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Sphingosine: A mediator of acute renal tubular injury and subsequent cytoresistance

(ceramide/sphingomyelinase/sphingosine 1-phosphate/dimethylsphingosine/acute tubular necrosis)

M. IWATA*, J. HERRINGTON†, AND R. A. ZAGER*‡

*Fred Hutchinson Cancer Research Center and †Department of Physiology and Biophysics, University of Washington, Seattle, WA 98104-2092

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ABSTRACT **The goal of this study was to determine whether sphingosine and ceramide, second messengers derived from sphingolipid breakdown, alter kidney proximal tubular cell viability and their adaptive responses to further damage. Adult human kidney proximal tubular (HK-2) cells were cultured for 0–20 hr in the presence or absence of sphingosine, sphingosine metabolites (sphingosine 1-phosphate, dimethylsphingosine), or C2, C8, or C16 ceramide. Acute cell injury was assessed by vital dye exclusion and tetrazolium dye transport. Their subsequent impact on superimposed ATP depletion/Ca^{2+} ionophore-induced damage was also assessed. Sphingosine (≥10 μM), sphingosine 1-phosphate, dimethylsphingosine, and selected ceramides (C2 and C8, but not C16) each induced rapid, dose-dependent cytotoxicity. This occurred in the absence of DNA laddering or morphologic changes of apoptosis, suggesting a necrotic form of cell death. Prolonged exposure (20 hr) to subtoxic sphingosine doses (≤7.5 μM) induced substantial cytoresistance to superimposed ATP depletion/Ca^{2+} ionophore-mediated damage. Conversely, neither short-term sphingosine treatment (≤8.5 hr) nor 20-hr exposures to any of the above sphingosine/ceramide derivatives/metabolites or various free fatty acids reproduced this effect. Sphingosine-induced cytoresistance was dissociated from the extent of cytosolic Ca^{2+} loading (indo-1 fluorescence), indicating a direct increase in cell resistance to attack. We conclude that sphingosine can exert dual effects on proximal renal tubular viability: in high concentrations it induces cell necrosis, whereas in low doses it initiates a cytoresistant state. These results could be reproduced in human foreskin fibroblasts, suggesting broad-based relevance to the area of acute cell injury and repair.**

It has long been recognized that plasma membrane phospholipids play crucial roles in cell signaling events. While most attention has been focused on glycerophospholipids and their metabolic byproducts, in recent years an important role for membrane sphingolipids has begun to emerge (e.g., refs. 1–4). Exploration of this area has been stimulated by observations that ceramide and sphingosine, lipid derivatives of sphingomyelin, can exert profound effects on cellular homeostasis. As examples, ceramide can inhibit cellular proliferation, induce differentiation, inhibit protein trafficking/secretion, and trigger apoptotic cell death (1–4). That these effects are likely mediated by secondary phosphorylation events (5–7) and that selected cytokines/hormones activate sphingomyelinases suggest that a ceramide/sphingosine signaling pathway exists (8–11).

A characteristic feature of acute toxic and ischemic renal tubular cell injury is partial degradation of membrane lipid constituents, leading to the accumulation of lipid by-products (12–17). This suggests that the sphingomyelinase/ceramide/sphingosine pathway could be involved in acute cell injury and repair. That ischemia and cytosolic Ca^{2+} loading may activate sphingomyelinase(s) (18, 19) further suggests this possibility. However, no evidence for ceramide or sphingosine effects on the expression of renal tubular cell injury and repair has been presented. Therefore, the present study has been conducted to address this possibility. Two specific issues have been addressed. (*i*) Does ceramide or sphingosine influence the expression of acute tubular cell injury, possibly by triggering apoptotic or necrotic cell death? (*ii*) Might these compounds help initiate the phenomenon of proximal tubular "cytoresistance" (acquired cellular resistance to further injury), a characteristic feature of the early recovery phase of acute renal failure (20–25)?

METHODS

Adult Human Kidney Proximal Tubule Cell Culture Conditions. Experiments were conducted using HK-2 cells, an immortalized proximal tubular cell line derived from normal adult human kidney proximal tubular epithelium by transduction with human papilloma virus 16 E6/E7 genes (26). The cells were grown in tissue culture flasks with keratinocyte serum-free medium (K-SFM) supplemented with epidermal growth factor and pituitary extract at 37°C under 95% O_2/5% CO_2 (26). Prior to achieving confluence, the cultures were trypsinized, transferred to six-well cluster plates (Costar) at a concentration of $\approx 1 \times 10^5$ cells per well, and cultured for an additional 24 hr. At that time, test reagent(s) was added as detailed below and the cultures were maintained by an additional 1½–20 hr. At the appropriate times, cell viability was assessed by determining percent vital dye exclusion (ethidium bromide; 100 cells in each of three to five fields counted) (25).

Assessment of Whether Ceramides, Sphingosine, and Sphingosine Derivatives Are Directly Cytotoxic. Twenty-four hours after establishing the subcultures, one of the following was added: C16 ceramide (a physiologic form); C8 ceramide; C2 ceramide (nonphysiologic forms, but with higher membrane permeability than C16 ceramide); sphingosine; or two naturally occurring sphingosine metabolites (dimethylsphingosine; sphingosine 1-phosphate). Three different concentrations of each were tested [5, 10, and 20 μM; added in ethanol (0.1%) or methanol (<2%), as per manufacturer's instructions; Biomol, Plymouth Meeting, PA]. In all instances, cocultured cells maintained under normal culture conditions, except for alcohol vehicle addition (which had no independent effects), served as controls.

Since the above experiments indicated that sphingosine caused HK-2 cell death, its time course was assessed. Cells were cultured in the presence of 0, 7.5, 15, or 20 μM sphin-

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Abbreviation: MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide.

‡To whom reprint requests should be addressed at: Fred Hutchinson Cancer Research Center, Room M 621, 1124 Columbia Street, Seattle, WA 98104-2092.

gosine, and after either 1, 3, 8, 15, or 24 hr, cell viability was assessed.

To determine whether sphingosine-mediated cell death occurred via an apoptotic or necrotic pathway, two features of apoptosis were sought: (*i*) DNA "laddering," and (*ii*) morphologic changes of apoptosis (27). To these ends, cultures were exposed to either 0 or 20 μM sphingosine for 45 or 90 min and then they were used for either DNA analysis or morphologic assessments. DNA was isolated and electrophoresed through 2% agarose containing ethidium bromide (27). The remaining cultures were treated with ethidium bromide (staining the nucleus), counterstained with acridine orange, and examined with a fluorescence microscope to characterize any nuclear morphologic changes.

Assessment of Whether Sphingosine or Ceramide Can Induce HK-2 Cytoresistance. Cells were cultured for 20 hr either with C2, C8, or C16 ceramide (5, 10, and 20 μM) or with sphingosine (1, 2, 5, 7.5, and 20 μM). The emergence of cytoresistance was then assessed by determining their vulnerability to an ATP depletion/Ca^{2+} overload protocol, as described (25, 28). In brief, this protocol induces >90% ATP depletion by antimycin A (7.5 μM) plus 2-deoxyglucose (20 mM) addition; superimposed Ca^{2+} overload is created by Ca^{2+} ionophore (A231867; 5 μM) addition. Loss of cell viability (vital dye uptake) was determined 90 min later. Cocultured cells maintained under normal conditions served as controls.

To define the time course of cell killing in the above experiments, additional cells were treated for 20 hr with either sphingosine (5 μM) or C16 ceramide (5 and 20 μM) addition and then subjected to the Ca^{2+} ionophore/ATP depletion challenge. Cell death was assessed every 30 min × 2.5 hr. Results were compared to those obtained with cocultured controls.

Assessment of Relative Specificity of Sphingosine-Induced HK-2 Cytoresistance. HK-2 cells were cultured with either sphingosine-1-phosphate (20 nM and 0.5, 1, and 2 μM) or dimethylsphingosine (20 nM and 1, 2, and 5 μM), and 20 hr later they were subjected to the Ca^{2+} ionophore/ATP depletion challenge. These doses were chosen because they were below directly toxic concentrations (analogous to 5 μM sphingosine). Whether free fatty acids (C16:0, C18:0, C18:1, C18:2, and C20:4, 5 μM) could reproduce sphingosine's cytoprotective effect was also assessed ($n \geq 3$ each). Cocultured normal cells were controls.

Time Course for the Emergence of Sphingosine-Triggered Cytoresistance. Previous studies have indicated that an approximate 18- to 24-hr lag time is required for the complete expression of *in vivo/in vitro* tubular cytoresistance (21–25). The following experiment tested whether sphingosine-triggered cytoresistance has the same time requirement. HK-2 cells were cultured with 5 μM sphingosine for either 90 min, 8.5 hr, or 20 hr and then they were subjected to the Ca^{2+} ionophore/ATP depletion injury protocol. Cocultured HK-2 cells without sphingosine exposure served as controls.

Confirmation of Sphingosine-Induced Cytoresistance: 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium Bromide (MTT) Assay. Cells were cultured with 0, 1, 5, or 10 μM sphingosine for 20 hr and then they were subjected to the Ca^{2+} ionophore/ATP depletion challenge. After 90 min, cell viability was assessed by a tetrazolium dye uptake assay (MTT), as described (26).

Cytosolic Free Ca^{2+} Measurements. The following experiment assessed whether sphingosine-induced cytoresistance was mediated by a decrease in cytosolic Ca^{2+} loading. HK-2 cells were cultured on glass coverslips for 20 hr with 0 or 5 μM sphingosine. Then, single cell free Ca^{2+} levels were measured either before or 30–45 min after applying the ATP depletion/Ca^{2+} ionophore challenge ($n \geq 20$ determinations for each). The intracellular fluorescent probe indo-1 AM (Molecular Probes) was used for this purpose, as described (25). Ionomycin (5 μM) was substituted for A23187 since the latter interferes with indo-1 fluorescence (each ionophore has the same effect on HK-2 viability).

Foreskin Fibroblast Experiments. To assess whether the above sphingosine effects were HK-2 cell specific, cultures of human foreskin fibroblasts (0.5×10^5 cells per well), maintained in RPMI medium with 10% fetal calf serum, were subjected to either 0 or 10 μM sphingosine exposure. Twenty hours later, direct cytotoxicity was assessed (vital dye exclusion). Other cultures were incubated with either 0 or 4 μM sphingosine for 20 hr, followed by the Ca^{2+} ionophore/ATP depletion challenge to test for cytoresistance. Cell viability (vital dye uptake) was assessed 2.5 hr later.

Calculations and Statistics. All values are presented as means ± 1 SEM. Comparisons were performed by the unpaired Student's *t* test. If multiple comparisons were made, the Bonferroni correction was applied. Significance was judged by a *P* value of <0.05.

RESULTS

Direct Ceramide/Sphingosine/Sphingosine-Derivative Effects on HK-2 Cell Viability. Under normal culture conditions, >95% of cells excluded ethidium bromide. After a 20-hr exposure to either 5, 10, or 20 μM C16 ceramide, no loss of cell viability occurred (Fig. 1). C8 ceramide was slightly toxic, but only at the 20 μM concentration (≈10% cell death). In contrast, C2 ceramide induced dose-dependent cell killing, apparent even at the 5 μM concentration. Sphingosine, sphingosine 1-phosphate, and dimethylsphingosine were highly toxic, each inducing 100% cell death when added in a 20 μM concentration. However, differing degrees of toxicity were apparent (sphingosine 1-phosphate > dimethylsphingosine > sphingosine), as evidenced by differing degrees of cell killing at the 5 and 10 μM concentrations (Fig. 1).

Sphingosine cytotoxicity was rapidly expressed and concentration dependent (see Fig. 2). This appeared to occur via a necrotic, not an apoptotic, pathway: although ≈50% of cells were nonviable after 90 min of 20 μM sphingosine exposure, no DNA laddering was apparent (Fig. 3*A*), and no nuclear morphologic changes of apoptosis were observed (Fig. 3*B*).

Impact of Sublethal Doses of Sphingolipid Derivatives on HK-2 Susceptibility to Injury. Only ≈20% of the control cells (0 sphingosine) retained their viability 90 min after applying the Ca^{2+} ionophore/ATP depletion challenge (Fig. 4*A*). Sphingosine pretreatment for 20 hr induced dose-dependent cytoprotection, reaching a maximum with the 5 μM concentration (Fig. 4*A*). This cytoprotection was lost with 10 μM sphingosine, undoubtedly because this concentration was directly cytotoxic (Fig. 1). C16 ceramide induced no cytoresistance, irrespective of the concentration employed (Fig. 4*B*). As

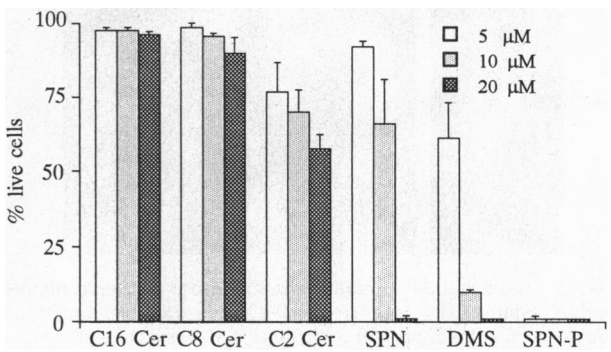

FIG. 1. Percentage of live cells (excluding vital dye) 20 hr following addition of ceramides (Cer), sphingosine (SPN), dimethylsphingosine (DMS), or sphingosine 1-phosphate (SPN-P). Dose-dependent cytotoxicity was noted with each, except C16 ceramide ($n \geq 3$ for each determination).

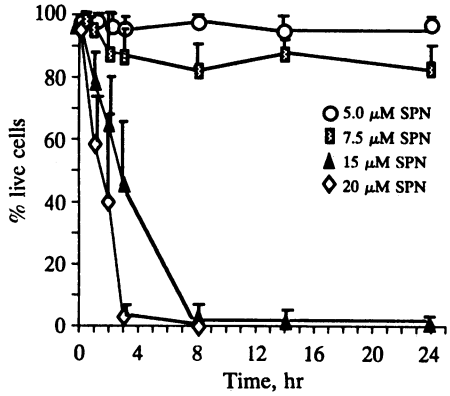

FIG. 2. Time course for sphingosine (SPN)-induced cell killing. The rate and extent of cell killing were highly concentration dependent (n = 3 at each time point).

shown in Fig. 5, 20 hr of 5 μM sphingosine pretreatment mitigated Ca^{2+} ionophore/ATP depletion injury throughout the 2½-hr time course experiments. Conversely, C16 ceramide (5 or 20 μM) continued to show no protective effect.

Neither 1 μM sphingosine 1-phosphate nor 2 μM dimethylsphingosine reproduced sphingosine's cytoprotective effect (Fig. 6*A*). Rather, dimethylsphingosine exacerbated cell killing, despite its being used in a concentration that was not directly toxic (see time zero data). Similarly, neither C2 nor C8 ceramide had a protective influence (Fig. 6*B*). Further experiments indicated that reducing the ceramide and sphingosine metabolite dosages from the micromolar to the nanomolar range (20–500 nM) also failed to trigger a cytoresistant state (data not depicted). None of the five test fatty acids either induced direct toxicity or initiated cytoresistance (data not shown). Thus, these results suggest that sphingosine induced-cytoresistance is highly compound specific.

Exposing HK-2 cells to 5 μM sphingosine for either 90 min or 8.5 hr failed to induce a significant cytoresistant state (data not shown). That long-term (20 hr), but not short-term (<8.5 hr), exposure was required for cytoresistance indicates that sphingosine does not act as a direct cytoprotectant (i.e., a secondary cellular response to it is required).

Cellular Ca^{2+} Uptake. Sphingosine treatment did not alter baseline HK-2 cytosolic Ca^{2+} levels (52 ± 3 nM, vs. 48 ± 3 nM for controls). Furthermore, sphingosine did not attenuate

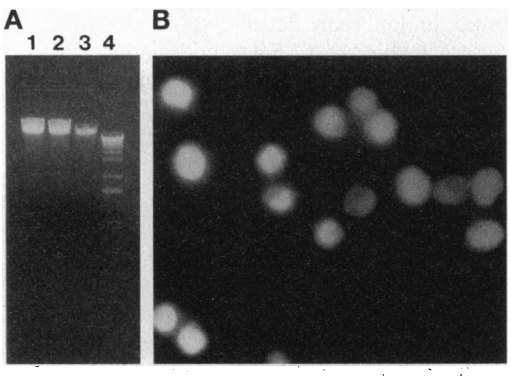

FIG. 3. (*A*) Ethidium bromide-stained agarose gel electrophoresis of DNA samples obtained from HK-2 cells exposed to 20 μM sphingosine for 0, 45, and 90 min (lanes 1–3, respectively). Lane 4, DNA molecular size standard (1-kb DNA ladder; GIBCO/BRL). Despite the fact that ≈50% of the cells were nonviable after this treatment, no "laddered" DNA was apparent. (*B*) Intact HK-2 nuclear morphology in these sphingosine-treated cells (ethidium bromide nuclear staining of nonviable cells), consistent with nonapoptotic cell death.

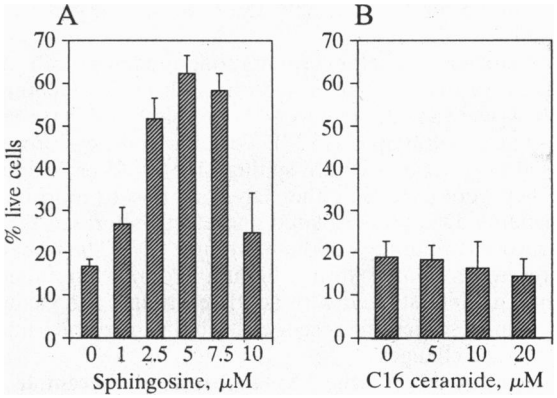

FIG. 4. Percentage of live cells (ethidium bromide exclusion) 90 min after addition of the Ca^{2+} ionophore/ATP depletion challenge. (*A*) Sphingosine treatment for 20 hr induced a bell-shaped pattern of cytoresistance (maximal at 5 μM; $P < 0.005$). (*B*) Conversely, C16 ceramide had no effect ($n \geq 3$ for each determination).

Ca^{2+} loading in response to the Ca^{2+} ionophore/ATP depletion challenge (3164 ± 109 nM vs. 3139 ± 162 nM for controls).

MTT Assay. The Ca^{2+} ionophore/ATP depletion challenge produced ≈85% HK-2 cell death, as reflected by decreased MTT cell uptake (Table 1). Increasing doses of sphingosine induced a bell-shaped pattern of cytoprotection (maximum at 5 μM), mirroring the previous vital dye exclusion data (Fig. 4*A*). Direct sphingosine cytotoxicity was documented at the 10 μM concentration (Table 1), again substantiating the vital dye exclusion results.

Foreskin Fibroblast Experiments. Exposing foreskin fibroblasts to 10 μM sphingosine for 20 hr induced 90% ± 2% vital dye uptake (vs. 0% for the cocultured controls; n = 3 each). Incubation with a subtoxic sphingosine dose (4 μM) for 20 hr protected against Ca^{2+} ionophore/ATP depletion-induced cell death (sphingosine, 18% ± 4%; control, 57% ± 7%; $P < 0.02$; n = 3).

DISCUSSION

It has been widely suggested that phospholipase A_2-generated phospholipid by-products, most notably fatty acids and lysophospholipids, participate in the evolution of acute renal tubular cell injury (12–17). However, that sphingolipid metabolites/breakdown products have potent signaling effects raises the possibility that they, too, could be involved. Therefore, cultured human proximal tubular cells were exposed to biologically relevant doses of ceramide and sphingosine (29) to assess whether altered cellular viability might result. In addition, their impact on cellular resistance to subsequent injury (ATP depletion/Ca^{2+} ionophore treatment) was determined.

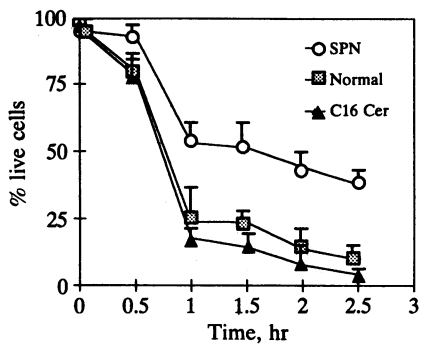

FIG. 5. Sphingosine (5 μM × 20 hr)-induced cytoresistance was expressed throughout a 2.5-hr observation period (n = 20). C16 ceramide had no effect.

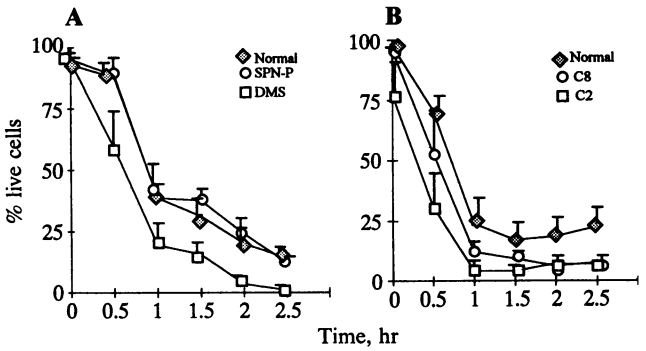

FIG. 6. (*A*) Unlike sphingosine, neither sphingosine 1-phosphate (SPN-P) nor dimethylsphingosine (DMS) conferred any cytoresistance to the Ca^{2+} ionophore/ATP depletion challenge despite being used in subtoxic concentrations. Dimethylsphingosine slightly exacerbated Ca^{2+} ionophore/ATP depletion-induced cell killing, as did C2 and C8 ceramide (*B*).

The rationale for using the latter injury model is that ATP depletion and cytosolic Ca^{2+} loading are critical mechanisms underlying diverse forms of acute tubular damage (16, 17).

The first notable result from these investigations was that sphingosine induced dose-dependent HK-2 cytotoxicity: as little as 10 μM sphingosine caused $\approx$25% loss of viability during a 20-hr incubation, whereas 20 μM sphingosine triggered rapid cell death ($\approx$40% and 100% after 1- and 3-hr exposures, respectively). That sphingosine 1-phosphate and dimethylsphingosine were more toxic than sphingosine raises the possibility that sphingosine metabolism to these naturally occurring products could be a prerequisite for full expression of its cytotoxicity. In contrast, physiologic (C16) ceramide caused no apparent toxic effects, whereas C8 and C2 ceramide exerted mild and moderate toxicity, respectively. That C8 and C2 ceramide are cell membrane permeable/water soluble, whereas C16 ceramide is not, strongly suggests that cell entry, rather than just plasma membrane exposure, is required for the expression of ceramide/sphingosine toxicity.

Since ceramide and/or sphingosine can trigger apoptosis in lymphocytes, neutrophils, and in tumor cell lines (e.g., refs. 30–34), we evaluated whether sphingosine induced HK-2 cell death via this pathway. To this end, DNA laddering and morphologic features of apoptosis were sought from 0 to 90 min following 10–20 μM sphingosine addition. Despite the fact that this time frame corresponded to a period of rapidly evolving, lethal cell injury, neither laddered DNA nor morphologic features of apoptosis resulted. This strongly suggests that a necrotic, rather than an apoptotic, form of cell death had developed. That these compounds can insert into cellular/subcellular membranes, potentially inducing a lytic effect, suggests one pathway by which this might occur.

Table 1. MTT assay assessments of sphingosine effects on HK-2 viability

Sphingosine, μM	Challenge		% survival
	Before	After	
0	0.738 ± 0.007	0.095 ± 0.003	12.9
1	0.747 ± 0.011	0.115 ± 0.007	15.4
5	0.731 ± 0.004	0.315 ± 0.010	43.1
10	0.625 ± 0.034	0.172 ± 0.028	27.5

Values represent 570-nm OD readings, a quantitative index of MTT uptake, and, hence, the number of viable cells. % survival = (after challenge value/before challenge value) × 100. Twenty hours of 5 μM sphingosine exposure increased % HK-2 cell survival from 12.9% to 43.1% ($P < 0.0001$; $n = 3$). At a 10 μM concentration, sphingosine caused slight direct cytotoxicity (i.e., before challenge; $P < 0.01$).

The second goal of this study was to address the possibility that the ceramide/sphingosine pathway might help initiate proximal tubular cytoresistance, a characteristic feature of the early recovery phase of acute renal failure (20–25). At present, the following characteristics define this phenomenon: (*i*) an approximate 18-hr lag time is required before the sublethally injured kidney becomes resistant to further insults (21–25); (*ii*) it is expressed directly at the proximal tubular cell level, since tubules extracted from injured kidneys manifest resistance to diverse forms of *in vitro* damage (22–24); (*iii*) it can be dissociated from a tubular regenerative response (23); (*iv*) it is not dependent on new protein synthesis (25); and (*v*) it appears to result from a direct increase in plasma membrane resistance to attack (23–25). The following data suggest that sphingosine might help initiate this state: *First*, pretreatment with subtoxic sphingosine doses protected against ATP depletion/Ca^{2+} overload injury; thus, it clearly can trigger a cytoresistant state. *Second*, sphingosine-induced cytoresistance had a prerequisite $\approx$20-hr lag time to develop; that this is highly analogous to all previous studies of this phenomenon suggests a common mechanistic pathway. *Third*, sphingosine-induced cytoresistance was not simply due to decreased cytosolic Ca^{2+} loading, indicating a direct increase in cellular/plasma membrane resistance to attack. That the latter is a uniform feature of the cytoresistant state (23–25) suggests sphingosine's potential mechanistic involvement.

The pathway by which sphingosine initiates cytoresistance remains to be defined. Since sphingosine can induce protein kinase C inhibition (5), we considered that this pathway could be involved. However, in experiments not presented, it was found that staurosporine, a protein kinase C inhibitor, did not reproduce sphingosine's cytoprotective state, presumably excluding this hypothesis. Another possibility is that sphingosine, when present in a subtoxic dose, merely induces a nonspecific "stress response," the result of which is a cytoresistant state. However, that sphingosine 1-phosphate, dimethylsphingosine, and C8/C2 ceramides were also toxic, but that none reproduced sphingosine's cytoprotective effect, rules out this possibility. A third hypothesis is that sphingosine selectively triggers the expression of specific cytoprotective proteins (e.g., heat shock proteins, antioxidant enzymes), which render the cells resistant to attack. However, we have recently demonstrated that HK-2 cytoresistance can develop *even in the presence of >98% protein synthesis inhibition* (e.g., induced with cycloheximide, or verrucarin A therapy; ref. 25). Indeed, that 24 hr of treatment with either of these protein synthesis inhibitors *independently* induced HK-2 cytoresistance (25) suggests that cell injury-induced protein synthesis blockade, rather than protein synthesis, is more likely to be responsible (25). Regarding this latter possibility, it is noteworthy that the sphingosine/ceramide pathway can inhibit cellular protein trafficking (35–37). Thus, if depletion of specific protein(s) at a particular biologic sight were responsible for cytoresistance, either a blockade in protein synthesis (e.g., with cycloheximide) or defective protein trafficking (e.g., with sphingosine) might produce this result. This theory could also explain why a substantial lag time is required for the emergence of the cytoresistant state, since time is required for altered intracellular protein expression to develop.

In conclusion, the present results provide compelling evidence that sphingosine can elicit dramatic effects on proximal renal tubular cell integrity: in high doses, cell necrosis results; conversely, after an $\approx$20-hr exposure to sublethal concentrations, a cytoresistant state is induced. That these results could be reproduced in cultured human foreskin fibroblasts, grown in the presence of serum, suggests that these observations could have broad-based relevance to the area of acute cell injury and repair.

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