experiments. *p < 0.05 compared with the control (Cont).

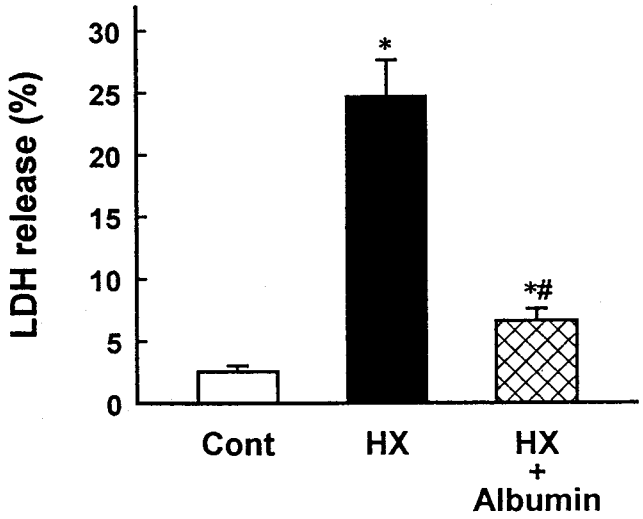

Fig. 9. Effect of albumin on hypoxia-induced LDH release in renal cortical slices. Slices were incubated for 30 min at 37°C under 100% O_2 (Cont) or 100% N_2 (HX) atmospheres in the presence or absence of 1% albumin, and then LDH release was measured. Data are mean ± SE of four experiments. *p < 0.05 compared with the control (Cont).

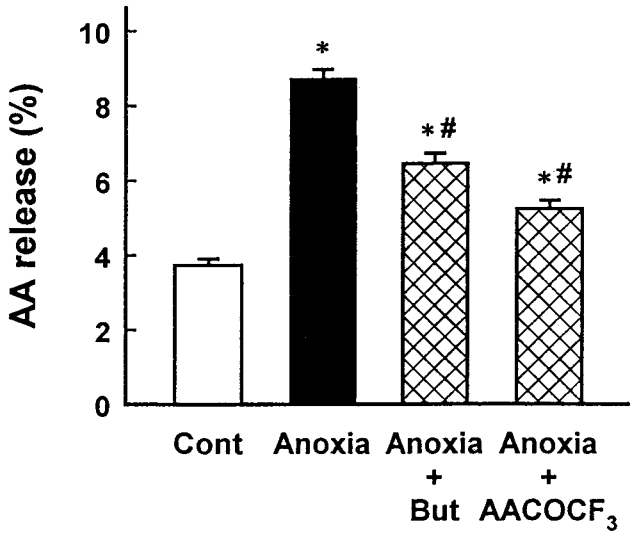

Fig. 10. Effects of PLA_2 inhibitors on chemical anoxia-induced arachidonic acid (AA) release in primary cultured proximal tubules. Cells were labelled with 0.1 μCi [^{3}H]AA/ml per well for 20 hr in a 24-well plate, and AA release was measured in the presence or absence of 250 μM butacaine or 20 μM $AACOCF_3$ for 2 hr at 37°C. Chemical anoxia was induced by exposure cells to 5 mM KCN/0.1 mM iodoacetic acid. Data are mean ± SE of four experiments. *p < 0.05 compared with the control (Cont). #p < 0.05 compared with anoxia alone.

2.82% during 30 min of hypoxia, but the increase was significantly retarded to 17.23±1.48% by addition of 20 μM $AACOCF_3$.

Effects of AA on LDH release in renal cortical slices

In order to ascertain whether AA causes directly renal cell injury, the LDH release was measured in slices exposed to 200 or 500 μM of AA. The results depicted in Fig. 8. indicated that AA increased LDH release in a dose-dependent manner. When slices were exposed to hypoxia in the presence of albumin, a free fatty acid absorbent, the LDH release was markedly prevented (Fig. 9).

Effects of PLA_2 inhibitors hypoxia-induced AA release in primary cultured cells

In the last series of experiments, effects of PLA_2 inhibitors on chemical anoxia-induced AA release was examined in primary cultures proximal tubules, in order to detemine if these inhibitors alter the hypoxia-induced activation of PLA_2 enzyme. The release of AA from membrane phospholipids has been accepted as a measure of PLA_2 activity (Harrison et al, 1991; Wang et al, 1996). The results are depicted in Fig. 10. When cells were exposed to 5 mM KCl and 0.1 mM iodoacetic acid for 60 min, AA release significantly increased, which was partially prevented by addition of 0.25 mM butacaine and 20 μM $AACOCF_3$. These results suggest that PLA_2 is activated during hypoxia and the activation is prevented by these inhibitors.

DISCUSSION

Increased accumulation of free fatty acids, particularly unsaturated fatty acids such as AA, is believed to contribute to cell injury in renal proximal tubules (Humes et al, 1989; Wetzels et al, 1993). PLA_2 is known to be activated during hypoxia (Portilla et al, 1992), and may be responsible, at least in part, for accumulation of unsaturated fatty acids (Choi et al, 1995). Most isoforms of PLA_2 have been shown to require Ca^{2+} for enzyme activity (Bonventre, 1993; Choi et al, 1995). However, Ca^{2+}-independent isoforms of the enzyme have also been described (Portilla et al, 1994). In addition, certain forms of PLA_2 translocate to the plasma membrane during hypoxia and are released into extracellular space; the extent of released PLA_2 activity parallels that of LDH release, suggesting that extracellular PLA_2 may act in a paracrine way to induce cell injury (Choi et al, 1995). However, the role of PLA_2 activation in hypoxia-induced renal cell injury has not been clearly established (Weinberg, 1991; Bunnachak et al, 1994; Edelstein et al, 1997).

To determine whether or not the PLA_2 activation is involved in hypoxia-induced cell injury, the effects of PLA_2 inhibitors were examined in the present study. Three inhibitors, mepacrine, dibucaine, and butacaine, all prevented hypoxia-induced LDH release (Fig. 5), and the effect of butacaine was dose- dependent (Fig. 6). Similar results have been reported by others in renal proximal tubular cells (Sheridan et al, 1993; Bunnachak et al, 1994) and hepatocytes (Harrison et al, 1991). These results suggest that PLA_2 activation is an important step in hypoxia-induced cell injury.

Three groups of mammalian PLA_2s have been reported, namely, the 14-kD Ca^{2+}-dependent secreted PLA_2s, the 85-kD-dependent and *sn*-2 arachidonyl-specific cytosolic PLA_2 ($cPLA_2$), and the Ca^{2+}-independent PLA_2s (Mayer & Marshall, 1984; Dennis, 1994; Mukherjee et al, 1994). In the kidney, multiple forms of the enzyme have been reported (Nakamura et al, 1991; Choi et al, 1995), but it is not clear which group plays a role in hypoxic injury. Since $cPLA_2$ is the major enzyme regulating the arachidonic acid cascade (Mayer & Marshall, 1984; Clark et al, 1991), it could be involved in hypoxia-induced fatty acid release and consequent cell injury. Activation of 85-kD $cPLA_2$ has also been associated with the agonist-induced AA release in kidney cells (Gronich et al, 1990; Xing & Insel, 1996) and ovary cells (Lin et al, 1992). In the present study, hypoxia-induced LDH release was significantly inhibited by $AACOCF_3$, a potent inhibitor of the 85-kD $cPLA_2$ (Street et al, 1993; Huang et al, 1994) (Fig. 7), suggesting that $cPLA_2$ activation may be responsible for hypoxia-induced renal cell injury.

Although the drugs such as mepacrine and dibucaine are known as PLA_2 inhibitors, their ability to protect hypoxia-induced cell injury may be associated with actions other than PLA_2 inhibition. These agents partitioned into the membrane may have protective effects by altering membrane structure, thereby making the membrane less permeable to Ca^{2+}. In fact, these agents are known to block increased Ca^{2+} entry induced by hypoxia in renal proximal tubules (Bunnachak et al, 1994). Furthermore, mepacrine is also known to impede Ca^{2+} entry (Favus et al, 1989). Since an increase in intracellular Ca^{2+} has been suggested to be an important step in hypoxic injury of renal proximal tubules (Edelstein et al, 1997), an

inhibition of Ca^{2+} entry may be considered as a mechanism of the protection of hypoxic cell injury by these drugs. However, in the present study, hypoxia-induced LDH release was not altered by omission of extracellular Ca^{2+} (Fig. 4), although buffering intracellular Ca^{2+} with quin-2/AM partially reduced the hypoxia-induced LDH release. These data suggest that Ca^{2+} entry from extracellular medium is not involved in hypoxia-induced cell injury in renal cortical slices. It thus follows that the protective effect of mepacrine observed from the present study could not be attributed to its inhibition of Ca^{2+} influx. Sheridan et al (Sheridan et al, 1993) observed that mepacrine reduces cell injury and fatty acid accumulation induced by chemical anoxia, which may suggest a direct effect of mepacrine on PLA_2 enzyme.

Since AA is a fatty acid contained exclusively in phospholipid, an increase in AA release represents an increased PLA_2 activity. In this study, AA release was significantly increased by chemical anoxia in primary cultures of rabbit proximal tubules and the increase was significantly prevented by PLA_2 inhibitors, butacaine and $AACOCF_3$. Such results strongly suggest that these inhibitors exert protective effect against hypoxic injury by inhibiting PLA_2 activity.

Another controversial issue in hypoxic cell injury is the accumulation of free fatty acids. Wetzels et al (1993) reported that hypoxia causes an increase in fatty acids, but the degree of increase is not sufficient to induce cell injury. Humes et al (1989) claimed that free fatty acid change is not a major factor in hypoxia-induced cell injury, since a fatty acid absorbent albumin does not prevent the cell injury. In the present study, we observed that AA, a modest injurious fatty acid (Matthys et al, 1984), increased LDH release and albumin attenuated hypoxia-induced LDH release, which suggest that the cell injury is mediated by fatty acids. Similar protective effect of albumin has been observed in proximal tubular cells exposed to chemical anoxia (Sheridan et al, 1993).

In conclusion, PLA_2 inhibitors in renal cortical slices prevented hypoxia-induced LDH release. PLA_2 inhibitors significantly reduced hypoxia-induced AA release in primary cultures of proximal tubules. These results suggest that PLA_2 activation is involved in hypoxia-induced cell injury in renal tubules.

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