Altered ceramide and sphingosine expression during the induction phase of ischemic acute renal failure

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Altered ceramide and sphingosine expression during the induction phase of ischemic acute renal failure. Recent evidence indicates that a "sphingomyelin signaling pathway" exists: in response to heterogeneous influences, sphingomyelin is hydrolyzed, liberating ceramide, and subsequently its sphingoid base, sphingosine. Ceramide and sphingosine can influence diverse cellular processes, including cell differentiation, proliferation, protein trafficking, and apoptosis. Each of these processes have important implications for post-ischemic acute renal failure (ARF). However, sphingosine and ceramide expression during the induction of ischemic/reperfusion injury have not been previously assessed. To this end, CD-1 mice were subjected to 45 minutes of unilateral renal ischemia ± reperfusion, followed by cortical sphingosine, ceramide, and sphingomyelin assessments. Contralateral kidneys served as controls. Ischemia caused ~50% sphingosine and ceramide decrements. During reperfusion, sphingosine rebounded to normal values. Conversely, ceramide rose to, and was maintained at, supranormal levels (~175% of controls). Subsequent studies performed with hypoxic or oxygenated isolated proximal tubules suggested that these changes: (*1*) had a multifactorial basis; (*2*) were partially simulated by enhanced PLA_2 activity; (*3*) and were dissociated from alterations in net sphingomyelin content. To assess the potential pathogenic relevance of the documented ceramide increments, cultured human proximal tubule (HK-2) cells were subjected to ATP depletion/Ca^{2+} ionophore- or PLA_2-induced attack with or without exogenous C2 ceramide loading. Ceramide worsened both forms of injury without exerting an independent lethal effect. Conversely, ceramide markedly attenuated arachidonic acid cytotoxicity. This occurred without any decrease in arachidonate uptake, suggesting a direct cytoprotective effect. In conclusion: (*1*) sphingosine and ceramide fluxes are hallmarks of early ischemic/reperfusion injury; (*2*) these changes occur via divergent metabolic pathways; and (*3*) that ceramide increments can affect divergent injury pathways, and that sphingosine and ceramide have potent cell signaling effects, suggest that the currently documented sphingosine/ceramide fluxes could have important implications for the induction phase and evolution of post-ischemic ARF.

Sphingomyelin traditionally has been viewed simply as a structural component of plasma membranes, comprising approximately 5 to 15% of total phospholipid content [1]. However, during the past 10 years it has become increasingly clear that sphingomyelin is not just a membrane "building block," since it also serves as a source of cell signaling molecules [1–5]. Under a variety of influences (such as, TNF α, arachidonic acid, interleukin-1, γ interferon, complement, vitamin D, corticosteroids), sphingomyelin is hydrolyzed by plasma membrane or lysosomal sphingomyelinases, resulting in ceramide release [1–5]. Ceramide can then be deacylated by ceramidase(s), liberating its sphingoid base, sphingosine. In turn, sphingosine can undergo phosphorylation via a sphingosine kinase, sphingosine-1-phosphate formation being the result. A burgeoning experimental literature indicates that each of these sphingomyelin derivatives can have potent, and sometimes divergent, effects on cellular homeostasis. For example, the addition of sphingolipid derivatives to cultured cells may alter cell proliferation and differentiation [6–9], inhibit protein trafficking [10–13], modulate cell adhesion/motility [14, 15], and initiate apoptosis (such as, [16–22]). Since diverse biological agents seemingly mediate their effects through sphingomyelin hydrolysis, it has been suggested that a sphingomyelin "signaling pathway" exists [1–5]. This may be analogous to PLA_2-initiated cell signaling through glycerolipid hydrolysis products (such as, via diacylglycerol, inositol triphosphate, and eicosanoids). Indeed, it has been postulated that the glycerolipid- and sphingolipid signaling pathways may have interactive, and potentially counter-regulatory, effects [4].

This laboratory recently demonstrated that exogenous sphingomyelin derivatives can have a marked impact on proximal tubular cell integrity, as assessed with a cell culture system [23]. For example, when sphingosine, sphingosine-1-phosphate, or cell membrane permeable ceramides were added to HK-2 cells (a proximal tubular cell line from an adult human kidney), a dose dependent loss of viability resulted [23]. Conversely, if the cells were exposed to subtoxic doses of sphingosine for ~18 hours, they became relatively resistant to superimposed ATP depletion/Ca^{2+} ionophore-induced attack (that is, a "cytoresistant state" emerged). Neither ceramide nor sphingosine-1-phosphate was able to reproduce this sphingosine-initiated cytoresistance. This suggested that it was a compound specific result, and not just a downstream consequence of a cellular "stress response" [24, 25].

Given these observations, we previously speculated that if perturbations in the sphingomyelin signaling pathway were to develop during renal injury, they could potentially impact on tubular cell integrity [23]. However, no evidence existed at that time to indicate that alterations in sphingosine or ceramide expression do, in fact, exist during the evolution of acute renal tubular damage. The present study was undertaken to test for this possibility. To this end, mouse kidneys or isolated proximal

tubular segments (PTS) were subjected to *in vivo* or *ex vivo* injury and then sphingosine, ceramide, and sphingomyelin concentrations were determined. Since these studies indicated that alterations in sphingosine and ceramide expression do develop during acute tubular injury, additional studies were undertaken to assess some possible reasons for these changes and to determine their potential impact on the evolution of acute tubular damage. Since interactions between glycerophospholipid and sphingomyelin by-products have been suggested [4], the impact of ceramide on the expression of PLA_2 and arachidonic acid mediated cytotoxicity was also addressed.

METHODS

In vivo ischemic/reperfusion injury protocol

Male CD-1 mice (25 to 35 grams; Charles River Laboratories, Wilmington, MA, USA) maintained under standard vivarium conditions were used for all whole kidney and isolated proximal tubule segment (PTS) experiments. They were injected with pentobarbital ($\sim$2 mg i.p.), and after the development of deep anesthesia they underwent a midline abdominal incision to expose both renal pedicles. During this procedure, normothermia was maintained by placing the animals in a 37°C warm room. Three sets of experiments were performed, as follows:

In vivo ischemia. Ten mice were subjected to total left renal ischemia by passing a silk ligature around the left renal pedicle and then inducing total vascular occlusion. After a 45-minute ischemic period, both kidneys were resected. The right kidney served as a control. Once resected, each kidney was cooled to 4°C, cortical tissues samples were obtained with a razor blade, the tissues were snap frozen in liquid N_2, and then they were stored frozen until sphingosine analysis (see below).

Ex vivo ischemia. Pilot data indicated that intrarenal heme trapping, a result of renal pedicle occlusion, caused an artifactual lowering of tissue ceramide concentrations. Therefore, it was necessary to induce whole kidney ischemia while avoiding this artifact. To this end, four mice were anesthetized and bilateral nephrectomy was performed (allowing for blood efflux). The right (control) kidney was immediately cooled, and cortical tissue samples were snap frozen in liquid N_2 and stored frozen until ceramide assay (see below). The left kidney was wrapped in a warm saline soaked gauze and then it was subjected to 45 minutes of *ex vivo* ischemia by placing it in a 37°C warm room. At the completion of this *ex vivo* ischemic period, it was frozen as above and stored until ceramide assay.

In vivo ischemia/reperfusion. Twelve mice were anesthetized and subjected to left renal pedicle occlusion, performed with a 1 cm nontraumatic vascular clamp. After completing 45 minutes of ischemia, the clamp was removed and a 30-minute reperfusion period was permitted. At its completion, both the post-ischemic kidney and the contralateral (control) kidney were resected and frozen for subsequent assay. Eight of the paired kidneys were used for ceramide determination. The remaining four pairs were assayed for sphingosine.

Since the above experiments detected significant ceramide increments at 30 minutes of reperfusion, four additional mice were subjected to 45 minutes of unilateral ischemia plus a longer reperfusion period (1 hr). This was done to ascertain whether the post-ischemic ceramide increases were merely transient or relatively sustained in duration.

Hypoxic injury in isolated proximal tubular segments (PTS)

Given the heterogeneity of cell types in cortical tissues, an *in vitro* cell injury model was employed to help substantiate that oxygen deprivation injury does, in fact, cause alterations in proximal tubular sphingosine and ceramide content. To this end, proximal tubular segments (PTS) were harvested from normal mouse kidneys by a previously described technique [26]. In brief, the kidneys were resected, cooled, the cortices dissected and finely minced, and then the pieces were subjected to 30 minutes of collagenase digestion at pH 6.5. The digest was passed through a stainless steel sieve, the recovered material was centrifuged, washed, and subjected to centrifugation through 32% Percoll. The viable PTS, recovered in the pellet, were washed twice and re-suspended in an experimentation buffer (in mmol/liter: NaCl, 100; KCl, 2.1; $NaHCO_3$, 25; KH_2PO_4, 2.4; $MgSO_4$, 1.2; $MgCl_2$, 1.2; $CaCl_2$, 1.2; glucose, 5; alanine, 1; Na lactate, 4; 10 Na butyrate; dextran, 0.6%; gassed with 95% O_2/5% CO_2; pH 7.4).

The preparations were re-warmed to 37°C over 15 minutes, they were divided into two 2 ml aliquots, and each aliquot was placed into 25 ml Erlenmeyer flasks maintained within a shaking 37°C water bath. One PTS aliquot was subjected to 20 minutes of hypoxia (gassing with 95% N_2/5% CO_2); the second PTS aliquot underwent simultaneous oxygenated incubation (95% O_2/5% CO_2). At the completion of these treatments, the extent of lethal cell injury was determined by calculating % lactate dehydrogenase (LDH) release [26]. The samples were then assayed for either sphingosine or ceramide concentrations ($N = 8$ and $N = 5$ control and hypoxic pairs for sphingosine and ceramide, respectively).

Combined in vivo/in vitro myohemoglobinuric injury protocol

The following experiment was undertaken to ascertain whether alterations in sphingosine/ceramide expression occur during the evolution of nephrotoxic, as well as ischemic tubular injury. To this end, a combined *in vivo/in vitro* model of myohemoglobinuric tubular damage was used, as previously described [27, 28]. In brief, 11 mice were anesthetized with pentobarbital and subjected to intramuscular glycerol injection (8.5 ml/kg of 50% glycerol; in 2 divided doses into the upper hind limbs). The mice were placed in a 37°C warm room to maintain body temperature. After one hour (sufficient time to permit myohemoglobin tubular uptake), PTS were isolated, as noted above. A baseline % LDH release was obtained and then the PTS were incubated for one hour under oxygenated conditions (allowing for the *in vitro* expression of myohemoglobin-induced injury [27, 28]). After this period, % LDH release was determined as a marker of lethal cell injury. Then, the PTS were immediately extracted and analyzed for either sphingosine ($N = 7$) or ceramide content ($N = 4$). PTS harvested from normal mice and subjected to 60 minutes of oxygenated incubation were used to establish normal sphingosine and ceramide concentrations for each of the assays ($N = 4$ for each).

Potential impact of PLA_2 activity on sphingosine and ceramide concentrations

The following experiment was undertaken to ascertain whether increased PLA_2 activity, a consequence of acute ischemic tubular damage [29–31], might be mechanistically linked to altered sphingosine/ceramide expression. To this end, 13 sets of PTS were prepared from normal mice and each preparation was divided into

two aliquots: control oxygenated incubation or oxygenated incubation in the presence of exogenous PLA_2 (5 U/ml; from porcine pancreas; P 6534; Sigma Chemicals, St. Louis, MO, USA). After completing the 20-minute oxygenated incubations, the % LDH release was determined. The samples were then immediately extracted and analyzed for either sphingosine or ceramide ($N = 8$ and $N = 5$ paired determinations, respectively).

The above experiment indicated that PLA_2 caused a marked reduction in sphingosine concentrations without significantly altering ceramide levels. The following experiment assessed whether PLA_2 caused this sphingosine lowering by enhancing its flux through its two known major metabolic pathways: (1) acylation via ceramide synthase; or (2) phosphorylation via sphingosine kinase. To achieve this end, the ability of a ceramide synthase inhibitor (fumonisin B1; FB1; Sigma) [21, 32–34] or a sphingosine kinase inhibitor (dimethylsphingosine; DMS; Biomol, Plymouth Meeting, PA, USA) [35] to block the PLA_2 initiated sphingosine depressions was assessed. Six PTS preparations were used to create the following experimental treatments: (1, 2) oxygenated incubation under control conditions $\pm$ 5 U/ml PLA_2; (3, 4) oxygenated incubation with 25 μM fumonisin B1 $\pm$ PLA_2; and (5, 6) oxygenated incubation with 5 μM dimethylsphingosine $\pm$ PLA_2. After completing the 20 minute incubations under these conditions, an aliquot was removed for LDH assay and the rest of the sample was used for the sphingosine assay.

Ceramide assay

Ceramide concentrations in renal cortex or proximal tubules were determined enzymatically by measuring the formation of ceramide-1-^{32}P in the presence of [γ-^{32}P] ATP under the influence of bacterial diacylglycerol (DG) kinase [36, 37]. Since this enzyme phosphorylates ceramide, and not just DG, the amount of ceramide-1-^{32}P formation serves as a quantitative index of ceramide concentrations. The assay of Younes et al was used [37]. In brief, samples of renal cortex (50 mg) were homogenized in 4 volumes of phosphate buffered saline. In the case of PTS, an aliquot equivalent to 3 to 6 mg/ml of PTS protein was centrifuged and the pellet was used for assay. Lipids from the samples were extracted in chloroform:methanol according to the method of Bligh and Dyer [38]. The chloroform phase of each was dried under N_2. Samples of each (1/10th of the cortical extract; 1/5th of the PTS extract) were solubilized by sonication in 20 μl of 7.5% n-octyl β-D-glucopyranoside, 5 mM cardiolipin, and 1 mM diethylene triamine penta-acidic acid. Each sample was added to a reaction buffer (20 μl; 250 mM Tris-HCl, 500 mM NaCl, 10 mM EGTA, 25 mM $MgCl_2$, pH 7.0). Next, 6 μl of bacterial DG kinase, as supplied by the manufacturer (Calbiochem, La Jolla, CA, USA; 6.3 units per ml) was added. The reaction was started by adding 20 μl of 10 mM ATP (unlabeled ATP + labeled ATP: [γ-^{32}P] ATP, 2.5 mCi/ml; in 20 mM Tris-HCl, 10 mM DTT, 1.5 M NaCl, 250 mM sucrose, 15% glycerol, pH 7.4). After incubating at 22°C for 40 minutes (the reaction was shown to be linear for at least 40 min with the amount of employed lipid), the reaction was stopped by extracting the lipids with 1 ml chloroform:methanol:HCl (100:100:1) + 30 μl 100 mM EDTA + 170 μl of buffered saline (135 mM NaCl, 1.5 mM $CaCl_2$, 0.5 mM $MgCl_2$, 5.6 mM glucose, 10 mM HEPES, pH 7.2). The lower organic phase was recovered, washed $\times$ 3 with the buffered saline and dried under N_2. The ^{32}P-labeled ceramide was separated from the rest of the lipids by thin layer chromatography (TLC), using chloroform:acetone: methanol:acetic acid:water (100:40:20:20:10) as solvent. The plates were analyzed by autoradiography. The ceramide-1-^{32}P band was recovered by scraping and then counted in scintillation fluid. The amount of ceramide was calculated from a standard curve constructed with 0, 1.12, 2.24, 5.6, 8.4, 11.2, and 12.5 nmol of type III ceramide (from bovine brain; Sigma Chemicals).

Sphingosine assay

Renal cortical tissues and PTS were assayed for sphingosine by a modification of the HPLC technique of Merrill et al [39]. In brief, renal cortical samples were homogenized as noted above and then these samples and the PTS samples were extracted by the method of Bligh and Dyer [38]. The chloroform extract was washed, dried and incubated at 37°C for one hour in 3 ml of 0.2 M NaOH in methanol at 37°C for one hour to cleave the acylglycerolipids. Then, 3 ml of chloroform and 3 ml of 1 M NaCl was added and the free long chain bases were recovered in the chloroform phase. The chloroform phase was washed with 5 ml of a wash solution (3 ml chloroform: 48 ml methanol: 47 ml water) and then the chloroform phase was dried. The long chained bases were derivatized with o-phthalaldehyde (OPA) [39]. The derivatized bases were quantitated by HPLC using a C18 column (Alltech Adsorbosphere OPA HR; 5 μ, 150 $\times$ 4.6 mm; Alltech Corp., Deerfield, IL, USA) and a methanol-5 mM potassium phosphate (pH 7.0) solvent system (82:18 vol:vol). Detection was performed with a spectrofluorometer (Hewlett Packhard, Wilmington, DE, USA; excitation and emission wavelengths, 340 nm and 455 nm, respectively). The values were calculated from a standard curve constructed with 0, 10, 25, 50, 100, 200, 500 pmole sphingosine standards (Biomol).

Sphingomyelin assay

The following experiments assessed the impact of the *in vivo* ischemia/reperfusion injury protocol and the *in vitro* hypoxic injury protocol on sphingomyelin content. Three mice were subjected to unilateral renal ischemia (45 min of left renal pedicle occlusion) and 30 minutes of vascular reflow. Then the cortex of the post-ischemic kidney and the contralateral control kidney were isolated and stored frozen until assay. In other experiments, four sets of PTS were prepared and each was divided into two aliquots: oxygenation $\times$ 40 minutes or 20 minutes hypoxia + 20 minutes of reoxygenation, followed by assessment of LDH release. Lipids were extracted from kidney cortex ($\sim$50 mg/wet wt) or from pelleted PTS (2 to 6 mg PTS protein) by the method of Bligh and Dyer [38]. The lipid phase was dissolved in 2:1 chloroform:methanol (1 and 0.5 ml used for cortex and PTS, respectively) and 90 μl were saved for phosphorus quantitation [40]. The rest of the lipids were dried under N_2 and resuspended in 50 μl of 2:1 chloroform:methanol. Lipid samples (10 to 15 μl) were subjected to TLC using chloroform:methanol:acetic acid: water (50:30:8:5) as the solvent. The sphingomyelin bands were visualized with 0.03% primulin spray, scraped, and quantitated by its phosphorus content [40].

Cell culture experiments

The overall goal of these experiments was to assess whether ceramide accumulation (such as, during ischemia), can impact on the evolution of renal tubular damage. Three questions were addressed:

(1) Since ischemic tubular injury is mediated, in part, by ATP

depletion + Ca^{2+} overload [30, 31], might increased cell ceramide levels alter the extent of this form of tissue damage?

(2) Since PLA_2 activation is a consequence of ischemic injury [29], might increased ceramide levels alter PLA_2 effects on cellular integrity?

(3) Might ceramide accumulation potentially impact on "downstream" consequences of PLA_2 activation (such as, altering cellular responses to arachidonic acid accumulation)?

These issues were addressed using cultured HK-2 cells, a proximal tubular cell line derived from a normal human kidney [41]. [Note: PTS were not used for these experiments because their isolation causes membrane damage, arachidonic acid accumulation, and potentially ceramide release, complicating data interpretation.] The cells were maintained in keratinocyte serum free medium (K-SFM; Gibco/BRL, Grand Island, NY, USA) to which was added 1 mM glutamine, 5 ng/ml epidermal growth factor, 40 μg/ml bovine pituitary extract, 25 U/ml penicillin, and 25 μg/ml streptomycin. Prior to experimentation, the cells, grown in T 75 Costar flasks (Cambridge, MA, USA), were trypsinized and transferred to 24-well Costar cluster plates. After an approximate 18-hour recovery period, they were used for experimentation, as described below. Cell viability in each experiment was assessed by determining LDH release [42]. Each experiment represented an $N \geq 10$, performed on at least three separate occasions.

Does ceramide alter the expression of ATP depletion/Ca ionophore-induced cell death?

Simultaneously cultured HK-2 cells were exposed to one of the following conditions: (*1*) control culture conditions; (*2*) 25 μM C2 ceramide addition (a synthetic cell membrane permeable ceramide); or (*3*) C16 (relatively impermeant) ceramide. The ceramides (obtained from Biomol) were added in 0.125% ethanol. After completing 10 minutes ceramide exposures, the cells in each well were subjected to a combined ATP depletion/Ca^{2+} overload injury protocol (7.5 μM antimycin + 20 mM 2-deoxyglucose + 10 μM Ca^{2+} ionophore A23187) [43]. One hr later, % LDH release was determined. Co-incubated cells exposed to 25 μM C2 ceramide, 25 μM C16 ceramide, or to routine culture conditions (with 0.125% ethanol addition) and not challenged with ATP depletion/Ca ionophore addition were used to establish independent ceramide effects on cell viability.

Does ceramide alter the expression of exogenous PLA_2 toxicity?

HK-2 cells were subjected to incubation under one of the following conditions: (*1*) control conditions; (*2*) 25 μM C2 ceramide addition; or (*3*) 25 μM palmitic acid addition (as a lipid control). After completing 10 minutes exposures, the cells were challenged with 10 U/ml of exogenous PLA_2. After completing four hours incubations, % LDH release was determined. Concomitantly treated cells exposed to C2 ceramide or palmitic acid but not to PLA_2 served to establish independent effects of the test agents.

Does ceramide alter arachidonic acid toxicity?

HK-2 cells were cultured under: (*a*) control conditions; (*b*) with a toxic dose of arachidonic acid (50 μM, in 0.25% ethanol); (*c*) with 25 μM C2 ceramide, followed 10 minutes later by 50 μM arachidonic acid addition; or (*d*) with 25 μM palmitic acid,

followed 10 minutes later by 50 μM arachidonic acid addition (the palmitate serving as a lipid control). After completing two hours arachidonic acid exposures, % LDH release was determined. Co-incubated cells maintained under control conditions or with two hours exposures to either 25 μM C2 ceramide or 25 μM palmitic acid established independent effects of these test agents on cell viability.

Potential determinants of ceramide's protective effect against arachidonic acid toxicity

C2 ceramide was found to markedly mitigate arachidonic acid toxicity. The following two experiments were undertaken to assess potential determinants of this effect.

(*a*) *Is cell ceramide uptake required for cytoprotection?* C16 ceramide, unlike C2 ceramide, undergoes little cellular uptake. Therefore, if cell ceramide uptake were critical to its ability to blunt arachidonic acid toxicity, C2 ceramide should be much more protective than C16 ceramide. C2 or C16 ceramide (25 μM) was added to HK-2 cells, followed 10 minutes later by the arachidonic acid challenge. Two hours later, % LDH release was assessed. The results were compared to co-incubated cells challenged only with arachidonic acid addition.

(*b*) *Does ceramide block arachidonic acid uptake by HK-2 cells?* The following experiment was undertaken to assess whether C2 ceramide blocks arachidonic acid toxicity by inhibiting its cellular uptake. HK-2 cells were cultured either with 25 μM C2 ceramide or under control conditions (with 0.125% ethanol as the ceramide vehicle). After 10 minutes exposures, 5 μM unlabeled arachidonic acid + ^{3}H labeled arachidonic acid (5,6,7,8,11,12,14,15-3H(N)C20:4; New England Nuclear, Wilmington, DE, USA; $\sim$50,000 cpm) were added. After a 30 minute incubation to permit ^{3}H arachidonic acid uptake, the media was removed, the cells were washed with Hank's balanced salt solution (with Mg and Ca), and then the cells were lysed with Triton 100X. The lysates were then counted in a scintillation counter. Non specific (background) binding of ^{3}H arachidonic acid was determined by repeating the above experiments in culture plates treated exactly as above but which contained no cells. This amount of background (not affected by the presence of C2 ceramide) was then subtracted from the values obtained with the lysed cultures.

Calculations and statistics

All values are presented as means ± 1 SEM. Statistical comparisons were performed by either paired or unpaired Student's *t*-test, as appropriate. If multiple comparisons were made, the Bonferroni correction was applied. Renal cortical ceramide and sphingosine concentrations were expressed per mg wet wt, per mg dry weight, or as nmoles/total phospholipid phosphate. The concentrations in PTS were either expressed per mg PTS protein or as nmoles/total phospholipid phosphate.

RESULTS

Ceramide levels during oxygen deprivation injury

Whole kidney ischemia. As shown in Figure 1, left, ceramide concentrations fell $\sim$33% during 45 minutes of *ex vivo* ischemia (I), when factored by tissue wet wt (Fig. 1A). Significant reductions were also observed when ceramide values were expressed
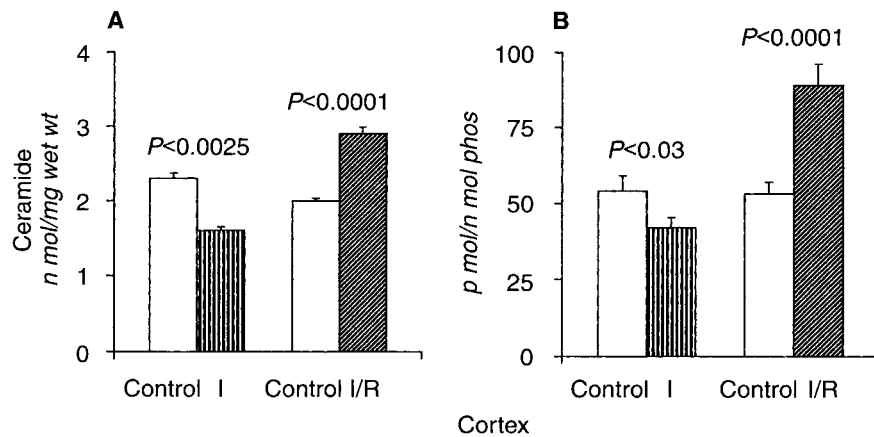
Fig. 1. Ceramide concentrations in mouse renal cortex under control conditions, after completing 45 minutes of ischemia (I), or after 45 minutes of ischemia + 30 minutes of reperfusion (I/R). The values are expressed per mg of tissue wet wt (*A*) or per nmol of phospholipid phosphate (*B*). Ischemia caused a significant reduction in ceramide concentrations. During reperfusion, ceramide rebounded, reaching supra-normal concentrations. [Not depicted, the post-ischemic ceramide increases were also maintained (at ~175% of control values) after 60 minutes of reflow, as discussed in the text].
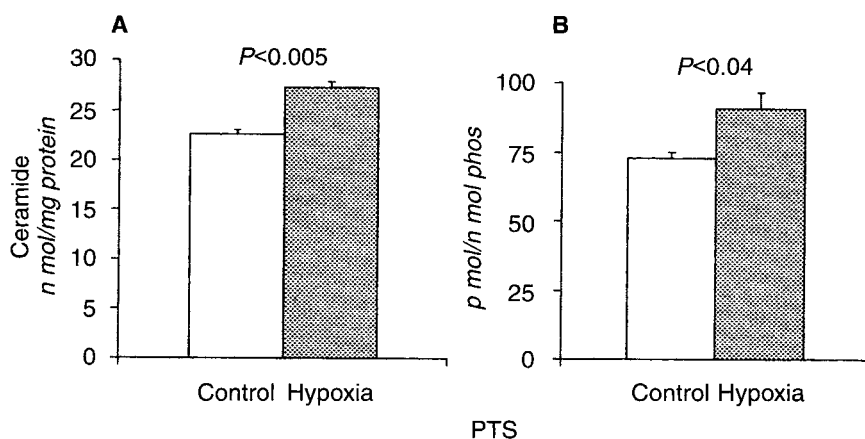

Fig. 2. Ceramide concentrations following induction of hypoxic injury to isolated proximal tubular segments (PTS). The values were expressed either as nmol/mg PTS protein (*A*) or as pmoles/nmoles phospholipid phosphate (*B*). In both instances, 20 minutes of hypoxia produced ~20% ceramide increments (simulating the change observed following *in vivo* ischemic/reperfusion injury, as per Fig. 1). The hypoxic challenge caused 61 ± 1% LDH release (11 ± 1% for controls).

either per total phospholipid phosphate (Fig. 1B) or per tissue dry weight (control, 9.9 ± 1.1; ischemia, 6.4 ± 1.2 nmol/mg; $P < 0.02$; not depicted).

Whole kidney reperfusion. After completing 30 minutes of reperfusion, ceramide concentrations rebounded from the ischemic depressions, rising ~40 to 80% above control concentrations (Fig. 1). This was true whether the values were expressed per wet wt (Fig. 1A), per phospholipid phosphate (Fig. 1B) or per tissue dry weight (controls 8.4 ± 0.8, vs. ischemia-reperfusion 15.3 ± 1.1 nmol/mg dry wt; $P < 0.0001$; not depicted).

After completing 60 minutes of reflow (data not depicted), the ceramide levels remained ~75% higher than control values (control 65 ± 6, vs. reperfusion, 110 ± 8 pmol/nmol phosphate; $P < 0.02$; control, 8.8 ± 1.0; vs. reperfusion, 14.3 ± 1.4 nmol/mg dry wt; $P = 0.03$). This indicates relatively stable post-ischemic ceramide elevations, theoretically permitting ceramide to impact early reperfusion events.

PTS hypoxic injury. When hypoxic injury was induced in PTS, a 20 to 35% increase in ceramide levels was apparent, whether expressed per mg PTS protein (Fig. 2A) or as PTS phospholipid phosphate (Fig. 2B). This hypoxic challenge induced dramatic cell injury, as denoted by 61 ± 1% LDH release (vs. 11 ± 1% for controls).

Sphingosine concentrations during oxygen deprivation injury

By the completion of 45 minutes of *in vivo* ischemia (I), sphingosine concentrations decreased by approximately 60% (Fig. 3A; $P < 0.0001$). When hypoxic injury was induced in PTS, comparable sphingosine reductions were observed (Fig. 3B). The latter change was associated with 54 ± 3% LDH release (vs. 12 ± 1% for controls).

During reperfusion (I/R) of *in vivo* ischemic kidneys, a prompt increase in sphingosine occurred. This resulted in a normalization of sphingosine concentrations whether expressed per tissue wet wt (Fig. 3A) or dry weight (not depicted).

Sphingomyelin determinations

Despite the fact that ischemia-reperfusion increased ceramide concentrations, no corresponding decrease in renal cortical sphingomyelin content resulted (controls 18.8 ± 1.25 nmol/mg dry wt; vs. ischemia/reperfusion 20.4 ± 1.54). Similarly, no decrease in PTS sphingomyelin content was noted in post-hypoxic PTS (0.10 ± 0.003 vs. controls 0.10 ± 0.004 nmol/nmol phosphate). This was despite the fact that the post-hypoxic tubules manifested 63 ± 2% LDH release (control oxygenated PTS 14 ± 1%). The failure to document a decrease in PTS sphingomyelin content was not due to a parallel reduction in phospholipid phosphate since
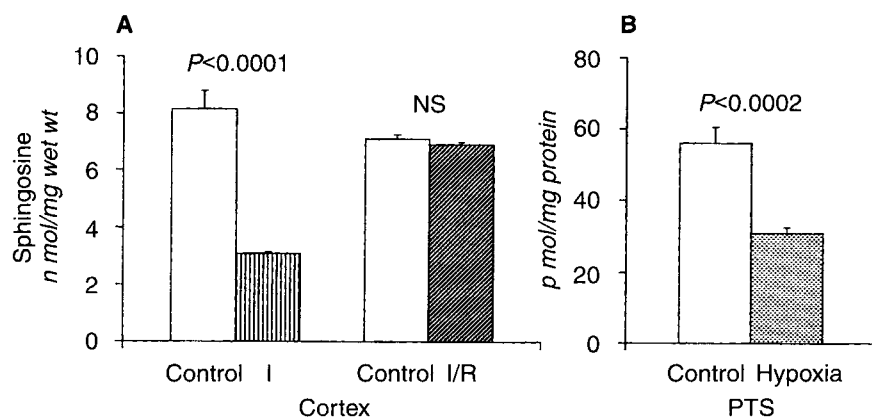
A

Sphingosine n mol/mg wet wt

10 — 8 — 6 — 4 — 2 — 0

$P<0.0001$

NS

Control I Control I/R

Cortex

B

p mol/mg protein

80 — 60 — 40 — 20 — 0

$P<0.0002$

Control Hypoxia

PTS

Fig. 3. Sphingosine concentrations in renal cortex (A) and in proximal tubule segments (B) in response to O_2 deprivation injury. In vivo renal ischemia (I) caused > 50% declines in sphingosine concentrations. These losses were rapidly corrected during reperfusion (I/R), with complete normalization of sphingosine levels occurring within 30 minutes. The ability of O_2 deprivation to deplete proximal tubular sphingosine was confirmed in the PTS experiments (~50% sphingosine depressions resulting with 20 min of hypoxia). The hypoxic challenge caused 54 ± 3% LDH release (vs. 12 ± 1% for controls).

A

Sphingosine p mol/mg protein

100 — 80 — 60 — 40 — 20 — 0

$P<0.005$

Control Glycerol

PTS

B

Ceramide p mol/n mol phos

100 — 80 — 60 — 40 — 20 — 0
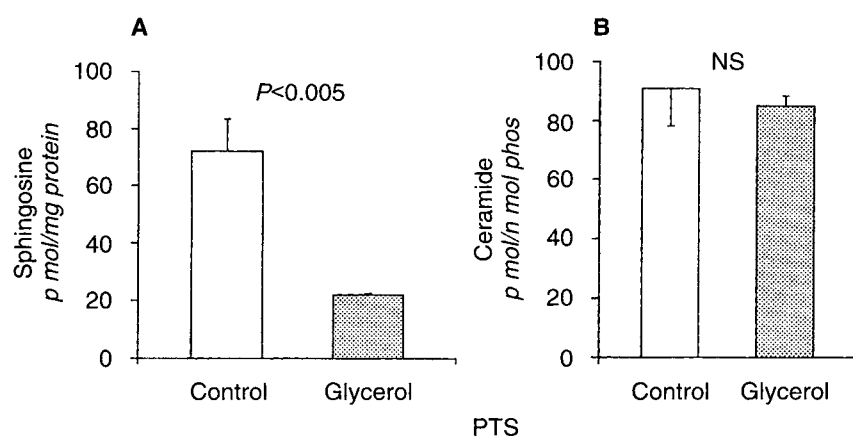
NS

Control Glycerol

Fig. 4. Sphingosine (A) and ceramide (B) concentrations in normal PTS and in PTS harvested from mice subjected to intramuscular glycerol injection. PTS extracted from the glycerol treated mice manifested markedly depressed sphingosine concentrations after completing a one hour in vitro incubation. In contrast, ceramide concentrations did not statistically differ from those observed in co-incubated normal PTS preparations. The glycerol PTS manifested 59 ± 3% LDH release by the end of the experiments (vs. 17 ± 1% for the controls). (That no ceramide increases were observed despite this profound lethal cell injury underscores that the ceramide increments observed in the hypoxic PTS were not simply a secondary manifestation of lethal cell injury).

the latter did not significantly change in response to hypoxic damage (202 ± 22 vs. 215 ± 29 nmol phosphate/mg PTS protein; post-hypoxic and oxygenated PTS, respectively).

Sphingosine/ceramide levels in response to in vivo/in vitro myohemoglobinuric injury

PTS harvested from control and glycerol injected mice manifested comparable % LDH release prior to the start of the one hour experimental incubations (5 ± 1% and 8 ± 1%, respectively). By completion of the in vitro incubations, the myohemoglobin loaded PTS had sustained 59 ± 3% LDH release, a result of iron mediated oxidant stress [24, 25]. In contrast, the control PTS manifested only 17 ± 1% LDH release ($P < 0.001$).

By the end of the in vitro incubations, sphingosine concentrations for the heme loaded PTS were depressed by ~70%, compared to control PTS values (Fig. 4A). However, no associated change in ceramide concentrations resulted (Fig. 4B).

Effects of exogenous PLA_2 on proximal tubule sphingosine and ceramide expression

PLA_2 treatment of PTS resulted in an approximate 50% reduction in sphingosine concentrations (Fig. 5A). This was quantitatively similar to the reductions seen with hypoxic and heme protein-induced PTS injury. However, the PLA_2-induced sphingosine reductions occurred without any increase in cell death (the PLA_2 treated PTS and the control PTS each had 12 ± 1%

LDH release). PLA_2 treatment had no significant impact on PTS ceramide concentrations (Fig. 5B).

Figure 6 depicts the sphingosine levels in PTS challenged with PLA_2 in the absence or presence of dimethylsphingosine (DMS; the sphingosine kinase inhibitor) or fumonisin B1 (FB1; the ceramide synthase inhibitor). DMS did not alter sphingosine levels under normal incubation conditions, and it did not attenuate the PLA_2-induced sphingosine depressions (suggesting that PLA_2 did not depress sphingosine levels by increasing its conversion to sphingosine-1-phosphate). FB1 almost doubled sphingosine concentrations, compared to normal values ($P < 0.01$; Fig. 6), indicating that ceramide synthase is a critical determinant of PTS sphingosine concentrations. However, FB1 did not block the PLA_2-induced sphingosine depressions (strongly suggesting that the reason for the PLA_2-induced depression was not increased sphingosine → ceramide conversion).

HK-2 cell experiments

Ceramide effect on ATP depletion/Ca^{2+} ionophore-induced cell death. As shown in Figure 7A, neither cell permeant (C2) nor cell impermeant (C16) ceramide increased LDH release in the absence of the ATP depletion/Ca^{2+} ionophore challenge. When control cells were challenged with ATP depletion/Ca^{2+} ionophore addition for one hour, only a mild increase in LDH release occurred under normal culture conditions (6% increase over control values; $P = 0.001$; Fig. 7B). However, when this challenge
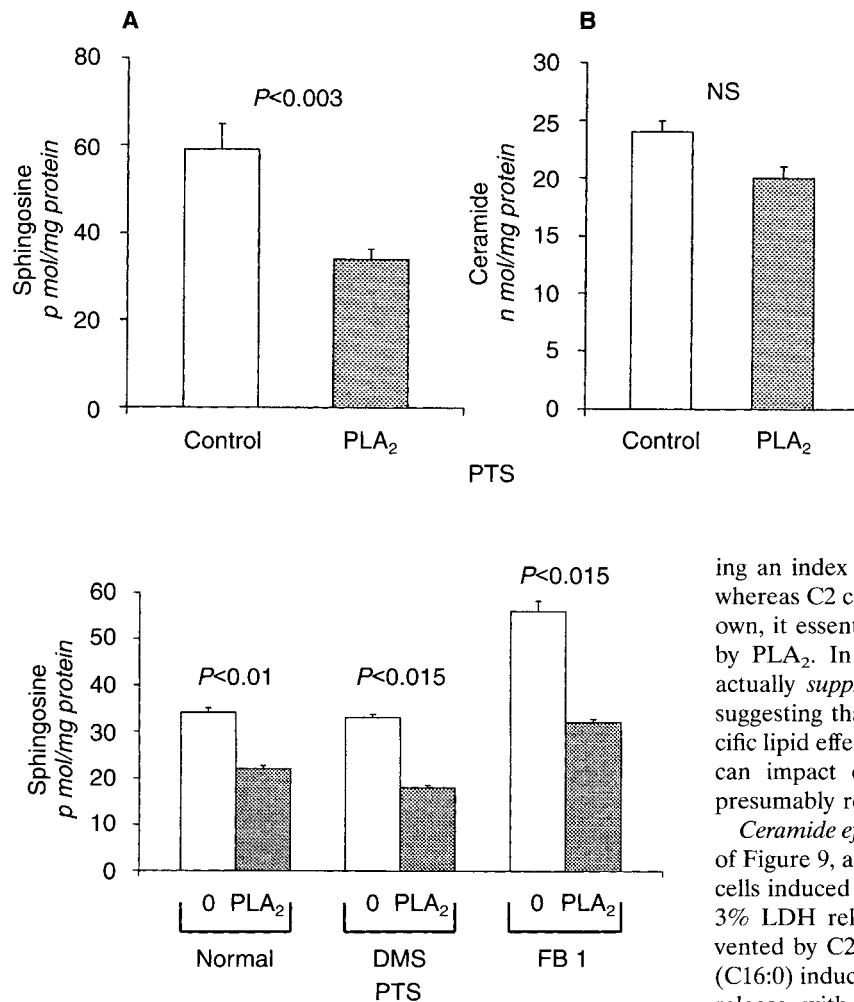
Fig. 5. Effects of exogenous PLA$_2$ on PTS sphingosine and ceramide expression. Addition of 5 U/ml of porcine pancreatic PLA$_2$ caused an approximate 50% decrease in PTS sphingosine levels (A). The PLA$_2$ challenge did not alter % LDH release (12 ± 1% with or without PLA$_2$), dissociating the sphingosine decrements from lethal cell injury. In contrast to the PLA$_2$ effects on sphingosine, it had no significant impact on PTS ceramide expression (B).
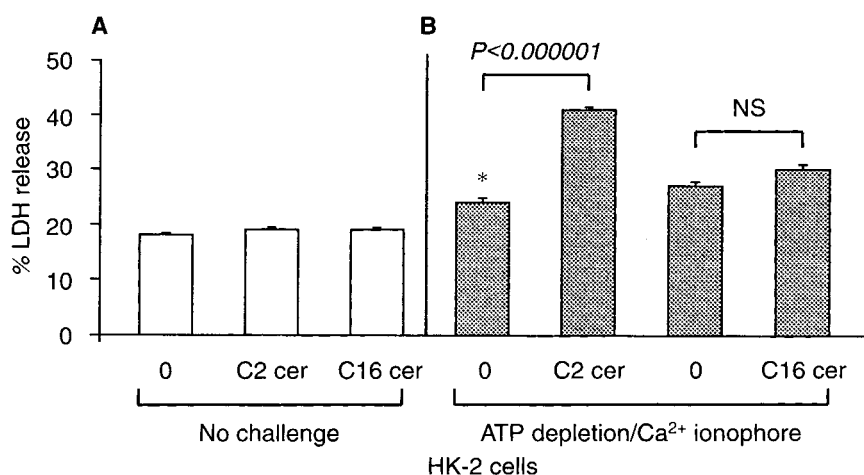

Fig. 6. Effect of sphingosine kinase inhibition (with dimethylsphingosine, DMS) or ceramide synthase inhibition (with fumonisin B1, FB1) on PTS sphingosine levels in the presence or absence of PLA$_2$. Under normal incubation conditions, FB1 treatment led to an approximate doubling of sphingosine concentrations (*P < 0.01). Conversely, DMS did not alter sphingosine levels. PLA$_2$ induced ~50% depressions in sphingosine levels whether it was added in the presence or absence of DMS or FB1. This suggests that PLA$_2$ does not lower sphingosine by increasing its flux via either of its two well defined metabolic pathways.

was conducted in the presence of C2 ceramide, a 24% increase in LDH release above control values resulted (P < 0.0001). In contrast, C16 ceramide failed to alter the extent of ATP depletion/ Ca^{2+} ionophore-induced LDH release.

Ceramide effect on PLA$_2$ toxicity. Four hour exposures to PLA$_2$ caused minimal cell lysis (LDH release) either in the presence or absence of C2 ceramide or palmitic acid (≤ 14%; not depicted). However, PLA$_2$ treatment did cause sublethal injury, as evidenced by an obvious shape change in the cells ("rounding up"), followed by cell detachment from the culture plates. This process appeared to be increased by ceramide but not palmitic acid treatment.

To quantitate the degree of cell detachment observed during these experiments, the culture media (containing the detached cells) were subjected to freeze thawing and sonication (causing cell lysis) and % LDH release was re-calculated (thereby provid-

ing an index of the % detached cells). As depicted in Figure 8, whereas C2 ceramide caused no significant cell detachment on its own, it essentially doubled the extent of cell detachment evoked by PLA$_2$. In contrast, the control lipid (palmitic acid; C16:0) actually *suppressed* PLA$_2$-induced cell detachment (P < 0.02), suggesting that the C2 ceramide result was not simply a nonspecific lipid effect. Thus, this experiment indicated that C2 ceramide can impact on PLA$_2$-induced toxicity (since cell detachment presumably reflects a sublethal, or a pre-lethal, event).

Ceramide effects on arachidonic acid toxicity. As shown at the left of Figure 9, addition of 50 μM arachidonic acid (C20:4) to control cells induced massive cell injury within a two hours period (85 ± 3% LDH release). Approximately 80% of this injury was prevented by C2 ceramide pre-treatment. Conversely, palmitic acid (C16:0) induced only a slight cytoprotective effect (65 ± 4% LDH release with palmitate vs. 26 ± 3% LDH release with C2 ceramide). As shown in the middle of Figure 9 (black bars), neither test agent exerted an independent effect on LDH release (that is, in the absence of arachidonic acid).

The comparison of C2 versus C16 ceramide effects on arachidonic acid toxicity are presented at the right of Figure 9. Unlike C2 (cell permeable) ceramide, C16 ceramide, which has minimal membrane permeability, exerted only a weak protective effect. These data suggest that cell ceramide uptake is critical to its ability to block arachidonic acid's cytotoxicity.

C2 ceramide addition to HK-2 cells did not appear to attenuate ^{3}H-arachidonic acid accumulation by HK-2 cells (control cell uptake, 690 ± 45 cpm per well; ceramide treated cells, 745 ± 62 cpm per well; N = 16 comparisons; NS). This suggests that ceramide exerted a direct protective action, rather than simply decreasing cellular arachidonate uptake.

DISCUSSION

It has been well documented that during the early stages of ischemic renal injury, marked alterations in cellular phospholipid homeostasis exist. The most characteristic changes noted to date are: (*1*) the rapid accumulation of unesterified ("free") fatty acids and lysophospholipids during ischemia (due to PLA$_2$-induced glycerophospholipid deacylation); (*2*) concomitant small reductions in total glycerophospholipid mass, most notably affecting phosphatidylcholine; and (*3*) a rapid decline in the accumulated

Fig. 7. Effect of ceramides on the expression of ATP depletion/Ca^{2+} ionophore-mediated HK-2 cell injury. Neither C2 nor C16 ceramide exposures x one hour under control conditions altered LDH release (A). The ATP depletion (antimycin/2-deoxyglucose)/Ca^{2+} ionophore A23187 challenge (B) caused only a 6% increase in LDH release above these control values ($*P < 0.01$). However, when this challenge was conducted in the presence of C2 ceramide, a marked increase in LDH release resulted (that is, C2 ceramide worsened the expression of ATP depletion/Ca^{2+} ionophore-initiated cell death). In contrast, C16 (cell impermeant) ceramide had no effect on the expression of ATP depletion/Ca^{2+} ionophore induced cell death.
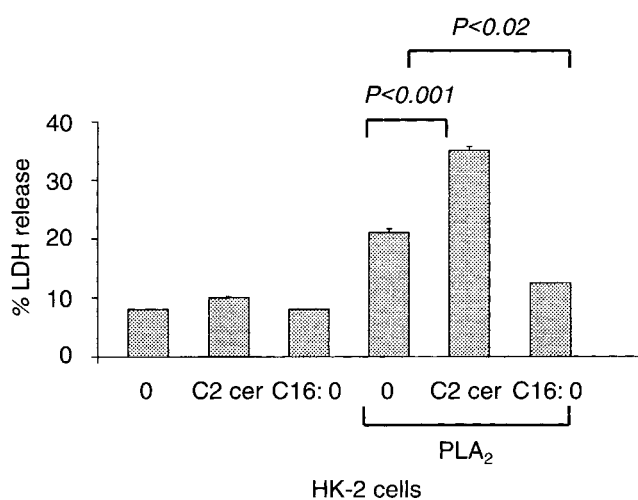

Fig. 8. Effect of C2 ceramide on the expression of PLA_2 toxicity. As discussed in the text, PLA_2 ± the lipid challenges induced no lethal injury, as assessed by LDH release. However, variable degrees of cell detachment were observed, and this was quantitated by lysing the detached cells in the medium. Thus, these derived % LDH release values (depicted in the figure) reflected the extent of this detachment process. As shown at the left of the figure, neither C2 ceramide nor the lipid control (C16:0, palmitate) caused increased detachment. PLA_2 caused ~20% detachment, and its extent was doubled in the presence of C2 ceramide. This was presumably not just a non specific lipid effect, since the control lipid (C16:0) actually decreased ($P < 0.02$), rather than increased, cell detachment in the presence of PLA_2.

fatty acid/lysophospholipid burden during early reperfusion, presumably due to metabolism [31, 44, 45]. While the overall importance of these changes to the evolution of lethal cell injury has been debated [31], there is no question that each of these alterations can affect cell homeostasis. For example, free fatty acids and lysophospholipids can exert direct membrane lytic effects [44, 46]; arachidonic acid accumulation negatively impacts cellular energetics [47]; and alterations in membrane phospholipid composition can alter the physical nature of the lipid bilayer, and presumably, the function of the homeostatic proteins which reside in it.

In contrast to an abundant literature documenting dramatic

fluxes in glycerophospholipid derivatives during ischemic injury, to our knowledge, no previous studies have addressed whether changes in sphingolipid metabolite expression also result. Since sphingomyelin has been reported to be relatively well preserved following renal ischemic/reperfusion injury [44, present data] accumulation of sphingolipid breakdown products might seem unexpected. However, the recent explosion of information concerning the existence of a sphingomyelin (ceramide/sphingosine) signaling cascade [1–4], plus the fact that sphingosine and ceramide levels might change independent of net sphingomyelin breakdown (*vide infra*) prompted the present series of experiments.

Indeed, the results of our studies clearly show that marked alterations in both ceramide and sphingosine expression occur during the evolution of renal tubular ischemic damage. When whole kidneys were subjected to ischemia, essentially parallel (~35 to 50%) reductions in renal cortical sphingosine and ceramide concentrations resulted. These did not reflect static changes, since during 30 minutes of reperfusion, a dramatic rebound in both sphingosine and ceramide concentrations occurred. In the case of sphingosine, the post-ischemic increase resulted in a return to control concentrations. However, ceramide rebounded to ~150 to 175% of normal values. Since these increases were noted at both 30 and 60 minutes of reperfusion, they theoretically were of sufficient duration to impact the evolution of reperfusion damage. It is noteworthy that these sphingosine and ceramide changes (↓ with ischemia/ ↑ with reperfusion) are opposite in direction to those which occur with free fatty acids and lysophospholipids (↑ with ischemia/ ↓ with reperfusion). This indicates that fundamentally different mechanisms must control the accumulation and disposal of glycerolipid and sphingolipid breakdown products. That is, unlike the fatty acid/lysophospholipid changes, the ceramide/sphingosine alterations cannot simply be explained first by parent compound hydrolysis and then subsequent metabolism of the generated catabolites.

One potential caveat of the above discussed cortical tissue assessments is that the observed ceramide and sphingosine fluxes might not have reflected proximal tubular cell events. This is because of the heterogeneity of cell types within renal cortex, making it impossible to conclude that perturbations in sphingosine or ceramide concentrations existed within the proximal
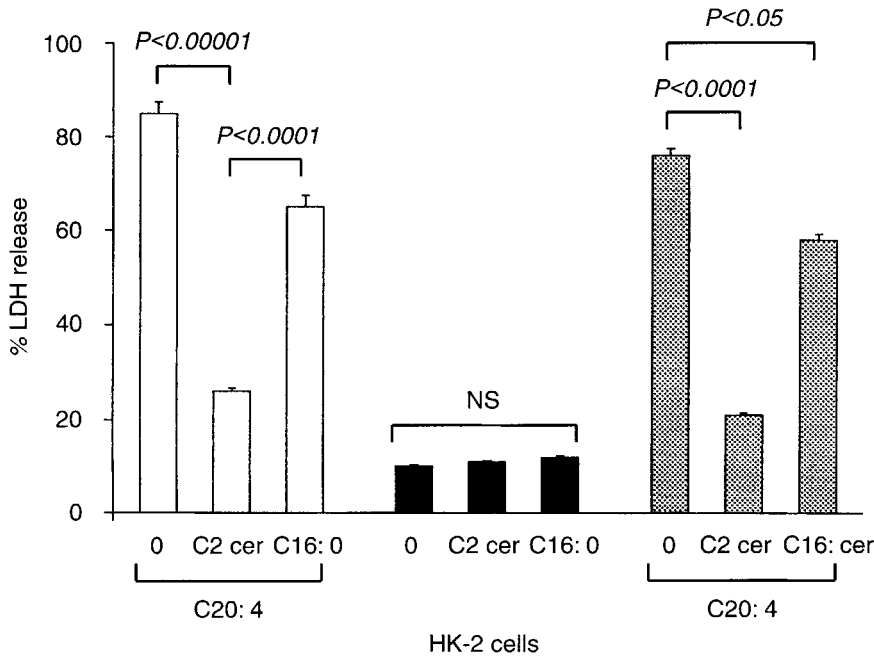
Fig. 9. Effects of C2 ceramide on the expression of arachidonic acid (C20:4) toxicity in HK-2 cells. When C20:4 was added to HK-2 cells under control conditions, ~85% cell lysis (LDH release) developed within two hours. C2 ceramide (10 min pre-treatment + its presence during the C20:4 challenge) almost completely eliminated C20:4's lytic effect. In contrast, palmitic acid (C16:0; the dominant saturated fatty acid released during ischemic/hypoxic PTS injury) had only a slight effect on the expression of C20:4's cytotoxic effect (serving as a lipid control for the C2 ceramide). Neither C2 ceramide nor C16:0 had an independent effect on LDH release, as determined over the two hours incubation period (■). As shown at the right of the figure, C2 ceramide was far more cytoprotective than was C16 ceramide. Since C2, but not C16, ceramide penetrates cells, these findings are consistent with cell uptake as an important determinant of ceramide's ability to mitigate C20:4 toxicity.

tubular epithelium. Since the latter is viewed by most investigators as the critical target of ischemic renal damage, we sought confirmation that oxygen deprivation does, in fact, perturb sphingosine/ceramide expression directly at the proximal tubular level. Therefore, ceramide and sphingosine levels were studied during the course of hypoxic injury in isolated PTS. After completing 20 minutes of hypoxia, sphingosine concentrations were depressed by ~50%. This strongly suggests that the sphingosine depressions observed during *in vivo* ischemia reflected, at least in part, a proximal tubular cell event. Unlike the renal cortical results, ceramide levels slightly rose, rather than fell, during O_2 deprivation PTS injury (in a sense, recreating the ceramide increments observed during early *in vivo* reperfusion). However, this difference in timing of the ceramide increments should not necessarily be considered contradictory given inherent differences between *in vitro* and *in vivo* proximal tubular damage. For example, during *in vitro* hypoxic injury, cell death is predominantly expressed during the oxygen deprivation period; in contrast, *in vivo* tubular cell death is generally considered to be an early post-ischemic event [48]. Thus, in both the *in vivo* and *in vitro* model systems, the ceramide increases seemed to denote the lethal injury phase.

It is noteworthy that the employed hypoxic PTS challenge induced ~50 to 60% LDH release. This raises the possibility that the observed alterations in sphingosine and ceramide expression were merely secondary reflections of tubular cell death. For example, dying cells might fail to maintain normal sphingosine concentrations, and ceramide increments might simply reflect nonspecific ceramide release from lethally damaged plasma membranes. However, the available data argue against this view. First, addition of exogenous PLA_2 to PTS induced ~50% sphingosine depressions, but there was no corresponding loss of cell viability. Second, tubules subjected to hypoxia or to myohemoglobin loading manifested comparable degrees of lethal cell injury (~50 to 60% LDH release), and yet, only hypoxia caused ceramide increments. Third, if the sphingosine depressions observed during

in vivo ischemia were simply due to lethal cell injury, a dramatic recovery of sphingosine would not be expected during the early reperfusion period. In sum, these observations indicate that the altered sphingosine and ceramide levels were not simply secondary consequences of tubular cell death.

Although fluxes in both sphingosine and ceramide content appear to be hallmarks of O_2 deprivation-mediated tubular injury, the triggers for these changes and the metabolic pathways through which they are effected remain to be defined. These are highly complex issues to resolve since alterations in synthesis, catabolism, and metabolic conversion to alternative compounds might each be involved. However, the available data suggest the following: First, rapid reductions in sphingosine and ceramide during renal ischemia must imply their consumption ± a concomitant block in production. Whether metabolic conversion or simple degradation of sphingosine and ceramide is involved remains unknown. However, the failure of either DMS or fumonisin B1 to preserve sphingosine content during PLA_2-induced PTS injury suggests that simple compound flux via the sphingosine kinase or ceramide synthase pathway is probably not involved. Second, the ischemic sphingosine and ceramide reductions probably do not simply stem from a single metabolic event (such as, sphingomyelinase inhibition). This is suggested by the fact that the heme loaded PTS and PLA_2-exposed PTS demonstrated marked sphingosine depressions in the absence of any corresponding reduction in ceramide content. Third, PLA_2 activation could be one stimulus that initiates sphingosine depletion during ischemic tubular damage. This is suggested by the fact that renal ischemia activates PLA_2 [29], and that PLA_2 addition to PTS caused prompt reductions in sphingosine content. (This new insight raises the intriguing possibility that some of PLA_2-induced signaling could conceivably involve secondary sphingosine depletion); and Fourth, post-ischemic increments in ceramide concentrations are not simply a result of sphingomyelin catabolism. This is because no decrement in sphingomyelin content was observed in either the

in vivo or the *in vitro* experiments. Thus, either increased sphingomyelin turnover (parallel increases in production and hydrolysis), or breakdown of alternative ceramide containing molecules (such as, glycosphingolipids) are seemingly involved. That PTS ceramide levels slightly rose during hypoxia (when synthesis should have been blocked) is consistent with the latter possibility.

Since ceramide concentrations were found to rise during early reperfusion, the final goal of this study was to gain some support for the concept that such increments could potentially contribute to the evolution of renal tubular damage. Towards this end, the impact of an increased cell ceramide burden on a well characterized model of ATP depletion/Ca^{2+} overload injury [43] was assessed. When applied in a physiologically relevant and sublethal dose, cell membrane permeable (C2) ceramide dramatically increased the extent of ATP depletion/Ca^{2+} ionophore-induced cell death. That cell impermeant (C16) ceramide did not reproduce this result suggests that cell ceramide uptake, and not simply membrane contact, is required for this effect. We next questioned whether ceramide might also affect cellular responses to PLA_2- and arachidonic acid-mediated attack. Although PLA_2 induced no lethal HK-2 cell injury (not surprising since it also failed to do so in the PTS), sublethal damage did occur, as denoted by a "rounding up" of the cells with subsequent detachment. Although C2 ceramide alone induced no cell detachment, it doubled cell detachment during PLA_2 attack. In contrast, the palmitic acid control slightly decreased PLA_2-induced cell detachment, suggesting that the ceramide effect was not simply a nonspecific lipid effect. Finally, and most dramatically, C2 ceramide paradoxically protected against arachidonic acid-mediated cytotoxicity, decreasing LDH release from 85% to 26%. This could not simply be explained by a block in HK-2 cell arachidonate uptake, since no decrease in ^3H arachidonic acid accumulation was noted. Furthermore, a nonspecific physical alteration of the arachidonic acid challenge within the culture medium seems unlikely, since neither C16 ceramide nor palmitic acid reproduced C2 ceramide's protective effect. Thus, it appears that cell ceramide insertion may have stabilized the plasma membrane against arachidonic acid attack. (In a sense, this protective action could potentially offset some of ceramide's injury provoking effects, as noted above). In sum, when these three sets of cell injury experiments are viewed together, they support the hypothesis that cell ceramide content may, in fact, modulate the expression of renal tubular damage. Although these results remain largely "descriptive" at this time, they nevertheless seem important: they provide a rationale for further exploration of ceramide effects on acute renal tubular injury and repair. That ceramide is known to trigger apoptosis [1–5, 16–22], a well documented process within the post-ischemic kidney [49, 50], provides an additional impetus for such investigations.

In conclusion, the present studies provide the first evidence that sphingosine and ceramide expression are markedly altered during the induction stage of ischemic acute renal failure. During ischemia, ~35 to 50% depressions in ceramide and sphingosine result. Conversely, during reperfusion, sphingosine and ceramide levels rapidly rebound to either normal (sphingosine) or supranormal (ceramide) levels. These fluxes occur independent of alterations in total sphingomyelin content, suggesting that rates of sphingomyelin hydrolysis are not the prime, or only, determinant of these changes. Since sphingosine and ceramide each exert potent and diverse cell signaling effects, these newly documented changes

could have important implications for the induction and evolution of post-ischemic acute renal failure. That PLA_2, an important determinant of ischemic injury, causes dramatic sphingosine decrements, and that ceramide loading of cultured human proximal tubular cells alters the severity of three different forms of tubular injury (ATP depletion/Ca^{2+} overload, PLA_2 attack, and arachidonate toxicity) strongly support this hypothesis. Given these observations, further definition of sphingosine and ceramide effects on ischemic/reperfusion injury appear to be important areas for future investigation.

ACKNOWLEDGMENTS

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