Importance of Adenosine Triphosphate in Phospholipase A_2-induced Rabbit Renal Proximal Tubule Cell Injury

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Abstract

The pathogenesis of ischemic renal tubular cell injury involves a complex interaction of different processes, including membrane phospholipid alterations and depletion of high-energy phosphate stores. To assess the role of membrane phospholipid changes due to activation of phospholipases in renal tubule cell injury, suspensions enriched in rabbit renal proximal tubule segments were incubated with exogenous phospholipase A_2 (PLA_2). Exogenous PLA_2 did not produce any significant change in various metabolic parameters reflective of cell injury in control nonhypoxic preparations despite a significant decrease in phosphatidylethanolamine (PE) and moderate increases in lysophosphatidylcholine (LPC) and lysophosphatidylethanolamine (LPE). In contrast, exogenous PLA_2 treatment of hypoxic tubules resulted in a severe degree of cell injury, as demonstrated by marked declines in tubule K^+ and ATP contents and significant decreases in tubule uncoupled respiratory rates, and was associated with significant phospholipid alterations, including marked declines in phosphatidylcholine (PC) and PE and significant rises in LPC, LPE, and free fatty acids (FFA). The injurious metabolic effects of exogenous PLA_2 on hypoxic tubules were reversed by addition of ATP-$MgCl_2$ to the tubules. The protective effect of ATP-$MgCl_2$ was associated with increases in tubule PC and PE contents and declines in LPC, LPE, and FFA contents. These experiments thus indicate that an increase in exogenous PLA_2 activity produces renal proximal tubule cell injury when cell ATP levels decline, at which point phospholipid resynthesis cannot keep pace with phospholipid degradation with resulting depletion of phospholipids and accumulation of lipid by-products. High-energy phosphate store depletion appears to be an important condition for exogenous PLA_2 activity to induce renal tubule cell injury.

Introduction

The pathogenesis of ischemic renal tubular cell injury involves a complex interaction of various processes, including phospholipid alterations, depletion of high-energy phosphate stores, accumulation of oxygen free radicals, and calcium alterations (1). Most evidence favors the idea that the critical events determining the course of cellular injury develop from damage to plasma and subcellular membranes (1). Phospholipids provide the major structural framework for cell membranes (2) and also participate in the regulation of membrane enzyme activity, permeability, and hormone activation (3–6). Thus, phospholipids play a major role in the maintenance of membrane function and cell viability. Ischemic injury has been associated with depletion of major phospholipids and accumulation of phospholipid by-products in liver, heart, and renal cortex (7–10). The alterations in membrane phospholipids have been attributed to the action of endogenous membrane phospholipases which are presumably activated by ischemia-induced increases in the cytosolic free calcium concentration (11). Accordingly, the activation of membrane phospholipases, especially phospholipase A_2 (PLA_2),[1] has been suggested to be a critical early event in the pathogenesis of ischemic cell injury.

To evaluate the possible role of PLA_2 activation in developing renal proximal tubule cell injury, we undertook a series of studies to evaluate the effects of exogenous PLA_2 on the viability of rabbit proximal tubule segments in suspension and to define the conditions in which exogenous PLA_2 produces cell injury in this preparation.

Methods

Tubule isolation. Suspensions enriched in separated proximal tubule segments were prepared as described for this laboratory (12, 13). New Zealand white rabbits weighing an average of 2.5 kg were used for these studies. For a typical experiment, four rabbits were used. In brief, suspensions of renal proximal tubule segments were obtained after collagenase (type I, Worthington Biochemical Corp., Freehold, NJ) treatment of minced renal cortices followed by Percoll (Pharmacia, Inc., Piscataway, NJ) gradient centrifugation. By morphologic criteria this preparation has been found to be composed of a highly enriched suspension of proximal tubule segments with virtually no contamination with glomeruli or more distal nephron segments.

The final tubule pellet was resuspended to a concentration of 5–7.5 mg tubule protein/ml in a 95% O_2/5% CO_2 gassed ice-cold solution containing 105 mM NaCl, 2.2 mM KCl, 24.6 mM $NaHCO_3$, 2.3 mM KH_2PO_4, 1.3 mM $CaCl_2$, 2.4 mM $MgSO_4$, 0.6% dialyzed dextran (Pharmacia T40), and metabolic substrates including 5 mM glucose, 4 mM lactic acid, 1 mM alanine, and 10 mM butyric acid (pH 7.0) (solution A). 5-ml aliquots of this tubule suspension were placed in 25-ml Erlenmeyer flasks which were then gassed with 95% O_2/5% CO_2, sealed, and kept on ice until use.

Measurement of tubule respiration. A sample of tubule suspension was placed in a 1.9-ml sealed, temperature-controlled (37°C) chamber

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1. *Abbreviations used in this paper:* CCCLP, carbonyl cyanide *m*-chlorophenylhydrazone; LPC, lysophosphatidylcholine; LPE, lysophosphatidylethanolamine; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PLA_2, phospholipase A_2; TAN, total adenine nucleotides.

equipped with a magnetic stirrer and a Clarke O_2 electrode (Gilson Medical Electronics, Inc., Middleton, WI) and oxygen consumption was measured. Basal tubule respiration was measured in the presence of 5 mM glucose, 4 mM sodium lactate, 1 mM alanine, and 10 mM butyric acid (pH 7.0) (14). Uncoupled tubule respiration was measured in the presence of supramaximal levels (10 μM) of the mitochondrial uncoupler carbonyl cyanide *m*-chlorophenylhydrazone (CCCLP). Respiratory rates were then calculated and expressed as nanoatoms of oxygen per minute per milligram of protein.

Measurement of tubule electrolyte content. For measurement of total tubule cell cation levels, the tubules were rapidly separated from their suspending medium by layering 0.5 ml of tubule suspension into a 1.5-ml microcentrifuge tube on 0.7 ml of bromododecane (Aldrich Chemical Co., Milwaukee, WI), which was previously layered on 0.2 ml of 275 mM sucrose containing 4% Ficoll (Pharmacia). The tube was then centrifuged in an Eppendorf microcentrifuge for 1.5–2.0 min to pellet the tubules through the bromododecane layer to the bottom sucrose layer. The layers above the tubule pellet were aspirated off and the pellet was then resuspended in 0.5 ml of deionized water. Electrolyte (K^+ and Ca^{2+}) levels were measured by atomic absorption spectrophotometry using methods previously reported (15).

Measurement of tubule adenine nucleotide metabolism. To determine tubule levels of adenine nucleotides, a procedure similar to that utilized for determination of tubule electrolytes was used, except that tubules were centrifuged through bromododecane into 0.4 ml 12% trichloroacetic acid (TCA). The volume of the TCA layer was brought up to 0.8 ml with solution A, and the samples were then remixed and recentrifuged. The supernatant was removed from the protein pellet, neutralized, and extracted by mixing with an equal volume of 0.5 M tri-*n*-octylamine (Aldrich Chemical Co.) in Freon (Matheson Gas Products, Inc., East Rutherford, NJ).

Separation of the Freon and aqueous layers was facilitated by a brief centrifugation. The aqueous layer was then removed, filtered, and frozen. Levels of AMP, ADP, and ATP were determined with use of a Whatman Partisil 10 SAX HPLC column (Whatman Chemical Separations Inc., Clifton, NJ). AMP, ADP, and ATP were eluted with a 5 to 0.5 mM ammonium phosphate gradient at a flow rate of 1.5 ml/min at room temperature. Total adenine nucleotide (TAN) tubule content was determined as the sum of ATP, ADP, and AMP contents.

Protein assay. Proteins were quantified by the Lowry assay (16).

Lipid extraction. Lipids from tubules were extracted using a procedure modified from Folch et al. (17). 4 ml of tubule suspension was added to 4 ml of cold buffer containing 117 mM NaCl, 2.3 mM KH_2PO_4, 2.2 mM KCl, 1.3 mM $CaCl_2$, 2.4 mM $MgSO_4$, and 24.6 mM $NaHCO_3$, previously gassed with and kept under 95% O_2/5% CO_2 and then centrifuged for 1.5 min at 1600 rpm. After the supernatant was removed, 4 ml of chloroform-methanol (2:1) were added to the tubule pellet and the extract mixture was then vortexed. After 60 min, 0.8 ml of distilled water was added, then the mixture was vortexed and centrifuged at 2,000 rpm for 20 min. The system separates into two layers (upper aqueous-methanol and lower chloroform) with the tissue residue in the interphase. The chloroform layer contains the extracted lipids. The two layers were removed separately using a Pasteur pipette. The tissue residue was reextracted a second time following similar steps. The combined chloroform layers were then dried under N_2 at room temperature. The lipid extract obtained was redissolved in chloroform and filtered through glasswool to remove impurities. After removal of one aliquot for determination of total lipid phosphorus, the extract was stored at −20°C for later phospholipid analysis.

Phospholipid separation. Phospholipids were separated by two-dimensional thin-layer chromatography (TLC) using 0.50-mm silica gel H plates with 10% magnesium acetate (Uniplate, Analtech, Inc., Newark, DE) following a procedure modified from Troyer et al. (18). The first solvent system was chloroform/methanol/ammonium hydroxide, 70:25:5.5 and the second chloroform/acetone/methanol/acetic acid/water, 30:40:10:10:5. The solvents were prepared and added to glass chambers lined with filter paper and left overnight to equilibrate. The following morning, the plate was first activated for 1 h at 110°C, cooled for 3 min, and spotted with 25 or 20% of total phospholipids (corresponding to ~ 3–7 mg tubule protein), and the chromatogram was developed in the chamber containing the first solvent. After 100 min, the plate was removed from the chamber, air dried at room temperature for 10 min, then placed in a vacuum oven for 45 min at 45–50°C. Every 5 min, dry air was run through the oven to allow evacuation of evaporated solvent. The plate was then cooled for 1 min at room temperature before placement in the chamber containing the second solvent for 60–75 min until the solvent ran to 0.25 cm from the top of the plate. The plate was then dried for 45 min at room temperature before exposure to iodine vapor for detection of spots. The identity of these spots was confirmed by co-chromatography with known lipid standards. These spots were scraped and phospholipid phosphorus was determined.

Phospholipid phosphorus determination. The mass of phospholipids separated by two-dimensional TLC was determined according to a modified method of Rouser et al. (19). The spots were scraped from TLC plates and digested with 0.3 ml of 70% perchloric acid in a borosilicate glass tube at 220°C for 1 h. After digestion, 0.94 ml of distilled water, 0.4 ml of 1.25% ammonium molybdate solution, and 0.4 ml of 5% ascorbic acid were added to the tube. The tube was heated in a water bath at 90°C for 7.5 min, then centrifuged at 2,000 rpm for 20 min. The sample was transferred to a cuvette and the optical density determined by spectrophotometer at 640 nm and 797 nm. The value was then converted to micrograms of phosphorus using a factor derived from a standard curve prepared using NaH_2PO_4. Recovery of lipid phosphorus by this methodology was ~ 85–95%.

Total free fatty acid determination. Free fatty acid (FFA) assay was based on the modified method of Matsubara et al. (20) using the acyl-coenzyme A (CoA) synthetase and acyl-CoA oxidase system, which produced hydrogen peroxide through the enzymatic oxidation of FFA. The Ti-PAR reagent [a mixture of Ti (IV) and 4-(2-pyridylazo) resorcinol] was used in the colorimetric determination of hydrogen peroxide generated by previous reactions. The absorbance was measured at 508 nm. A standard curve was generated using palmitic acid. All measurements were made with reference to a reagent blank. Each unknown sample's reading was also referenced to a corresponding turbidity blank. Data was expressed as micrograms of FFA per milligram of protein. The data obtained by this method were similar to those obtained by gas liquid chromatography.

Experimental design. After isolation, 25-ml flasks containing 5 ml of tubule suspension were transferred from ice to a shaking water bath at 37°C and gassed with 95% O_2/5% CO_2. After 15 min of warm incubation, the flasks were gassed for 2 min, with either 95% N_2/5% CO_2 or 95% O_2/5% CO_2 and various agents, including PLA_2 (0.15 U or 1.5 U/mg protein) and/or ATP-$MgCl_2$ (250 μM or 2 mM) with or without fatty acid-free bovine serum albumin (BSA) (10 mg/ml), were added and incubated for an additional period of 22.5 min. After a cumulative time of 37.5 min, all flasks were reoxygenated with 95% O_2/5% CO_2 and incubated again at 37°C for 37.5 additional min. Samples from various tubule suspensions were taken for measurement of tubule respiratory rates, cation levels, adenine nucleotide, and phospholipid contents at cumulative time of 75 min.

To assess the utilization of FFA in phospholipid synthesis during these experiments, 2 μCi [1-^{14}C]linoleic acid (21) was added to flasks containing tubule suspensions immediately after the 15 min preincubation period. At various time points after reoxygenation, samples were taken to assess the incorporation of the radiolabel into phospholipids.

Reagents. All reagents used were of the highest grade commercially available. All organic reagents were obtained from Sigma Chemical Co. (St. Louis, MO) unless otherwise indicated. PLA_2 extracts of bee venom or of *Naja mocambique-mocambique* venom, which are neutral active, devoid of proteolytic activity, and melittin free (22), were used for these experiments. [1-^{14}C]Linoleic acid was purchased from New England Nuclear Research Products (Boston, MA).

Statistical analysis. Statistical analysis was performed using analysis of variance (ANOVA) to identify significant differences between

treatment groups for each set of grouped experiments. ANOVA was considered significant if $\alpha < 0.05$. If significant differences between treatment groups were identified by ANOVA, specific comparisons between mean values for various parameters were accomplished using Tukey's multiple comparison procedure in conjunction with the Studentized Range Critical Values Table.

Results

Control conditions. The functional parameters of control preparations incubated for 75 min at 37°C are summarized in Table I. Average control basal respiratory rates were 66 natoms oxygen/min · mg protein. Respiratory rates increased to 221 natoms of oxygen/min · mg protein with CCCLP (CCCLP-uncoupled respiration). Average tubule K^+ content was 348 nmol/mg protein. Tubule Ca^{2+} levels averaged 14.6 nmol/mg protein after 75 min of incubation at 37°C. Cell ATP contents averaged 6.85 nmol/mg protein, average cell AMP levels were 0.17 nmol/mg protein, and ADP levels were 0.81 nmol/mg protein. Average cell TAN contents were 7.84 nmol/mg protein.

The percent distribution of phospholipid inorganic phosphorus (P_i) of tubule suspensions is summarized in Table II. Phosphatidylcholine (PC) and phosphatidylethanolamine (PE) were the major membrane phospholipids and accounted for 34.3 and 29.2% of total phospholipid P_i. The levels of the lysophospholipids, lysophosphatidylcholine (LPC) and lysophosphatidylethanolamine (LPE), were small and accounted for 0.33 and 0.31%. The percent distribution of various other phospholipids are reported in Table II. Total P_i averaged 8.41 μg/mg protein. The contents of individual phospholipids as micrograms P_i per milligram protein are summarized in Table III.

Table I. Effects of Exogenous PLA_2 Treatment on Tubule Metabolic Parameters under Oxygenated Conditions

Metabolic parameters	Control	PLA_2 (0.15 U/mg protein)	PLA_2 (1.5 U/mg protein)
		natoms O/min · mg protein	
Respiratory rates			
Basal	66±1.8	70±2.3	70±2.3
CCCLP (uncoupled)	221±8.7	227±6.3	238±6.5
		nmol/mg protein	
Cation contents			
K^+	348±6	378±11.9*	379±13*
Ca^{2+}	14.6±0.8	13.6±0.6*	12.1±0.7‡
		nmol/mg protein	
Adenine nucleotide contents			
AMP	0.17±0.01	0.19±0.02	0.21±0.02
ADP	0.81±0.05	0.88±0.03	0.88±0.05
ATP	6.85±0.21	7.42±0.25	7.25±0.32
TAN	7.84±0.24	8.50±0.26	8.62±0.23

All values given are means±SE (n = 4–8).
* $P < 0.05$ compared to control values.
‡ $P < 0.01$ compared to control values.

Table II. Alterations in the P_i Contents of Phospholipids and Lysophospholipids in Proximal Tubule Suspensions during Exposure to Exogenous PLA_2 under Oxygenated Conditions

Phospholipid P_i	Control	PLA_2 (0.15 U/mg protein)	PLA_2 (1.5 U/mg protein)
		%	
Major phospholipids			
PC	34.3±0.2	33.3±0.4	33.9±0.6
PE	29.2±0.2	27.8±0.3	26.1±1.0*
Lysophospholipids			
LPC	0.38±0.02	1.62±0.10*	2.28±0.30*
LPE	0.31±0.02	1.90±0.15*	2.55±0.24*
Other phospholipids			
Phosphatidic acid	0.57±0.05	0.52±0.05	0.55±0.03
Phosphatidylserine	6.77±0.10	6.78±0.13	6.86±0.24
Phosphatidylinositol	6.00±0.10	5.96±0.15	5.87±0.22
Sphingomyelin	15.23±0.31	15.75±0.28	14.48±1.20
Cardiolipin	7.23±0.10	7.25±0.10	7.33±0.14

All values given are means±SE (n = 8).
* $P < 0.01$ compared to control values.

Effects of exogenous PLA_2 on oxygenated tubules. Exogenous PLA_2 treatment (0.15 U/mg protein) did not result in any deleterious effects in metabolic parameters during oxygenated conditions (Table I). Even a higher dose of PLA_2 (1.5 U/mg protein) did not produce deleterious alterations. Instead, both basal and CCCLP-uncoupled respiratory rates showed minimal increases above control rates. Cell K^+ and cell Ca^{2+} levels showed small but significant changes from control values.

Although exogenous PLA_2 caused a mild but significant decline in PE and moderate significant increases in LPC and LPE compared to control values (Table II), there was no metabolic changes reflective of cell injury. The degree of changes in PE, LPC, and LPE were dose related.

Thus, treatment of oxygenated tubules with exogenous PLA_2 produced mild declines in tubule levels of PE and moderate accumulation of the lipid by-products, LPC and LPE, but did not lead to alterations in metabolic parameters suggestive of cell injury. Of note, these phospholipid alterations were

Table III. Membrane Phospholipid P_i Contents of Proximal Tubule Suspensions under Oxygenated Conditions

Phospholipids	Phospholipid P_i
	μg P_i/mg protein
PC	2.88±0.078
PE	2.45±0.059
LPC	0.03±0.002
LPE	0.03±0.002
Phosphatidic acid	0.05±0.004
Phosphatidylserine	0.57±0.016
Phosphatidylinositol	0.50±0.016
Sphingomyelin	1.28±0.048
Cardiolipin	0.61±0.015
Total phospholipid	8.41±0.220

Table IV. Metabolic Parameters of Proximal Tubule Suspensions Treated with Exogenous PLA_2 and ATP-$MgCl_2$ under Hypoxic Conditions

Metabolic parameters	Control	Hypoxia	Hypoxia + PLA_2	Hypoxia + PLA_2 + ATP-$MgCl_2$
			natoms 0/min · mg protein	
Respiratory rate				
CCCLP (uncoupled)	215±8.6	155±14.6‡	107±14.6‖	127±14.9
			nmol/mg protein	
Cation contents				
K^+	333±8	291±9*	174±18‖	244±17**
Ca^{2+}	12.4±0.7	14.9±0.6	13.4±0.9	12.2±0.7
			nmol/mg protein	
Adenine nucleotide contents				
AMP	0.20±0.02	0.23±0.03	1.58±0.19‖	0.98±0.16**
ADP	0.85±0.06	0.80±0.07	1.40±0.09‖	1.24±0.09
ATP	6.30±0.46	4.87±0.36	2.70±0.32‖	4.78±0.46**
TAN	7.36±0.53	5.70±0.47*	5.67±0.31	7.01±0.35

All values given are means±SE (n = 10–13). * $P < 0.05$; ‡$P < 0.01$ compared to control values. ‖ $P < 0.01$ compared to hypoxia values; ** $P < 0.01$ compared to hypoxia + PLA_2 values.

accompanied with modest improvement of these variables as reflected by mild increases in tubule K^+ content and decreases in Ca^{2+} levels.

Treatment of hypoxic tubules with exogenous PLA_2. Since tubule cells may resist the injurious effect of PLA_2 by increasing resynthesis of phospholipids, tubule suspensions were gassed with 95% N_2/5% CO_2 for 22.5 min at the beginning of the incubation with exogenous PLA_2 in order to decrease cell oxidative phosphorylation capacity, thereby potentially impairing resynthesis of phospholipids. Hypoxia alone caused moderate changes in metabolic parameters reflective of cell injury with moderate significant declines in uncoupled respiratory rates, cell K^+, and TAN contents compared to control values (Table IV). This short course of hypoxia did not cause any change in the composition of major phospholipids including PC, PE, and the by-products LPC and LPE (Table V). The changes in other phospholipids are summarized in Table V. Hypoxic tubules treated with exogenous PLA_2 (0.15 U/mg protein) developed severe cell injury as reflected by significant decreases in tubule uncoupled respiratory rates and K^+ and ATP contents compared to hypoxia values. The severe degree of cell injury seen in hypoxic tubules treated with exogenous PLA_2 was associated with major changes in membrane phospholipids with markedly significant declines in PC and PE and significant increases in LPC and LPE levels (Table V) compared to values seen in hypoxic tubules. Changes in other

Table V. Alterations in Contents of P_i of Various Phospholipids in Proximal Tubule Suspensions during Exposure to Exogenous PLA_2 under Hypoxic Conditions with and without Addition of ATP-$MgCl_2$

Phospholipid P_i (percent of total)	Control	Hypoxia	Hypoxia + PLA_2	Hypoxia + PLA_2 + ATP-$MgCl_2$
Major phospholipids				
PC	34.3±0.37	34.3±0.40	26.6±0.88‖	30.8±0.63
PE	29.6±0.38	30.0±0.28	19.0±0.52‖	23.1±0.75**
Lysophospholipids				
LPC	0.44±0.06	0.46±0.06	10.0±0.60‖	5.76±0.67**
LPE	0.33±0.03	0.43±0.06	10.9±0.60‖	6.75±0.66**
Other phospholipids				
Phosphatidic acid	0.64±0.07	0.61±0.08	0.63±0.24	0.62±0.05
Phosphatidylserine	6.78±0.12	6.35±0.09*	4.98±0.11‖	5.61±0.10**
Phosphatidylinositol	5.80±0.11	6.18±0.13*	6.36±0.19	5.87±0.12¶
Sphingomyelin	15.03±0.38	13.46±0.23‡	14.20±0.23§	14.51±0.18
Cardiolipin	6.94±0.15	8.24±0.17‡	7.46±0.50	7.02±0.31

All values are presented as means±SE (n = 11–13). * $P < 0.05$; ‡ $P < 0.01$ compared to control values. § $P < 0.05$; ‖ $P < 0.01$ compared to hypoxia values. ¶ $P < 0.05$; ** $P < 0.01$ compared to hypoxia + PLA_2 values.

Table VI. Alterations in the FFA Content and P_i Contents of Important Phospholipids and Lysophospholipids in Proximal Tubule Suspensions during Exposure to Exogenous PLA_2, Fatty Acid-free BSA and ATP-$MgCl_2$ under Hypoxic Conditions

Lipid group	Control	Hypoxia	Hypoxia + PLA_2	Hypoxia + PLA_2 + BSA	Hypoxia + PLA_2 + BSA + ATP-$MgCl_2$
			Phospholipid P_i (percent of total)		
PC	34.50±0.45	34.60±0.44	27.10±1.16‡	25.48±1.24	27.50±0.66
PE	29.70±0.48	29.96±0.34	19.45±0.72‡	18.77±0.98	20.64±0.77
LPC	0.48±0.08	0.44±0.08	9.82±0.85‡	7.73±1.02	5.95±0.63
LPE	0.33±0.04	0.40±0.07	10.76±0.86‡	10.16±1.04	8.26±0.79
			Total FFA (μg/mg protein)		
FFA	1.21±0.12	2.25±0.17	26.18±4.99‡	8.69±2.19‖	8.20±1.69

All values given are means±SE ($n = 6$). * $P < 0.05$; ‡ $P < 0.01$ compared to hypoxia values. § $P < 0.05$; ‖ $P < 0.01$ compared to hypoxia + PLA_2 values.

phospholipids are again displayed in Table V. Since PLA_2 specifically removes the fatty acid from the second carbon of glycerol with resulting increases in LPC and LPE and decreases in PC and PE, and since these changes were observed in these experiments, these findings are highly suggestive that cell injury was due to the direct effect of exogenous PLA_2 on membrane phospholipids rather than other potentially contaminating substances in the PLA_2 preparations.

Protective effects of ATP-$MgCl_2$. Since hypoxia potentiated the injurious effects of exogenous PLA_2 on proximal tubules, and this effect was associated with a decline in high-energy phosphate levels, 250 μM ATP-$MgCl_2$ was added to tubules treated with exogenous PLA_2 at the beginning of hypoxic period in order to replete tubule ATP contents and to assess whether this maneuver protected against this injury process. ATP-$MgCl_2$ provided significant protective effects as demonstrated by significant increases in tubule K^+ and ATP contents compared to values observed in hypoxic tubules treated with exogenous PLA_2 alone (Table IV). The improvement of these metabolic parameters was associated with increases in PE levels and significant decreases in LPC and LPE levels (Table V) compared to values observed in hypoxic tubules treated with exogenous PLA_2. Other phospholipids again showed some changes (Table V). Higher doses of ATP-$MgCl_2$ (2 mM) produced further increases in PC and PE contents to 31.8±0.5% and 26.7±0.8%, respectively, and further decreases in LPC and LPE contents of 2.5±0.4% and 4.0±0.6%, respectively, compared to values seen with 250 μM ATP-$MgCl_2$. In addition, 2 mM ATP-$MgCl_2$ addition to tubules in the presence of hypoxia and exogenous PLA_2 led to a significant reduction in FFA levels from 26±5 to 9±2 μg/mg protein ($P < 0.01$). This improvement in phospholipid levels towards normal values was associated with marked further improvement in uncoupled respiratory rates to 184±3 natoms oxygen/mg protein · min, which was significantly higher than the value of 117±6 observed in hypoxic tubules treated with exogenous PLA_2 ($P < 0.01$), but was still significantly lower than the control value of 234±7 ($P < 0.01$). In addition, 2 mM ATP-$MgCl_2$ also produced major increases in cell K^+ content to 360±25 nmol/mg protein, a level even higher than the value of 338±16 seen in control oxygenated tubules. Similarly, as expected, tubule contents of ATP reached supranormal values of 18.2±2.0 nmol/mg protein with 2 mM ATP-$MgCl_2$ and were significantly ($P < 0.01$) higher than the values of 7.6±0.5 observed in control oxygenated tubules. This large rise in tubule ATP content may not only be due to an increase in cellular stores but also to binding to the plasma membrane.

This protective effect of ATP-$MgCl_2$ on the magnitude of exogenous PLA_2 and hypoxia induced cell injury was associated with increases in PC and PE levels and declines in LPC, LPE, and FFA levels. These improvements in lipid levels towards normal values could be due to (*a*) an effect of ATP-$MgCl_2$ to inhibit PLA_2 activity, thereby diminishing PLA_2-induced phospholipid breakdown, or (*b*) an effect to provide energy during hypoxia, thereby enhancing phospholipid resynthesis from precursors formed from increased exogenous PLA_2 activity. To determine between these two possibilities, fatty acid-free BSA was utilized. BSA has the ability to avidly bind FFA so that FFA released from exogenous PLA_2-induced phospholipid degradation cannot be reutilized for resynthesis of phospholipids. In this regard, as demonstrated in Table VI, the presence of BSA in tubule preparations treated with hypoxia and exogenous PLA_2 resulted in a significant decline in accumulated FFA and no changes in PC, PE, LPC, and LPE levels, findings consistent with a BSA effect to bind FFA released from exogenous PLA_2 action. This decline in FFA availability with BSA would lead to differing results depending upon which mechanism ATP-$MgCl_2$ increases PC and PE levels and decreases LPC and LPE levels during exogenous PLA_2 and hypoxic exposure, as summarized in Table V. If ATP-$MgCl_2$ enhances phospholipid resynthesis during hypoxia and exogenous PLA_2 exposure, the presence of BSA in the tubule suspension treated with hypoxia, exogenous PLA_2, and ATP-$MgCl_2$ would lead to no increase in PC and PE levels and no decrease in LPC and LPE levels compared to those levels observed with hypoxia and exogenous PLA_2 without BSA due to the unavailability of released FFA (now bound to BSA) to be reutilized for phospholipid synthesis. In other words, BSA would block the phospholipid alterations induced by ATP-$MgCl_2$ in suspensions exposed to hypoxia and exogenous PLA_2. On the other hand, if ATP-$MgCl_2$ diminishes exogenous PLA_2 activity, the presence of BSA should not affect the increase in PC and PE and decline in LPC and LPE produced by ATP-$MgCl_2$ in tubule preparations exposed to exogenous PLA_2 and hypoxia, since the presumed inhibition of PLA_2 activity by ATP-$MgCl_2$ would still result in less PC and PE

breakdown. The binding of released FFA by BSA would not alter this presumed inhibitory effect of ATP-$MgCl_2$ on PLA_2 activity. In this regard, as demonstrated in Table VI, BSA addition to the tubule suspensions treated with hypoxia, exogenous PLA_2, and ATP-$MgCl_2$ resulted in no change in PC, PE, LPC, and LPE levels. This finding thus suggests that ATP-$MgCl_2$ promotes phospholipid synthesis rather than suppresses exogenous PLA_2 activity. This effect of ATP-$MgCl_2$ to increase phospholipid resynthesis, which diminishes the degree of phospholipid depletion and lysophospholipid accumulation, was then associated with less deleterious alterations in the metabolic parameters reflective of tubule cell injury.

To further assess the role of ATP-$MgCl_2$ to enhance phospholipid synthesis during concurrent exogenous PLA_2 and hypoxia exposure, the rate of incorporation of radiolabeled linoleic acid into phospholipids immediately after hypoxia and during the reoxygenation period was assessed. ATP-$MgCl_2$ (2 mM) addition to suspensions exposed to both exogenous PLA_2 and hypoxia resulted in significantly ($P < 0.05$ or better) greater incorporation of [^{14}C]linoleic acid into phospholipids at all time points (0, 7.5, 15, 30 min) of reoxygenation from an average of $70.56 \pm 16.88 \times 10^3$ dpm/ml of tubule suspension in the absence of ATP-$MgCl_2$ to $141.94 \pm 20.75 \times 10^3$ dpm/ml in the presence of ATP-$MgCl_2$ ($n = 3$).

Discussion

Alterations in phospholipid metabolism during ischemia have been suggested to be a critical process in the pathogenesis of ischemic cell injury in a variety of tissues, including liver, heart, and kidney (7–10). Interruption of the blood supply to rat liver produces a progressive loss of phospholipids from ischemic cells (7). Whole homogenates and microsomes from livers made ischemic for 3 h developed 40% and 55% declines in total phospholipid levels, respectively, compared to control values. PC and PE were predominantly affected without accumulation of either LPC or LPE. This phospholipid depletion was due to an accelerated rate of degradation of phospholipids (7). The accelerated phospholipid degradation was most likely due to increases of membrane phospholipase activity. Stimulated phospholipase activity has also been suggested to occur in renal ischemia with the observation that accumulation of FFA and lysophospholipids (9), which are hydrolysis products of PLA_1 and PLA_2 which specifically remove fatty acid esters from glycerol, develops after renal ischemia. A relatively larger increase in polyunsaturated FFA (9, 23) than in saturated FFA has been observed in cerebral and renal ischemic injury, indicating a predominant action of PLA_2 since the acyl group attached to the second carbon of glycerol is usually an unsaturated fatty acid. Evidence of increased phospholipase activity during ischemia has also been demonstrated in rat hepatocytes in culture (24) and canine myocardium (8, 25–27).

Our study provides further insight into the role of phospholipases in renal ischemia by assessing the effect of exogenous PLA_2 on renal proximal tubule segments in vitro. Exogenous PLA_2 treatment of oxygenated proximal tubules did not cause any alterations in a variety of metabolic parameters reflective of cell injury despite mild depletion of PE and moderate accumulation of lysophospholipids.

This observation may be due to the fact that intact tubular cells in the presence of oxygen are able to increase oxidative phosphorylation and maintain high-energy phosphate stores allowing effective resynthesis of membrane phospholipids including reacylation of lysophospholipids and preservation of membrane function. Concurrently, the increased synthesis of phospholipids will reduce the accumulation of lipid by-products, including lysophospholipids and FFA which may be potentially toxic to cells (9, 18).

When oxygen is removed from the incubation medium, the cessation of oxidative phosphorylation causes a decline in high-energy phosphate stores, potentially preventing effective resynthesis of membrane phospholipids in renal proximal tubule cells which are almost totally dependent upon oxidative phosphorylation for energy production. Thus, accelerated phospholipid degradation due to the activity of phospholipase in the face of decreased phospholipid synthesis will eventually lead to marked phospholipid depletion and significant accumulation of lipid by-products. These lipid alterations did, in fact, develop in renal tubule suspensions exposed to exogenous PLA_2 during a brief hypoxic stress. Associated with these alterations in phospholipid levels, concomitant hypoxia and exogenous PLA_2 exposure led to marked changes in metabolic parameters reflective of severe cell injury. The critical role of high-energy phosphate stores in maintaining cell integrity during hypoxia and exogenous PLA_2 exposure was supported by the significant protective effect of exogenous ATP-$MgCl_2$ on the degree of cell injury occurring in hypoxic tubules treated with exogenous PLA_2. This protection by exogenous ATP was dose dependent, since 2 mM ATP-$MgCl_2$ provided better protection than 250 μM and was associated with significantly greater increases in tubule PC and PE levels and greater declines in the phospholipid by-products levels of LPC, LPE, and FFA. This ATP-related maintenance of tubule phospholipid levels and increased cellular resynthesis of membrane phospholipids, as evidenced by the higher incorporation rates of linoleic acid into phospholipids, allowed better preservation of membrane structure and function and cell viability parameters despite high exogenous PLA_2 activity. It is interesting to note that 2 mM ATP provided almost complete protection against this injurious process with return of most metabolic parameters back to normal or supranormal levels, although the levels of major phospholipids and lysophospholipids did not completely return to control levels. This finding indicates that ATP may protect cell integrity by additional effects on other cellular metabolic functions (13). A variety of studies have clearly demonstrated the protective effect of ATP-$MgCl_2$ in renal ischemia in vivo as well (28–30).

Thus, acute renal cell injury induced by exogenous PLA_2 under hypoxic conditions is associated with depletion of major membrane phospholipids along with accumulation of various lipid by-products. However, this association does not necessarily establish a cause and effect relationship between lipid alterations and ischemic cell injury. In fact, declines in phospholipid content may not be a critical event in the pathogenesis of ischemic cell injury. It has been demonstrated recently that the time course of the loss of phospholipids is too slow to account for the presumably much more rapid loss of viability of the renal cortex and myocardium in ischemia (10, 26). Instead, the accumulation of a variety of products of abnormal lipid metabolism during ischemic cell injury, such as FFA (26, 31), acylcarnitine, acyl-CoA, and lysophospholipids may be more critical in the pathogenesis of cellular injury in ischemia. The general propensity for these lipid metabolites to induce cellular damage probably rest, in large part, on their amphiphilic na-

ture, i.e., the presence of both hydrophilic and hydrophobic components within their molecular structure. This property allows their interaction with and incorporation into cellular membranes in a variety of ways depending on the size, configuration, and quantity of amphiphile present. This interaction may markedly alter the structural and functional properties of these membranes (9, 18, 32–38). The resulting membrane alterations may be the critical event leading to cell injury and death.

The data presented in this report indicate that increases in exogenous PLA_2 activity produces cell injury when cell ATP levels decline. This circumstance may arise due to an imbalance of the rates of phospholipid resynthesis and degradation resulting in depletion of phospholipids and accumulation of lipid by-products. Improvement in high-energy phosphate stores with exogenous ATP-$MgCl_2$ resulted in less cell injury, increases in phospholipid levels, and reduction in the levels of lipid by-products. High-energy phosphate store depletion, simultaneous to increases in exogenous PLA_2 activity, was associated with marked renal tubule cell injury. The mechanism by which exogenous PLA_2 induces cell injury appears to be membrane alterations caused by either depletion in major membrane structural phospholipids or accumulation of potentially toxic lipid by-products.

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