Ceramide Accumulation During Oxidant Renal Tubular Injury: Mechanisms and Potential Consequences

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Abstract. Ceramide is an important signaling molecule that is typically generated via sphingomyelinase (SMase)-mediated sphingomyelin (SM) hydrolysis. Although diverse forms of renal injury elicit ceramide accumulation, the molecular determinants of this change and its contribution to tissue damage are poorly defined. The present study uses iron (Fe/hydroxyquinoline)-mediated injury of cultured human proximal tubular (HK-2) cells to gain additional insights into these issues. A 4-h Fe exposure doubled ceramide levels in the absence of cell death. This was independent of de novo synthesis, since ceramide synthase inhibition (with fumonisin B1) had no effect. Oxidant stress directly suppressed, rather than stimulated, SMase activity by: (1) decreasing SMase levels; (2) depleting SMase-stimulating glutathione; and (3) increasing SM resistance to SMase attack. Fe suppressed cell sphingosine levels (3 to 4 times ceramidase/sphingosine ratio increments), suggesting a possible ceramidase block. Fe did not directly affect HK-2 ceramidase levels. However, arachidonic acid (C20:4) accumulation, a consequence of oxidant-induced phospholipase A2 (PLA2) activation, markedly suppressed ceramidase and stimulated SMase activity. Exogenous C20:4, as well as PLA2 (in doses simulating Fe-induced decylation) recapitulated Fe’s ceramide-generating effect. Because C20:4 is directly cytoxic, it was hypothesized that ceramide might offset some of C20:4’s adverse effects. Supporting this possibility were the following: (1) C20:4 exacerbated Fe toxicity; (2) this was abrogated by ceramide treatment; and (3) ceramide blunted Fe-mediated cell death. Conclusions: (1) ceramide accumulation during acute cell injury can be an adaptive response to PLA2 activation/C20:4 generation; (2) C20:4-induced ceramidase inhibition, coupled with SMase stimulation, may trigger this result; and (3) these ceramide increments may exert a “biostat” function, helping to offset C20:4/PLA2- and “catalytic” iron-mediated tubular cell death.

During the past decade, it has become clear that sphingomyelin (SM), which accounts for approximately 15% of renal cortical phospholipid content (1), is a source of important cell signaling molecules (2-9). The classic view of this signaling cascade is that when cells are confronted with a diverse array of physiologic or pathophysiologic stimuli, one or more sphingomyelinases (SMases), e.g., within plasma or lysosomal membranes, are activated. The result is cleavage of phosphorycholine from SM, forming ceramide. Ceramide can then undergo deacylation to sphingosine, which in turn is phosphorylated, forming sphingosine-1-phosphate (see Figure 1). Each of these molecules can exert important, and potentially offsetting, biological effects. In general, ceramide causes cell cycle arrest and may trigger apoptosis (6). Conversely, sphingosine and sphingosine-1-phosphate can initiate cell dedifferentiation and exert either mitogenic or cytotoxic effects (10,11).

Given these protean influences, it seems quite plausible that were the “SM pathway" activated during ischemic or toxic renal injury, it could have an impact on the expression of acute renal failure. Several recent pieces of information support this view. First, increased renal cortical ceramide levels have been observed after the induction of diverse forms of kidney damage (e.g., ischemic and toxic tubular injury; anti-glomerular basement membrane antibody-induced acute glomerulonephritis) (12). Second, ceramide loading of cultured renal tubular cells can either induce tubular necrosis (13) or exacerbate ATP depletion-mediated tubular cell death (14,15). And third, if tubular cells are exposed to sphingosine for 18 to 24 h (permitting sufficient time for its metabolic conversion to ceramide or other sphingolipids), the cells acquire a “cytoresistant” state (i.e., they become resistant to ATP depletion-induced attack) (13).

Despite this evidence suggesting the potential importance of the sphingomyelin pathway to the evolution of acute renal injury, the biochemical determinants that give rise to altered sphingolipid expression during acute cell injury remain poorly defined. Although the classic explanation for ceramide accumulation during physiologic “stress" is SMase activation, several pieces of evidence suggest that this is an overly simplistic view. First, data gathered in our laboratory have dissociated injury-induced ceramide accumulation from SMase activity, as assessed by SMase assay of cortical tissue extracts (11). Second, although SMase-mediated SM hydrolysis should dictate a secondary increase in sphingosine (i.e., its proximate downstream catabolite) (Figure 1), we have observed that acute cell injury typically depresses, rather than increases, sphingosine content (14). And third, there are recent suggestions that de
Sphingomyelin Pathway During Oxidant Stress

novoceramide synthesis, rather than increased SM hydrolysis, may, in selected circumstances, be responsible for ceramide accumulation during cellular stress (15,16).

In light of the above findings, the present study was undertaken to better define some of the biochemical determinants, and potential consequences, of altered ceramide and sphingosine expression during the evolution of acute renal tubular damage. To this end, a single injury model was selected to permit in-depth analysis. The model chosen was iron-mediated oxidant injury, since intracellular "catalytic" iron has been implicated as a critical mediator of diverse forms of both toxic and ischemic tubular cell damage (17-23). Thus, it was hypothesized that results obtained with this particular injury model could have broad-based biological relevance.

Materials and Methods

Cell Culture Conditions

HK-2 cells (an immortalized proximal tubule cell line derived from normal human kidney) (24) were used for all of the following experiments. They were cultured in T75 Costar flasks (Cambridge, MA) with keratinocyte serum-free medium (K-SFM, Life Technologies, Grand Island, NY) containing 1 mM glutamine, 5 ng/ml epidermal growth factor, 40 μg/ml bovine pituitary extract, 25 U/ml penicillin, and 25 μg/ml streptomycin (37°C; 5% CO2). At near confluence, the cells were trypsinized and transferred to either additional T75 flasks or to Costar cluster plates (6 or 24 wells). Cells growing in the T75 flasks were used for experiments requiring ceramide and sphingosine quantification. Cells grown in 6- or 24-well plates were used for specific cell injury experiments, as delineated below.

Does Fe-Induced Oxidant Stress Alter Tubular Cell Ceramide and Sphingosine Content?

Iron Challenge. Iron-mediated oxidant stress was induced for a 4-h period by addition of 10 μM ferrous ammonium sulfate complexed to 10 μM 8-hydroxyquinoline (Fe/HQ). HQ functions as a siderophore, allowing for ready intracellular iron access (25). The 10 μM Fe/HQ dose was first demonstrated to induce nonlethal cell injury within a 4-h period (<5% increase in percentage of cell lactate dehydrogenase [LDH] release; no morphologic evidence of apoptosis). Hence, this protocol permitted assessment of ceramide and sphingosine changes during the evolution of cell injury (rather than reflecting changes inherent to cell death). At the end of the 4-h challenge, these flasks and an equal number of control flasks were harvested for biochemical analyses. They were immediately iced, the culture medium decanted, and the medium was centrifuged to capture a small number of detached cells. Hanks' balanced salt solution (+ Mg/Ca; HBSS) was added (3 ml) to the flasks, and the cells were detached with a rubber policeman followed by centrifugation. This process was repeated twice. Finally, the recovered cells were pelleted and subjected to chloroform/methanol extraction (14), the lipid phase was dried under N2, and the samples were saved for ceramide or sphingosine analysis (see below). Separate flasks were used for either ceramide (n = 10) or sphingosine (n = 6) quantification. The results were contrasted with those observed in an equal number of cocultured flasks maintained under normal incubation conditions.

Effects of Deferoxamine on Fe/HQ-Induced Ceramide Changes. To prove that the Fe/HQ-induced changes in sphingolipid expression were secondary to cellular iron (and not simply HQ) exposure, six T75 flasks of cells were prepared and treated for 4 h, as follows (n = 2 each): (1) control incubation; (2) Fe/HQ exposure, as denoted above; and (3) Fe/HQ exposure + an iron chelator (2 mM deferoxamine [DFO]). HK-2 cell ceramide content was then assessed. (Note: One cannot simply add HQ to cells to assess an independent HQ effect because HQ binds trace free iron in the culture medium, thereby producing the Fe/HQ complex and allowing intracellular Fe access.)

Ceramide Assay. Ceramide was quantified by the bacterial diacylglycerol kinase assay of Younes et al. (26), as used previously in this laboratory (12,14). This assay is based on the principle that ceramide is phosphorylated to ceramide-1-32P in the presence of [γ-32P]ATP and diacylglycerol kinase. The amount of ceramide-1-32P formed is directly related to tissue ceramide content. The ceramide-1-32P is then separated from other lipids/reactants by thin layer chromatography (TLC), followed by autoradiographic analysis (14). The ceramide-1-32P band is recovered by scraping, followed by counting in a scintillation counter. The amount of ceramide present is calculated from a standard curve constructed with type three ceramide standards (14). Values were expressed as pmol of ceramide/nmol of phospholipid phosphate (27).

Sphingosine Assay. Sphingosine concentrations within cell extracts were determined by the method of Merrill et al. (28), as performed previously in this laboratory (14). In brief, after cell lipid extraction, free long chain bases are derivatized with o-pthalaldehyde-hydride. The derivatized products are then separated by HPLC with detection by spectrofluorometry. Sphingosine values are calculated from a standard curve constructed with sphingosine standards (14). Sphingosine values were expressed as pmol/nmol of sample phospholipid phosphate.

Does Fe-Mediated Oxidant Stress Alter HK-2 Cell SMase Content?

The following experiment was undertaken to determine whether iron-mediated ceramide accumulation could be a result of an upregulation of SMase activity. T75 flasks were exposed to the 4-h Fe/HQ challenge or to control incubation conditions (n = 9). Then, the media were decanted and saved, iced HBSS was added, the flasks were scraped and the detached cells were recovered by centrifugation. The samples were divided into two aliquots for either neutral (Mg2+-dependent; plasma membrane-associated) SMase assay (n = 9) or acidic (lysosomal-associated) SMase assay (n = 5), as described previously (12). In brief, the cells were lysed in either a neutral or acidic lysis buffer, followed by sample centrifugation at 1,000 rpm or 14,000 rpm, respectively (12). SMase activities were assessed by the degree to which the acidic or neutral extracts hydrolyzed sphingomyelin (a mixture of 0.2 μCi of N-methyl 14C-sphingomyelin + either 7.5 or 10 nmol of unlabeled sphingomyelin) under acidic or neutral pH conditions, respectively (29,30). Enzyme activities were expressed as nmol sphingomyelin hydrolyzed/mg extract protein per h (i.e., 14C-phosphorylcholine released from SM).

Does Oxidant Stress Alter the Ability of SMase to Hydrolyze SM?

The following experiments assessed whether oxidant stress alters the ability of pre-formed SMases to induce SM hydrolysis. To this end, neutral and acidic SMase-containing tissue extracts were obtained from normal male CD-1 mouse kidneys (approximately 50 g; Charles River Laboratories, Wilmington, MA), as described previously (12). Enzyme activities within 15 or 25 μg of acidic or neutral protein extract, respectively, were then assessed as noted above under one of the following conditions: (1) normal incubation conditions; (2) in the presence of either 1 or 5 mM H2O2 (added to the reaction); or
(3) when SM was first pretreated with 5 mM H$_2$O$_2$, and then the H$_2$O$_2$ was removed before its addition to the SMase assay. (The latter was done by adding the H$_2$O$_2$ to the labeled and unlabeled SM, followed by drying under N$_2$ to remove the H$_2$O$_2$; SM subjected to these same drying conditions, but without H$_2$O$_2$ exposure, was used for the paired control reactions.) Each of the above experiments was conducted with at least six paired determinations (experimental and control conditions).

Do Glutathione Levels Alter Renal Tubular Cell SMase Activity?

Free thiols (glutathione, cysteine) have previously been reported to suppress neuronal SMase activity (31). Because glutathione (GSH) depletion develops during iron-induced oxidant stress (32), the following experiment assessed whether a loss of GSH could explain the Fe/HQ-induced ceramide increments. To this end, acidic and neutral mouse SMase extracts were assayed for acidic or neutral SMase activity, respectively, either under normal incubation conditions or in the presence of exogenous GSH (2.5 or 5 mM; i.e., physiologic concentrations) (32). Six paired assessments under each condition were made.

Does de Novo Synthesis Cause the Iron-Induced Ceramide Increments?

HK-2 cells were cultured under one of the following conditions (three flasks each): (1) normal conditions; (2) with 10 μM Fe/HQ; or (3) with Fe/HQ + a ceramide synthase inhibitor (50 μM fumonisin B$_1$ [FB$_1$]) (15) (Sigma Chemicals, St. Louis, MO). Four hours later, cell ceramide concentrations were assessed, as noted above.

Does Sphingosine → Ceramide Flux Cause the Iron-Induced Sphingosine Decrements?

Ten flasks of HK-2 cells were divided into four treatment groups: (1) control incubation conditions (n = 2); (2) 4-h control incubations in the presence of 50 μM FB$_1$ (n = 2); (3) the 4-h Fe/HQ challenge (n = 3); and (4) the 4-h Fe/HQ challenge in the presence of FB$_1$ (n = 3). The cells were then harvested and assayed for sphingosine content, as noted above.

Does PLA$_2$ Activity Alter HK-2 Ceramide Expression and SMase Activity?

PLA$_2$ and SMase have been suggested to have interrelated signaling effects (33–36). The goal of the following four sets of experiments was to ascertain whether interactions between these two pathways might be responsible for the Fe/HQ-induced ceramide increments.

Does PLA$_2$ alter HK-2 Cell Ceramide Content?. T75 flasks of HK-2 cells were subjected to ceramide analysis after the following 4-h incubations: (1) control conditions; (2) in the presence of 5 U/ml PLA$_2$ (from porcine pancreas; P6534; Sigma); or (3) with 10 U/ml PLA$_2$ (n = 6, 5, and 4 flasks, respectively).

Does C20:4 Reproduce PLA$_2$'s Effects on HK-2 Cell Ceramide Content?. Flasks of HK-2 cells were assayed for ceramide content after 4-h incubations under the following conditions: (1) control incubation; (2) in the presence of 10 μM C20:4 (A9673; Sigma; in 0.1% ethanol final concentration); or (3) in the presence of 25 μM C20:4 (n = 3 each).

Does C20:4 Affect Renal Tubular Cell SMase Activity?. To assess the direct effect of C20:4 on renal SMase activity, mouse cortical extracts were assayed for acidic and neutral SMase activity either under control conditions or in the presence of exogenous C20:4 (50, 100, or 250 μM) (n = 6 each).

Does the Fe/HQ Challenge Cause C20:4 Release?. Oxidant injury is known to activate PLA$_2$, causing C20:4 release (37–41). The following experiment was undertaken to test whether the 10 μM Fe/HQ challenge caused comparable C20:4 release as the above PLA$_2$ challenge, thereby allowing the PLA$_2$ and Fe/HQ effects on ceramide accumulation to be compared. HK-2 cells were cultured in 6-well plates for 24 h, and then $^3$H-C20:4 (approximately 100,000 dpm; New England Nuclear, Wilmington, DE) was added. After allowing 18 h for $^3$H-C20:4 incorporation, the cells were washed to remove the unincorporated label, and then they were challenged with either: (1) no additions; (2) 5 U/ml PLA$_2$; or (3) 10 μM Fe/HQ. After a 2-h incubation, 1 mg/ml fatty acid free bovine serum albumin was added (to bind released C20:4). After another 2 h, the percentage of C20:4 release (42) was determined (increase above control incubation samples; n = 3 samples each).

Does C20:4 Alter Ceramidase Activity?

The following experiment was undertaken to evaluate whether C20:4 affects ceramidase activity, potentially affecting both ceramide and sphingosine expression during acute cell injury. Mouse kidney cortex was iced, minced, washed twice in HBSS, and then homogenized in 0.25 M sucrose + 1 mM ethylenediaminetetra-acetic acid, pH 7.0. Neutral and acidic ceramidase activities were then assayed in the presence or absence of exogenous C20:4 (43). This assay is based on the ability of tissue extracts to deacylate $^3$H-ceramide, forming $^3$H-sphingosine. In brief, [3-$^3$H]-N-hexanoyl-sphingosine (i.e., $^3$H-ceramide; Amersham, Buckinghamshire, England; specific activity, 6435 counts per nmole) + 50 nmol unlabeled C6 ceramide (Biomol; Plymouth Meeting, PA) were incubated with tissue extract (approximately 150 μg of protein) in the presence or absence of exogenous C20:4 (see below) under either acidic (pH 4.5) or neutral (pH 7.4) assay conditions (43). The reaction mixture contained 0.1 to 0.25 mg of Triton X-100 and 0.2 to 0.4 mg of sodium cholate (final volume, 200 μl). The reactions were incubated at 37°C for 90 min and then stopped by the addition of eight parts chloroform:methanol (2:1) + 1 part H$_2$O. The lower phase partition was washed and dried under N$_2$. The recovered lipids were subjected to TLC with a solvent system consisting of chloroform/methanol/5N NH$_4$OH/H$_2$O (80/20/0.5/0.5). After completion, the plates were stained with 3% primulin dye and the lipids were visualized by ultraviolet light. The band corresponding to sphingosine (determined by including 15 μg of unlabeled sphingosine standard at the time of each TLC run) was cut and then counted in a scintillation counter. Ceramidase activities were expressed as nmol of deacylated ceramide/mg protein extract per h. Using the above approach, neutral and acidic ceramidase activities were determined on the following samples: normal tissue extract or tissue extract + either 50, 100, or 250 μM exogenous C20:4 (n = 6 paired determinations for each).

Does Fe-Mediated Oxidant Stress Suppress HK-2 Ceramidase Levels?

Flasks of HK-2 cells were cultured either in the presence (n = 4) or absence (n = 4) of Fe/HQ for 4 h. The cells were then harvested as described for the ceramide assay, and a portion of the whole lysed cell extract was saved for neutral ceramidase assay as noted above. (Note: It was first demonstrated that there was only trace acidic ceramidase, and hence, only neutral ceramidase was assessed.)
Does Ceramide Affect C20:4/Fe/HQ-Mediated Injury?

C20:4 Effects on Fe Cytotoxicity: Modulation by Ceramide.
The following experiment assessed whether C20:4 accumulation adversely affects Fe/HQ cytotoxicity, and, if so, what the effect of concomitant ceramide accumulation might be on this reaction. HK-2 cells, grown in 24-well cluster plates, were incubated for 4 h, as follows: (1) normal conditions; (2) 10 μM C20:4 (in ethanol, final concentration, 0.1%); (3) 10 μM C2 ceramide (Biomol); (4) 10 μM Fe/HQ; (5) Fe/HQ + C20:4; (6) Fe/HQ + C2 ceramide; (7) Fe/HQ + C2 ceramide + C20:4. Cell injury was assessed by percentage LDH release after completing 4-h incubations (n = 8 samples each).

Ceramide Effect on Fe Cytotoxicity. The following experiment assessed whether ceramide alters the degree of Fe/HQ toxicity, as assessed over a 24-h period. HK-2 cells were incubated as follows: (1) control conditions; (2) 10 μM Fe/HQ; or (3) Fe/HQ + 10 μM C2 ceramide. At the completion of the 24-h incubations, percentage LDH release was assessed (n = 8 samples each).

Statistical Analyses

All values are given as means ± 1 SEM. Statistical comparisons were performed by either paired or unpaired t test, as appropriate. If multiple comparisons were made, the Bonferroni correction was applied. Statistical significance was judged by a P value of <0.05.

Results

Iron-Induced Changes in HK-2 Cell Ceramide and Sphingosine Content

Figure 1 depicts the metabolic pathways that can lead to altered ceramide and sphingosine expression. As shown in Figure 2 (left panel), 4 h of Fe/HQ exposure caused an approximate 125% increase in HK-2 cell ceramide content. When the Fe/HQ challenge was conducted in the presence of DFO, no ceramide increment was observed.

In contrast to the Fe/HQ-induced ceramide increments, Fe/HQ induced an approximate 35% decrease in HK-2 cell sphingosine content (Figure 2, right panel). This led to an approximate 3.5 to 4 times increase in ceramide/sphingosine ratio (suggesting decreased ceramidase activity).

Iron Effects on HK-2 Cell SMase Content

The 4-h Fe/HQ challenge caused a 60% reduction in neutral (plasma membrane-associated) SMase activity, as assessed in HK-2 cell extracts (Figure 3, left panel). However, acidic (lysosomal) SMase activity was unaffected (Figure 3, right), indicating the relative specificity of the neutral SMase results.

Effect of Oxidant Stress on SMase-Mediated SM Hydrolysis

Addition of 1 or 5 mM H₂O₂ to the neutral SMase reaction caused statistically significant, dose-dependent reductions in

![Figure 2](image21x3to591x789)

*Figure 2.* Ceramide and sphingosine expression in HK-2 cells exposed to ferrous ammonium sulfate complexed to 8-hydroxyquinoline (Fe/HQ)-induced oxidative stress. (Left Panel) Within 4 h of Fe/HQ exposure, an approximate doubling of ceramide concentrations resulted. Cont, control incubation conditions. This increase was completely abrogated by concomitant treatment with an Fe chelator (deferoxamine [DFO]), indicating that the ceramide increase was due specifically to Fe, rather than to HQ, exposure. (Right Panel) In contrast to the ceramide increments, the Fe/HQ challenge significantly decreased HK-2 cell sphingosine content. These changes resulted in an approximate 3.5 to 4 times increase in the ceramide/sphingosine ratio (consistent with ceramidase inhibition).

![Figure 3](image21x3to591x789)

*Figure 3.* SMase levels in HK-2 cell extracts under normal conditions and after 4 h of Fe/HQ-mediated attack. Fe/HQ induced an approximate two-thirds reduction in neutral SMase in HK-2 cell extracts (left panel). The relative specificity of this result was indicated by the fact that Fe/HQ had no effect on acidic SMase levels (right panel).
neutral SMase activity (15 and 24%, respectively) (Table 1). Conversely, H₂O₂ did not affect acidic SMase-mediated SM hydrolysis.

When SM was first exposed to 5 mM H₂O₂ (and then the H₂O₂ was removed), a significant (26%) reduction in neutral SMase-mediated hydrolysis resulted (Table 1). However, this same SM pretreatment with H₂O₂ did not affect acidic SMase-mediated hydrolysis.

GSH Effects on SMase Activity
As shown in Figure 4, GSH induced a dose-dependent in vitro stimulation of neutral SMase activity (left panel). At the 5 mM (normal tubular cell) GSH concentration, a doubling of enzyme activity was apparent. In contrast, GSH had no effect on the acidic SMase reaction (right panel).

Ceramide Synthase Inhibition: Impact on Ceramide and Sphingosine Levels
As illustrated in Figure 5, left panel, Fe/HQ doubled ceramide levels (i.e., recapitulating the Figure 2 results). This ceramide increase was completely unaffected by FB₁ treatment.

When added to control cells, FB₁ doubled sphingosine concentrations (Figure 5, right). (This confirmed that the FB₁ did, in fact, induce ceramide synthase inhibition [i.e., it blocked sphingosine conversion to ceramide, thereby increasing sphingosine concentrations].) When the Fe/HQ challenge was added

Table 1. Effects of oxidant stress on the ability of SMase to induce SM hydrolysis

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>1 mM H₂O₂</th>
<th>5 mM H₂O₂</th>
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<tr>
<td>H₂O₂ (in reaction)</td>
<td></td>
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<tr>
<td>neutral SMase activity</td>
<td>9.5 ± 0.8</td>
<td>8.1 ± 0.6c</td>
<td>7.2 ± 0.6c</td>
</tr>
<tr>
<td>acidic SMase activity</td>
<td>85 ± 0.2</td>
<td>84 ± 1</td>
<td>85 ± 1</td>
</tr>
<tr>
<td>H₂O₂ (SM Pre-Rx)</td>
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<td></td>
</tr>
<tr>
<td>neutral SMase activity</td>
<td>9.4 ± 0.8</td>
<td>NT</td>
<td>7.2 ± 0.4c</td>
</tr>
<tr>
<td>acidic SMase activity</td>
<td>81 ± 2</td>
<td>NT</td>
<td>83 ± 1</td>
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</table>

* Effect of oxidant stress on the ability of mouse acidic or neutral SMase to induce in vitro SM hydrolysis. All enzyme activities are expressed as nmol SM hydrolyzed/mg mouse kidney protein extract per h. Values are means ± 1 SEM. n = 6 to 8 paired reactions per group. SMase, sphingomyelinase; SM, sphingomyelin; SM, sphingomyelin; NT, not tested.

b When either 1 or 5 mM H₂O₂ was added to the neutral SMase reaction, a significant depression in enzyme activity resulted. Conversely, when H₂O₂ was added to the acidic SMase reaction, no effect was apparent.

c P = 0.02 versus results obtained in the absence of H₂O₂.

d When SM was pretreated with 5 mM H₂O₂ and then the H₂O₂ was removed, the SM had increased resistance to neutral SMase-mediated hydrolysis. Conversely, H₂O₂ pretreatment did not alter acidic SMase-mediated hydrolysis.

Figure 4. Effect of glutathione (GSH) on mouse kidney SMase activity. Addition of GSH to mouse renal cortical extracts caused a prominent, dose-dependent increase in neutral SMase activity (left panel). In contrast, GSH had no effect on acidic SMase activity (right panel).

Figure 5. Effect of ceramide synthase inhibition on Fe/HQ-mediated ceramide and sphingosine changes. (Left Panel) Addition of FB₁, a ceramide synthase inhibitor, did not prevent ceramide accumulation during Fe/HQ-mediated HK-2 cell attack. Cont, control incubation conditions. (Right Panel) FB₁ significantly increased sphingosine concentrations in non-iron-exposed cells (P < 0.04), indicating that the FB₁ treatment did, in fact, inhibit ceramide synthase activity (i.e., it blocked sphingosine conversion to ceramide). When these FB₁-treated cells were challenged with Fe/HQ, an approximate 50% decrease in sphingosine levels resulted (depicted by the downward vertical arrow). This indicates that Fe/HQ did not lower sphingosine levels by driving its conversion to ceramide. Taken together, these experiments exclude de novo ceramide synthesis as the cause of the iron-triggered ceramide increments.

in the presence of FB₁, sphingosine concentrations fell by approximately 50% (depicted by the vertical arrow in Figure 5).

PLA₂ and Arachidonic Acid (C20:4) Effects on Ceramide Content
Addition of PLA₂ to HK-2 cells induced striking dose-dependent increases in HK-2 cell ceramide content (Figure 6). When exogenous C20:4 was added to HK-2 cells, statistically significant, dose-dependent ceramide increments were also observed (compared with the control cells) (Figure 6).
CERAMIDE

![Graph showing the effect of phospholipase A2 (PLA2) and arachidonic acid (C20:4) addition on HK-2 cell ceramide content.](Image)

**Figure 6.** Effect of phospholipase A2 (PLA2) and arachidonic acid (C20:4) addition on HK-2 cell ceramide content. Addition of PLA2 induced significant (*), dose-dependent increments in HK-2 cell ceramide levels (P < 0.015 and <0.005 at the 5 and 10 U/mL doses, respectively). These increments were largely reproduced by substituting C20:4 for PLA2 (P < 0.02 versus control values).

**C20:4 Effects on SMase Activity**

C20:4 addition to the neutral SMase reaction induced a striking dose-dependent increase in SM hydrolysis (Figure 7, left). At the highest C20:4 dose tested, a threefold increase in neutral SMase activity was observed (P < 1 x 10^-4). In contrast, C20:4 exerted no stimulatory effect on the acidic SMase reaction (Figure 7, right).

**C20:4 Effects on Ceramidase Activities**

As shown in Figure 8, C20:4 additions to mouse kidney cortical tissue extracts induced dose-dependent depressions in both neutral and acidic ceramidase activities. At the highest doses tested, approximately 35% and approximately 50% neutral and acidic ceramidase depressions, respectively, were observed (P < 0.002).

**Fe/HQ Effects on HK-2 Cell Neutral Ceramidase Activity**

Neutral ceramidase activity in control HK-2 cells was 0.49 ± 0.02 nmol ceramide degraded/mg protein per h. This activity did not statistically differ from that observed in HK-2 cells after 4 h of Fe/HQ-mediated attack (0.51 ± 0.02).

**C20:4 Release after PLA2 Addition and During Fe/HQ-Mediated HK-2 Cell Stress**

The addition of 5 U/ml of PLA2 resulted in a 1.3 ± 0.2% release of the 3H-C20:4 label from the HK-2 cells. In contrast, Fe/HQ caused 2.8 ± 0.17% 3H-C20:4 release (P = 0.005 versus PLA2). Thus, these results indicate that the Fe/HQ challenge induced at least, if not more, deacylation than exogenous PLA2.

**Effect of Ceramide on the Expression of C20:4/Fe-Mediated Attack**

As shown in Figure 9, left panel, addition of C20:4 or ceramide (cer) to HK-2 cells caused no increase in LDH release compared with coincubated cells. Fe/HQ addition alone (shaded bar, 0 additions) also did not increase percentage LDH release versus controls over the 4-h incubation period. Adding C20:4 to the iron challenge unmasked iron’s cytopathic effect. Ceramide partially blocked C20:4’s potentiation of the iron-mediated injury (consistent with the fact that ceramide has previously been shown to protect against high C20:4 cytotoxicity without affecting cell C20:4 uptake) (14).

After a 24-h exposure to Fe/HQ, approximately 55% LDH release resulted (Figure 9, right). When the Fe/HQ challenge was conducted in the presence of ceramide, a significant decrease in Fe/HQ toxicity resulted. (Note: This result cannot simply be explained by a nonspecific, lipid-mediated reduction in cell iron uptake because in experiments not presented, high
Figure 9. Lethal cell injury in response to Fe/HQ attack: effects of C20:4 and ceramide. (Left Panel) Addition of 10 μM C20:4 or C2 ceramide did not increase HK-2 cell lactate dehydrogenase (LDH) release over a 4-h period. Fe/HQ also did not increase LDH release over this 4-h period. However, in the presence of C20:4, increased LDH resulted (indicating synergistic C20:4/Fe/HQ toxicity). Ceramide was able to mitigate this C20:4/Fe/HQ interaction. (Right Panel) Fe/HQ caused approximately 55% cell death over the course of a 24-h incubation. C2 ceramide addition blunted this cytotoxicity.

dose ceramide [as well as C20:4, see above] acutely worsened, rather than protected against, Fe/HQ-mediated cell death.)

Discussion

Iron-mediated oxidant stress has been widely implicated as a critical pathogenetic mediator of diverse forms of acute renal tubular injury (e.g., ischemia-reperfusion; mercuric chloride, cisplatin, myohemoglobin, and aminoglycoside toxicity; cold storage-induced tissue damage) (17–23,44). Although the source of this pathogenic “free” iron may vary according to the specific type of insult (e.g., release from cytochromes; degradation of endocytosed heme proteins) (17,20,21), the final pathway leading to cell injury is generally accepted to be iron-catalyzed oxidant tissue stress (45). Given the broad-based importance of these iron-catalyzed oxidant reactions, the present study has sought to: (1) delineate critical changes in sphingolipid expression that they might trigger; (2) characterize the biochemical pathways that underlie these results; and (3) address some of the potential “downstream” pathophysiological consequences of the resultant sphingolipid changes. Because ceramide accumulation has been the most consistently documented sphingolipid alteration after in vivo acute renal injury (12,14,15), it was the principal focus of the current investigation.

In a previous study, we demonstrated that renal cortical ceramide levels double within approximately 24 h of glycerol-induced (rhabdomyolysis) acute renal failure (12). Because this particular form of renal injury has a multifactorial basis (e.g., iron-mediated oxidant stress, renal hypoperfusion/ATP depletion, tumor necrosis factor/cytokine release) (46), it was not previously possible to conclude that iron loading was the proximate cause of the ceramide increments. Furthermore, because whole renal cortical tissues were assayed (12), it was not certain whether the proximal tubular epithelium was, indeed, the site of the ceramide accumulation. The present study resolves these two issues because a 4-h Fe/HQ exposure doubled HK-2 cell ceramide content. It is noteworthy that DFO completely blocked these ceramide elevations, indicating that iron (rather than the HQ carrier) was responsible. Although iron loading caused approximately 55% cell death over a 24-h period, at the time of ceramide assay (4 h) no increased LDH release was observed. This indicates that the ceramide elevations reflect a prelethal event. Hence, they had the potential to alter evolving injury pathways (to be discussed below).

Having documented that cell iron loading triggers ceramide accumulation, we next sought to delineate potential underlying mechanism(s) for this change. The first hypothesis tested was that oxidant stress increases HK-2 cell SMase activity (e.g., by inducing new enzyme synthesis or “activating” the preexistent SMase pool). However, the available data seemingly exclude this possibility: Iron loading did not increase acidic SMase levels, and it lowered neutral SMase levels by approximately 50%. It is noteworthy that in our previous in vivo study (12), renal cortical SMase depressions were observed at 24-h post-glycerol injection. That Fe/HQ reproduced this result within just 4 h in cultured tubular cells indicates that these SMase losses were, indeed, an early and presumably direct consequence of oxidant tissue damage.

Because the Fe/HQ-mediated ceramide increments were associated with decreased, rather than increased, cell SMase content, we next questioned whether SM oxidation might increase its susceptibility to SMase-mediated attack. If true, then oxidant stress could theoretically increase SM hydrolysis despite a decrease in SMase content. To test this possibility, SM was exposed to H₂O₂ and then its susceptibility to either neutral or acidic SMase was determined. Surprisingly, H₂O₂ exposure lowered SM susceptibility to plasma membrane (neutral) SMase, whereas the acidic (lysosomal) SMase reaction was unaffected. It is noteworthy that identical results were obtained whether SM exposure to H₂O₂ was confined to before, or during, the SMase reaction. This indicates that the depressed SM hydrolysis was almost certainly due to oxidative modification of the SM target, and not simply H₂O₂-mediated damage to SMase during the course of the reaction.

A consistent consequence of renal tubular oxidant stress is a fall in cellular GSH content (32). Because GSH has been shown to inhibit neuronal SMase (31), we speculated that Fe-induced GSH depletion (32) would remove a normal inhibitor of SMase activity and thereby cause ceramide accumulation. To test this possibility, GSH was added to the SMase reactions to see whether enzyme inhibition would result. However, GSH induced a marked, dose-dependent stimulation, rather than a depression, in neutral (but not acidic) SMase activity. Thus, it seems clear that GSH depletion is an untenable explanation for ceramide accumulation during oxidant tubular stress. The reason why neuronal and tubular SMases differ vis à vis their regulation by thiol content remains unknown. Nevertheless, these data strongly suggest that important organ-specific differences, as well as organelle differences (e.g., plasma membrane versus lysosomes), in SMase regula-
tion exist which need to be carefully considered when studying this signaling pathway.

In composite, the above three sets of experiments argue that oxidant stress is not a direct stimulus for SMase-mediated SM hydrolysis. Rather, oxidant stress may actually downregulate SM hydrolysis by: (1) partially destroying neutral SMase; (2) decreasing SM susceptibility to neutral SMase; and (3) depleting GSH levels, as discussed above. Given these observations, alternate explanations for the Fe/HQ-induced ceramide accumulation were sought. In light of recent data suggesting that cell “stress” can stimulate de novo ceramide synthesis (15,16), we questioned whether iron-induced oxidant injury might increase ceramide synthase activity, thereby triggering ceramide accumulation. To address this issue, we tested whether FB1, a ceramide synthase inhibitor, would block the iron-induced ceramide increments. Despite the fact that FB1 was clearly active in the HK-2 cells (i.e., it raised sphingosine levels) (Figure 5), it did not attenuate the iron-mediated ceramide accumulation. Furthermore, FB1 did not prevent the iron-induced sphingosine decrements, proving that the reason for the latter was not conversion to ceramide. Therefore, these two FB1 experiments definitively exclude de novo synthesis as the cause of ceramide buildup during Fe-mediated attack.

Another potential explanation for ceramide accumulation during oxidant stress could be a block in ceramidase activity. Under such a circumstance, any ongoing ceramide production (e.g., via residual SMase activity) would cause ceramide increments due to a block in its conversion to sphingosine. To test for this possibility, sphingosine concentrations were measured during Fe-mediated cell attack. That sphingosine levels were significantly depressed, despite an increase in precursor ceramide levels (i.e., a 3 to 4 times increase in ceramide/sphingosine ratio existed), suggested a partial ceramidase blockade. By analogy with neutral SMase, a simple explanation for a possible reduction in ceramidase activity could be enzyme destruction via oxidant attack. However, that Fe/HQ did not lower HK-2 cell neutral ceramidase levels favors a functional block in ceramidase activity.

In a previous study from this laboratory, we observed that addition of exogenous PLA2 to isolated mouse proximal tubular segments caused abrupt declines (within 20 min) in cell sphingosine content (14). This led us to speculate that PLA2 activation/C20:4 accumulation (a result of oxidant stress) (37–41) could potentially block ceramidase activity. If PLA2/C20:4 were also able to stimulate the residual SMase pool (33), this, in concert with a ceramidase block, could explain ceramide accumulation during 4 h of Fe-mediated attack. Strong experimental support for this possibility has been obtained. First, we demonstrated that PLA2 induced dramatic, dose-dependent increments in HK-2 cell ceramide content. Second, exogenous C20:4 reproduced this ceramide accumulation (indicating that C20:4 was the likely secondary mediator of this PLA2 effect). Third, C20:4 induced dose-dependent reductions in both neutral and acidic ceramidase activities. Fourth, C20:4 evoked fourfold increments in neutral SMase activity. And fifth, Fe/HQ caused as much, or more, 3H-C20:4 release from HK-2 cells as did PLA2. This suggests that like PLA2, Fe/HQ generated sufficient C20:4 to raise HK-2 cell ceramide content. Thus, these data strongly support the following pathogenetic schema: (1) iron-mediated oxidant stress triggers PLA2 activation/C20:4 generation (37–41); (2) C20:4 then inhibits ceramidase and stimulates SMase (which is otherwise depressed by oxidant injury); and (3) the combination of SMase stimulation + ceramidase inhibition induces ceramide accumulation. An integrated overview of this schema is depicted in Figure 10.

The final goal of this study was to gain some support for the concept that ceramide accumulation is not simply an epiphenomenon, but rather, it can potentially affect the evolution of iron-mediated tubular cell damage. The first hypothesis tested was that because C20:4 can be highly toxic to tubular epithelium (1,14), a C20:4-induced ceramide increment might serve a “negative feedback” function, offsetting some of C20:4’s cytotoxic effects. To gain support for this hypothesis, two experiments were performed. First, HK-2 cells were exposed to Fe/HQ for 4 h in the presence or absence of exogenous C20:4. That an overall subtoxic dose of C20:4 exacerbated Fe-mediated injury supports the concept that PLA2 activation/C20:4 accumulation can contribute to oxidant-initiated tubular cell death. Second, we addressed whether ceramide treatment might negate C20:4’s ability to promote Fe-mediated attack. Indeed, this was the case because ceramide virtually eliminated C20:4’s adverse effect. The exact mechanism for this ceramide-induced protection remains a subject for further investigation. However, that ceramide can block C20:4 cytotoxicity even in the absence of an iron challenge (14), and that it does not decrease C20:4 cell uptake (14), strongly suggest that ceramide is able to directly oppose at least some of C20:4’s direct cytotoxic effects.

If ceramide is able to block C20:4 toxicity, and if the latter contributes to iron-induced tubular cell death, it should be possible to demonstrate that ceramide can protect against the Fe/HQ challenge. To explore this possibility, HK-2 cells were incubated with Fe/HQ for 24 h either in the presence or absence of exogenous ceramide. Under these circumstances, ceramide reduced Fe/HQ toxicity by approximately 50%. Although this does not necessarily implicate an “anti-C20:4” effect as the reason for this protection, it is at least consistent with this concept. It is noteworthy that diverse forms of acute cell injury can cause C20:4 accumulation (e.g., ischemia-reperfusion), and under these circumstances, it can contribute to lethal cell damage (1,42). Hence, the current observations that C20:4 can stimulate ceramide accumulation, and that ceramide can then induce “feedback inhibition” of C20:4 toxicity, suggest a cellular adaptive response with relevance beyond the confines of the present Fe/HQ results.

From the above discussion, it should be obvious that multiple factors can potentially affect cellular ceramide expression, and that ceramide can exert a panoply of downstream signaling and nonsignaling effects. Hence, the current findings are by no means all-encompassing, and certainly, alternative possibilities and hypotheses exist. It is also possible that results obtained with exogenous ceramide additions to cells, as used in these studies, may not accurately reproduce ceramide effects stemming from pathophysiologic events. Finally, although the
Figure 10. Integrated schema by which oxidant stress may alter the sphingomyelin pathway, resulting in increased ceramide and decreased sphingosine expression in HK-2 cells. Right of the dotted line: Fe-mediated oxidant stress causes a relative decrease in sphingomyelin (SM) hydrolysis by: (1) partially destroying neutral SMase; (2) oxidizing SM, thereby decreasing its susceptibility to neutral SMase-mediated attack; and (3) decreasing GSH levels, a normal stimulant of neutral SMase activity. Each of these should partially suppress ceramide production. Left of the dotted line: Oxidative stress activates PLA₂, which causes C20:4 accumulation. The latter then stimulates residual SMase activity (partially depressed by the events depicted at right), allowing for continued ceramide production. Ceramide then accumulates because C20:4 induces a functional block in both neutral and acidic ceramidase activities. This partial block in ceramidase then lowers sphingosine levels by inhibiting its formation from ceramide. Bottom: Factors favoring ceramide accumulation (left) are greater than the factors decreasing ceramide production (right), and thus, ceramide accumulates. One potential biological implication of the ceramide accumulation is to decrease C20:4 cytotoxicity (14), which can then decrease the overall severity of C20:4/Fe-mediated attack.

The present study has focused on iron-induced cell necrosis, differing mechanisms and consequences could be operative in the setting of apoptotic cell death. Therefore, as with all studies, the current results need to be interpreted in light of these experimental limitations.

With these caveats, we believe that the current study provides the following new insights. (1) Iron-mediated oxidant stress is a potent inducer of ceramide accumulation within proximal tubular cells. (2) This ceramide accumulation appears to be a secondary, rather than a primary, response to oxidant injury because the latter can suppress SM hydrolysis (via decrements in neutral SMase, increments in SM resistance to SMase, and GSH depletion). (3) Oxidant stress-induced PLA₂ activation, and resultant C20:4 accumulation, may be at least partially responsible for the iron-initiated ceramide increments. This may stem from two C20:4 effects: stimulation of residual SMase and induction of a functional block in ceramidase activity. Thus, ongoing SMase activity/ceramide generation, coupled with decreased ceramide catabolism, would be expected to raise ceramide levels. (4) Ceramide can mitigate C20:4 toxicity, as well as iron-mediated tubular cell death.

These findings suggest that although ceramide is typically viewed as “death signal” (4–6), it may also exert cytoprotective effects. Indeed, it is probably a balance between these two opposing forces that ultimately determines ceramide’s final impact on cell “life versus death.”

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