Kidney International, Vol. 49 (1996), pp. 620–625

LABORATORY INVESTIGATION

Arachidonic acid protects against hypoxic injury in rat proximal tubules

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Arachidonic acid protects against hypoxic injury in rat proximal tubules. Free fatty acids (FFA) and lysophospholipids accumulate during hypoxia (H) in rat proximal tubular epithelial cells partly as a result of increased phospholipase A_2 (PLA_2) activity. The role of FFA in mediating hypoxic injury and modulating PLA_2 activity is not clear. In the present study, the effect of several FFA including arachidonic acid (AA, 20:4) on hypoxia-induced injury and PLA_2 activity was assessed in freshly isolated rat proximal tubules. Hypoxia (H) was induced in the presence of either an unsaturated free fatty acid (uFFA) [AA or linoleic acid (LA, 18:2)] or a saturated FFA (sFFA) [palmitic (PA, 16:0) or myristic acid (MA, 14:0)]. Cell membrane injury was assessed by measuring lactate dehydrogenase release (LDH). AA markedly reduced LDH release during hypoxia in a dose dependent manner, while sFFA had no protective effect. LA showed similar protection to that observed with AA. AA did not affect buffer calcium concentration, buffer pH, intracellular pH or intracellular calcium concentration. Neither inhibiting the cyclooxygenase pathway with indomethacin, nor the lipoxygenase pathway with nordihydroguaiaretic acid (NDGA) had any effect on the AA observed cytoprotection. *In vitro* PLA_2 activity in the control tubular extracts was compared to that following addition of AA or PA. PLA_2 activity decreased significantly with AA but not with PA in a dose dependent manner. These data suggest that: (1) AA protects against hypoxic injury in rat proximal tubules. (2) This cytoprotection is not specific for AA and other uFFA have a similar effect. (3) AA significantly inhibits PLA_2 activity. (4) AA induced cytoprotection may be related to a negative feedback inhibition of PLA_2 activity.

Accumulation of unesterified fatty acids has been observed during ischemic renal injury [1, 2] and models of ATP depletion in rat and mouse proximal tubular epithelial cells [3–5]. This accumulation of FFA has been attributed to breakdown of membrane phospholipids which is believed to be secondary to increased PLA_2 activity [1, 6, 7]. Intracellular Ca^{2+} has been suggested to be an important mediator of hypoxic injury possibly through activation of certain cytosolic enzymes such as PLA_2, cysteine proteases and nitric oxide synthase [8–10]. Our laboratory has reported that cytosolic free Ca^{2+} increases significantly by five minutes of hypoxia [11]. Multiple isoforms of PLA_2 have been identified in proximal tubules, including both Ca^{2+}- dependent [7] and Ca^{2+}-independent [12] isoforms. The contribution of individual isoforms in mediating cell injury remains controversial.

Received for publication September 5, 1995
and in revised form October 24, 1995
Accepted for publication October 26, 1995

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The role of PLA_2 activation in hypoxia-induced proximal tubular injury has been evaluated using phospholipase inhibitors. Dibucaine, a known PLA_2 inhibitor has been shown to reduce LDH release in the hypoxic rat proximal tubules [3]. Protection against injury was also observed with a selective inhibitor of Ca^{2+} independent PLA_2 [12]. However, such studies do not distinguish between injurious effects of the end products of PLA_2 that is, FFA or lysophospholipids. To further underscore the uncertainty in this area, some investigators have demonstrated that exogenous PLA_2 added to rabbit renal proximal tubules in an oxygenated suspension did not produce cell injury [13], while others have shown a protective effect of exogenous PLA_2 against hypoxic injury in rat proximal tubules [14]. The aim of the present study was to examine the effects of one of the products of PLA_2, namely AA on cell membrane injury during hypoxia and on the activation of PLA_2 in rat proximal tubules.

Methods

Materials

Arachidonic acid (AA, 20:4), linoleic acid (LA, 18:2), palmitic acid (PA, 16:0), myristic acid (MA, 14:0) (all sodium salts), hyaluronidase, indomethacin, and nordihydroguaiaretic acid (NDGA) were obtained from Sigma Chemical Co. (St. Louis, MO, USA). Radioactively labeled phospholipid substrate, 1-palmitoyl-2-(1,^{14}C) arachidonoyl phosphatidyl ethanolamine (PE) was obtained from New England Nuclear (Boston, MA, USA). Collagenase (lot No F141499) was purchased from Boehringer Mannheim (Indianapolis, IN, USA). Bovine serum albumin was purchased from ICN (Cleveland, OH, USA).

Preparation of tubules

Proximal tubules were isolated from kidney cortex of male Sprague-Dawley rats (200 to 300 g) as described previously [15, 16]. The rats were anesthetized with pentobarbital sodium (60 mg/kg body wt i.p.). The kidneys were perfused for three minutes with 30 ml of a 4°C oxygenated solution containing (in mM) 112 NaCl, 18 $NaHCO_3$, 5 KCl, 1.6 $CaCl_2$, 2 $NaHPO_4$, 1.2 $MgSO_4$, 5 glucose, 2.5 HEPES, 10 mannitol, 1 glutamine, 1 sodium butyrate, 1 sodium lactate (buffer A), and 800 U heparin. Perfusion was continued for three minutes with 30 ml of buffer A containing 15 mg collagenase and 10 mg hyaluronidase. The kidneys were then decapsulated, removed and the cortex was minced on a cold petri

dish. After three washes in buffer A, the tissue was incubated at 37°C for 35 minutes in buffer A containing collagenase (40 mg in 60 ml of buffer A). Following collagenase digestion, the tissue was incubated for 10 minutes in 30 ml of ice-cold buffer A containing bovine serum albumin (1 g in 30 ml of buffer A). The tissue was then filtered through a tea strainer, then washed three times in buffer A to remove albumin and collagenase. Tubules were then suspended in 45% percoll and layered on top of 100% percoll. After centrifugation at 15,000 g for 10 minutes, the proximal tubules were recovered from the lowest band at a purity of greater than 95%. Purity of the tubule preparation has been determined in our laboratory using microscopic examination and distribution of proximal and distal tubule markers according to Gesek et al [17].

The tubules were then washed three times in buffer A to remove the percoll and resuspended in buffer B containing (in mM): 106 NaCl, 18 $NaHCO_3$, 1 $CaCl_2$, 5 KCl, 2 Na_2HPO_4, 1 $MgSO_4$, 5 glucose, 2.5 HEPES, 2 glutamine, 10 sodium butyrate and 4 sodium lactate. Six milliliter aliquots of tubule suspension (~1 to 2 mg protein/ml) were placed in siliconized 25 ml Erlenmeyer flasks for a recovery period consisting of the following: (1) gassing with 95% O_2–5% CO_2 for five minutes on ice; (2) flasks were then capped with rubber stoppers and kept at room temperature for five minutes; and (3) flasks were then placed in a shaking water bath at 37°C for 10 minutes. To create hypoxia, the tubule suspension was regassed for five minutes with 95% N_2–5% CO_2 at a rate of 3 liter/min. After regassing, the flasks were closed and kept in the shaking water bath for the period of hypoxia studied. At the end of the hypoxic period, 1 ml of tubule suspension was sampled from controls and hypoxic tubules for measurement of LDH release.

Exogenous fatty acids studies

FFA were added to the suspension at the end of the recovery period for 10 minutes prior to the hypoxia study time. Aliquots of the concentrated stock solutions (20 mM) in ethanol in the case of AA and LA and methanol in the case of PA and MA were used to achieve a final concentration ranging from 15 to 600 μM. FFA were dried down under N_2, resuspended in buffer B and sonicated for 20 seconds with a probe sonicator prior to addition to the renal tubule suspension. Hypoxia was induced for 20 minutes in the presence or absence of FFA. Uptake of ^{3}H-labeled fatty acids was used to assess incorporation of the FFA into the tubular cells during hypoxia.

LDH measurement

LDH release was measured to evaluate cell damage as previously described [15, 18]. The percentage LDH released from tubules was calculated by determining the ratio of LDH in the supernatant compared to that in the lysed tubule pellet plus the supernatant.

PLA_2 assay

Proximal tubules were isolated and frozen at −80°C until the time of the experiment. PLA_2 activity was measured as previously described with some modifications [19]. 1-palmitoyl-2-(1,^{14}C) arachidonyl phosphatidyl ethanolamine (PE) was used as a substrate. Protein contents were measured using a protein analysis kit (Bio-Rad Laboratories, Richmond, CA, USA) with bovine serum albumin as a standard. Cell pellets were resuspended in homogenization buffer (50 mM HEPES, pH 7.5, 1 mM EDTA, 1 mM EGTA, 250 mM sucrose plus a cocktail of protease inhibitors: leupeptin, pepstatin, aprotonin, PMSF), and homogenized with a Dounce dome homogenizer. Extracts were centrifuged at 100,000 g for one hour and supernatants were matched for protein. Pellets contained little PLA_2 activity and were discarded. Substrates were then dried down under N_2 and resuspended in dimethylsulfoxide. Two microliters of substrate (final concentration 15 μM) was pipetted into an Eppendorf microcentrifuge tube. Extracts were preincubated with FFA for 10 minutes at 4°C. Separate reactions were initiated by the addition of high (5 mM) and low (200 nM) Ca^{2+} concentration to each sample. The mixture was incubated for 30 minutes at 37°C and the reaction was terminated by the addition of ethanol containing 2% (vol/vol) acetic acid and 100 μg/ml free AA. Release of AA was analyzed using thin layer chromatography. Fifty microliters of the reaction mixture was spotted onto heat-activated silica gel thin-layer chromatography plates and developed in an organic phase consisting of ethyl acetate/isooctane/H_2O/acetic acid (55:75:100:8). The phospholipids and free AA bands were stained with iodine, scraped and radioactivity was counted with a liquid scintillation counter (Beckman). Specific activity of PLA_2 was expressed as pmole of AA released per min per mg of protein at 37°C.

Video imaging fluorescence microscopy (VIFM)

Quantitative fluorescence video microscopy of the isolated renal tubules was performed on a customized VIFM system which has been previously described in detail by our laboratory [11, 20]. Briefly, a Nikon epifluorescence microscope is interfaced to a PC computer with Universal Imaging Image/FL software, which allows for multiple excitation ratio imaging. A microperfusion chamber was mounted onto the stage of the microscope. The design and configuration of the perfusion chamber has been described previously by our laboratory [20]. It allows for continuous perfusion of normoxic or hypoxic tubules at (2 ml/min) and meticulous control of the environment in which the tubules are visualized during microscopy. The perfusion buffer contained (in mM): 113 NaCl, 18 $NaHCO_3$, 5 KCl, 1 $CaCl_2$, 2 Na_2HPO_4, 1 $MgSO_4$, 5 glucose, 1 glutamine, 1 sodium butyrate, 1 sodium lactate.

Intracellular pH measurements

Intracellular pH (pH_i) measurements were made using the dual-excitation ratioable pH fluorophore SNAFL-2 as described previously [21]. SNAFL-2 fluoresces at 620 nm and the ratio of fluorescence intensities when excited at 500 nm and 550 nm is proportional to pH_i. pH_i calibration curves were performed by adding 5 μg/ml nigericin to proximal tubules in a high K^+ buffer (in mM): 120 KCl, 30 NaCl, 0.5 $MgSO_4$, 1 $CaCl_2$, 1 $NaHPO_4$, 5 glucose, 10 HEPES, 10 PIPES. The high K^+ buffer was adjusted with 1 or 10 nM NaOH to varying pHs to generate a calibration curve for each experiment.

Cytosolic Ca^{2+} measurement

Alterations of cytosolic Ca^{2+} due to experimental maneuvers were measured with the VIFM system using the calcium sensitive dye, Fura-2, as previously described by our laboratory [20].

Buffer Ca^{2+} measurement

An AVL-984-S electrolyte analyzer (AVL Scientific Corporation, Roswell, GA, USA) was used to determine the buffer ionized calcium concentration in the different tubule preparations.

Cellular K^+ measurement

Cellular K^+ was measured using atomic absorption spectroscopy as described previously by our laboratory [22].

Statistical analysis

Multiple group comparisons were done using the analysis of variance (ANOVA) with post-test according to Newman-Keuls. The commercially available Graph Pad In Stat software version 2.0, 1990-1993 was used to perform the statistical tasks. A P value of less than 0.05 was considered statistically significant. Values are expressed as means ± standard error (SEM).

Results

Effect of exogenous FFA on hypoxic injury

To examine the effect of FFA on cell injury, freshly isolated rat proximal tubules were incubated with FFA prior to the onset of hypoxia. Figure 1A demonstrates the effect of 20 minutes hypoxia on LDH release with or without pretreatment with AA. AA significantly decreased LDH release at 20 minutes of hypoxia in a dose dependent fashion. Maximum protection was achieved with 200 μM of AA. No further protection was seen with 400 μM. Therefore, a dose of 200 μM or lower was used in all other experiments to assess cytoprotection. LA (200 μM) had a similar protective effect on LDH release as depicted in Figure 1A. To determine whether sFFA offer similar protection to that of the uFFA, AA or LA, we studied the effect of PA and MA on LDH release during 20 minutes of hypoxia. No cytoprotection could be observed in the presence of either PA or MA as compared with control hypoxic tubules (Fig. 1B). The intracellular incorporation of PA after 20 minutes of hypoxia was threefold less than that of AA as assessed by ^{3}H-labeled fatty acid uptake. Repeat experiments using a threefold higher dose of PA, that is, 600 μM, were therefore undertaken and also did not show any protection against hypoxic injury. LDH = 9 ± 1.1% during N, 43 ± 4.4% during 20 minutes of H, $P < 0.05$ versus N; and 41 ± 3.7 during H + PA (600 μM), P = NS versus H, all $N = 3$.

Effect of cyclooxygenase and lipoxygenase inhibition on AA related cytoprotection

To determine whether cyclooxygenase or lipoxygenase products rather than AA itself play a role in the observed cytoprotection we studied the effect of indomethacin (125 nM) and NDGA (50 μM) on LDH release. Neither indomethacin (Fig. 2) nor NDGA (Fig. 3) altered the protective effect of AA.

We found similar results to that observed with indomethacin using flurbiprufen (250 nM), another cyclooxygenase inhibitor. Therefore, inhibiting the cyclooxygenase or the lipoxygenase pathway with these agents did not affect the cytoprotection of AA. Both indomethacin and NDGA are by themselves protective to some degree, suggesting that inhibition of endogenous AA degradation through cyclooxygenase or lipoxygenase pathway may result in cytoprotection.

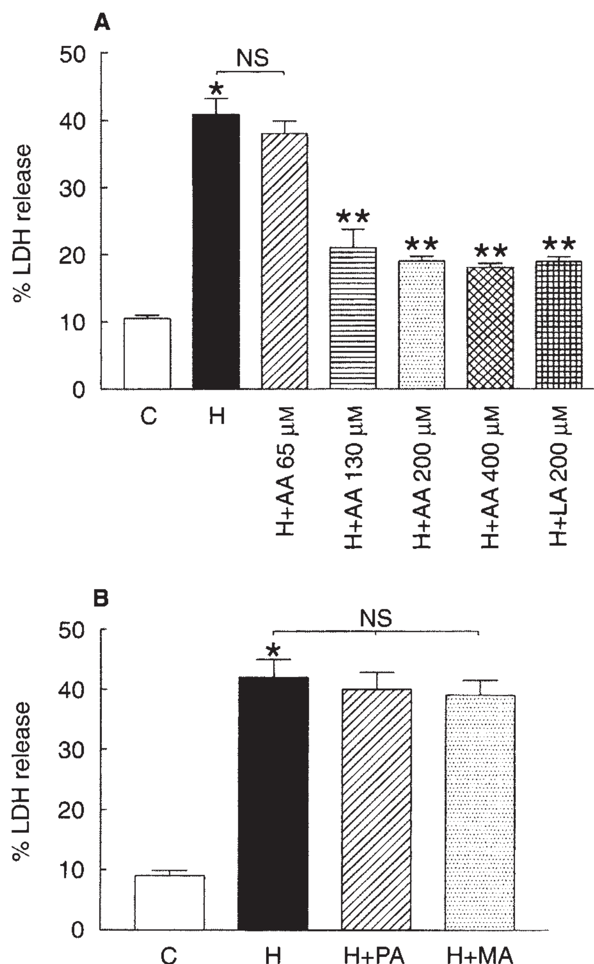

Fig. 1. A. *Effect of arachidonic acid (AA) (dose dependent effect) and linoleic acid (LA) on hypoxic cell injury.* Abbreviations are: C, control (N = 20); H, hypoxia 20 minutes (N = 20); H + AA 65 μM (N = 4); H + AA 130 μM (N = 4); H + AA 200 μM (N = 20); H + AA 400 μM (N = 4); H + LA 200 μM (N = 4). **B.** Lack of effect of palmitic acid (PA, 200 μM), or myristic acid (MA, 200 μM) on hypoxic cell injury (N = 4). Similarly, no effect was seen using 600 μM of PA. *$P < 0.001$ versus C **$P < 0.001$ versus H.

Effect of bovine serum albumin (BSA) on AA related protection

To rule out possible contaminants in the FFA preparation as possible mediators of the cytoprotective effect observed with AA, we preincubated the tubule preparation with BSA at a concentration of 1 g% for five minutes prior to AA administration. BSA has a strong binding affinity for FFA. In the presence of BSA, AA related cytoprotection was completely reversed. LDH = 10 ± 0.8% during N, 41 ± 4.1% during 20 minutes H, $P < 0.05$ versus N; 16 ± 1.2% during H + AA, $P < 0.05$ versus H; and 45 ± 3.4 during H + AA + BSA, P = NS versus H, all $N = 3$.

Effect of AA on buffer Ca^{2+}, cytosolic Ca^{2+} concentration, buffer pH and intracellular pH (pH_i)

To eliminate any possible artifact in the model which might explain the observed protection, the effect of AA on buffer Ca^{2+}, cytosolic Ca^{2+}, buffer pH and pH_i was determined. pH_i averaged 7.32 ± 0.007 (N = 28), 7.39 ± 0.012 (N = 28), and 7.42 ± 0.007

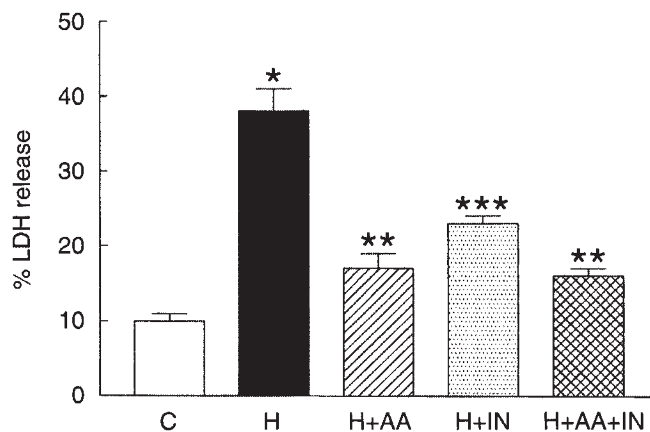

Fig. 2. *Effect of indomethacin (IN, 125 nM) on arachidonic acid (AA, 200 µM) related cytoprotection against hypoxic cell injury (H, 20 min), (all* N = *4).* *$P < 0.001$ versus control (C); **$P < 0.001$ versus H; ***$P < 0.01$ versus H.

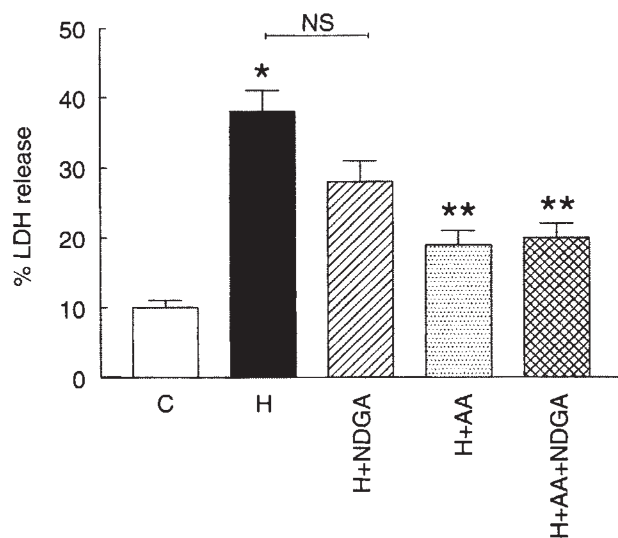

Fig. 3. *Effect of NDGA (50 µM) on arachidonic acid (AA,200 µM) related protection against hypoxic cell injury (H, 20 min) (all* N = *4).* *$P < 0.001$ versus control (C); **$P < 0.001$ versus H.

($N = 20$) in control, vehicle and tubules exposed to AA, respectively. Buffer pH averaged 7.4 at the end of the experiment in all tubule preparations. The ionized calcium concentration was 0.61, 0.62 and 0.64 mM in the control buffer, buffer plus AA and buffer plus PA, respectively. There was also no difference in the cytosolic calcium concentration in the tubules with and without treatment with AA.

Effect of FFA on cellular K^+

In our system K^+ starts to leak out of the cells within two minutes of hypoxia and reaches a nadir at 10 minutes. To determine whether AA prevents cellular K^+ depletion as a possible mechanism of the observed cytoprotection, we measured the effect of various FFA on cellular K^+ depletion during hypoxia as demonstrated in Table 1. No effect of FFA was seen at 7.5 or 20 minutes of hypoxia on the fall of cellular K^+.

Table 1. Lack of effect of arachidonic acid (AA), linoleic acid (LA), and palmitic acid (PA) on intracellular K^+ during hypoxia (H)

	K^+ *nmol/mg protein*	
	7.5 min	20 min
C	280 ± 8.5	220 ± 10.2
H	200 ± 9.6[a]	90 ± 5.4[a]
H+AA	180 ± 7.6[b]	88 ± 6.7[b]
H+LA	205 ± 10.2[b]	92 ± 4.6[b]
H+PA	170 ± 6.8[b]	87 ± 5.3[b]

All $N = 6$
[a] $P < 0.001$ vs. control (C)
[b] P = NS vs. H

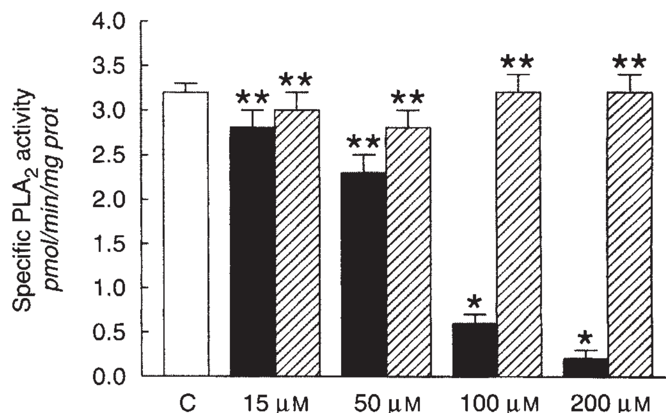

Fig. 4. *Effect of arachidonic acid (AA, solid bar) and palmitic acid (PA, hatched bar) on PLA_2 activity in vitro.* Control (C) = open bar. (All N = 4). *$P < 0.001$ versus C; **P = NS versus C.

Effect of AA and PA on PLA_2 activity

To determine whether FFA have any effect on PLA_2 activity in proximal tubules, AA or PA were preincubated with soluble extracts prepared from normoxic tubules, and PLA_2 activity was measured using ^{14}C-phosphatidylethanolamine as exogenous substrate. Figure 4 demonstrates the effect of the uFFA, AA, and the sFFA, PA on PLA_2 activity. AA resulted in a significant decrease in PLA_2 activity in a dose dependent manner. At doses of 100 and 200 µM, AA resulted in 80 and 90% inhibition of PLA_2 activity, respectively. No inhibition of PLA_2 activity was observed with a PA dose up to 200 µM.

Discussion

The rationale for studying AA and other FFA is based on the conflicting data on the role of FFA and PLA_2 in mediating hypoxic damage. Membrane phospholipid breakdown with accumulation of FFA, diacylglycerols, and lysophospholipids has been reported in different organs such as liver, heart and kidney following ischemia [1, 23, 24], and during hypoxia in isolated rabbit [25] and rat proximal tubules [15]. Various studies have suggested that the accumulation of these agents is secondary to the activation of PLA_2 [1, 6, 7]. Impaired phospholipid reacylation during hypoxia could also contribute to the accumulation of these compounds [7]. There is some evidence to suggest that the accumulation of lysophospholipids plays a pathophysiological role in proximal renal tubules during hypoxia [26]. Some investigators have shown that the addition of exogenous PLA_2 to hypoxic

isolated rabbit proximal tubules causes severe membrane phospholipid breakdown [13, 25]. On the other hand, in contrast to the evidence of a pathogenic role of PLA_2 during oxygen deprivation, others have reported a protective effect of exogenous PLA_2 against hypoxic injury [14].

The physiological role of FFA accumulation during hypoxic injury is not clear, and different results have been reported in different organs [1, 15, 23, 25–29]. We started our experiments using AA, the most important long chain uFFA released during hypoxia. The effect of AA was compared to that of other unsaturated and saturated FFA. Our data show that AA added to isolated rat proximal tubules significantly protects against hypoxic damage. AA is metabolized in the kidney through three different pathways, namely, cyclooxygenase, lipoxygenase and cytochrome P-450 pathway [30]. It was not clear initially whether the protective effect was secondary to AA itself or secondary to it's metabolites; thus, further experiments using cyclooxygenase and lipoxygenase inhibitors were performed. Inhibiting the cycloxygenase pathway with indomethacin or florbiprufen and the lipoxygenase pathway with NDGA did not reverse the cytoprotection of AA, thus eliminating prostaglandins, thromboxane A_2, prostacyclins and leukotrienes as potential mediators in this process. We did not investigate the cytochrome P 450 (C-P 450) pathway; however, the observation that another uFFA, LA, which is probably not metabolized by the C-P 450 pathway [31], had similar protection, strongly suggests that the protection is not caused by a metabolite of this pathway. We observed moderate cytoprotection with indomethacin alone, which could possibly be explained by the inhibition of AA degradation through the cyclooxygenase pathway thereby leading to accumulation of endogenous AA. NDGA alone also showed mild protection; however, that effect did not reach statistical significance.

To investigate whether the observed protective effect is specific for uFFA or can be seen with all long chain FFA, we studied the role of sFFA, PA and MA, in the same model of hypoxic proximal renal tubules. Neither of these sFFA had a protective effect against hypoxic injury in our model.

There is a difference in the physical properties of the different FFA in terms of solubility in aqueous solutions with the uFFA being more water soluble than the sFFA. To eliminate this solubility confounding factor in the observed difference in cytoprotection, we measured ^{3}H-labeled FFA uptake in the hypoxic tubules to assess intracellular accumulation of these agents. Intracellular accumulation of uFFA was higher than that of sFFA, and using a threefold larger dose of PA than AA still did not have any effect against hypoxic injury. Thus, our results indicate that the cytoprotective effect observed with AA and LA is specific for uFFA rather than being a property of all long chain FFA.

Several investigators have observed different biologic effects of uFFA as compared to sFFA on K^+ channel activation [27, 28, 32], GABA-gated chloride channel function [33], cytoskeleton [29], Na,K-ATPase activity [34], and platelet aggregation [35]. To investigate whether the cytoprotection in our model is related to inhibition of cellular K^+ depletion, we measured cellular K^+ in the presence of various FFA during hypoxia. There was clearly no effect of any of the FFA on cellular K^+ depletion during hypoxia. We then looked at the possible negative feedback inhibition of endogenous PLA_2 activity in the proximal renal tubules. Our results show that the uFFA, AA, in a dose dependent manner, but not the sFFA, PA, significantly inhibited PLA_2 activity in the proximal tubules. Inhibition of PLA_2 activity was observed at doses of AA between 50 to 100 μM. However, two- to fourfold higher concentrations of PA did not have any effect on PLA_2 activity.

Our laboratory has reported that decreasing the accelerated rate of calcium influx into tubular cells using the Ca^{2+} channel blocker verapamil protects against hypoxic damage [16, 22]. Extracellular acidosis has also been reported to provide cytoprotection against hypoxic injury in various organs including the proximal renal tubules [36–38]. Therefore, to determine whether AA affected Ca^{2+} in the buffer or cytosol or induced a change in pH so as to be responsible for the observed protection, we measured buffer pH, intracellular pH, buffer Ca^{2+} and intracellular Ca^{2+}. AA did not affect any of these parameters.

Matthys et al [1] and Wetzels et al [15] have shown a cytotoxic effect of AA when used in calcium free medium. In our model of isolated proximal tubules, calcium is essential for the viability of cells and the damage observed in those studies may have been attributed to the increased cellular susceptibility caused by the total absence of calcium in the medium rather than by AA itself. In support of our finding, Humes et al have reported that addition of bovine serum albumin (BSA) to hypoxic tubules actually potentiated the degree of injury despite a reduction of FFA values close to control values [25]. BSA is known to have a strong affinity to bind FFA. In our model BSA added at a concentration of 1 g% also reversed the cytoprotection of AA. Thus both the concentration of AA and extracellular calcium appear to be important variables which may determine whether or not hypoxia-induced cell damage is observed.

Zager et al have recently shown a protective effect of exogenous pancreatic PLA_2 when added to hypoxic rat proximal tubules [14]. They observed injurious effects of snake and bee venom PLA_2 added at a large dose to hypoxic tubules; however, smaller doses were also protective in their model. In that model it is conceivable that a small dose of exogenous PLA_2 was able to release enough uFFA including AA from the cell membranes to inhibit endogenous PLA_2 activity without causing significant damage. On the other hand, a higher dose of exogenous PLA_2 may have caused severe membrane breakdown, therefore overriding any effect of AA to inhibit endogenous PLA_2 activity. In the present study, *in vitro* measurement of PLA_2 clearly showed that AA reduced PLA_2 activity. These results suggest that AA may have modulated the activity of endogenous PLA_2 during hypoxia.

Our data confirm previous observations that uFFA exhibit different functional activity compared with sFFA. The exact mechanism by which AA and other uFFA protect against cell injury is not clear. Our finding that PLA_2 activity is significantly inhibited by AA supports the hypothesis that there is an enzyme substrate feedback inhibition between PLA_2 and AA. Although other effects of uFFA may have a role in this cytoprotection, the current results strongly implicate inhibition of PLA_2 as a potential mechanism of cytoprotection. PLA_2 inhibition would lead to decreased generation of lysophospholipids, which are believed to cause cell membrane disruption and cytotoxicity [26, 39]. Exogenous uFFA also may inhibit the production of lysophospholipids by enhancing reacylation of membrane phospholipids. Such potential effects of lysophospholipids in mediating hypoxia-induced proximal tubular damage and its modulation by AA are in need of further research.

In summary, accumulation of FFA during hypoxia does not

appear to be injurious to rat renal tubular cells. Rather AA protects against hypoxia-induced cell injury. This cytoprotection is not specific for AA but also observed with other uFFA, that is, LA, and may be related to inhibition of phospholipase A_2 activity.

Acknowledgments

This research was supported by grants from the NIH, DK 35098, DK 39902. Dr. Alkhunaizi was the recipient of a Colorado National Kidney Foundation (NKF) 560 fellowship.

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