Ceramide synthase is essential for endonuclease-mediated death of renal tubular epithelial cells induced by hypoxia-reoxygenation

Alexei G. Basnakian,¹ Norishi Ueda,¹ Xiaoman Hong,¹ Valentin E. Galitovsky,¹ Xiaoyan Yin,¹ and Sudhir V. Shah¹,²

¹University of Arkansas for Medical Sciences and ²Central Arkansas Veterans Healthcare System, Little Rock, Arkansas

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Both hypoxia-reoxygenation of renal cells in vitro and kidney ischemia-reperfusion injury in vivo are strongly associated with cell death (3). Ceramide, a metabolite of sphingolipids, participates in cell death signaling pathways (16, 17, 22). Although the role of ceramide has been studied in apoptotic cell death in response to a variety of stimuli, there is limited information of a role of ceramide in hypoxia-reoxygenation injury. In vivo studies of ischemic injury to the kidney (39, 40) or brain (19) as well as in vitro study of hypoxic injury to cultured PC12 cells (37) have shown an increase in ceramide level. However, the cause-effect relationship between an alteration of ceramide and cytotoxicity is not known in hypoxia-reoxygenation injury to renal tubular epithelial cells, and the pathways for ceramide generation in ischemia-reperfusion or hypoxia-reoxygenation injury remain to be elucidated.

Ceramide is generated by the two major pathways: condensation of sphingosine or sphinganine and fatty acyl-CoA by ceramide synthase or by hydrolysis of sphingomyelin by sphingomyelinases (16, 17, 22). Several studies using an apoptotic model of cellular injury have shown that an increased ceramide level is mediated by activation of sphingomyelinases (10, 16). It was shown that hypoxia to PC12 cells results in enhanced ceramide generation through the activation of neutral sphingomyelinase (37). In contrast, other studies have shown that in ischemic injury to kidneys or cultured kidney cells the activity of sphingomyelinases is decreased rather than increased (39). Currently, there is no information on the role of ceramide synthase in the regulation of ceramide in hypoxia-reoxygenation injury.

It was demonstrated that ceramide activates apoptotic signals through mitochondria (9, 26). Ceramide induces cytochrome c release from mitochondria in a caspase-independent fashion, leading to the activation of executioner caspases (9). Studies with isolated mitochondria showed that ceramides induce a destabilization and permeabilization of the outer mitochondrial membrane, which allows a leakage of mitochondrial proteins up to 60 kDa (24, 25). This leads to the release of small proteins like 11-kDa cytochrome c, 26-kDa adenylate kinase, or recently identified 27-kDa apoptotic endonuclease G (EndoG), which resides in the mitochondrial intermembrane space, into the cytoplasm (13, 25). Being released from mitochondria, EndoG was shown to accumulate in nuclei, leading to DNA fragmentation and caspase-independent cell death (21). There is no information on whether an activation of the ceramide pathway may lead to EndoG leakage from mitochondria during hypoxia-reoxygenation injury.

The present study was undertaken to examine a role of ceramide synthase in ceramide generation responsible for endonuclease-mediated DNA damage and cell death in hypoxia-reoxygenation injury to normal rat kidney NRK-52E tubular epithelial cells. Our data provided the evidence that the insult of hypoxia-reoxygenation increases the activity of ceramide synthase but not sphingomyelinases, resulting in enhanced ceramide generation, and that the ceramide synthase-dependent pathway is a key modulator of hypoxia-reoxygenation injury to renal tubular epithelial cells. This injury is associated with the release of EndoG from mitochondria and the induction of...
endonuclease-mediated DNA fragmentation, both preventable by fumonisin B1, the inhibitor of ceramide synthase.

METHODS

Materials. Dihydrosphingosine (sphinganine) was purchased from Biomol Research Laboratory (Plymouth Meeting, PA). Dicylglycerol kinase of Escherichia coli strain N4830/pJW10 and fumonisin B1 were purchased from Calbiochem (La Jolla, CA). [γ-32P]ATP and N-methyl-[14C]sphingomyelin were obtained from Amersham (Arlington Heights, IL). Silica gel 60 plates for thin-layer chromatography (TLC) were purchased from Whatman (Hillsboro, OR). All of the other agents were supplied by Sigma (St. Louis, MO).

Cell culture. NRK-52E cells obtained from the American Type Culture Collection (Rockville, MD) were cultured as in our previous studies (4, 18). Cells were maintained in a humidified incubator gassed with 95% air-5% CO2 at 37°C, fed at intervals of 48–72 h, and used 2 days after confluence.

In vitro hypoxia-reoxygenation and fumonisin B1 treatment. We first characterized an in vitro model of hypoxia-reoxygenation injury using a Molecular Incubator Chamber (Billups-Rothenberg, CA). Cells were rinsed with serum-free DMEM, pH 7.4, followed by glucose-free DMEM, pH 7.4, and then incubated in serum-free, glucose-free DMEM bubbled with 100% N2 for 30 min just before the experiment. Our experiments during elaboration of the model showed that without glucose deprivation cells were tolerant to hypoxia-reoxygenation injury. However, we have not observed any effect of glucose-free media alone on the renal tubular epithelial cells. Because the presence of glucose alone was not important, we chose not to add glucose or change the media during the reoxygenation period. This allowed us to study the cumulative effect of hypoxia and reoxygenation (for example, released LDH) in the same media. Induction of oxygen deprivation was carried out using a gas chamber with 95% N2-5% CO2 as previously described (5, 14, 37). Reoxygenation was carried out using a gas chamber with 95% air-5% CO2 (for example, released LDH) in the same media. Induction of the presence of glucose alone was not important, we chose not to add glucose-free media alone on the renal tubular epithelial cells. Because reoxygenation injury. However, we have not observed any effect of glucose-free media alone on the renal tubular epithelial cells. Because the presence of glucose alone was not important, we chose not to add glucose or change the media during the reoxygenation period. This allowed us to study the cumulative effect of hypoxia and reoxygenation (for example, released LDH) in the same media. Induction of oxygen deprivation was carried out using a gas chamber with 95% N2-5% CO2 as previously described (5, 14, 37). Reoxygenation was carried out using a gas chamber with 95% air-5% CO2 as previously described (5, 14, 37).

Assay for ceramide synthase. Ceramide synthase activity was measured as previously described (6) with a minor modification. In brief, protein (50 μg) was incubated in a final volume of 1 ml reaction mixture containing 2 mM MgCl2, 20 mM HEPES, pH 7.4, 20 μM defatted bovine serum albumin, 20 μM dihydrosphingosine (sphingamine), 70 μM unlabeled palmitoyl-coenzyme A, and 0.2 μCi of [1-14C]palmitoyl-CoA (specific activity, 53.6 mCi/mmol). The reaction was stopped by extraction of lipids using 2 ml of chloroform/methanol (1:2 vol/vol). The lower phase was dried under nitrogen gas and applied to a 20-cm silica gel TLC plate. Radiolabeled dihydro-ceramide was resolved using a solvent system of chloroform/methanol/1 N ammonium hydroxide (40:10:1 vol/vol/vol), identified by iodine vapor staining based on comigration with ceramide type III standards, and quantified by a liquid scintillation counting. Data are expressed as picomoles of incorporated [14C]palmitoyl-CoA per milligram of protein per minute.

Assay for acid or neutral sphingomyelinase. At the end of the experiment, cells were washed twice with PBS at 4°C. Cells were lysed in 200 μl of either neutral buffer containing 20 mM HEPES, pH 7.4, 10 mM MgCl2, 2 mM EDTA, 5 mM dithiothreitol, 100 mM Na3VO4, 100 mM NaF, 10 mM β-glycerophosphate, 750 μM ATP, 1 mM PMFS, 1 mg/ml leupeptin, 1 mg/ml pepstatin, and 0.5% CHAPS, or acid buffer containing 0.5% CHAPS, 1 mM PMSF, 1 mg/ml leupeptin, and 1 mg/ml pepstatin. After incubation for 20 min at 4°C, cells were homogenized and centrifuged at 14,000 g for 5 min to obtain the protein extract for acid sphingomyelinase assay and 800 g for 5 min for neutral sphingomyelinase assay, respectively. The supernatants were kept at −80°C until used for the assay. The activity of neutral sphingomyelinase was measured as described (35, 38). For both acid and neutral sphingomyelinase assay, 10 nmol of unlabeled sphingomyelin and 0.02 μCi N-methyl-[14C]sphingomyelin (specific activity, 56 mCi/mmol) were added to a glass tube, and the lipids were dried under N2. For acid sphingomyelinase assay, 50 μl of acid buffer containing 250 mM sodium acetate, 1 mM EDTA, pH 5.0, were added to the dried labeled and unlabeled sphingomyelin and resolved. The protein extract (50 μg) was then added, and the volume was brought up to 150 μl. For neutral sphingomyelinase assay, the reaction was carried out in a neutral buffer containing 20 mM HEPES, pH 7.4, 1 mM MgCl2, pH 7.4. The samples were then incubated for 90 min and 2 h at 37°C for acid and neutral sphingomyelinase assay, respectively. At the end of incubation, the reaction was stopped by adding 0.8 ml of chloroform/methanol (2:1, vol/vol) and 0.25 ml H2O. The samples were then vortexed, centrifuged, and the upper phase (0.3 ml) containing radioactive phosphatidylcholine, a product of hydrolysis of [14C]sphingomyelin, was measured by scintillation counting. The data are expressed as nanomoles of hydrolyzed [14C]sphingomyelin per milligram of protein per hour.
Western blot analysis. Immunoblotting was used for identification and measurement of cytochrome c and EndoG release from mitochondria. To obtain cytosolic extract, cells were homogenized in a Teflon-glass homogenizer as described previously (4) and pelleted at 20,000 g for 20 min. Proteins (10–30 μg/well) were subjected to SDS-PAGE in reducing conditions (20), electrophoretically transferred to nitrocellulose membranes (Millipore, Bedford, MA), and reacted with diluted 1/500 anti-EndoG antibody (Abcam), 1/1,000 anti-cytochrome c (Santa Cruz Biotechnology), or 1/1,000 anti-β-actin (Santa Cruz Biotechnology) as described by Towbin et al. (27).

Endonuclease activity assay. Endonuclease activity was measured using a pBR322 plasmid incision assay as previously described (2, 4). One endonuclease unit was defined as the amount of the enzyme required to convert 1 μg covalently closed circular pBR322 DNA to open circular DNA or linear DNA.

DNA fragmentation assay. DNA was isolated from cells and endonuclease-mediated DNA fragmentation was measured by quantification of 3’OH DNA strand breaks using a random oligonucleotide-primed synthesis (ROPS) assay as previously described (2).

Cell death assays. Cell viability was determined using trypan blue exclusion or LDH release as previously described (18, 29). For LDH release, the CytoTox 96 Nonradioactive Cytotoxicity Assay (Promega, Madison, WI) was used. The cytotoxicity is expressed as the percentage of absorption in treated cell medium to that of the maximal LDH release.

Statistics. Results were expressed as means ± SE. Statistical analysis was performed with ANOVA and Student’s t-test and corrected using Bonferroni’s procedure. A value of P < 0.05 was considered significant.

RESULTS

Effect of hypoxia-reoxygenation on ceramide synthase activity. To determine which pathway, namely, activation of ceramide synthase or hydrolysis of sphingomyelin mediated by sphingomyelinases, is involved in hypoxia-reoxygenation-induced ceramide generation, we first examined the effect of hypoxia-reoxygenation on ceramide synthase activity in NRK-52E cells. Exposure of cells to hypoxia for 60 min resulted in a significant increase in ceramide synthase activity (121.7 ± 21.9 pmol·mg protein⁻¹·min⁻¹, n = 6, P < 0.05) compared with control value at 120 min (73.3 ± 9.5 pmol·mg protein⁻¹·min⁻¹, n = 6) and further increased after reoxygenation for 60 min (145.4 ± 20.9 pmol·mg protein⁻¹·min⁻¹, n = 6, P < 0.01; Fig. 1). These data indicate that hypoxia-reoxygenation-induced ceramide generation in NRK-52E cells is likely to be due to the activation of ceramide synthase.

Effect of hypoxia-reoxygenation on sphingomyelin content and sphingomyelinase activity. To determine the contribution of sphingomyelinases to hypoxia-reoxygenation-induced increase in ceramide, we measured the sphingomyelin content in NRK-52E cells subjected to hypoxia-reoxygenation. As shown in Fig. 2, sphingomyelin content was not decreased but rather increased in the cells subjected to hypoxia-reoxygenation compared with control cells, suggesting little contribution of sphingomyelinases to hypoxia-reoxygenation-induced ceramide generation. We then measured the activity of acid or neutral sphingomyelinase in NRK-52E cells subjected to hypoxia-reoxygenation. In contrast to previous studies that suggested activation of neutral sphingomyelinase for hypoxia-induced ceramide generation in PC12 cells (37), exposure of NRK-52E cells to hypoxia-reoxygenation did not result in any significant change in acid or neutral sphingomyelinase activity (Fig. 3, left and right, respectively). Taken together, these data strongly indicate that hypoxia-reoxygenation results in an increase in ceramide synthase activity rather than sphingomyelinases in renal tubular epithelial cells. The data also show that the pathway for ceramide generation may vary with cell types even in response to same stimuli.

Generation of ceramide and the effect of ceramide synthase inhibitor, fumonisin B1, on hypoxia-reoxygenation-induced ceramide generation. The exposure of NRK-52E cells to hypoxia for 60 min resulted in increased ceramide generation from the basal level of 24 ± 3 (n = 6) to 48 ± 4 pmol/nmol

![Fig. 1. Effect of hypoxia-reoxygenation on ceramide synthase activity. Cells were exposed to hypoxia for 60 min followed by reoxygenation for 60 min. Ceramide synthase activity was measured as described in METHODS. Results are means ± SE, n = 6. *P < 0.05, **P < 0.01, compared with control.](http://ajprenal.physiology.org/)

![Fig. 2. Effect of hypoxia-reoxygenation on sphingomyelin content. Cells were exposed to hypoxia for 60 min followed by reoxygenation for 60 min, and then the sphingomyelin content was measured as described in METHODS. Results are means ± SE, n = 3–5. *P < 0.05, compared with control.](http://ajprenal.physiology.org/)
phosphate ($n = 3$, $P < 0.01$), and the ceramide level further increased ($56 \pm 4$ pmol/nmol phosphate, $n = 7$, $P < 0.0001$) after 60-min reoxygenation. We then examined the ability of a specific inhibitor of ceramide synthase, fumonisin B1, to suppress hypoxia-reoxygenation-induced ceramide generation in NRK-52E cells. As shown in Fig. 4, fumonisin B1 prevented hypoxia-reoxygenation-induced ceramide generation at 120 min, control: $23.7 \pm 2.7$ pmol/nmol phosphate, hypoxia (60 min)/reoxygenation (60 min) alone, $29.0 \pm 3.0$ pmol/nmol phosphate, hypoxia-reoxygenation + fumonisin B1, $29.0 \pm 3.0$ pmol/nmol phosphate, $n = 3–7$, $P \leq 0.01$]. These data confirm the role of ceramide synthase in hypoxia-reoxygenation-induced ceramide generation.

Effect of fumonisin B1 on hypoxia-reoxygenation-induced cell death. We examined the effect of fumonisin B1 on hypoxia-reoxygenation-induced cell death. As shown in Fig. 5A, fumonisin B1 provided marked protection against hypoxia-reoxygenation-induced cell death (as measured by trypan blue exclusion; control, $12 \pm 1\%$; hypoxia-reoxygenation alone, $39 \pm 1\%$; hypoxia-reoxygenation + $50 \mu M$ fumonisin B1, $18 \pm 1\%$, $n = 5$, $P < 0.0001$). We confirmed that fumonisin B1 did not alter the uptake of trypan blue dye by the cells (data not shown). In another approach, an LDH release assay was applied to measure irreversible cell death (Fig. 5B). This assay also showed that fumonisin B1 is protective against hypoxia-reoxygenation injury (control, $10.5 \pm 1.3\%$; hypoxia-reoxygenation alone, $32.4 \pm 1.9\%$; hypoxia-reoxygenation + $50 \mu M$ fumonisin B1, $18.1 \pm 1.4\%$, $n = 4$, $P < 0.0001$). The protection by fumonisin B1 was dose dependent (Fig. 5C).

Effect of fumonisin B1 on endonuclease-mediated DNA fragmentation. Our previous studies showed a strong correlation between the accumulation of DNA breaks induced by endonucleases and cell death in different models (4, 29, 32). We applied the ROPS assay (2), which is designed to measure 3'OH DNA termini produced by an endonuclease (Fig. 6). This assay showed that hypoxia-reoxygenation led to the accumulation of endonuclease-mediated DNA strand breaks ($4.2 \pm 0.3 \times 10^3$ cpm vs. $0.8 \pm 0.1 \times 10^3$ cpm in control, $n = 5$, $P < 0.01$). Fumonisin B1 provided a strong protection against DNA fragmentation in a dose-dependent manner ($2.3 \pm 0.2 \times 10^3$ cpm at $25 \mu M$, $1.8 \pm 0.1 \times 10^3$ cpm at $50 \mu M$, and $1.3 \pm 0.2 \times 10^3$ cpm at $100 \mu M$ fumonisin B1, $P < 0.01–0.001$ compared with hypoxia-reoxygenation alone).

Effect of fumonisin B1 on hypoxia-reoxygenation-induced EndoG release from mitochondria. Ceramide was shown to induce permeabilization of the outer mitochondrial membrane for proteins and release of cytochrome c and EndoG from mitochondria, and EndoG was shown to cause DNA fragmentation during apoptosis (21, 24, 25). In the next set of experiments using Western blotting, we determined that EndoG and cytochrome c were released from mitochondria into the cyto-
plasm during hypoxia-reoxygenation of NRK-52E cells, and this release was suppressed by fumonisin B1 (Fig. 7, A, B, and D). In support of the observed EndoG release and DNA fragmentation after hypoxia-reoxygenation, which could be protected by the inhibition of ceramide synthase, our data showed that endonuclease activity measured in cytosolic extracts was increased during hypoxia-reoxygenation and inhibited by fumonisin B1 (Fig. 7E). The quantification of the endonuclease activity in the cytosolic extracts using a plasmid incision assay showed that it was increased from 6.8 ± 1.0 U/g protein in control to 43.5 ± 4.6 U/g protein during hypoxia-reoxygenation (n = 4, P < 0.001 vs. control) and decreased to 8.8 ± 2.2 U/g protein after fumonisin B1 pretreatment (n = 4, P < 0.001 vs. hypoxia-reoxygenation).

**DISCUSSION**

Ceramide is generated through a condensation of sphingosine or sphinganine and fatty acyl-CoA by ceramide synthase or by a hydrolysis of sphingomyelin by sphingomyelinases (16, 17, 22). Earlier studies using an apoptotic model of cell injury showed that increased ceramide generation is mediated by activation of sphingomyelinases (10, 16). In terms of the pathway for ceramide generation in hypoxic injury, an in vitro study showed that hypoxic injury to PC12 cells results in enhanced ceramide generation through activation of neutral sphingomyelinase (37). In contrast, the study by Zager et al. (40) showed that the ceramide level is increased in an in vivo model of ischemia-reperfusion injury to kidneys or in an in vitro model of hypoxic injury to cultured kidney cells accompanied by a decrease in the activity of neutral sphingomyelinase. However, recent studies suggested the role of ceramide synthase for enhanced generation of ceramide in different models (8, 23, 28). In our previous study, we demonstrated that chemical hypoxia induced by a mitochondrial respiratory chain inhibitor, antimycin A, resulted in an increase in ceramide generation in LLC-PK1 cells due to the activation of ceramide synthase (30).

The present study was aimed to determine whether ceramide synthase is important in “true” hypoxia-reoxygenation of renal tubular epithelial cells and to analyze the mechanism of cell death downstream of ceramide generation. We showed that exposure of NRK-52E cells to hypoxia-reoxygenation in glucose-free media resulted in an increase in ceramide generation and ceramide synthase activity. Our experiments during elaboration of the model showed that without glucose deprivation, cells were tolerant to hypoxia-reoxygenation injury, whereas...
mid DNA was converted to open circular or linear forms (E).

**Fig. 7.** Fumonisin B1 protects against hypoxia-reoxygenation-induced mitochondrial endonuclease G (EndoG) release. Protein extracts were obtained and subjected to Western blotting with anti-EndoG (A), anti-cytochrome c (B), or anti-β-actin (C) as described in METHODS. The amounts of EndoG and cytochrome c were determined by densitometry and presented as percentage of control (D). Results are means ± SE, n = 4 in each group. **P < 0.01 and ***P < 0.001, compared with hypoxia-reoxygenation alone. Endonuclease activity of ceramide synthase is increased in response to hypoxia-reoxygenation without glucose deprivation (15). Thus it appears likely that the pathway for ceramide generation triggered after exposure to insults may be dependent on the nature of stimuli or cell types even in response to the same stimuli.

To examine a role of ceramide synthase-dependent ceramide generation in hypoxia-reoxygenation injury, we used a specific inhibitor of ceramide synthase, fumonisin B1. This compound has a very similar structure to sphingosine or sphinganine, which is a substrate for ceramide synthase, and thus blocks ceramide synthase, resulting in a decrease in intracellular ceramide level (6, 33, 34, 36). Because of the nature of the action of fumonisin B1, it appears unlikely that this compound can inhibit sphingomyelinases. This is supported by the inability of fumonisin B1 to prevent cell death in a model in which sphingomyelinases have been implicated (6, 37). Multiple recent studies showed the protective effect of fumonisin B1 against apoptotic cell death in response to stimuli in which ceramide synthase might be involved for ceramide generation (6, 8, 12, 23, 28). In fact, our data showed the ability of fumonisin B1 to suppress hypoxia-reoxygenation-induced ceramide generation in NRK-52E cells. Furthermore, the present study demonstrated that fumonisin B1 provided a marked protection against hypoxia-reoxygenation-induced cell death. This cell death was associated with increased release of EndoG from mitochondria, higher endonuclease activity in cytoplasm, and increased fragmentation of DNA. Taken together, these data strongly indicate that the ceramide synthase pathway plays a major role in hypoxia-reoxygenation-induced ceramide generation and that ceramide synthase-dependent ceramide is a modulator of hypoxia-reoxygenation injury to renal tubular epithelial cells that involves EndoG as a mechanism of DNA fragmentation.

The mechanism(s) of activation of ceramide synthase and sequential consequences of ceramide on hypoxia-reoxygenation injury remains to be elucidated. In a model of hypoxia-reoxygenation injury, a role of reactive oxygen metabolites has been implicated (1). In our recent study, we demonstrated that activity of ceramide synthase is increased in response to oxidant stress (29). Oxidant stress has been shown to result in DNA fragmentation in various types of cells (7). Similarly, we previously showed that oxidant stress induces endonuclease activation as an early event, leading to DNA damage and cell death in renal tubular epithelial cells (29, 31). Thus activation of the ceramide synthase-dependent pathway may be one of the mechanisms by which oxidant stress causes endonuclease activation, leading to DNA damage and cell death in renal tubular epithelial cells. It is possible that the generation of reactive oxygen metabolites after exposure to hypoxia-reoxygenation might trigger the activation of ceramide synthase, resulting in an increase in ceramide, which in turn causes endonuclease release from mitochondria, causing cytotoxicity.

In summary, the present study demonstrated that hypoxia-reoxygenation injury increased ceramide synthase activity but not sphingomyelinases, resulting in enhanced ceramide production by NRK-52E cells. The inhibition of ceramide synthase was able to suppress hypoxia-reoxygenation-induced ceramide production and prevented EndoG release from mitochondria, DNA fragmentation, and cell death. Our data provided the first evidence that the ceramide synthase-dependent pathway for ceramide generation is a key modulator of hypoxia-reoxygenation injury in renal tubular epithelial cells.
REFERENCES


GRANTS

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