Functional Modulation of Mitochondria by Eicosapentaenoic Acid Provides Protection against Ceramide Toxicity to C6 Glioma Cells

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Mitochondrial dysfunction and associated apoptosis have been reported in the pathogenesis of neuron degeneration. The effects of eicosapentaenoic acid (EPA) and arachidonic acid (AA) on the mitochondrial membrane potential, mitochondrial biogenesis, and mitochondrial function of rat C6 glioma cells were determined in this study. Increased cytochrome *c* release and activated caspase-3 expression were determined in cells treated with >20 μM C_2 ceramide. There were significant repressive effects on ceramide-induced cell death with 25−100 μM EPA and 25 μM AA pretreatment. However, significantly increased membrane potentials were detected in cells pretreated with 25 and 50 μM EPA compared to ceramide-treated cells, but not in AA pretreatment groups. In cells pretreated with EPA, ATP production loss was prevented from ceramide-induced mitochondrial dysfunction. In mitochondrial biogenesis related assay, both EPA and AA enhanced peroxisome proliferator-activated receptor γ-coactivator-1α (PGC-1α) and mitochondrial transcription factor A (Tfam) transcriptional activities. However, elevated PGC-1α transcriptional activities in groups pretreated with 25, 50, and 100 μM EPA and only in the 100 μM AA group were analyzed. The Tfam transcriptional activities were enhanced in groups pretreated with 25 and 50 μM EPA and AA. Increased NADH dehydrogenase subunit 6 (ND6) mRNA expression was determined in cells pretreated with 25 and 50 μM EPA and 25 μM AA. Elevated protein levels of Tfam, flavoprotein, and cytochrome oxidase subunit III (COX III) were determined in cells pretreated with 25 and 50 μM EPA. The EPA-provided a more protective effect than AA against ceramide-induced cell death, which might mainly be due to maintaining the membrane potential and sustaining the mitochondrial ATP production function. EPA has more potential to elevate mitochondrial biogenesis through enhanced PGC-1α, and Tfam transcriptional activities may provide partial protection against ceramide cytotoxicity.

KEYWORDS: Arachidonic acid; ceramide; eicosapentaenoic acid; mitochondrial biogenesis; mitochondrial transcription factor

INTRODUCTION

Neurodegeneration is a common disease and a cause of dementia in aging populations. Mitochondrial dysfunction and associated apoptosis have been reported in the pathogenesis of neuron degeneration. There are different clinical features in a variety of neurodegenerative disorders, including Alzheimer's disease, Parkinson's disease, amyotrophic lateral sclerosis (ALS), schizophrenia, and depression (*1*, *2*). The main energy source of neurons is supplied from mitochondrial oxidative metabolism, and a unified pathogenetic mechanism of neurodegeneration has been suggested. Reactive oxygen species (ROS) generation, deficiencies in energy supply, and compromised Ca^{2+} buffering or apoptosis regulation can all theoretically contribute to a progressive decline in the central nervous system (*3*).

Beneficial effects on various neuron functions and mitochondrial biogenesis of n-3 polyunsaturated fatty acids (PUFAs) have been reported. Eicosapentaenoic acid (EPA) is one of the n-3 PUFAs and is essential for the human body because mammals are incapable of synthesizing fatty acids with a double bond

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converted from the n-9 position (*4*). The literature during the past decade reported the effects of EPA on neuroprotection. Epidemiological studies showed that a decreased prevalence of Alzheimer's disease was found in people with a high frequency of fish consumption (*5*). Supplementation of n-6 and n-3 fatty acids also improved the life quality of Alzheimer's disease patients (*6*). n-3 fatty acids are reported to be neuroprotective against excitotoxic processes (*7*, *8*). Furthermore, in both in vitro and in vivo studies, Lonergan showed that EPA protected rat hippocampal neurons from lipopolysaccharide (LPS)-induced neurotoxicity (*9*). It is also known that n-3 fatty acids, such as docosahexaenoic acid (DHA) and EPA, have anti-inflammatory effects (*10*). In addition, it was shown that DHA can counteract glutamate-induced excitotoxicity (*11*). Arachidonic acid (AA) is the precursor of eicosanoids that produce prostaglandins (PGs), thromboxanes (TXs), leukotrienes (LTs), and other oxidized derivatives and is also related to the synthesis of proinflammatory cytokines. However, the cellular mechanisms of the neuroprotective effects of EPA and AA remain unclear.

Human mitochondrial (mt) DNA is a circular, histone-free molecule composed of 16.6 kb of DNA, present in one or more copies in every mitochondrion. It encodes 13 of about 80 protein subunits constituting the oxidative phosphorylation system, with the remainder being encoded by nuclear genes and imported into mitochondria. Human mtDNA also encodes two rRNAs and 22 tRNAs. The oxidative phosphorylation capacity of mitochondria is determined by the interplay between nuclear and mitochondrial genes. Mitochondrion-related proteins are synthesized from two cellular locations. A minor proportion of mitochondrial proteins are encoded by mtDNA and are transcribed/translated by their own machinery. The other predominant fractions of mitochondrial proteins are encoded by nuclear DNA and are translated in the cytoplasm. Although mtDNA encodes 13 polypeptides that are components of the respiratory system, nuclear DNA encodes the majority of respiratory chain proteins and all of the proteins and enzymes that regulate replication and transcription of mtDNA (*12*).

In mammals, an essential component of the mitochondrial transcription initiation complex is mitochondrial transcription factor A (Tfam), a nuclear-encoded 25 kDa protein that bends and unwinds mtDNA upon binding and which belongs to the high-mobility-group (HMG)-box family of proteins (*13*, *14*). Tfam regulates both mitochondrial transcription and replication by permitting commencement of transcription of the RNA primer from the unwinding L-strand of DNA (*15*). The Tfam gene has been isolated in humans and is composed of seven exons and six introns (*16*). Knockdown of Tfam gene expression may down-regulate the mtDNA copy number and expression and compromise mitochondrial function and subsequent cell growth and morphology (*17*). Peroxisome proliferator-activated receptor γ−coactivator-1α (PGC-1α) belongs to a small family of transcriptional coactivators that possess a common function in the thermogenic response and mitochondrial physiology (*18*). PGC-1α is composed of multiple protein complexes dependent on the target gene and various signals. The PGC-1α complex has been studied and found to regulate various PGC-1α target genes including gluconeogenic-related genes and mitochondrial oxidative phosphorylation genes (*19*).

Decreased mitochondrial content and reduced mRNA expression of mtDNA resulting in mitochondrial dysfunction and altered cellular characteristics have been reported in various kinds of cells. mtDNA depletion is a frequent cause of bioenergetic defects in human neurological diseases (*20*, *21*). Ceramide accumulation in neurons is one of the factors correlated to acute and chronic neurodegeneration (*22*, *23*). Ceramide-induced loss of the mitochondrial membrane potential, overproduction of ROS, and ATP depletion result in necrosis of lymphoid cells (*24*).

In this study, the neuroprotective potential of EPA and AA on rat C6 cells exposed to ceramide was evaluated. The effects on mitochondrial gene regulation, membrane potential, and mitochondrial function by fatty acids were determined. We investigated whether EPA can protect against injury to C6 cells from ceramide-induced cell death by modulating mitochondrial function.

MATERIALS AND METHODS

Cell Culture and Drug Treatment. Rat C6 glioma cells (ATCC CCL 107) were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 100 U/mL penicillin, and 100 μg/mL streptomycin. Cells were grown at 37 °C in a humidified atmosphere with 5% CO_2 for 3 days before the experimental treatment began. According to the literature and manufacturer's instructions, C2 ceramide, EPA, and AA were dissolved in EtOH. C2 ceramide, EPA, and AA were purchased from Sigma-Aldrich (St. Louis, MO). Cells were seeded in 96-well culture plates with various concentrations of ceramide alone or presupplemented with EPA or AA. Control cells were cultured in the equivalent concentration of carrier (0.1% EtOH), and no significant difference was found with cells grown in medium without the carrier.

Reporter Gene Construct and Transient Transfection. Promoter constructs were generated using rat C6 glioma cell genomic DNA as the template. The 5′-flanking region of the rat PGC-1α gene was PCR-amplified (−1783 to +1; forward, ATGAGATCTACCATTCTGCTGTCTTGAA; and reverse, TATAAGCTTCAACTCCAATCCACTCTGAC) and cloned into the luciferase reporter gene vector, pGL3-basic (Promega, Madison, WI), to generate the reporter vector, pGL3-PGC-1α. The reporter plasmid, pGL3-Tfam, contains the Tfam promoter sequence (−462 to +49; forward, TGCTCGAGAAAGATTAGATAAACAAAGCGATACAT; and reverse, TATTAAAAGCTTGACACTGTGCCTACACACAGC). All constructs were confirmed by automated sequencing.

C6 glioma cells (5×10^5) were seeded onto 6-well culture plates before transfection. Each reporter vector was transfected into cells using the SuperFect transfection reagent (Qiagen, Valencia, CA) according to the manufacturer's protocol. Twelve hours after transfection, the medium was replaced and test agents were added. Twenty-four hours after the addition of the test agents, cells were harvested by passive lysis buffer (Promega, San Luis Obispo, CA). Luciferase activity assay was performed in a Victor2 1420 multilabel counter (PerkinElmer, Waltham, MA) using a dual-luciferase reporter assay system (Promega), and then Renilla luciferase activity was used to normalize the transfection efficiency.

MTS Assay of Cell Viability. The reduction metabolism of a tetrazolium compound (MTS) assay evaluated the cell viability in C6 glioma cells. The MTS colorimetric assay is based on the reduction of MTS (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2*H*-tetrazolium) to a colored formazan product. The absorbance was measured at 492 nm and is directly proportional to the number of living cells from the tested samples. Cells were seeded in 96-well plates for 24 h. To investigate the effects of ceramide, EPA, and AA on cell viability, several concentrations of the drugs were added to the culture medium to a total volume of 200 μL in each well. After 24 or 48 h of incubation, the medium was aspirated, and cells were washed with 100 μL of PBS twice. MTS reagent (20 μL) mixed with 100 μL of fresh medium without drug was added to each well, and cells were incubated at 37 °C in a humidified, 5% CO_2 atmosphere for 2 h. The plates were read on a Microplate Reader (VersaMax, Molecular Devices, Sunnyvale, CA). Cell viability was expressed as the percentage of viable cells in treated samples relative to the nontreated control.

RNA Extraction and Real-Time Reverse-Transcription Polymerase Chain Reaction (RT-PCR) Analysis. Total RNA was extracted from cells with the Trizol reagent according to the manufacturer's instructions (Invitrogen, Carlsbad, CA). Total RNA (1−5 μg) was reverse-transcribed into cDNA using oligo (dT) 18 (Protech Technology, Taipei, Taiwan) as a primer and into MMLV reverse transcriptase (Epicenter Biotechnology, Madison, WI), and 2 μL of the cDNA template

was separately used to amplify the different messenger (m)RNAs. A LightCycler PCR machine (Roche Diagnostics, Mannheim, Germany) was used to perform the real-time PCR. PCR conditions were 95 °C for 10 min, followed by 40 cycles of 95 °C for 10 s, 60 °C for 5 s, and 72 °C for 10 s. Primers were designed using Primer Express (Applied Biosystems). The sequences of the oligonucleotide primers used in this study were ND6 (forward, CTGCTATGGCTACTGAGG; and reverse, CCCGCAAAC-AATGACC) and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (forward, GAGAGGCAATGAAAAGGTA; and reverse, ACATTG-TTGCATCAGCTCAGGTCT).

Western Blot Analysis. Total protein was extracted from harvested cells using lysis buffer [25 mM Tris-phosphate (pH 7.8), 2 mM DTT, 10% glycerol, and 1% Triton X-100] with protease inhibitors. The cell lysate was cleared of cell debris by centrifugation at 10000*g* for 5 min. The protein concentration was measured using a BCA protein assay kit (Pierce, Rockford, IL). Proteins were resolved on polyacrylamide gels, and then proteins were transferred onto a polyvinylidene difluoride (PVDF) membrane (Amersham Pharmacia, Piscataway, NJ). Membranes were blocked for 1 h at 4 °C with 10% skim milk in TBST buffer (1 M Tris-HCl, 100 mM NaCl, and 1% Tween-20). Blots were probed with the following primary antibodies: monoclonal antibodies (mAbs) against COX III and flavoprotein (Molecular Probes, Eugene, OR) and Tfam (GlycoNex, Taipei, Taiwan) or a polyclonal antibody against α-tubulin and GAPDH. Blots were then incubated with the appropriate horseradish peroxidase-conjugated anti-immunoglobulin G (IgG) antibody. Antibody-bound protein was detected using the Western blotting chemiluminescence luminol reagent (Santa Cruz, Santa Cruz, CA) and exposure to film.

Analysis of Caspase-3 Activities. Caspase-3 activities were evaluated by measuring the proteolytic cleavage of their fluorogenic substrates Z-DEVD-R110. A commercial caspase-3 assay kit (Molecular Probes) was used according to the manufacturer's instructions to determine the caspase-3 activity. Absorbance was measured at excitation/emission ~496/520 nm using a plate reader. The activated caspase-3 was also measured by Western blotting with primary antibody against the active form of caspase-3 (Sigma-Aldrich).

Analysis of Cytochrome *c* Release. For analysis of cytochrome *c* release, the treated cells were harvested in their culture medium, pelleted by centrifugation at 300*g* for 5 min, rinsed with PBS, and resuspended in 5 mL of 0.25 M sucrose, 1 mM EGTA, and 10 mM Hepes−NaOH (pH 7.4) containing 0.5% BSA, and the suspensions were centrifuged at 500*g* for 2 min. The cloudy supernatant was discarded, and the cells were resuspended in 5 mL of the same medium. Cells were homogenized with a tight-fitting glass−Teflon homogenizer glass and centrifuged at 1200*g* for 10 min to remove nuclei and unbroken cells. The supernatant was retained, and the nuclear pellet was re-extracted with a further 5 mL of homogenizing medium; the two supernatants were then combined. The resulting supernatant was subjected to 12000*g* centrifugation for 10 min at 4 °C, and the pellet, corresponding to the mitochondrial fraction, was solubilized in 50 L of 10 mM Tris buffer (pH 8), 0.1% NP- 40, and 5 mM $CaCl_2$. The resulting supernatant fraction was centrifuged at 100000*g* for 1 h at 4 °C to obtain the cytosol. The ctyochrome *c* contents in cytosolic and mitochondrial fractions were determined by Western blotting assay.

Assessment of the Mitochondrial Membrane Potential. Ceramide-induced changes in the mitochondrial membrane potential were verified with the JC-1 (5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzimidazolyl-carbocyanine iodide) cationic fluorophore. The JC-1 dye is redistributed between the inside and outside of the mitochondrial matrix depending on the membrane potential separating the inner and outer membranes. In polarized membranes, it forms red fluorescent aggregates, and when depolarized, JC-1 stays dispersed as a monomer and fluoresces green. Carbonyl cyanide *m*-chlorophenylhydrazone (CCCP) causes mitochondrial uncoupling and produces a drastic decrease in the fluorescence of JC-1. After 24 h of incubation, cells were trypsinized, centrifuged, and resuspended in 1 mL of PBS. JC-1 (50 μM) was added for 20 min at 37 °C; CCCP (5 μg/mL) was used as a positive control for mitochondrial depolarization. Cells were illuminated with blue (488 nm) excitation, and light green and red fluorescence emissions were detected with 530/30 and 585/42 nm filters, respectively, in a FACS Calibur system using CellQuest software (BD Biosciences, San Jose, CA). The proportion of red-to-green fluorescence of individual cells was calculated.

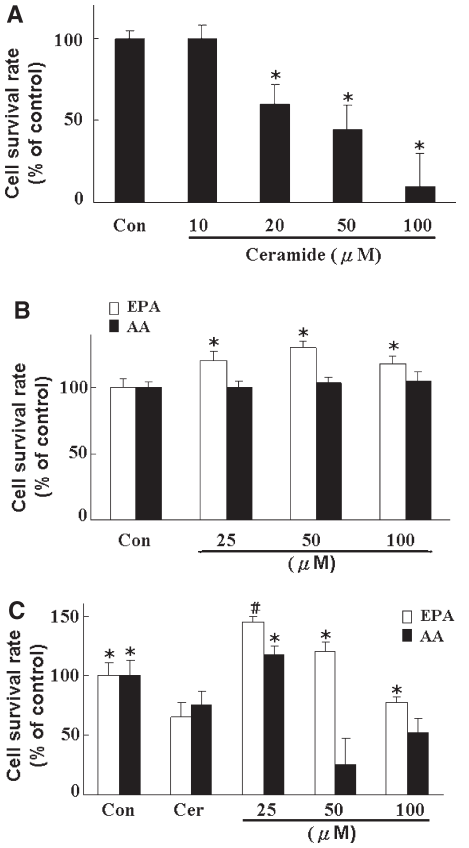

Figure 1. Cell viabilities were determined by an MTS assay after ceramide, EPA, and AA treatment. (**A**) C6 glioma cells were treated with various concentrations of C_2 ceramide (Cer) for 24 h. (**B**) Cells were treated various concentrations of EPA or AA for 24 h. (**C**) Cells were pretreated with various concentrations of EPA or AA for 24 h followed by ceramide treatment for 24 h. Data are expressed as the mean ± SD of three separate experiments. ∗, $p < 0.05$; #, $p < 0.01$, significantly different from control (Con) or ceramide-treated cells.

Adenosine Triphosphate (ATP) Determination. Cells were harvested and lysed in 200 μL of lysis buffer [25 mM Tris−phosphate (pH 7.8), 20 mM DTT, 2 mM 1,2-diaminocycloexane-*N*,*N*,*N*,*N*-tetraacetic acid, 10% glycerol, and 1% Triton X-100]. ATP was measured using an ATP assay kit (Molecular Probes); 100 μL of each sample or ATP standard was mixed with an equal amount of the ATP assay mix, containing luciferase, luciferin, dithiothreitol, reaction buffer including tricine buffer (pH 7.8), $MgSO_4$, ethylenediaminetetraacetic acid (EDTA), and sodium azide. The light emitted at 560 nm was measured in a luminometer and was proportional to the ATP present.

RESULTS

Effects of Ceramide, EPA, and AA on Cell Viability. To test the effects of ceramide on cell viability, C6 glioma cells were treated with different concentrations of ceramide of 10−100 μM. After 24 h of treatment, the percentage of living cells was determined, and it was shown that 20, 50, and 100 μM ceramide induced decreases in cell viability (**Figure 1A**). The effect of EPA or AA on cell viability was also determined at three different concentrations of 25, 50, and 100 μM for 24 h. Increased cell viabilities were determined for 25, 50, and 100 μM EPA incubations, but this was not observed in cells incubated with the same concentrations of AA (**Figure 1B**). To further determine whether EPA or AA could prevent ceramide-induced cell death, cell viabilities were also determined after ceramide was used alone or when cells were pretreated with EPA or AA. Three different concentrations of 25, 50, and 100 μM of EPA or AA were used to pretreat cells for 24 h

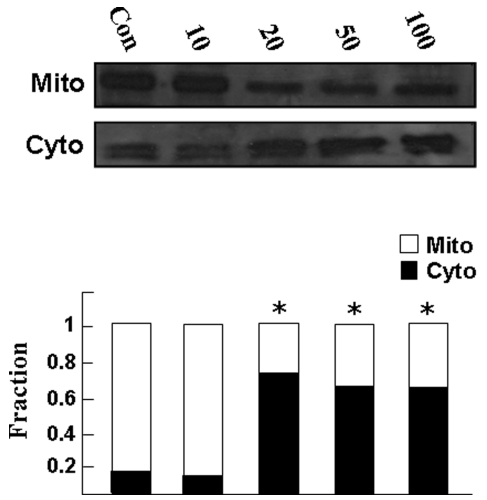

Figure 2. Mitochondrial cytochrome *c* release assay. The proportions of cytochrome *c* in mitochondrial and cytosolic fractions from control or various concentrations (μM) of ceramide-treated cells were determined by Western blotting. Data are expressed as the mean ± SD of three separate experiments. *, $p < 0.05$, significantly different from control (Con) cells.

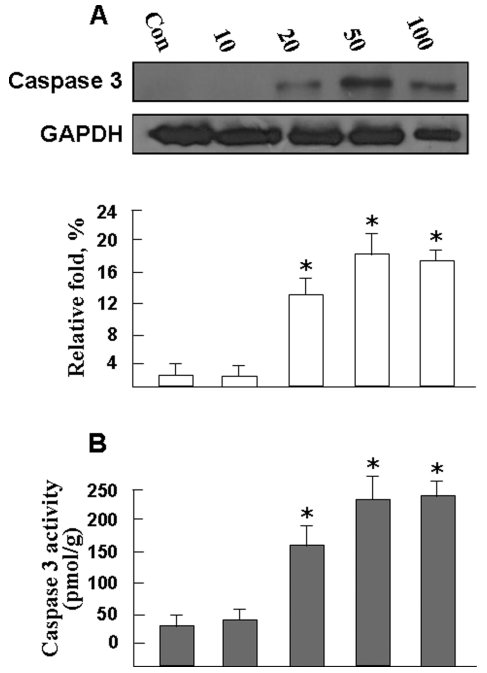

Figure 3. Caspase-3 activation in ceramide-treated cells: (**A**) Western blotting assay for the caspase-3 acitve form; (**B**) caspase-3 activity measured by enzymatic assay. Data are expressed as the mean ± SD of three separate experiments. *, $p < 0.05$, significantly different from control (Con) cells.

followed by treatment with 50 μM ceramide for another 24 h. There were significant repressive effects on ceramide-induced cell death from 25, 50, and 100 μM EPA but only from 25 μM AA treatment. Furthermore, with 25, 50, and 100 μM, there were enhanced effects on cell viability by EPA compared to AA incubation (**Figure 1C**).

Ceramide Induced Cytochrome *c* Release and Caspase-3 Activation. To confirm the caspase-3-dependent apoptosis in ceramide-treated cells, cytochrome *c* release and caspase-3 activation were measured. Increased contents of cytochrome *c* in the cytosolic fraction from 20, 50, and 100 μM ceramide-treated cells were determined by Western blotting (**Figure 2**). Ceramide-induced caspase-3 activation is presented as **Figure 3A**. Consistent with the Western blotting analysis, the enzymatic assay also showed an increase in caspase-3 activity in >20 μM ceramide-treated cells (**Figure 3B**).

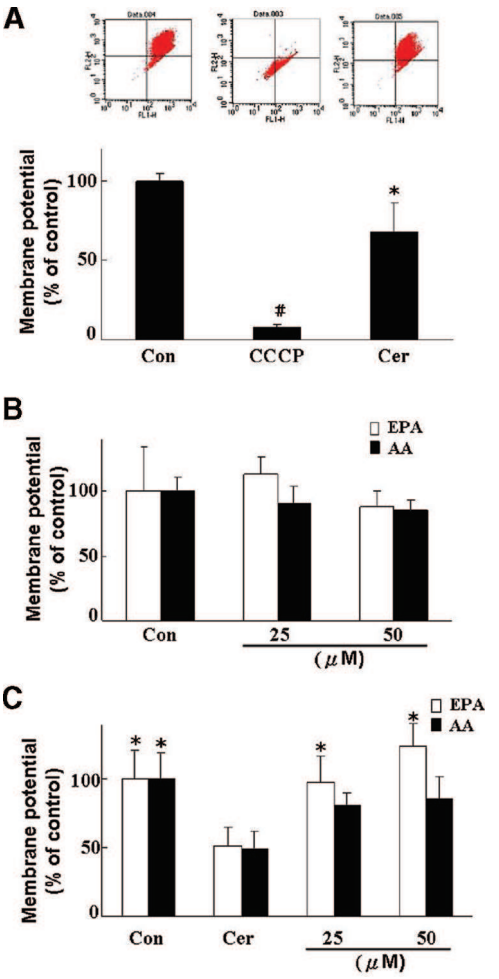

Figure 4. Mitochondrial membrane potential. Cells were incubated with EPA, AA, and ceramide for 24 h, labeled by a JC-1 fluorescent dye, and analyzed by flow cytometry. (**A**) C6 glioma cells were treated with ceramide for 24 h. Cells were incubated with carbonyl cyanide *m*-chlorophenylhydrazone (CCCP) as the negative control. (**B**) Cells were treated with 25 and 50 μM EPA or AA for 24 h. (**C**) Cells were pretreated with 25 and 50 μM EPA or AA for 24 h followed by ceramide treatment for 24 h. Data are expressed as the mean ± SD of three separate experiments. *, $p < 0.05$; #, $p < 0.01$, significantly different from control or ceramide-treated cells.

Loss of the Mitochondrial Membrane Potential from Ceramide-Damaged Cells. To investigate whether the observed ceramide-induced cell death might be due to loss of the mitochondrial membrane potential, we analyzed its effect on the membrane potential using a JC-1 fluorescent probe and monitoring by flow cytometer. As shown in **Figure 4A**, ceramide decreased the mitochondrial membrane potential. Membrane potentials were maintained in groups treated with 25 and 50 μM EPA and AA (**Figure 4B**). To assess whether EPA and AA can inhibit the phenomenon of ceramide-induced membrane potential loss, the effects of separate EPA and AA pretreatments followed by ceramide incubation on cells were determined. There were significantly increased membrane potentials in cells pretreated with

25 and 50 μM EPA compared to ceramide-treated cells (**Figure 4C**). However, no significant difference between cells pretreated with 25 and 50 μM AA and ceramide-treated cells was found.

EPA Retained the Mitochondrial ATP Production Ability of Treated Cells. There was significant decrease in ATP contents in ceramide-treated cells (**Figure 5**). Because ceramide decreased the mitochondrial membrane potential resulting in mitochondrial dysfunction, we characterized the ATP production ability of mitochondria from treated cells. To determine whether EPA and AA could prevent ceramide-induced loss of the ATP production ability, ATP contents in cells were analyzed. In cells pretreated with 25, 50, and 100 μM EPA, the ATP production ability was preserved against ceramide-induced mitochondria dysfunction. ATP contents showed significant decreases in AA-pretreated cells (**Figure 5**).

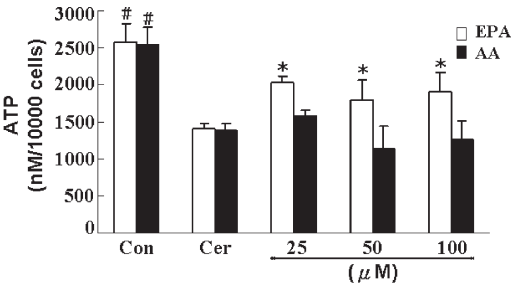

Figure 5. ATP determination. Cells were pretreated with 25, 50, and 100 μM EPA or AA for 24 h followed by 50 μM ceramide treatment for 24 h. Data are expressed as the mean ± SD of three separate experiments. *, $p < 0.05$; #, $p < 0.01$, significantly different from ceramide-treated cells.

EPA and AA Promote PGC-1α and Tfam Transcriptional Activities. A transcriptional reporter assay was performed to determine whether EPA and AA are capable of activating mitochondrial biogenesis and thus to provide a protective effect against ceramide-induced damage. PGC-1α and Tfam are two important transcription regulators of mitochondrial biogenesis, and the transcriptional activities of PGC-1α and Tfam were determined. Both EPA and AA at 25, 50, and 100 μM enhanced PGC-1α and Tfam (except 100 μM AA) transcriptional activities (**Figure 6A**,**B**). Increased PGC-1α transcriptional activities were found in groups pretreated with 25, 50, and 100 μM EPA and 100 μM AA (**Figure 6C**). The Tfam transcriptional activities were enhanced in groups pretreated with 25 and 50 μM EPA and AA (**Figure 6D**).

Mitochondrial RNA and Protein Expression in EPA- and AA-Treated Cells. Because both EPA and AA can enhance PGC-1α and Tfam transcriptional activities, to determine whether EPA- and AA-promoted PGC-1α and Tfam transcription could enhance mitochondrial RNA expression, mitochondrial gene expression was analyzed by real-time RT-PCR. We examined the RNA expression of mitochondrial ND6, which is a subunit of the first enzyme complex in the electron transfer chain and is transcribed by mtDNA. Increased ND6 mRNA expression was determined in cells pretreated with 25 and 50 μM EPA and 25 μM AA compared to ceramide-incubated cells (**Figure 7A**). The protein levels of the mitochondrial transcription factor, Tfam, the electron transfer chain subunit of nuclear-encoded flavoprotein, and mtDNA-encoded COX III were also determined to characterize mitochondrial biogenesis. Increased protein levels of Tfam were found in cells pretreated with 25 and 50 μM EPA compared to ceramide-incubated cells. However, no significant difference was noted between cells pretreated with AA and cells treated with ceramide alone (**Figure 7B**). There were significantly

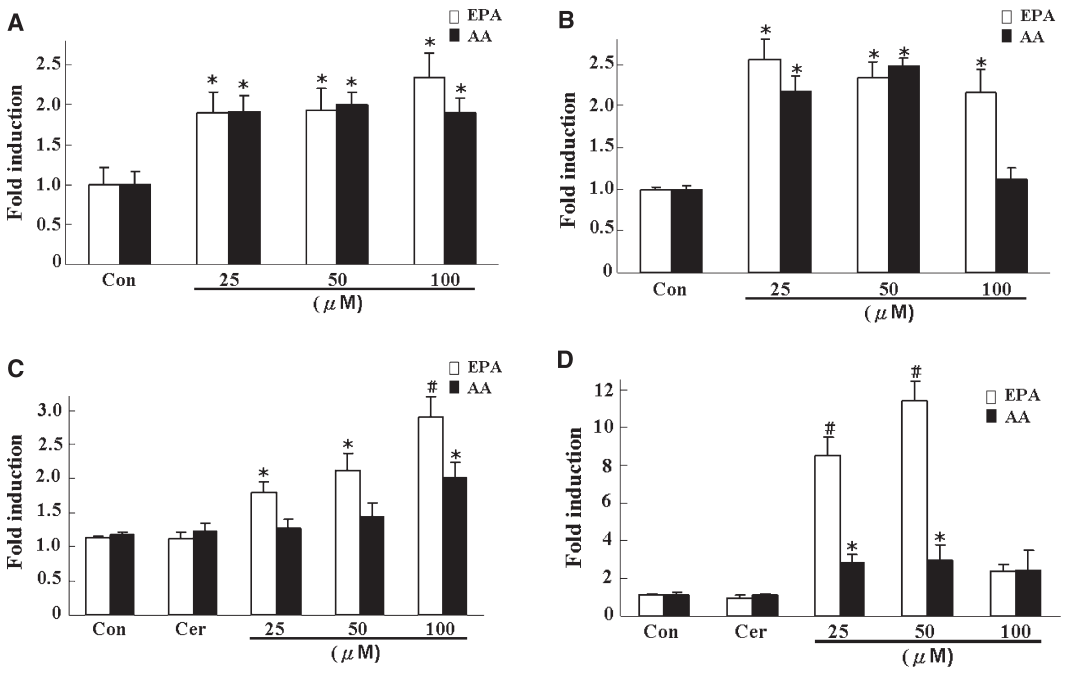

Figure 6. Effects of treatment with EPA or AA on PGC-1α or Tfam transcriptional activities in C6 cells investigated by a reporter assay. (**A**) In the PGC-1α transcriptional activity assay, cells were treated with 25, 50, and 100 μM EPA or AA for 24 h. (**B**) In the Tfam transcriptional activity assay, cells were treated with 25, 50, and 100 μM EPA or AA for 24 h. (**C**) In the PGC-1α transcriptional activity assay, cells were pretreated with 25, 50, and 100 μM EPA or AA for 24 h followed by ceramide treatment for 24 h. (**D**) In the Tfam transcriptional activity assay, cells were pretreated with 25, 50, and 100 μM of EPA or AA for 24 h followed by ceramide treatment for 24 h. Data are expressed as the mean ± SD of three separate experiments. *, $p < 0.05$; #, $p < 0.01$, significantly different from control or ceramide-treated cells.

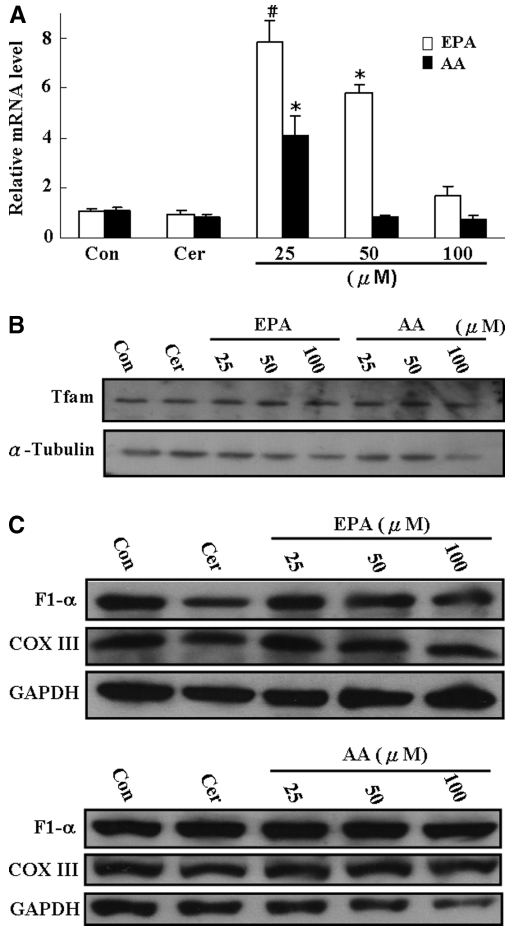

Figure 7. Expression levels of mitochondrial RNA and protein. (**A**) Cells were pretreated with 25, 50, and 100 μM EPA or AA for 24 h followed by 50 μM ceramide treatment for 24 h. ND6 gene expression was determined by real-time RT-PCR analysis. (**B**) Protein levels of Tfam were determined from cells pretreated with 25, 50, and 100 μM EPA or AA. (**C**) Protein levels of flavoprotein and COX III were determined. Data are expressed as the mean $\pm$ SD of three separate experiments. $*$, $p < 0.05$; #, $p < 0.01$, significantly different from control or ceramide-treated cells.

increased flavoprotein and COX III expressions in cells pretreated with 25 and 50 μM EPA (**Figure 7C**).

DISCUSSION

There is growing knowledge about the role of glial cells in neurogenesis, neuronal development, and maintenance of synaptic contacts (*25*). The glial cell strain, C6, was cloned from a rat glial tumor (*26*) and used as an experimental model system for the study of depressive disorder and neuronal development (*27*, *28*). In this study, the protective potential of EPA and AA on rat C6 cells from ceramide-induced cytotoxicity was evaluated. The current results show that 25 and 50 μM ceramide treatment for 24 h may cause significant changes in cell morphology. Ceramide also decreased cell viability after a 24 h incubation at concentrations of 20−100 μM (**Figure 1A**). Ceramide-induced cytochrome *c* release, and caspase-3 activation may trigger cell apoptosis (**Figures 2** and **3**). In this study, we also suggested that ceramide-induced cell death might have been due to disruption of mitochondrial membrane potential (**Figure 4A**), ATP production (**Figure 5**), and mitochondrial biogenesis (**Figures 6** and **7**). Consistent with our observation, ceramide-induced mitochondrial depolarization was reported in both cultured cardiomyocytes and retina photoreceptors (*29*, *30*). Loss of the mitochondrial membrane potential, overproduction of ROS, and ATP depletion were reported, which resulted in necrosis of ceramide-treated lymphoid cells (*24*). Increased ceramide synthesis in the endoplasmic reticulum can initiate apoptosis by permeabilizing the mitochondrial outer membrane to proapoptotic proteins (*31*). Ceramide treatment initiates a cascade of biochemical alterations including mitochondrial depolarization and cytochrome *c* release, and caspase-3 activation associated with cell death was determined in cerebellar granule cells and primary cortical neuronal cells (*32*,*33*). From 25 to 100 μM, EPA and AA treatment maintained or increased cell viability (**Figure 1B**). However, there were significantly decreased cell viabilities in groups treated with 200 and 400 μM EPA and AA (data not shown). It seems that the lipotoxic effect was expressed with higher concentrations of fatty acids.

Different effects of saturated and unsaturated fatty acids contributing to mitochondrial membrane potential have been described. Palmitate incubation induced decreased membrane potentials, cytochrome *c* release, and mitochondrial apoptosis (*34*). Long-term exposure to palmitate sensitizes pancreatic β-cells to mitochondrial permeabilization, resulting in cell lipotoxicity (*35*). Pretreatment with 25, 50, and 100 μM EPA or 25 μM AA followed by ceramide incubation showed significant effects on prevention of ceramide-induced cell death (**Figure 1C**). These results demonstrated that various concentrations of EPA could provide protective effects against ceramide-induced cell death. Furthermore, EPA was stronger at maintaining cell survival after ceramide-induced damage than was AA pretreatment.

To test which effect was induced by EPA or AA in preventing cell death, mitochondrial function, the mitochondrial membrane potential, ATP production, mitochondrial biogenesis, PGC-1 and Tfam transcriptional activities, and mitochondrial RNA expression were characterized. In a study of the mitochondrial membrane potential, results showed that ceramide decreased the membrane potential, and both EPA and AA treatment alone helped maintain it (**Figure 4B**). EPA pretreatment prevented ceramide-induced membrane potential loss (**Figure 4C**). However, no significant preventive effect was detected in the AA-pretreated groups. EPA had a stronger effect on maintaining the membrane potential than did AA. These results demonstrate that ceramide caused a decrease in the mitochondrial membrane potential in C6 glioma cells, and the protective effect against ceramide-induced cell death provided by EPA might have been due to its ability to maintain the membrane potential. The mitochondrial membrane potential maintained by electron transfer passed through respiratory chain and mitochondrial depolarization may compromise ATP production. Consistently, EPA elevated ATP production by ceramide-damaged cells, and that ability was lost with AA treatment (**Figure 5**). These data support EPA protecting mitochondrial function from ceramide-induced damage.

In characterizing whether the ability to maintain the membrane potential is due to up-regulation of mitochondrial biogenesis, it was found that both EPA and AA enhanced PGC-1α and Tfam transcriptional activities (**Figure 6A**,**B**). EPA induces mitochondrial proliferation and reduces intracellular lipids (*36*). EPA/DHA enhances β-oxidation and up-regulates mitochondrial biogenesis. A 3-fold stimulation of the expression of the mitochondrial regulatory factors, PGC-1α and nuclear respiratory factor-1 (NRF1), was also reported in that study (*37*). EPA pretreatment at 25−50 μM also enhanced PGC-1α and Tfam transcriptional activities (**Figure 6C**,**D**). The elevated level of mitochondrial ND6 RNA observed was consistent with the above observation. Increased protein expression levels of Tfam,

flavoprotein, and COX III were also found in cells pretreated with 25 and 50 μM EPA (**Figure 7C**). This study indicated the EPA pretreatment elevated mitochondrial RNA and protein expression levels possibly through enhanced PGC-1α and Tfam transcriptional activities.

EPA provides two different protective roles, maintenance of the mitochondrial membrane potential and elevation of mitochondrial biogenesis, to prevent a ceramide-induced decrease in cell viability. At concentrations of 25, 50, and 100 μM EPA or AA, there was significantly greater cell viability with EPA than with AA incubation (**Figure 1C**). EPA may prevent the effects of LPS-induced deterioration of synaptic function in the rat hippocampus. This neuroprotective effect of EPA may be dependent on its ability to inhibit the downstream consequences of JNK activation (*9*). Leonardi et al. (*38*) demonstrated that EPA and DHA induce alterations in the glucose-6-phosphate dehydrogenase activity to modulate oxidative status that could affect the glial function. Kou et al. (*39*) also reported that EPA exerts beneficial effects on cell survival through modulating neurotrophin receptor expression. In addition, EPA increases the activities of carnitine palmitoyltransferase (CPT)-1 and β-oxidation in adipocytes by altering the structure or dynamics of mitochondrial membranes (*40*). EPA treatment lowered plasma triacylglycerol and increased hepatic mitochondrial fatty acid oxidation and CPT-1 activity in both the presence and absence of malonyl-CoA (*41*). AA metabolism of eicosanoids, which are inflammatory intermediates, did not prevent ceramide-induced membrane potential loss and may also have resulted in membrane potential loss. Despite AA enhancing mitochondrial biogenesis, there was no effect on maintaining the membrane potential. EPA prevents platelet aggregation and inhibits the conversion of AA into thromboxane A2 and prostaglandins (*42*). These results suggest that EPA has greater potential to prevent ceramide-induced C6 glioma cell death. This study is the first report to demonstrate that EPA provides greater protective effects for cells damaged by ceramide.

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LITERATURE CITED

(1) Sasaki, S.; Iwata, M. Impairment of fast axonal transport in the proximal axons of anterior horn neurons in amyotrophic lateral sclerosis. *Neurology* **1996**, *47*, 535–540.
(2) Mandemakers, W.; Morais, V. A.; De Strooper, B. A cell biological perspective on mitochondrial dysfunction in Parkinson disease and other neurodegenerative diseases. *J. Cell. Sci.* **2007**, *120*, 1707–1716.
(3) Schon, E. A.; Manfredi, G. Neuronal degeneration and mitochondrial dysfunction. *J. Clin. Invest*. **2003**, *111*, 303–312.
(4) Sarsilmaz, M.; Songur, A.; Ozyurt, H.; Kuş, I.; Ozen, O. A.; Ozyurt, B.; Söğüt, S.; Akyol, O. Potential role of dietary omega-3 essential fatty acids on some oxidant/antioxidant parameters in rats' corpus striatum. *Prostaglandins, Leukotrienes Essent. Fatty Acids* **2003**, *69*, 253–259.
(5) Grant, W. B. Dietary links to Alzheimer's disease: 1999 update. *J. Alzheimer's Dis*. **1999**, *1*, 197–201.
(6) Yehuda, S.; Rabinovtz, S.; Carasso, R. L.; Mostofsky, D. I. Essential fatty acids preparation (SR-3) improves Alzheimer's patients quality of life. *Int. J. Neurosci.* **1996**, *87*, 141–149.
(7) Lauritzen, I.; Blondeau, N.; Heurteaux, C.; Widmann, C.; Romey, G.; Lazdunski, M. Polyunsaturated fatty acids are potent neuroprotectors. *EMBO J*. **2000**, *19*, 1784–1793.
(8) Blondeau, N.; Widmann, C.; Lazdunski, M.; Heurteaux, C. Polyunsaturated fatty acids induce ischemic and epileptic tolerance. *Neuroscience* **2002**, *109*, 231–241.
(9) Lonergan, P. E.; Martin, D. S.; Horrobin, D. F.; Lynch, M. A. Neuroprotective actions of eicosapentaenoic acid on lipopolysaccharide-induced dysfunction in rat hippocampus. *J. Neurochem*. **2004**, *91*, 20–29.
(10) Mori, T. A.; Beilin, L. J. Omega-3 fatty acids and inflammation. *Curr. Atheroscler. Rep*. **2004**, *6*, 461–467.
(11) Wang, X.; Zhao, X.; Mao, Z. Y.; Wang, X. M.; Liu, Z. L. Neuroprotective effect of docosahexaenoic acid on glutamate-induced cytotoxicity in rat hippocampal cultures. *Neuroreport* **2003**, *14*, 2457–2461.
(12) Taanman, J. W. The mitochondrial genome: structure, transcription, translation and replication. *Biochim. Biophys. Acta* **1999**, *1410*, 103–123.
(13) Chang, D. D.; Clayton, D. A. Precise identification of individual promoters for transcription of each strand of human mitochondrial DNA. *Cell* **1984**, *36*, 635–643.
(14) Fisher, R. P.; Clayton, D. A. Purification and characterization of human mitochondrial transcription factor 1. *Mol. Cell. Biol*. **1988**, *8*, 3496–3509.
(15) Montoya, J.; Perez-Martos, A.; Garstka, H. L.; Wiesner, R. J. Regulation of mitochondrial transcription by mitochondrial transcription factor A. *Mol. Cell. Biochem*. **1997**, *174*, 227–230.
(16) Larsson, N. G.; Barsh, G. S.; Clayton, D. A. Structure and chromosomal localization of the mouse mitochondrial transcription factor A gene (Tfam). *Mamm. Genome* **1997**, *8*, 139–140.
(17) Jeng, J. Y.; Yeh, T. S.; Lee, J. W.; Lin, S. H.; Fong, T. H.; Hsieh, R. H. Maintenance of mitochondrial DNA copy number and expression are essential for preservation of mitochondrial function and cell growth. *J. Cell. Biochem*. **2008**, *103*, 347–357.
(18) Rodgers, J. T.; Lerin, C.; Gerhart-Hines, Z.; Puigserver, P. Metabolic adaptations through the PGC-1 α and SIRT1 pathways. *FEBS Lett*. **2008**, *582*, 46–53.
(19) Cooper, M. P.; Qu, L.; Rohas, L. M.; Lin, J.; Yang, W.; Erdjument-Bromage, H.; Tempst, P.; Spiegelman, B. M. Defects in energy homeostasis in Leigh syndrome French Canadian variant through PGC-1α/LRP130 complex. *Genes Dev*. **2006**, *20*, 2996–3009.
(20) Lamantea, E.; Tiranti, V.; Bordoni, A.; Toscano, A.; Bono, F.; Servidei, S.; Apadimitriou, A.; Spelbrink, H.; Silvestri, L.; Casari, G.; Comi, G. P.; Zeviani, M. Mutations of mitochondrial DNA polymerase γA are a frequent cause of autosomal dominant or recessive progressive external ophthalmoplegia. *Ann. Neurol*. **2002**, *52*, 211–219.
(21) Mancuso, M.; Salviati, L.; Sacconi, S.; Otaegui, D.; Camaño, P.; Marina, A.; Bacman, S.; Moraes, C. T.; Carlo, J. R.; Garcia, M.; Garcia-Alvarez, M.; Monzon, L.; Naini, A. B.; Hirano, M.; Bonilla, E.; Taratuto, A. L.; DiMauro, S.; Vu, T. H. Mitochondrial DNA depletion: mutations in thymidine kinase gene with myopathy and SMA. *Neurology* **2002**, *59*, 1197–1202.
(22) Cutler, R. G.; Kelly, J.; Storie, K.; Pedersen, W. A.; Tammara, A.; Hatanpaa, K.; Troncoso, J. C.; Mattson, M. P. Involvement of oxidative stress-induced abnormalities in ceramide and cholesterol metabolism in brain aging and Alzheimer's disease. *Proc. Natl. Acad. Sci. U.S.A*. **2004**, *101*, 2070–2075.
(23) Lee, J. T.; Xu, J.; Lee, J. M.; Ku, G.; Han, X.; Yang, D. I.; Chen, S.; Hsu, C. Y. Amyloid-β peptide induces oligodendrocyte death by activating the neutral sphingomyelinase−ceramide pathway. *J. Cell Biol*. **2004**, *164*, 123–131.
(24) Villena, J.; Henriquez, M.; Torres, V.; Moraga, F.; Díaz-Elizondo, J.; Arredondo, C.; Chiong, M.; Olea-Azar, C.; Stutzin, A.; Lavandero, S.; Quest, A. F. Ceramide-induced formation of ROS and ATP depletion trigger necrosis in lymphoid cells. *Free Radical Biol. Med*. **2008**, *44*, 1146–1160.
(25) Páv, M.; Kovárů, H.; Fiserová, A.; Havrdová, E.; Lisá, V. Neurobiological aspects of depressive disorder and antidepressant treatment: role of glia. *Physiol. Res*. **2008**, *57*, 151–164.
(26) Benda, P.; Lightbody, J.; Sato, G.; Levine, L.; Sweet, W. Differentiated rat glial cell strain in tissue culture. *Science* **1968**, *161*, 370–371.

(27) Sullivan, N. R.; Burke, T.; Siafaka-Kapadai, A.; Javors, M.; Hensler, J. G. Effect of valproic acid on serotonin-2A receptor signaling in C6 glioma cells. *J. Neurochem.* **2004**, *90*, 1269–1275.
(28) Benítez, J. A.; Arregui, L.; Cabrera, G.; Segovia, J. Valproic acid induces polarization, neuronal-like differentiation of a subpopulation of C6 glioma cells and selectively regulates transgene expression. *Neuroscience* **2008**, *156*, 911–920.
(29) Parra, V.; Eisner, V.; Chiong, M.; Criollo, A.; Moraga, F.; Garcia, A.; Härtel, S.; Jaimovich, E.; Zorzano, A.; Hidalgo, C.; Lavandero, S. Changes in mitochondrial dynamics during ceramide-induced cardiomyocyte early apoptosis. *Cardiovasc. Res.* **2008**, *77*, 387–397.
(30) German, O. L.; Miranda, G. E.; Abrahan, C. E.; Rotstein, N. P. Ceramide is a mediator of apoptosis in retina photoreceptors. *Invest. Ophthalmol. Vis. Sci.* **2006**, *47*, 1658–1668.
(31) Stiban, J.; Caputo, L.; Colombini, M. Ceramide synthesis in the endoplasmic reticulum can permeabilize mitochondria to pro-apoptotic proteins. *J. Lipid Res.* **2008**, *49*, 625–634.
(32) Miñano, A.; Caballero-Benítez, A.; Lluch, M.; Morán, J.; Rodríguez-Alvarez, J. C2-ceramide mediates cerebellar granule cells apoptosis by activation of caspases-2, -9, and -3. *J. Neurosci. Res.* **2008**, *86*, 1734–1747.
(33) Stoica, B. A.; Movsesyan, V. A.; Lea, P. M.4th; Faden, A. I. Ceramide-induced neuronal apoptosis is associated with dephosphorylation of Akt, BAD, FKHR, GSK-3β, and induction of the mitochondrial-dependent intrinsic caspase pathway. *Mol. Cell. Neurosci.* **2003**, *22*, 365–382.
(34) Buratta, M; Castigli, E.; Sciaccaluga, M.; Pellegrino, R. M.; Spinozzi, F.; Roberti, R.; Corazzi, L. Loss of cardiolipin in palmitate-treated GL15 glioblastoma cells favors cytochrome *c* release from mitochondria leading to apoptosis. *J. Neurochem.* **2008**, *105*, 1019–1031.
(35) Koshkin, V.; Dai, F. F.; Robson-Doucette, C. A.; Chan, C. B.; Wheeler, M. B. Limited mitochondrial permeabilization is an early manifestation of palmitate-induced lipotoxicity in pancreatic β-cells. *J. Biol. Chem.* **2008**, *283*, 7936–7948.
(36) Kjaer, M. A.; Vegusdal, A.; Gjøen, T.; Rustan, A. C.; Todorcević, M.; Ruyter, B. Effect of rapeseed oil and dietary n-3 fatty acids on triacylglycerol synthesis and secretion in Atlantic salmon hepatocytes. *Biochim. Biophys. Acta* **2008**, *1781*, 112–122.
(37) Flachs, P.; Horakova, O.; Brauner, P.; Rossmeisl, M.; Pecina, P.; Franssen-van Hal, N.; Ruzickova, J.; Sponarova, J.; Drahota, Z.; Vlcek, C.; Keijer, J.; Houstek, J.; Kopecky, J. Polyunsaturated fatty acids of marine origin upregulate mitochondrial biogenesis and induce β-oxidation in white fat. *Diabetologia* **2005**, *48*, 2365–2375.
(38) Leonardi, F.; Attorri, L.; Di Benedetto, R.; Di Biase, A.; Sanchez, M.; Nardini, M.; Salvati, S. Effect of arachidonic, eicosapentaenoic and docosahexaenoic acids on the oxidative status of C6 glioma cells. *Free Radical Res.* **2005**, *39*, 865–874.
(39) Kou, W.; Luchtman, D.; Song, C. Eicosapentaenoic acid (EPA) increases cell viability and expression of neurotrophin receptors in retinoic acid and brain-derived neurotrophic factor differentiated SH-SY5Y cells. *Eur. J. Nutr.* **2008**, *47*, 104–113.
(40) Guo, W.; Xie, W.; Lei, T.; Hamilton, J. A. Eicosapentaenoic acid, but not oleic acid, stimulates β-oxidation in adipocytes. *Lipids* **2005**, *40*, 815–821.
(41) Madsen, L.; Rustan, A. C.; Vaagenes, H.; Berge, K.; Dyrøy, E.; Berge, R. K. Eicosapentaenoic and docosahexaenoic acid affect mitochondrial and peroxisomal fatty acid oxidation in relation to substrate preference. *Lipids* **1999**, *34*, 951–963.
(42) Bas, O.; Songur, A.; Sahin, O.; Mollaoglu, H.; Ozen, O. A.; Yaman, M.; Eser, O.; Fidan, H.; Yagmurca, M. The protective effect of fish n-3 fatty acids on cerebral ischemia in rat hippocampus. *Neurochem. Int.* **2007**, *50*, 548–554.

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