Role of Cytosolic Calcium-independent Plasmalogen-selective Phospholipase A_2 in Hypoxic Injury to Rabbit Proximal Tubules

Didier Portilla,* Sudhir V. Shah,* Paul A. Lehman,‡ and Michael H. Creer§
With the technical assistance of **Aditi Bandyopadhyay* and Janet H. Jones§**
*Divisions of Nephrology and *Dermatology, and Departments of ‡Medicine and §Pathology, University of Arkansas for Medical Sciences, and John L. McClellan Memorial Veterans Administration Hospital, Little Rock, Arkansas, 72205*

Abstract

Although the activation of calcium-independent phospholipase A_2 (PLA_2) enzymes has been described in the heart, the pathogenetic role of this enzyme(s) in hypoxic cell injury has not been previously examined in any tissue. Therefore, we characterized the time course of activation of calcium-independent PLA_2 using both plasmalogen and diacylglycerophospholipid substrates during hypoxia in rabbit proximal tubules and examined whether inhibition of calcium-independent PLA_2 activity is associated with a cytoprotective effect. Subjecting rabbit proximal tubules to hypoxia for 5 min resulted in at least a threefold increase in cytosolic calcium-independent PLA_2, which was selective for plasmalogen substrates (control 444±69 vs hypoxia 1,675±194 pmol · mg protein^{-1} · min^{-1}, n = 5). In contrast, no changes in PLA_2 activity were observed in the presence of 4 mM EGTA in the membrane fraction using plasmenylcholine substrates. 20 min of hypoxia resulted in an increase in arachidonate from 3±1 to 28±4 ng/mg protein and lactate dehydrogenase release from 7.5±2% to 38±5%, n = 4. Pretreatment of proximal tubules with 10 μM Compound I, a specific inhibitor of calcium-independent PLA_2, resulted in reduction in the magnitude of both hypoxia-induced arachidonic acid release (11±3 ng/mg protein) and lactate dehydrogenase release (18±4%). Our data indicate that a significant fraction of PLA_2 activity in the proximal tubule is calcium-independent and selective for plasmalogen substrates. Furthermore, the activation of this enzyme plays an important role in the pathogenesis of membrane injury during hypoxia in the proximal tubule. (*J. Clin. Invest.* 1994. 93:1609–1615.) Key words: phospholipid hydrolysis • ischemic cell injury • hypoxia • calcium-independent phospholipase A_2

Introduction

Activation of intracellular phospholipase A_2 (PLA_2)[1] has been implicated as an important biochemical mechanism responsible for accelerated phospholipid hydrolysis during renal ischemia (1–5). The vast majority of previous studies of the PLA_2 activated during ischemia or anoxia in the kidney have been based on measurements of PLA_2 activity using exogenous diacylglycerophospholipid substrates in the presence of supraphysiological concentrations of calcium (1, 5). Thus, these studies have focused on the identification of calcium-dependent PLA_2 enzymes.

Recently, a novel class of intracellular, calcium-independent, plasmalogen-selective PLA_2 enzymes have been characterized in canine myocardium and bovine brain (6–8). Plasmalogens represent a distinct subclass of diradylglycero-phospholipids that contain an alkenyl ether (i.e., vinyl ether) covalent linkage at the sn-1 position, as compared to the *O*-acyl ester linkage at the sn-1 position of diacylglycerophospholipids (9). Although plasmalogens are important constituents of kidney cortex membranes (10–11), the potential role of plasmalogens as substrates for PLA_2(s) activated during renal ischemia has not been previously examined.

Using diacylglycerophospholipid substrates, we recently described the rapid activation of a novel cytosolic calcium-independent PLA_2 enzyme during anoxia in rabbit proximal tubules (4). At the present time, however, it is not known whether this cytosolic calcium-independent enzyme(s) in the kidney uses plasmalogen as a substrate. Although ischemia-induced activation of a membrane-associated form of plasmalogen-selective PLA_2 has been observed in rabbit myocardium (12, 13), the activation of cytosolic calcium-independent, plasmalogen-selective PLA_2 enzyme(s) in response to hypoxia has not been previously studied in any tissue. In addition, the effect of inhibiting calcium-independent PLA_2 on hypoxia-induced cell death has also not been previously examined in any model system of hypoxic injury.

The present study was undertaken to (*a*) characterize the calcium-independent PLA_2 activity in rabbit proximal tubules with regard to substrate preference (plasmalogen versus diacylglycerophospholipid); (*b*) compare the total activity of PLA_2 obtained using plasmalogen and diacylglycerophospholipid substrates in the absence and presence of calcium to determine whether the majority of PLA_2 activity requires calcium or is calcium-independent; (*c*) characterize the time course of activation of calcium-independent PLA_2 using both plasmalogen and diacylglycerophospholipid substrates during hypoxia in rabbit proximal tubules and compare to the time course of lactate dehydrogenase (LDH) release as a marker of ischemic cell injury; and (*d*) determine whether inhibition of calcium-independent PLA_2 activity is associated with a cytoprotective effect.

Methods

Radioactive lipids were from Du Pont-New England Nuclear Research Products (Boston MA). Cell culture media reagents, Percoll, and fatty acid–free albumin were all obtained from Sigma Chemical Co. (St.

Address correspondence and reprint requests to Didier Portilla, M.D., University of Arkansas for Medical Sciences, Department of Medicine, Division of Nephrology, 4301 West Markham Street, Slot 501, Little Rock, AR 72205-7199.

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1. *Abbreviations used in this paper:* AA, arachidonic acid; Compound I, 1-3-(E)-6-(bromomethylene)-(tetrahydro-3-(1-naphthalenyl)-2H-pyran-2-one; LDH, lactate dehydrogenase; PLA_2, phospholipase A_2.

Louis, MO). Compound I (1-3-(E)-6-(bromomethylene)-(tetrahydro-3-(1-naphthalenyl)-2H-pyran-2-one) prepared as previously described (14, 15), was a generous gift of Hoffman-LaRoche Inc. (Nutley, NJ).

Proximal tubule isolation. Rabbit proximal tubules were isolated as previously described (4) and resuspended in DME/F12 medium at a protein concentration of 1.5 mg/ml. Isolated proximal tubules were then transferred from ice to a 37°C shaking water bath and preequilibrated for 20 min in a 95% O_2/5% CO_2 atmosphere. In the experiments using the plasmalogen-selective PLA_2 inhibitor (Compound I), proximal tubules were incubated with Compound I for 30 min before starting the experiments. Hypoxia was achieved by subjecting the tubules to a 95% N_2/5% CO_2 atmosphere that was maintained for variable time periods at 37°C. Samples were obtained before hypoxia and at different hypoxic intervals for measurements of cytosolic LDH release.

For the measurement of LDH release, 1 ml of tubule suspension was centrifuged through a 0.4-ml of a mixture of dibutyl phthalate and dioctyl phthalate (2:1) to separate the tubules from the extracellular medium. LDH activity was measured spectrophotometrically on Triton-permeabilized 0.5 ml of tubule suspensions and corresponding supernatants as previously described (16).

Subcellular fractionation. Tubules subjected to either normoxia or hypoxia were disrupted with 50 strokes of a Dounce homogenizer in an ice-cold buffer comprised of 50 mM Tris (hydroxymethyl)-aminomethane hydrochloride, 4 mM EGTA, pH 7.3, containing 1% (10 mg/ml) aprotinin, and 0.1 mM phenylmethylsulfonyl fluoride. After homogenization, nuclei were sedimented at 500 *g* for 5 min, and the resulting supernatant was centrifuged at 100,000 *g* for 1 h to obtain membrane (pellet) and cytosolic (supernatant) fractions.

Arachidonic acid (AA) release. AA was measured after a modified method previously described by us (4). Briefly, AA was extracted from a 2-ml aliquot of the 2 mg/ml tubule suspensions after a Bligh Dyer procedure (17) after the addition of 20 ng of octadeuterated (d8) AA as internal standard. AA isolated on C18 columns was then converted to the pentafluorobenzoyl ester, evaporated to dryness, and dissolved in acetonitrile for mass spectrometry. The quantity of AA was determined by high pressure liquid chromatography/particle beam/mass spectrometry in the electron capture negative ion chemical ionization mode and monitoring m/z 303 for arachidonic acid and m/z 311 for deuterated AA as previously described (4, 18).

Phospholipase A_2 assays. Phospholipase A_2 activity was assayed using synthetic choline diacylglycerophospholipids specifically radiolabeled at the sn-2 position with [^{3}H]oleate acid or [^{3}H]arachidonic acid. 1-*O*-hexadecanoyl-2-*cis*-octadec-[9,10-^{3}H]enoyl-sn-glycero-3-phosphocholine (^{3}H-labeled phosphatidylcholine) and 1-*O*-hexadec-1′-enyl-2-*cis*-octadec-[9,10-^{3}H]enoyl-sn-glycero-3-phosphocholine (^{3}H-labeled plasmenylcholine) were prepared by dimethylaminopyridine-catalyzed acylation of 1-*O*-hexadecanoyl-sn-glycero-3-phosphocholine and 1-*O*-hexadec-1′-enyl-sn-glycero-3-phosphocholine, respectively, using [^{3}H]oleic anhydride as previously described (19). To compare PLA_2 activity against arachidonic acid-labeled phosphatidylcholine substrate, we used commercially available 1-palmitoyl-2-[^{14}C]-arachidonyl-sn-glycero-3-phosphocholine.

Tritium-labeled 1-*O*-hexadec-1′-enyl-2-*cis* eicosatetra-5′,8′,11′,14′-enoyl-sn-glycero-3-phosphocholine (^{3}H-labeled plasmenylcholine with esterified radiolabeled arachidonic acid at the sn-2 position) was prepared exactly as described above for the synthesis of ^{3}H-labeled plasmenylcholine containing esterified oleate at the sn-2 position by substituting [^{3}H]arachidonic acid for [^{3}H]oleic acid. Phospholipase A_2 activity in proximal tubule fractions was assessed by incubating enzyme (typically 50 μg of microsomal protein or 200 μg of cytosolic protein) with 100 μM sn-2–radiolabeled choline glycerophospholipid (specific activity = 40 Ci/mol). The substrate was introduced by ethanolic injection of 10 μl to a tube containing 200 μl of assay buffer consisting of 50 mM Tris HCl and either 4 mM EGTA (to define calcium-independent PLA_2 activity) or 10 mM $CaCl_2$ (to define calcium-dependent PLA_2 activity), pH 7.3, at 37°C for 15 min. All measurements of PLA_2 activity were made under initial reaction conditions where the observed rates of fatty acid release were linear with respect to time (0–30 min) and protein content (0–200 μg). The reaction products were recovered by Bligh and Dyer extraction, separated by thin-layer chromatography (TLC), and quantified by liquid scintillation spectrometry (17, 20).

Statistics. Results are means±SE. All experiments were performed in at least four independent cell preparations. Statistical significance was determined by the paired Student's *t* test. $P < 0.05$ were considered statistically different.

Results

Characterization of phospholipase A_2 activity. To characterize phospholipase activity in proximal tubules, we used phosphatidylcholine and plasmenylcholine substrates of identical aliphatic carbon chain length at the sn-1 position and containing either [^{3}H]oleic acid or [^{3}H]arachidonic acid regiospecifically esterified at the sn-2 position. In initial experiments, we characterized the radiolabeled reaction products generated after incubation with phosphatidylcholine and plasmenylcholine substrates. Using two different solvent systems for TLC, with appropriate neutral lipid and phospholipid standards to identify the corresponding isolated zones, unesterified fatty acid was the only detectable radioactive product identified after incubation of proximal tubule subcellular fractions with sn-2 radiolabeled plasmenylcholine and phosphatidylcholine substrates. With both substrates, there was no evidence for production of significant amounts of other potential radiolabeled products including diradylglycerols, lysophospholipids (including lysoplasmalogens) or diradylglycerolphosphates (phosphatidic acids). Furthermore, radioactivity recovered in the fatty acid region accounted for > 95% of the decrease in total radioactivity observed in the region of the TLC plate corresponding to the unhydrolyzed choline diradylglycerophospholipid substrate. On the basis of these observations, we conclude that the overwhelming majority of activity measured as radiolabeled fatty acid release in our experiments is mediated by a phospholipase with regiospecificity for hydrolysis of the *O*-acyl ester linkage at the sn-2 position of diradylglycerophospholipids. Thus, in our experiments, the rate of radiolabeled fatty acid release specifically represents PLA_2 activity.

Substrate specificity, subcellular distribution, and calcium dependence of proximal tubule PLA_2 activity. In preliminary studies, we determined that > 90% of the total activity present in whole homogenates of proximal tubules was recovered in the cytosolic and membrane fractions, indicating not only that the majority of activity resides in these fractions, but also that the isolation of these fractions did not result in substantial loss of activity. For quantification of PLA_2 activity, we selected "near saturating" concentrations of substrate (100 μM) to minimize the effect of isotope dilution of exogenous radiolabeled substrate by endogenous choline diradylglycerophospholipids. Based on measurements of choline diradylglycerophospholipid mass in the isolated subcellular fractions, under our assay conditions, endogenous choline diradylglycerophospholipids contributed < 1% of the total plasmenylcholine mass and < 1% of the total phosphatidylcholine mass present during the assay of cytosolic PLA_2 activity. In the assay of membrane-associated PLA_2 activity, endogenous choline phospholipids contributed < 5% of the total plasmenylcholine and < 20% of the total phosphatidylcholine mass. Accordingly, measurements of PLA_2 activity in the cytosol and membranes are not significantly affected by the presence of endogenous substrate.

Table I summarizes the results of our characterization of the substrate specificity, calcium-ion requirements, and subcellular distribution of enzyme activity. The majority of PLA_2 activity in proximal tubules is calcium independent and is present in the cytosolic fraction. This calcium-independent cytosolic PLA_2 activity displays a distinct preference for plasmalogen substrates. The PLA_2 activity defined with substrates containing esterified arachidonic acid was approximately two- to threefold greater than that obtained using substrates containing esterified linoleate for both plasmalogen and diacylglycerophospholipid classes. The PLA_2 activity in the cytosol defined using either ^3H-labeled phosphatidylcholine or ^3H-labeled plasmenylcholine substrates is not affected by the presence of supraphysiologic concentrations of calcium. The membrane fraction also contains substantial amounts of calcium-independent PLA_2 activity selective for plasmalogen substrates; however, this activity is considerably lower than the activity found in the cytosolic fraction. In addition, in contrast to the cytosolic fraction, the majority of PLA_2 activity present in the membrane fraction, which uses choline diacylglycerophospholipid substrates, requires calcium for maximal activity.

Effect of hypoxia on the activity of proximal tubule cytosolic plasmalogen-selective PLA_2. The effects of hypoxia on PLA_2 activity in the cytosolic and membrane fractions in the absence and presence of calcium are shown in Figs. 1 and 2. In Fig. 1, after a 20-min period of hypoxia, PLA_2 activity was observed to increase more than twofold in the cytosolic fraction. The increase in cytosolic PLA_2 activity during hypoxia was demonstrable only when plasmenylcholine was used as a substrate. This cytosolic plasmalogen-selective PLA_2 did not require calcium for activity. In fact, calcium may have had a slight inhibitory effect on the hypoxia-induced PLA_2 activity. Total PLA_2 activity in the membrane fraction defined with either plasmenylcholine or phosphatidylcholine substrates was not affected by

Table I. Total Phospholipase A_2 Activity (pmol · mg protein^{-1} · min^{-1}) in Subcellular Fractions from Normoxic Rabbit Proximal Tubules

Cell fraction		4 mM EGTA	10 mM Ca^{2+}
Cytosol	Phosphatidylcholine		
	(16:0,[^{3}H]18:1) PtdCho	105±5	122±11
	(16:0,[^{14}C]20:4) PtdCho	216±21	223±19
	Plasmenylcholine		
	(16:0,[^{3}H]18:1) PlasCho	217±16	198±9
	(16:0,[^{14}H]20:4) PlasCho	444±69	457±74
Membrane fractions	Phosphatidylcholine		
	(16:0,[^{3}H]18:1) PtdCho	38±11	117±12
	(16:0,[^{14}C]20:4) PtdCho	95±16	268±23
	Plasmenylcholine		
	(16:0,[^{3}H]18:1) PlasCho	124±7	129±9
	(16:0,[^{14}H]20:4) PlasCho	297±82	302±55

Cytosol and membrane fractions were prepared from isolated rabbit proximal tubules and phospholipase A_2 activity was quantified by incubation of cytosolic (200 μg) or membrane (50 μg) protein with the indicated sn-2–radiolabeled substrate (100 μM) for 15 min in the presence of 4 mM EGTA or 10 mM $CaCl_2$ as shown. The radiolabeled fatty acid product was isolated by TLC and quantified by liquid scintillation spectrometry as described under Methods. (16:0,[^{3}H]18:1) and (16:0,[^{14}C]20:4) PtdCho, radiolabeled phosphatidylcholine substrates; (16:0,[^{3}H]18:1) and (16:0,[^{3}H20:4) PlasCho, plasmenylcholine substrates containing radiolabeled oleic acid (18:1) or arachidonic acid (20:4) esterified to the sn-2 position, respectively. Each value represents the mean±SEM for four independent experiments.

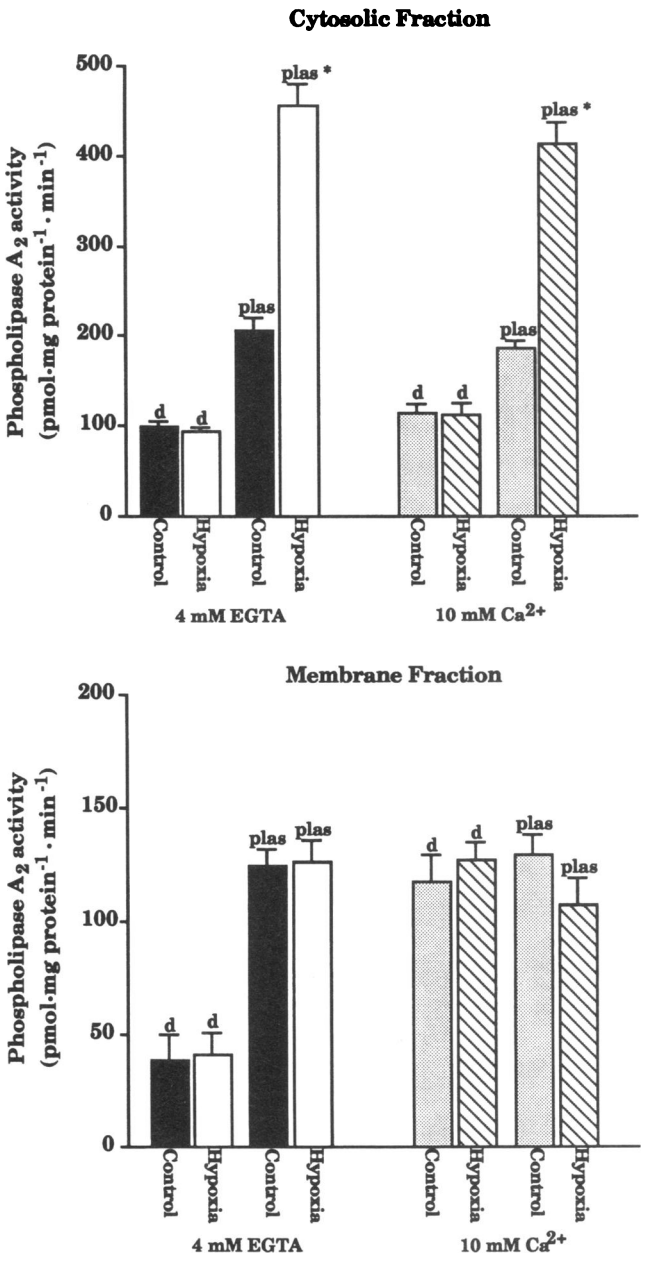

Figure 1. Effects of hypoxia on proximal tubule phospholipase A_2 activity. Isolated rabbit proximal tubules were exposed to 20 min of hypoxia after which cytosolic and membrane fractions were prepared as described under Methods. PLA_2 activity was quantified in the isolated subcellular fractions in the presence of 4 mM EGTA or 10 mM Ca^{2+} as indicated in the figure. *d*, phospholipase A_2 activity measured using [^{3}H]oleate-labeled phosphatidylcholine substrate; *plas*, phospholipase A_2 activity measured using [^{3}H]oleate-labeled plasmenylcholine substrates. All values represent the mean±SEM of five independent experiments. $*P < 0.005$ for comparisons between activity measurements in subcellular fractions prepared from control and hypoxic proximal tubules.

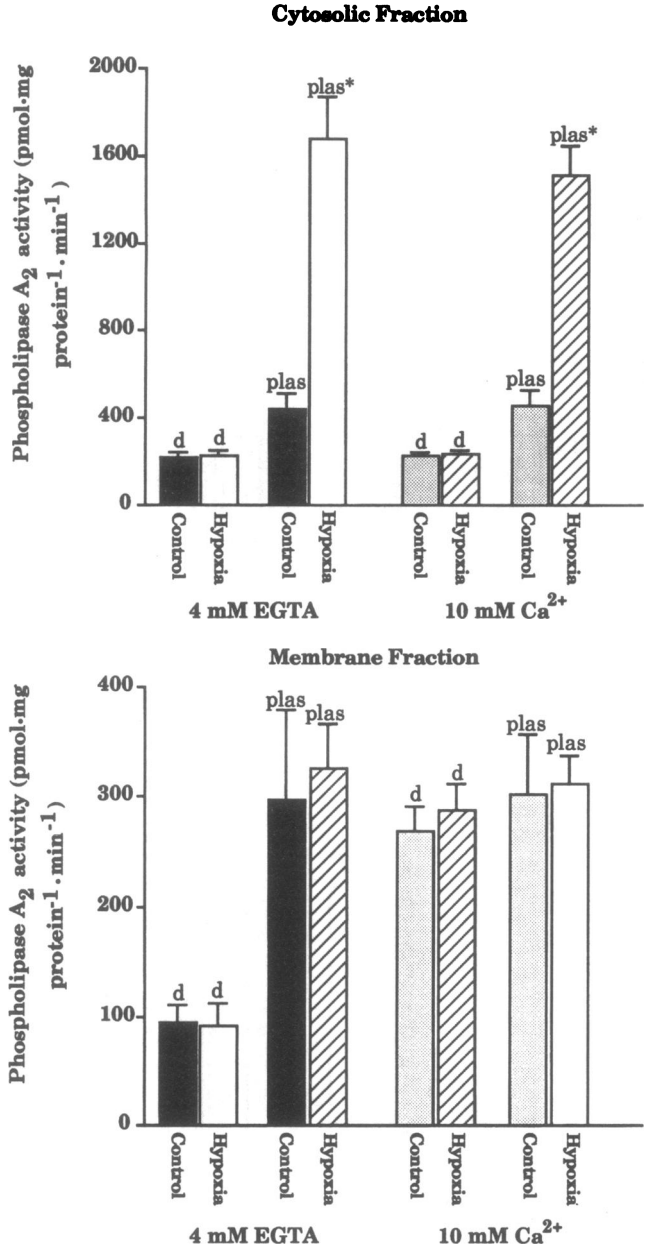

Figure 2. Effects of hypoxia on proximal tubule phospholipase A_2 activity. Isolated rabbit proximal tubules were exposed to 20 min of hypoxia, after which cytosolic and membrane fractions were prepared as described under Methods. PLA_2 activity was quantified in the isolated subcellular fractions in the presence of 4 mM EGTA or 10 mM Ca^{2+} as indicated in the figure. *d*, phospholipase A_2 activity measured using [^{14}C]arachidonate-labeled phosphatidylcholine substrate; *plas*, phospholipase A_2 activity measured using [^{3}H]arachidonate-labeled plasmenylcholine substrates. All values represent the mean±SEM of five independent experiments. $^*P < 0.005$ for comparisons between activity measurements in subcellular fractions prepared from control and hypoxic proximal tubules.

hypoxia. Hypoxia also had no effect on the apparent substrate specificity (plasmalogen vs diacylglycerophospholipid) or calcium ion requirements for membrane-associated PLA_2 activity. Thus, 20 min of hypoxia in proximal tubules is associated with a selective increase in cytosolic PLA_2 activity that does not require calcium (i.e., calcium independent) and demonstrates a distinct preference for plasmalogen substrates (i.e., plasmalogen selective). As shown in Fig. 2, virtually identical results were observed using substrates radiolabeled with arachidonic acid. The activity of plasmalogen-selective PLA_2 was approximately threefold greater with AA-containing substrates as compared to those containing esterified oleate under normoxic and hypoxic conditions.

Since plasmalogen-selective PLA_2 defined using (16:0,[^{3}H] 18:1) PlasCho or (16:0,[^{3}H] 20:4) PlasCho showed virtually identical time-dependent increases during hypoxia, we used the (16:0, [^{3}H] 18:1) PlasCho species for additional studies to characterize PLA_2 activity. (The synthesis of this substrate could be performed in a more cost-effective manner.)

During hypoxia in isolated proximal tubules, the increase in cytosolic plasmalogen-selective, calcium-independent PLA_2 is time dependent with a rapid and sustained increase in PLA_2 activity throughout the 20-min hypoxic interval (Fig 3). The activation of cytosolic plasmalogen-selective PLA_2 is essentially complete within 5 min after the onset of hypoxia. Measurements of PLA_2 activity in the presence of 4 mM EGTA in the membrane fraction using plasmenylcholine substrates failed to demonstrate any significant alterations in plasmalogen-selective, calcium-independent PLA_2 activity in this fraction throughout the 20-min interval of hypoxia (data not shown).

Effect of inhibition of plasmalogen-selective, calcium-independent PLA_2 on arachidonic acid release and hypoxia-induced membrane injury. Compound I is a haloenol lactone that belongs to a family of serine esterase inhibitors that have previously been shown to inhibit calcium-independent, plasmalogen-selective PLA_2 isolated from canine myocardium. This compound is a mechanism-based, irreversible inhibitor with a > 1,000-fold selectivity for inhibition of calcium-independent PLA_2 as compared to calcium-dependent PLA_2 activity (14). Compound I has previously been shown to inhibit calcium-independent PLA_2 at submicromolar concentrations (IC_{50} ~ 100 nM). Inhibition is also very rapid in onset (PLA_2 inacti-

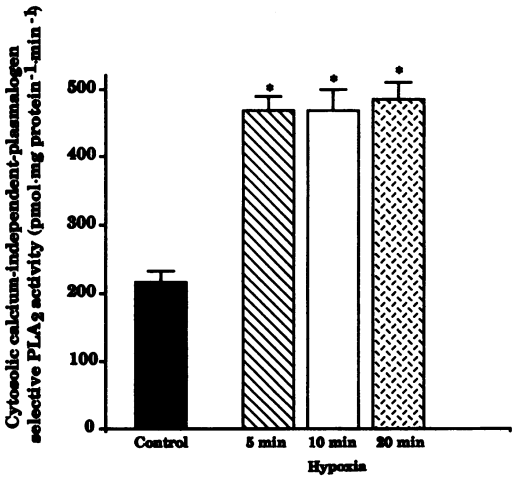

Figure 3. Time course of the effect of hypoxia on cytosolic calcium-independent, plasmalogen-selective PLA_2 in isolated rabbit proximal tubules. Phospholipase A_2 activity was measured in the presence of 4 mM EGTA using ^{3}H-labeled plasmenylcholine substrate in the cytosolic fractions prepared from control and hypoxic proximal tubules as described under Methods. Values shown represent the mean±SE for five independent experiments. $^*P < 0.005$ for comparisons between phospholipase A_2 activity measurements in the cytosolic fraction prepared from control and hypoxic rabbit proximal tubules.

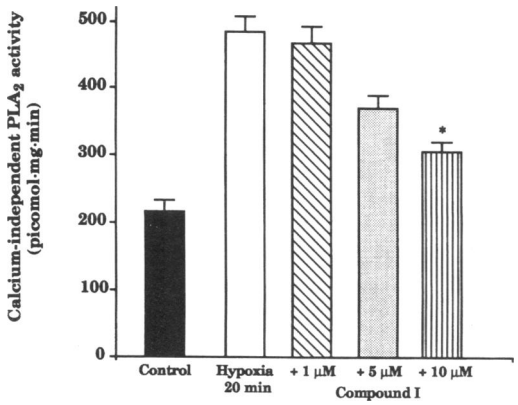

Figure 4. Effect of Compound I on hypoxia-induced activation of cytosolic calcium-independent, plasmalogen-selective phospholipase A_2. Proximal tubules were incubated in the absence and presence of various concentrations (1, 5, or 10 μM) of Compound I for 30 min, washed, resuspended in DMEM-F12 media, and then exposed to 20 min of hypoxia. PLA_2 activity was assayed in proximal tubule cytosolic fractions using ^{3}H-labeled plasmenylcholine substrate as described in Methods. Results represent the mean±SE of four independent experiments. $*P < 0.005$ for comparison with phospholipase A_2 activity measurements made in the cytosolic fraction after 20 min of hypoxia in the absence of Compound I.

vation rate constant of 0.15 min^{-1}). In preliminary experiments, we have found that incubation of proximal tubule cytosolic subcellular fractions with a 1-μM concentration of Compound I for 10 min at 37°C results in > 95% inhibition of cytosolic calcium-independent, plasmalogen-selective PLA_2 without having any significant effect on calcium-dependent PLA_2 tested against diacylglycerophospholipid substrates (data not shown).

To determine if Compound I is able to diffuse across the proximal tubule cell membrane to gain access to the catalytic site of the cytosolic plasmalogen-selective, calcium-independent PLA_2 to inhibit the enzyme in intact cells, we measured PLA_2 activity in hypoxic proximal tubules pretreated with Compound I. As shown in Fig. 4, pretreatment of the cells for 30 min with Compound I resulted in a dose-dependent inhibition of plasmalogen-selective, calcium-independent PLA_2 in proximal tubules with maximal (60%) inhibition of the hypoxia-induced increased in PLA_2 activity observed at 10 μM concentration of Compound I.

To determine whether inhibition of plasmalogen-selective, calcium-independent PLA_2 with Compound I would prevent the loss of surface membrane integrity during hypoxia, we examined the effect of Compound I on the magnitude of 20-min hypoxia-induced LDH release (Fig 5). In the absence of Compound I, 20 min of hypoxia resulted in an increase in the release of LDH from 7.5% to 38% ($n = 6$), demonstrating that hypoxia-induced tubule cell damage in these experiments was similar to previously described changes in hypoxic tubules (4). Pretreatment of proximal tubules with 10 μM Compound I for 30 min resulted in a 55% reduction in the magnitude of hypoxia-induced LDH release.

To establish whether inhibition of plasmalogen-selective, calcium-independent PLA_2 with Compound I, a maneuver that decreased cytotoxicity, was associated with decreased generation of free fatty acids, we examined the effect of Compound I on the magnitude of hypoxia-induced AA release (Fig 6). In the absence of Compound I, 20 min of hypoxia resulted in an increase in the release of arachidonic acid from 3±1 to 28±4 ng/mg protein, similar to previously described changes in hypoxic tubules (4). Pretreatment of proximal tubules with 10 μM Compound I for 30 min resulted in a concentration-dependent reduction in the magnitude of hypoxia-induced AA release ($n = 4$). With 10 μM Compound I, AA release during hypoxia was reduced > 50%. These results and those described above are consistent with the conclusion that inhibition of cytosolic plasmalogen-selective PLA_2 plays an important role in preventing both AA generation and LDH release during hypoxia by preserving the integrity of the cell surface membrane.

Discussion

The results of the present study are the first to demonstrate that the predominant PLA_2 activity in proximal tubules is mediated by a protein (or proteins) with a unique preference for plasmalogen substrates. Our studies also suggest that the amount of PLA_2 activity present in proximal tubules is likely to be underestimated in previous studies that used diacylglycerophospholipid substrates (1, 5). The exclusive use of diacylglycerophospholipid substrates would also account for the failure to recognize the rapid activation of proximal tubule cytosolic PLA_2

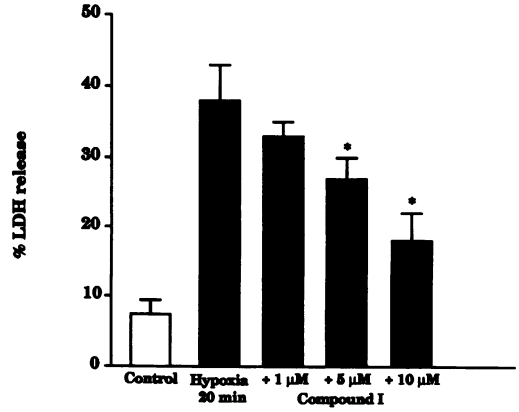

Figure 5. LDH release under normoxic control conditions and at 20 min of hypoxia in the absence and presence of various concentrations (1, 5, and 10 μM) of Compound I. Results are means±SE, $n = 4$. $*P < 0.005$ vs 20 min hypoxia in the absence of Compound I.

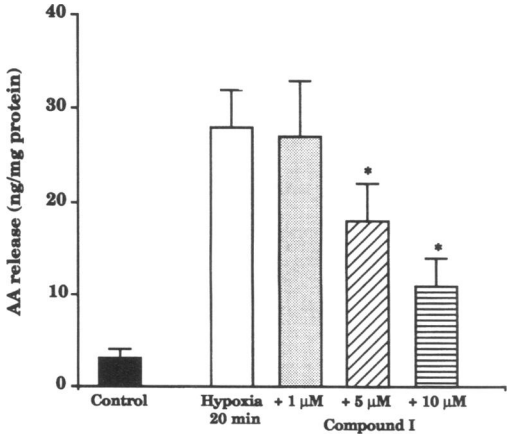

Figure 6. AA release under normoxic control conditions and at 20 min of hypoxia in the absence and presence of various concentrations (1, 5, and 10 μM) of Compound I. Results are means±SE, $n = 4$. $*P < 0.005$ vs 20 min hypoxia in the absence of Compound I.

during hypoxia, since this observation could only be demonstrated with the use of plasmalogen substrates. The relative preference of PLA_2 activity for plasmalogen substrates is not likely to be attributed entirely to differences in the physical state of diacyl vs plasmalogen substrates. For example, it can be demonstrated that calcium-independent, plasmalogen-selective PLA_2 from the heart displays a distinct preference for plasmalogen substrates when both diacyl and plasmalogen substrates are presented to the enzyme as a mixture in the same bilayer lipid structure (i.e., the enzyme obeys strict surface-dilution kinetics in mixed plasmalogen-diacyl phospholipid substrate vesicles [12]). Thus, while differences in molecular conformation of plasmalogen and diacyl phospholipids may contribute to differences in PLA_2-catalyzed hydrolytic rates, the relative preference of the enzyme for plasmalogen substrates may still be evident when both substrates are presented simultaneously to the enzyme in the same physical state.

The PLA_2 activity defined in proximal tubules with plasmalogen substrates was calcium-independent based on the observation that maximal activity was obtained in the presence of EGTA and that basal activity was not substantially affected by the presence of a high calcium ion concentration (10 mM). Calcium-independent PLA_2 was present in both cytosolic and membrane fractions prepared from isolated proximal tubules with the majority of activity present in the cytosol, where the reaction velocity observed with choline plasmalogens was greater than that observed with choline diacylglycerophospholipid substrates. The cytosolic localization, expression of maximal activity in the presence of EGTA, and relative specificity for plasmalogen substrates are features very similar to those previously described for plasmalogen-selective PLA_2 in canine myocardium (6). However, in contrast to the canine myocardial enzyme, proximal tubule cytosolic plasmalogen-selective PLA_2 is not inhibited by supraphysiologic calcium ion concentrations. In marked contrast to the previously described ischemia-stimulated, membrane-associated PLA_2 from rabbit myocardium (13), we found no changes in membrane-associated, plasmalogen-selective PLA_2 after hypoxic injury to proximal tubules. Instead, there was a threefold increase in cytosolic plasmalogen-selective PLA_2 after hypoxic injury to proximal tubules. Taken together, our data suggest that the proximal tubule and myocardial PLA_2 proteins represent distinct members of the calcium-independent, plasmalogen-selective class of intracellular PLA_2 enzymes that could be regulated during hypoxia by different mechanisms. In preliminary studies using rabbit kidney cortex tissue as starting material to partially purify soluble calcium-independent PLA_2, we have used DEAE ion exchange chromatography and have found that the fractions containing PLA_2 enzyme activity using phosphatidylethanolamine substrates coelutes with PLA_2 activity using choline plasmalogen substrate. SDS-PAGE of the active fractions shows a band at 40 kD that immunoreacts with the same pancreatic PLA_2 antibody described previously in proximal tubules (4). Thus, the plasmalogen-selective PLA_2 may represent the same protein that we described in our earlier study (4). Additional studies are in progress to complete the purification of this novel enzyme activity.

The mechanisms responsible for the hypoxia-induced activation of calcium-independent plasmalogen selective PLA_2 in rabbit proximal tubules are currently unknown. In the present study we can exclude a direct effect of Ca^{2+} on catalytic activity, since maximal activity could be achieved in the presence of 4 mM EGTA, and that activity was not altered significantly in the presence of 10 mM Ca^{2+}. In addition, the increase in plasmalogen-selective PLA_2 during hypoxia measured in the absence or presence of calcium was confined to the cytosolic compartment with minimal change in membrane-associated activity. Accordingly, there is no significant translocation of enzyme activity, a characteristic of the human monocytic and rat kidney PLA_2 enzyme activity using diacylglycerophospholipid substrates (21–28). Although Ca^{2+} is not required for catalytic activity and calcium-dependent translocation does not occur, it is still possible that calcium-dependent mechanisms such as protein kinase C–mediated phosphorylation of PLA_2 may play a role in the activation of cytosolic plasmalogen-selective PLA_2 in rabbit proximal tubules during hypoxia. Other potential mechanisms which may participate in the modulation of calcium-independent plasmalogen selective PLA_2 activity include phosphorylation by tyrosine-kinases (23, 24), interactions with G protein alpha subunits (29) and beta gamma heterodimers (30), synthesis of inhibitory or activating proteins (31, 32), and alterations in transcriptional regulation of PLA_2 genes (33). Further studies are required to define which of these mechanisms may participate in the upregulation of calcium-independent, plasmalogen-selective PLA_2 during hypoxia in rabbit proximal tubules.

Several lines of evidence suggest that the PLA_2 activity previously defined in proximal tubules using choline diacylglycerophospholipids, which is not activated during hypoxia, represents a distinct PLA_2 enzyme. Specifically, the cytosolic enzyme previously characterized in rat kidney (27) using diacylglycerophospholipid substrates requires calcium concentrations > 100 nM to be activated, and its activity increases with increased calcium concentrations. In contrast, the proximal tubule plasmalogen selective PLA_2 activity described in our studies can be demonstrated in the presence of 4 mM EGTA, and its activity is not increased by increased calcium concentrations.

The increase in cytosolic plasmalogen-selective PLA_2 during hypoxia precedes the accumulation of unesterified arachidonic acid previously observed during hypoxia in proximal tubules (4). Plasmalogens in many tissues, including isolated proximal tubules, are known to have a high content of arachidonic acid esterified at the sn-2 position (34, 35). Thus, the activation of a proximal tubule PLA_2 enzyme with a distinct preference for plasmalogen substrates may represent an important event in the generation of unesterified arachidonic acid and of biologically active eicosanoid derivatives previously observed during hypoxia in the kidney (2–4). Indeed, cytosolic proximal tubule plasmalogen-selective PLA_2 has several features that suggest that this enzyme activity plays a potentially important role in the pathogenesis of membrane injury during hypoxia. First, our preliminary studies demonstrate that plasmalogen molecular species comprise a significant fraction of proximal tubule phospholipids (35). Second, hypoxia in isolated proximal tubules in vitro results in the rapid and selective activation of cytosolic plasmalogen-selective, calcium-independent PLA_2. Third, activation of this PLA_2 activity occurs very rapidly before there is evidence of irreversible injury to the cell surface membrane based on measurements of LDH release. The activation of a cytosolic enzyme during hypoxia is likely to affect not only plasma membranes, but also membranes of other intracellular organelles containing plasmalogen phospholipids. Thus, accelerated phospholipolysis initiated by cytosolic calcium-independent PLA_2 activation during hypoxia could ultimately contribute to cell death.

The effect of inhibition of plasmalogen-selective, calcium-independent PLA_2 on hypoxia-induced cell death has not been previously examined in any tissue. Inhibition of proximal tubule cytosolic plasmalogen-selective, calcium-independent PLA_2 with Compound I is accompanied by a substantial reduction in both AA generation and total LDH release, as well as a delay in the time course of LDH release during hypoxia. This supports the conclusion that the activation of cytosolic plasmalogen-selective PLA_2 during hypoxia plays an important role in the processes responsible for generation of free fatty acids and loss of surface membrane integrity and cytosolic LDH release. Our direct demonstration that Compound I prevents the hypoxia-induced selective increase in proximal tubule plasmalogen-selective PLA_2, reduces production of free AA during hypoxia, and is accompanied by preservation of the permeability barrier function of the surface membrane is compelling evidence to suggest that accelerated hydrolysis of membrane plasmalogens may contribute to the development of membrane injury during hypoxia in proximal tubules. Further studies are needed to fully define the role of endogenous proximal tubule plasmalogens as substrates for calcium-independent, plasmalogen-selective PLA_2, and to determine whether activation of this enzyme activity accompanied by decreased phospholipid mass, increased production of unesterified fatty acids, or accumulation of lysoplasmalogen products may contribute to the pathogenesis of hypoxic cell injury in proximal tubules.

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