JNK3 Signaling Pathway Activates Ceramide Synthase Leading to Mitochondrial Dysfunction*

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A cardinal feature of brain tissue injury in stroke is mitochondrial dysfunction leading to cell death, yet remarkably little is known about the mechanisms underlying mitochondrial injury in cerebral ischemia/reperfusion (IR). Ceramide, a naturally occurring membrane sphingolipid, functions as an important second messenger in apoptosis signaling and is generated by de novo synthesis, sphingomyelin hydrolysis, or recycling of sphingolipids. In this study, cerebral IR-induced ceramide elevation resulted from ceramide biosynthesis rather than from hydrolysis of sphingomyelin. Investigation of intracellular sites of ceramide accumulation revealed the elevation of ceramide in mitochondria because of activation of mitochondrial ceramide synthase via post-translational mechanisms. Furthermore, ceramide accumulation appears to cause mitochondrial respiratory chain damage that could be mimicked by exogenously added natural ceramide to mitochondria. The effect of ceramide on mitochondria was somewhat specific; dihydroceramide, a structure closely related to ceramide, did not inflict damage. Stimulation of ceramide biosynthesis seems to be under control of JNK3 signaling: IR-induced ceramide generation and respiratory chain damage was abolished in mitochondria of JNK3-deficient mice, which exhibited reduced infarct volume after IR.

Mitochondria are known to be involved in both necrotic and apoptotic cell death, both of which have been identified in the ischemia/reperfusion (IR)-injured brain (1-3). Also, restricted respiratory chain function has been found to develop in various models of cerebral IR (4-8); specifically, mitochondrial respiration supported either by glutamate or succinate was decreased up to 40%, but ascorbate-supported respiration was not significantly altered (9, 10). Mitochondrial changes appear to be one essential step in tissue damage in cerebral IR. Treatments that slow tissue impairment were associated with better recovery of mitochondrial function (11, 12).

A number of cell death regulatory molecules have been implicated in neuronal injury in IR, including c-Jun N-terminal kinase (JNK) (13). Once activated, JNK can phosphorylate serine residues of several transcription factors, such as c-Jun, or non-nuclear proteins, including pro-apoptotic Bcl-2 family proteins (14-17). Among three JNK isoforms encoded by different genes, JNK1 and JNK2 are present in most tissues, whereas JNK3 is selectively expressed in the nervous system and in the heart. A critical role of JNK3 in cerebral ischemia has been implicated, targeted deletion of JNK3-protected mice from brain IR injury (18).

Numerous reports support a role for sphingolipids as second messengers in intracellular signaling pathways (19, 20), especially ceramide, which is critical in controlling cell death (20). Data describing the cellular effects of ceramide have been obtained mostly by employing synthetic short-chain analogs that may not fully mimic the properties of naturally occurring ceramides. Emerging evidence suggests that highly hydrophobic natural ceramides are generated by membrane-associated enzymes, and they exert their effects either in close proximity to the generation site or require specific transporter proteins to reach their targets (21-24). New evidence indicates a local action of ceramide on mitochondria in intact cells. Selective hydrolysis of a mitochondrial pool of sphingomyelin by overexpressed bacterial sphingomyelinase targeted to mitochondria resulted in apoptosis. In contrast, generation of ceramide in the plasma membrane, endoplasmic reticulum (ER), or Golgi apparatus by bacterial sphingomyelinase targeted to these compartments did not effect cell viability (25).

De novo synthesis of ceramide is thought to occur in the endoplasmic reticulum (ER), although recent studies describe activity in semi-purified liver mitochondria (26, 27). (Dihydro)ceramide synthase (EC 2.3.1.24) is a key enzyme in de novo synthesis of ceramide (28, 29), and in yeast, longevity assurance gene 1 (Lag1) was identified as a component of ceramide synthase.

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‡The abbreviations used are: IR, ischemia/reperfusion; JNK, c-Jun N-terminal kinase; WT, wild type; MCAO, middle cerebral artery occlusion; VDAC, voltage-dependent anion channel; ER, endoplasmic reticulum; FCCP, carbonyl cyanide p-trifluoromethoxyphenylhydrazone; MS, mass spectrometry; MS/MS, tandem mass spectrometry; TTC, 2,3,5-triphenyltetrazolium chloride; ICA, internal carotid artery; FB1, fumonisin B1.
Lag1 gene deletion in yeast cells resulted in pronounced increase (about 50%) in mean and maximum life span (30). Recently, mammalian homologs of Lag1, which belong to the LASS (longevity assurance gene homolog) family, were cloned, and characterized (21). Northern blot analysis revealed the expression of LASS1, LASS2, LASS4, LASS5, and LASS6 genes in brain (31). Each of six known LASS genes regulates synthesis of a specific subset of ceramides and displays a unique substrate specificity profile for chain length and/or saturation in fatty acid acyl-CoA (31). It has been shown that LASS1 generates C_{18:0}- and C_{18:1}-ceramide; LASS2 or LASS4 generates C_{20:0}-, C_{24:0}-, and C_{24:1}-ceramide. LASS5 generates C_{14:0}-, C_{16:0}-, C_{18:0}-ceramide and C_{18:1}-ceramide; and LASS6 produces C_{14:0}-, C_{16:0}-, and C_{18:0}-ceramide (31, 32). Although the protein exhibiting the ceramide synthase activity has been partially purified from liver mitochondria (27), the expression of LASS proteins in mitochondria remains to be elucidated.

A few studies describe endogenous ceramide accumulation in brain via activation of sphingomyelinase and sphingomyelin hydrolysis (33, 34) during severe and lethal cerebral IR. Consistent with these data, the extent of brain tissue damage was decreased in mice lacking acid sphingomyelinase (35).

The aim of this study was to determine the mechanism of mitochondrial ceramide accumulation and its role in cerebral IR-induced mitochondrial injury. We present evidence that ceramide biosynthesis rather than sphingomyelin hydrolysis mediates the accumulation of ceramide in mitochondria. We demonstrated that several key enzymes generating ceramide, including LASS1, LASS2, and LASS6, are localized in mitochondria, and LASS6 appears to be activated by IR. Furthermore, data indicate that excessive accumulation of ceramide could inhibit mitochondrial respiratory chain activity at the level of complex III. Importantly, we uncovered a novel mechanism of regulation of ceramide biosynthesis in IR in that IR-induced stimulation of ceramide synthase in mitochondria and the mitochondrial respiratory chain damage were abolished in JNK3-deficient mice. These data suggest that activation of ceramide synthase mediates cerebral IR-induced mitochondrial injury and is regulated by JNK3 signaling.

**MATERIALS AND METHODS**

**Animals and Reagents**—Male C57BL/6 mice (The Jackson Laboratories, 25–35 g each) were acclimated for 1 week prior to experimentation. JNK3-deficient mice (provided by Alex Kuan, University of Cincinnati) were bred in-house. All chemicals and reagents, unless stated otherwise, were purchased from Sigma. Anti-voltage-dependent anion channel (VDAC) rabbit polyclonal antibody was purchased from Calbiochem. Anti-calnexin rabbit polyclonal antibody was from Chemicon, San Diego. Monoclonal anti-LASS1, anti-LASS2, anti-LASS4, and anti-LASS6 antibodies were purchased from Abnova, Taiwan. Anti-LASS5 antibody was made by Sigma Genosys (Woodlands, TX). n-Octyl glucoside (n-octyl-β-D-glucopyranoside) was from LabScientific, Livingston, NJ. Stearoyl-CoA was obtained from Avanti, Alabaster, AL. \(\Delta\)-erythro-C_{18:0}-ceramide, \(\Delta\)-erythro-C_{18:0}-dihydroceramide, \(\Delta\)-erythro-C_{18:1}-ceramide, 17\(\Delta\)-dihydrophosphoglycerine, as well as all standards used for sphingolipid analysis, were synthesized by the Lipidomics Core Facility at the Medical University of South Carolina, Charleston.

**Middle Cerebral Artery Occlusion (MCAO) Surgery and Induction of Ischemia**—Experimental protocols were reviewed and approved by the Institutional Animal Care and Use Committee of Medical University of South Carolina, Charleston, and followed the National Institutes of Health guidelines for experimental animal use. Under temporary anesthesia, mice were subjected to MCAO as described previously (36). Briefly, the left common carotid artery was exposed through a midline incision in the neck. Sham control animals were treated identically but without MCAO. A microsurgical clip was placed around the origin of the internal carotid artery (ICA). The distal end of the ICA was ligated with 6-0 silk and transected. A 6-0 silk was tied loosely around the ICA stump. The clip was removed, and the fire-polished tip of a 5-0 nylon suture (poly-L-lysine-coated) was gently inserted into the ICA stump. The loop of the 6-0 silk was tightened around the stump and the nylon suture was advanced ~11 mm (adjusted for body weight) into and through the ICA after removal of the aneurysm clip, until it rested in the anterior cerebral artery, thereby occluding the anterior communicating and middle cerebral arteries. After 30 min of MCAO, the suture was removed; blood flow was restored to normal, and the incision was closed.

**Infarct Size Measurement**—Infarct size was measured by histological examination using 2,3,5-triphenyltetrazolium chloride (TTC) staining. Brains were dissected out and cut into four 3-mm-thick coronal sections, which were stained with 2% TTC for 90 min at 37 °C. The TTC-stained sections were placed in 10% neutral buffered formalin and kept in darkness at 4 °C for at least 24 h. The infarct area in each section was determined with a computer-assisted image analysis system, consisting of a computer equipped with a Quick Capture frame grabber card, a Hitachi CCD camera mounted on an Olympus microscope, and a camera stand. NIH Image Analysis software was used for data analysis. The images were captured, and the total area of damage was determined over the seven sections. A single operator blinded to treatment status performed all measurements. The infarct volume was calculated by summing the infarct volumes of the sections. Infarct size (%) was calculated by using the following formula: corrected infarct volume (mm³) = infarct volume − (total ipsilateral volume − total control volume). Total control volume was measured as the volume of the contralateral side; total ipsilateral volume was measured as the total volume of the ipsilateral side, and infarct volume was measured as the total infarct volume to eliminate effects of edema.

**Isolation of Mouse Brain Mitochondria**—All procedures were performed at 4 °C. Tissue was placed immediately in ice-cold isolation medium containing 230 mM mannitol, 70 mM sucrose, 10 mM HEPES, and 1 mM EGTA, pH 7.4. Tissue from four hemispheres (~1 g) was homogenized in 10 ml of isolation medium using a Teflon-glass homogenizer. The homogenate was centrifuged at 2,000 \(\times\) g for 10 min. The supernatant was then centrifuged at 12,000 \(\times\) g for 10 min in a Sorvall RC 5C centrifuge. The supernatant was saved for isolation of ER and cytosol. The pellet was resuspended in 10 ml of the isolation medium and centrifuged again at 12,000 \(\times\) g for 10 min. The pellet was resuspended in 2 ml of 15% Percoll (GE Healthcare)
and placed atop the discontinuous Percoll gradient consisting of a bottom layer of 4 ml of 40% Percoll and a top layer of 4 ml of 23% Percoll. The gradient was spun at 31,000 × g for 30 min in an SW-Ti40 rotor in a Beckman LE80K centrifuge. The final mitochondrial pellet was resuspended in 0.1 ml isolation medium. Protein concentration was determined with a bicinchoninic acid assay (Sigma) using bovine serum albumin as a standard.

Mitochondrial Respiratory Chain Activity—Mitochondrial respiration was measured by recording oxygen consumption at 25 °C in a chamber equipped with a Clark-type oxygen electrode (Instech Laboratories, Plymouth Meeting, PA) as described previously (37). Briefly, mitochondria are incubated in the medium containing 125 mM KCl, 10 mM HEPES, 2 mM KH2PO4, 5 mM MgCl2, and 0.5 mg/ml mitochondrial protein supplemented with either complex I substrate (mixture of 5 mM glutamate and 5 mM malate) or complex II substrate (5 mM succinate in the presence of 1 mM rotenone) or complex IV substrate (1 mM ascorbate in the presence of 250 μM N,N,N',N'-tetramethyl-p-phenylenediamine and 1 μM antimycin). The quality of mitochondrial preparation was tested by measuring a respiratory control ratio in mitochondria isolated from the contralateral (sham) hemisphere of the brain. Respiratory control ratio is measured as the oxygen consumption rate in the presence of the complex I substrate and 100 μM ADP (state 3) divided by the rate in the resting state (state 4) in the presence of 2 μg/ml oligomycin. Mitochondria preparations with respiratory control ratio values of at least five were used for these studies. Uncoupler-stimulated (state 3u) respiration was measured in the presence of 50 nM FCCP.

Sphingolipid Measurements (Tandem Mass Spectrometry)—Remarkable and recent progress in mass spectrometric techniques (20, 38, 39) resulted in new mass spectrometry (MS)-based methods of sphingolipid analysis that permit the assessment of multiple sphingolipid species simultaneously using reverse-phase high pressure liquid chromatography column coupled to electrospray ionization followed by separation using reverse-phase high pressure liquid chromatography column. The peaks for the target analyte were identified and quantified by tandem MS. Ceramide synthase-specific activity was determined by tandem MS. IR significantly elevated ceramide, dihydroceramide, and dihydrosphingosine, indicative of stimulation of de novo ceramide synthesis (47, 48) (Fig. 1). Impor-

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Lack of changes in sphingomyelin species content in brain

Sphingomyelin species content (pmol/mg protein) was determined in the ipsilateral (IR) and contralateral (sham) hemispheres of mouse brain 30 min of MCAO followed by 24 h of reperfusion. Values are means ± S.E., n = 12.

| Sphingomyelin Species | Sham | IR |
|-----------------------|------|----|
| C14:0                 | 5.3 ± 0.4 | 6.0 ± 0.6 |
| C16:0                 | 878.5 ± 57.0 | 982.1 ± 73.4 |
| C18:0                 | 12640 ± 174.7 | 12782 ± 120.8 |
| C18:1                 | 1158.6 ± 81.8 | 1383.8 ± 88.6 |
| C18:2                 | 773.5 ± 85.1 | 799.8 ± 94.2 |
| C24:0                 | 1184.6 ± 87.1 | 1390.8 ± 91.0 |
| C24:1                 | 1897.1 ± 89.9 | 2048.3 ± 77.4 |

Dihydrosphingosine (DHsph), dihydroceramide (DHCer), or ceramide (Cer) content was elevated in ischemic compared with sham hemisphere, whereas sphingomyelin (SM) content remained unchanged. Data are means ± S.E., *p < 0.05, n = 16.

FIGURE 1. IR triggered accumulation of ceramide during reperfusion phase. Sphingolipids were analyzed in ipsilateral (ischemic) and contralateral (sham) hemisphere after sham surgery (no ischemia) or 30 min of MCAO (ischemia) or 30 min of MCAO followed by 24 h of reperfusion (ischemia/reperfusion, IR). Dihydrosphingosine (DHsph), dihydroceramide (DHCer), or ceramide (Cer) content was elevated in ischemic compared with sham hemisphere, whereas sphingomyelin (SM) content remained unchanged. Data are means ± S.E., *p < 0.05, n = 16.

Notably, lack of change in content of sphingomyelin species does not support the notion that increased ceramide is because of activation of sphingomyelin hydrolysis (Table 1). The enhanced accumulation of sphingolipids seems to occur during the reperfusion phase; there were no changes in sphingolipid content after ischemia without reperfusion. This finding is in line with data that show that both ischemia and the restoration of blood flow to ischemic tissue (reperfusion) causes cellular damage by different molecular mechanisms (3). In contrast to short-chain artificial ceramides, endogenous ceramide species are characterized by long-chain fatty acid, including C14:0, C16:0, C18:0, C18:1, C20:0, C24:0, and C24:1. The content of all ceramide species was elevated after 30 min of cerebral ischemia followed by 24 h of reperfusion (Table 2).

Together, data are consistent with activation of ceramide biosynthesis in the brain after IR. However, it cannot be ruled out that IR has activated sphingomyelin synthases at the same time that there was activation of sphingomyelin turnover resulting in no net change in sphingomyelin but a localized change in ceramide from turnover in intracellular compartment. Therefore, we focused on mitochondria which are an important intracellular compartment involved in neural cell responses to IR.

**IR Stimulates Ceramide Synthase Activity of LASS in Mitochondria**—Emerging evidence suggests that ceramide synthesis occurs not only in the ER, but also in mitochondria or mitochondria-associated membranes (26, 27, 49). To determine whether mitochondrial ceramide biosynthesis is stimulated by cerebral IR, we purified mitochondria on a discontinuous 15:23:40% Percoll gradient as described previously (8, 50). Post-mitochondrial supernatant was centrifuged at 105,000 × g for 1 h to obtain the ER fraction. Western blot analysis of mitochondria and ER confirmed high quality separation between mitochondria and ER (Fig. 2A). To confirm the purity of mitochondria, the activity of the ER-specific marker enzyme, NADPH cytochrome c reductase, was measured in mitochondria and ER (Fig. 2B). The activity of the enzyme was 45.65 ± 0.22 nmol of cytochrome c oxidized per min/mg of protein in ER, whereas the activity was 0.23 ± 0.15 nmol of cytochrome c oxidized per min/mg of protein in mitochondria. The contamination of mitochondria with ER was less than 1%.

Sphingolipid analysis revealed that dihydrosphingosine, dihydroceramide, and ceramide were increased in both ER and mitochondria after IR (Fig. 2B). The lack of change in pro-apoptotic sphingolipid sphingosine in mitochondria and ER indicates a possible important role of excessive accumulation of ceramide in these intracellular compartments and, in particular, in mitochondria, which are crucial integrators of cell survival/death signals. There was no change in content of sphingomyelin either in ER or in mitochondria following IR (Fig. 2B). Thus, the sphingomyelin content was 4857 ± 175 pmol/mg protein in sham mitochondria, whereas sphingomyelin content was 5269 ± 134 pmol/mg protein in mitochondria from IR-injured brain. The data suggest that IR triggers the activation of de novo ceramide biosynthesis in mitochondria. Next, we analyzed the ceramide species profile in ER and mitochondria after IR. The content of all ceramide species was elevated in ER from an IR-damaged brain (Fig. 2C). In contrast, in mitochondria, the content of three ceramide species was increased, including C18:0, C18:1, and C16:0-ceramide. The results suggest that LASS could reside in mitochondria as well as in ER and is activated by IR. Given the relatively selective substrate profile of each LASS protein for chain length and saturation of fatty acyl-CoA (31), the data also suggest that different LASS family enzymes could be activated by IR in mitochondria compared with ER.

mRNA expression of several isoforms of ceramide synthase, including LASS1, LASS2, LASS5, and LASS6, has been detected in brain (31). We analyzed the expression of LASS family proteins in mitochondria and ER (Fig. 3). As expected, we found that LASS1, LASS2, LASS5, and LASS6 proteins reside in the ER. Moreover, LASS1, LASS2, and LASS6 were partly localized in the mitochondria. IR-induced accumulation of C18:0-, C18:1-, and C16:0-ceramide in mitochondria is consistent with activation of LASS6. However, concomitant activation of LASS1 could not be ruled out. LASS2 is not activated by IR in mitochondria; IR failed to elevate the very long-chain ceramides (Fig. 2C) such as C20:0-, C24:0-, or C26:1-ceramide that are generated by LASS2 (31).

To determine the mechanism of mitochondrial LASS activation in IR, the expression level of LASS proteins was assessed in mitochondria isolated from sham or IR-damaged brain. Western blot analysis revealed no changes in LASS6 or
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TABLE 2
IR-induced changes in ceramide species content in brain

Ceramide species content (pmol/mg protein) was determined in the ipsilateral (IR) and contralateral (sham) hemispheres of mouse brain after 30 min of MCAO followed by 24 h of reperfusion. Values are means ± S.E., n = 12.

| Ceramide Species | Sham | IR |
|------------------|------|----|
| C14:0            | 4.3 ± 0.3 | 6.9 ± 0.4* |
| C16:0            | 91.9 ± 2.6 | 193.3 ± 3.1* |
| C18:0            | 881.3 ± 24.6 | 1538.6 ± 38* |
| C18:1            | 763.0 ± 18.4 | 1435.2 ± 25.8* |
| C20:0            | 115.5 ± 9.7 | 183.7 ± 11.9* |
| C24:0            | 89.9 ± 7.5 | 142.6 ± 10.3* |
| C24:1            | 278.8 ± 19.3 | 367.5 ± 27.2* |

* Values are p < 0.05.

LASS1 protein expression in mitochondria following IR-induced brain injury (Fig. 4A). It appears that mitochondrial LASS activation was not because of stimulation of gene transcription that should result in elevated protein level in mitochondria after cerebral IR. Lack of change in protein expression suggested that IR could lead to augmentation of LASS activity in mitochondria via post-translational mechanisms.

Ceramide synthase activity of LASS was assayed under conditions determined to be linear for time and enzyme concentration and for which the substrate was not rate-limiting. Mitochondria from sham- or IR-injured brain were incubated with or without the ceramide synthase inhibitor FB1. Compelling evidence provided by Merrill et al. (51) indicate that FB1 is a potent and specific inhibitor of ceramide synthase, and the other enzyme of ceramide biosynthesis, serine palmitoyltransferase, is not affected by FB1. Indeed, IR-enhanced mitochondrial ceramide synthase activity was sensitive to FB1 (Fig. 4B). It is noteworthy that even though FB1 completely abrogated IR-induced LASS activity, ceramide synthase activity in sham mitochondria was FB1-insensitive. A similar IR-induced increase in ceramide synthase activity was observed in ER fractions (Fig. 4C). However, both the IR-activated ceramide synthase and base-line enzyme activity were sensitive to FB1. The results of these studies suggest that either IR triggers robust activation of mitochondrial ceramide synthase, which is normally not involved in ceramide biosynthesis in mitochondria, or IR confers FB1 sensitivity to mitochondrial ceramide synthase. Although both mitochondrial ceramide synthases, LASS1 and LASS6, could generate C₁₆:₀-dihydroceramide used to assess enzyme activity, only LASS6 can generate C₁₆:₀-ceramide (31) that is accumulated concomitantly with C₁₈:₀-ceramide in ischemic mitochondria. Moreover, it has been reported that LASS1 generated C₁₈:₀-ceramide in an FB1-insensitive manner, whereas it was overexpressed in cancer cells (52). Taken together, the data suggest that mitochondrial LASS6 is likely activated by IR via post-translational mechanisms.

Natural Ceramide Interaction with Respiratory Chain of Cerebral Mitochondria—After demonstrating excessive accumulation of ceramide species in mitochondria following IR, we addressed whether ceramide accumulation affects vital mitochondrial functions. We focused on mitochondrial respiratory chain dysfunction that has been reported previously after global or transient cerebral IR in the rat (6, 7). To establish the critical role of endogenous ceramide accumulation in mitochondrial respiratory chain dysfunction in cerebral IR, the effect of long-chain ceramide on respiratory chain activities was elucidated in base-line mitochondria purified from the mouse brain. It was important to demonstrate that C₁₈:₀-ceramide could inhibit respiratory chain activities in a manner identical to IR-imposed respiratory chain damage. Respiratory chain activities were determined using specific substrates donating electrons to different respiratory chain complexes (Fig. 5A), including glutamate, succinate, and ascorbate. Oxygen consumption by mito-
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The inability of exogenously added C18:0-dihydroceramide to inhibit mitochondrial respiration could be due to less efficient delivery of the compound to mitochondria. Therefore, we determined the concentration of C18:0-ceramide or C18:0-dihydroceramide in mitochondria after the organelles were incubated with 2 nmol/mg protein of either compound for 10 min at 25 °C and reisolated by centrifugation. There was 0.83 ± 0.09 nmol/mg protein of C18:0-ceramide and 1.55 ± 0.08 nmol/mg protein of C18:0-dihydroceramide in mitochondria. Dihydroceramide differs from ceramide only by reduction of 4, 5-trans-double bond. The data suggest that the ceramide-induced respiratory chain damage requires the presence of this double bond as dihydroceramide did not inhibit the respiratory chain function. This is consistent with the notion that ceramide mediates pro-apoptotic intracellular signaling, but dihydroceramide is not involved in promoting cell apoptosis (20, 53) and plays an important role in regulation of autophagy (23). Given the ability of long-chain ceramide to impose specific damage to the mitochondrial respiratory chain similar to IR-induced injury, ceramide accumulation in IR-injured mitochondria strongly implicates ceramide as a cause of mitochondrial respiratory chain defect in IR.

Activation of Ceramide Biosynthesis Is Diminished in Mitochondria of JNK3-deficient Mice—Involvement of JNK in regulating the mitochondrial death pathway is well documented by many subcellular fractionation studies showing localization of activated JNK and JNK-binding scaffold protein Sab (SH3BP5) to the mitochondria (54). Recently, a critical role of JNK3 for ischemic brain injury has been implicated. Although JNK1 is a major isoform responsible for the high level of basal activity in brain, targeted deletion of JNK3 not only reduced stress-induced JNK, but also protected mice from brain injury after cerebral ischemia (18). To determine whether JNK3 is involved in regulation of ceramide biosynthesis, ceramide accumulation was analyzed in brains of JNK3-deficient mice after IR. Wild-type or JNK3-deficient mice were exposed to 30 min of MCAO followed by 24 h of reperfusion. As reported previously (18), the infarct volume in JNK3-deficient mice was significantly reduced compared with wild-type mice (Fig. 6, A and B). Remarkably, IR-induced elevation of ceramide was profoundly diminished in JNK3-deficient mice brains (Fig. 6C). These data provide evidence that the JNK3 signaling pathway could mediate activation of ceramide biosynthesis in cerebral IR.

To establish that IR-induced activation of mitochondrial ceramide biosynthesis is mediated by JNK3 signaling, the ceramide species content was analyzed in mitochondria from JNK3-deficient mice. The accumulation of C16:0-, C18:0-, or C18:1-ceramide observed in the wild type was completely abolished in mitochondria from JNK3-deficient mice (Fig. 7A). The decreased accumulation of ceramide species in cerebral mitochondria of JNK3-deficient mice was not because of lower LASS protein expression. Thus, Western blot analysis revealed similar LASS protein expression in mitochondria from JNK3-deficient mice compared with wild type (Fig. 7B). The results of this study indicate that IR-induced stimulation of mitochondrial ceramide biosynthesis is under control of JNK3-mediated signaling.

**IR-induced Respiratory Chain Dysfunction Is Abolished in Mitochondria from JNK3-deficient Mice**—To demonstrate a vital role of JNK3 signaling in IR-induced ceramide-mediated mitochondrial dysfunction, we elucidated respiratory chain...
activities in mitochondria of JNK3-deficient mice after 30 min of MCAO/24 h of reperfusion. The respiratory chain activities were determined using specific substrates donating electrons to different respiratory chain complexes (Fig. 5A), including glutamate, succinate, and ascorbate. Oxygen consumption by mitochondria oxidizing the appropriate substrate was measured in the presence of uncoupler of oxidative phosphorylation, FCCP (state 3u). In mitochondria from wild-type mice, the decreased respiration rates of respiration supported by succinate indicated an ischemic defect either in complex II/III and/or in complex IV (Fig. 8). The lack of change in the respiration rate in the presence of ascorbate, a specific substrate for complex IV, suggested that IR damaged complex III. The IR-induced decrease in respiration in the presence of glutamate supports the defect being in complex III, but concomitant inhibition of complex I cannot be ruled out. In agreement with the reports on mitochondrial damage after global or transient cerebral IR in the rat (6, 7, 9, 10), the data suggest that IR damaged the mitochondrial respiratory chain at the level of complex III. Importantly, IR-induced mitochondrial respiratory chain dysfunction was completely abolished in mitochondria from JNK3-deficient mice. The data strongly suggest that JNK3-mediated signaling is responsible for IR-induced stimulation of LASS activity, ceramide accumulation in mitochondria, and mitochondrial respiratory chain damage.

**DISCUSSION**

The present studies provide evidence that cerebral IR could trigger the accumulation of ceramide in brain mitochondria via activation of ceramide synthase that resulted in the mitochondrial respiratory chain damage. The effect of ceramide on mitochondria was somewhat specific, because dihydroceramide, a closely related structure, failed to inflict the damage. These studies drew attention to mitochondrial localization of ceramide generation and its regulation by brain-specific JNK3 through post-translational mechanisms. The results revealed a critical role of JNK3 in mediating the IR-induced activation of the key enzyme of the ceramide biosynthetic pathway, ceramide synthase, which was found in mitochondria.

Two major pathways, namely, sphingomyelin hydrolysis and de novo ceramide synthesis, have been implicated in the generation of an apoptotic response through ceramide. Both pathways may be activated separately or in parallel depending on stimuli or on the cell type (55). The sphingomyelin pathway is a ubiquitous signaling system that links specific cell-surface receptors and environmental stresses to the nucleus (20, 56, 57). This pathway is initiated by hydrolysis of sphingomyelin, which is preferentially concentrated in the plasma membrane of mammalian cells. Sphingomyelin hydrolysis occurs after stimulation of sphingomyelinases to generate ceramide. An apoptotic signaling function for ceramide produced by de novo synthesis has also been identified (47, 58). Ceramide synthesized de novo increased after brief exposure of cultured brain cells to hypoxia, oxygen/glucose deprivation, or tumor necrosis factor-α (59, 60).

The prevailing hypothesis for the mechanism of ceramide generation in IR suggests a pivotal role of sphingomyelin hydrolysis and activation of sphingomyelinases. In earlier studies, a correlation was established between lower acidic sphingomyelinase activity and reduced tissue damage (about 25%) after severe ischemia (1 h) in acidic sphingomyelinase-deficient mice (35). Similarly, chronic cerebral ischemia caused ceramide accumulation because of activation of sphingomyelin degradation, accompanied by reduced ceramide utilization by glucosylceramide synthase (61). Our studies suggest an alternative mechanism of ceramide generation via activation of de novo synthesis and highlight novel determinants responsible for...
mitochondrial damage in IR. Our studies in brain tissue subjected to transient ischemia (30 min) followed by reperfusion show that sphingolipid intermediates of \textit{de novo} ceramide biosynthesis (dihydrosphingosine, dihydroceramide, and ceramide) increased concomitantly with a lack of sphingomyelin hydrolysis. Possibly, the severity of ischemic insult dictates the mechanism of ceramide generation. Further investigations will illuminate the precise roles of specific pathways of ceramide production in neural cell responses to IR.

Emerging data suggest that the subcellular location of ceramide generation plays a fundamental role in dictating its downstream targets and cell responses to pro-apoptotic stimuli (25, 62). In this regard, mitochondrial generation of ceramide via activation of sphingomyelin hydrolysis has been shown to mediate cell responses to UV irradiation (63) or tumor necrosis factor-\alpha (64). Current studies suggest for the first time that IR triggers activation of the key enzyme of ceramide biosynthesis, ceramide synthase, thereby leading to excessive accumulation of several ceramide species, including C\textsubscript{16:0}-, C\textsubscript{18:0}-, and C\textsubscript{18:1}-ceramide in mitochondria. At least three ceramide synthases, longevity-assurance homolog family members, namely LASS1, LASS2, and LASS6, partly reside in mitochondria. Importantly, lack of change in the expression of these proteins in the ischemic brain strongly indicates that activation of the enzyme(s) involves post-translational mechanisms. The profiles of ceramide species accumulated in mitochondria are consistent with IR-induced activation of LASS6, which has been shown to generate preferentially C\textsubscript{18:0} and C\textsubscript{18:1} ceramide (31). However, additional activation of LASS1 to generate C\textsubscript{18:0} ceramide (31, 32) has not been ruled out, whereas the activity of LASS2, generating mainly C\textsubscript{20:0} and C\textsubscript{24:0} ceramide (31), remained unchanged. Based on the different FB1 sensitivities of ceramide synthase in mitochondria isolated from IR-exposed and control brain, at least two enzymes might be involved in C\textsubscript{18:0} ceramide synthesis in mitochondria. IR-induced stimulation of mitochondrial ceramide synthase activity was completely sensitive to the inhibitor FB1.
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In contrast, the ceramide synthase activity was FB1-insensitive in control mitochondria, reminiscent of FB1-insensitive LASS1 activity that is overexpressed in cancer cells (52). Collectively, the data suggest that mitochondrial LASS1 mainly generates C_{18:0} ceramide in an FB1-insensitive manner, whereas mitochondrial LASS6 is activated by IR, and its activity is abrogated by FB1. This supports the concept that several ceramide synthases differ in fatty acid specificities and/or FB1 sensitivities may coexist in mitochondria (26). Furthermore, our results provide evidence that LASS are not entirely redundant despite some commonalities in fatty acyl-CoA specificity, and this suggests a selective mechanism modulating enzyme activity in IR.

In this study, we explored the mechanism of ceramide synthase regulation by examining the effects of JNK3 deletion on mitochondrial ceramide accumulation in response to IR. c-Jun N-terminal kinases represent one subgroup of mitogen-activated protein kinases activated in response to a variety of extracellular stimuli such as environmental stress, cytokines, and growth factors (65). JNKs have been characterized by their ability to phosphorylate and associate with numerous transcription factors (c-Jun, ATF-2, Elk-1, etc.). JNKs can also play a wider intracellular role through phosphorylation of non-nuclear proteins, including various proteins of the Bcl2 family (54). Recent identification of a scaffold protein that brings JNKs to the cytoplasmic surface of mitochondria emphasizes the importance of mitochondrial kinase signaling. In IR injury, CA1 hippocampal neurons had increased activated JNK that localized to the mitochondria prior to cell death (66). Overexpression of a JIP-1 (a JNK-interacting protein) that sequesters JNKs in the cytosol conferred protection against cardiac IR (67). Mammalian JNK proteins are encoded by three genes, jnk1, jnk2, and jnk3, that are spliced alternatively to create at least 10 JNK isoforms. Gene ablation studies in vivo suggest that different JNK isoforms perform distinct roles in cell survival. Thus, disruption of jnk1/jnk2 genes together results in embryonic lethality, whereas jnk3 null animals are normal (68, 69). However, targeted disruption of the jnk3 gene, but not jnk1 or jnk2, protected mice from IR brain injury, oxidative stress, and excitotoxicity (18, 70). The molecular significance of JNK3 isoforms remains to be elucidated. The scaffold proteins that assemble the molecules of JNK pathway in intracellular compartments play a fundamental role in orchestrating the stimulus- and compartment-specific JNK signaling complex (54). Our studies showing a link between JNK3 and activation of mitochondrial ceramide synthase shed some light on another downstream target of the JNK3 signaling pathway. IR-induced ceramide generation and respiratory chain damage were abolished in mitochondria of JNK3-deficient mice that had reduced infarct volume after IR. The inability of IR to up-regulate the expression of LASS suggests a post-translational mechanism of JNK-mediated regulation of LASS activity. Based on a distinct profile of accumulated ceramide species and FB1 sensitivity, the results support but do not definitively prove that JNK3 mediates activation of LASS6 in cerebral IR. Further investigations will shed more light onto the role of downstream targets of JNK3 in the neural cell responses to IR. Interestingly, LASS1 is predominantly expressed in brain (71), which normally contains much higher levels of C_{18:0} ceramide compared with other tissues and some cancer cell lines in which LASS1 expression is down-regulated (38). This implies restricted tissue distribution and regulation-specific LASS gene activity as important mechanisms for controlling the fatty acid composition of ceramides.

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