Cytokine-mediated Induction of Ceramide Production Is Redox-sensitive

IMPLICATIONS TO PROINFLAMMATORY CYTOKINE-MEDIATED APOPTOSIS IN DEMYELINATING DISEASES*

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The present study underlines the importance of reactive oxygen species in cytokine-mediated degradation of sphingomyelin (SM) to ceramide. Treatment of rat primary astrocytes with tumor necrosis factor-α (TNF-α) or interleukin-1β led to marked alteration in cellular redox (decrease in intracellular GSH) and rapid degradation of SM to ceramide. Interestingly, pretreatment of astrocytes with N-acetylcysteine (NAC), an antioxidant and efficient thiol source for glutathione, prevented cytokine-induced decrease in GSH and degradation of sphingomyelin to ceramide, whereas treatment of astrocytes with diamide, a thiol-depleting agent, alone caused degradation of SM to ceramide. Moreover, potent activation of SM hydrolysis and ceramide generation were observed by direct addition of an oxidant like hydrogen peroxide or a prooxidant like aminotriazole. Similar to NAC, pyrrolidinedithiocarbamate, another antioxidant, was also found to be a potent inhibitor of cytokine-induced degradation of SM to ceramide indicating that cytokine-induced hydrolysis of sphingomyelin is redox-sensitive. Besides astrocytes, NAC also blocked cytokine-mediated ceramide production in rat primary oligodendrocytes, microglia, and C6 gliial cells. Inhibition of TNF-α- and diamide-mediated depletion of GSH, elevation of ceramide level, and DNA fragmentation (apoptosis) in primary oligodendrocytes by NAC, and observed depletion of GSH, elevation of ceramide level, and apoptosis in banked human brains from patients with neuroinflammatory diseases (e.g. X-adrenoleukodystrophy and multiple sclerosis) suggest that the intracellular level of GSH may play a critical role in the regulation of cytokine-induced generation of ceramide leading to apoptosis of brain cells in these diseases.

Sphingomyelin is preferentially concentrated in the outer leaflet of the plasma membrane of most mammalian cells; it comprises sphingosine (a long chain sphingoid base backbone), a fatty acid, and a phosphocholine head group. Ceramide is composed of a sphingoid base with a fatty acid in amide linkage. Sphingomyelin was initially considered only a structural component of plasma membrane; however, several investigations established the involvement of sphingolipids and their metabolites in the key events of signal transduction associated with cell regulation, cell differentiation, and apoptosis (1–3). The sphingomyelin pathway-associated signal transduction pathway mediates the action of several extracellular stimuli that lead to important biochemical and cellular effects (4–8). This pathway is initiated by the activation of two distinct forms of sphingomyelinase (SMase),1 a membrane-associated neutral sphingomyelinase (9) and an acidic sphingomyelinase (10), which reside in the caveola and the endosomal-lysosomal compartment. Each type of SMase hydrolyzes the phosphodiester bond of sphingomyelin to yield ceramide and phosphocholine. Proinflammatory cytokines (tumor necrosis factor-α, TNF-α; interleukin-1β, IL-1β; interferon-γ, IFN-γ) and bacterial lipopolysaccharides have been shown as potent inducers of SMases. One of the products, ceramide, has emerged as a second messenger molecule that is considered to mimic most of the cellular effects of cytokines and lipopolysaccharides in terminal differentiation, apoptosis, and cell cycle arrest (4, 5).

Sphingomyelin turnover and ceramide generation in response to TNF-α and IL-1β occurs within minutes of stimulation; however, the sequence of events linking receptor stimulation and SMase activation remains largely unknown (7, 8, 11). In a number of cell systems, interaction of TNF-α with its membrane receptors (p75 and p55) has been found to activate phospholipase A2 and to induce release of arachidonic acid from phosphatidylcholine and phosphatidylethanolamine pools. This arachidonic acid has been shown as a mediator of sphingomyelin hydrolysis in response to TNF-α (11). In addition, proteases have also been implicated in the pathway leading from TNF-α to the activation of SMase (7, 12) recently. On the other hand, IL-1β and TNF-α are known to induce the production of reactive oxygen species (ROS), a class of highly diffusible and ubiquitous molecules, which have been suggested to act as second messengers (13–15). ROS encompassing species such as superoxide, hydrogen peroxide, and hydroxyl radicals are known to regulate critical steps in the signal transduction cascade and many important cellular events including protein phosphorylation, gene expression, transcription factor activation, DNA synthesis, and cellular proliferation (16, 17). A recent observation has shown that glutathione or similar molecules inhibit the activity of magnesium-dependent neutral SMase in vitro (18). However, surprisingly, the SH group of GSH was not required as S-methyl GSH and GSSG inhibited neutral SMase at lower concentrations than GSH (18). On the other hand, N-acetylcysteine has also been found to inhibit the

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1 The abbreviations used are: SMase, sphingomyelinase; SM, sphingomyelin; ROS, reactive oxygen species; TNF-α, tumor necrosis factor-α; IL-1β, interleukin-1β; IFN-γ, interferon-γ; NAC, N-acetylcysteine; POTC, pyrrolidinedithiocarbamate; DMEM, Dulbecco’s modified Eagle’s medium; FBS, fetal bovine serum; HBSS, Hank’s buffered saline solution; X-ALD, X-adrenoleukodystrophy; MS, multiple sclerosis; DAG, diacylglycerol; ATZ, aminotriazole.
synthesis of ceramide in cultured rat hepatocytes through the inhibition of dihydrolipoceramide desaturase (19). In the present study, we examined the possible involvement of ROS in cytokine-mediated activation of sphingomyelin breakdown and ceramide formation in rat primary glial cells. We show that intracellular GSH plays a crucial role in the breakdown of SM to ceramide, in that low GSH levels are required for ceramide generation and high GSH levels inhibit production of ceramide. Inhibition of cytokine-mediated breakdown of SM to ceramide by antioxidants like N-acetylcysteine (NAC) and pyrroldinedithiocarbamate (PDTC) and induction of ceramide production by oxidants or pro-oxidants like hydrogen peroxide, aminotriazole, diamide, and 1-buthione-(SR)-sulfoximine clearly delineate a novel function of ROS and GSH in regulation of the first step of sphingomyelin signal transduction pathway. Moreover, decreased levels of GSH and increased levels of ceramide correlate with the DNA fragmentation in rat primary oligodendrocytes as well as in the banked human brains from patients with neuroinflammatory diseases (e.g. multiple sclerosis and X-adrenoleukodystrophy).

MATERIALS AND METHODS
Reagents—DMEM/F-12 and fetal bovine serum (FBS) were from Life Technologies, Inc. Human IL-1β was from Genzyme. Mouse recombinant TNF-α was obtained from Boehringer Mannheim, Germany. Diamide, buthione-(SR)-sulfoximine, N-acetylcysteine, and pyrroldinedithiocarbamate were from Sigma.

Isolation and Maintenance of Rat Primary Microglia, Oligodendrocytes, and Astrocytes—Microglial cells were isolated from mixed glial cultures according to the procedure of Guilian and Baker (20). Briefly, after 7 days the mixed glial cultures were washed 3 times with DMEM/F-12 containing 10% FBS and subjected to a shake at 240 rpm for 4 h at 37°C on a rotary shaker. The floating cells were washed and seeded onto plastic tissue culture flasks and incubated at 37°C. After 30 min the non-attached cells (mostly oligodendrocytes) were removed by repeated washes, and the attached cells were used as microglia. These cells were seeded onto new plates for further studies. Ninety to ninety-five percent of this preparation was positive for nonspecific esterase, a marker for macrophages and microglia.

After 4 h shaking, the flasks were washed three times to remove the floating cells. Medium with 10% FBS was added, and flasks were subjected to another cycle of shaking for 24 h at 250 rpm. The suspended cells were spun at 200 g and incubated for 30 min in tissue culture dish. The non-attached or weakly attached cells (mostly oligodendrocytes) were removed by repeated washes, and the attached cells were used as microglia. These cells were seeded onto new plates for further studies. Ninety to ninety-five percent of this preparation was positive for galactocerebroside immunostaining.

Astrocytes were prepared from rat cerebral tissue as described by McCarthy and DeVellis (21). After 10 days of culture astrocytes were separated from microglia and oligodendrocytes by shaking for 24 h in an orbital shaker at 240 rpm. To ensure the complete removal of all oligodendrocytes and microglia, the shaking was repeated twice after a gap of 1 or 2 days. Attached cells were trypsinized (1 mM EDTA and 0.05% trypsin in 10 mM Tris-buffered saline, pH 7.4) and distributed into culture dishes. These cells were selected for the astrocyte marker glial fibrillary acidic protein to be found to be 95–100% positive. Glial cells obtained from ATCC were also maintained in DMEM/F-12 containing 10% FBS as indicated above.

Brain Tissue—Frozen and fixed X-adrenoleukodystrophy and multiple sclerosis brain tissue were obtained from Brain and Tissue Banks for Developmental Disorders, University of Maryland, Baltimore, MD 21201. Two X-ALD brains were from 7- and 9-year-old males, and two MS brains were from 30- and 33-year-old females. Control brain for MS studies was from an 8-year-old male (Control 1), and control brain for MS studies was from a 30-year-old female (Control 2).

Lipid Extraction—Approximately 106 cells were exposed to different cytokines in the presence or absence of antioxidants for different periods, and lipids were extracted according to the methods described by Welsh (22).

Quantification of Sphingomyelin by High Performance TLC and Denaturation—Sphingomyelin was separated from total lipid extracts by high performance TLC (LHPK plates from Whatman) as described by Ganser et al. (23) for phospholipids with the following modification: the plate was overrun for 30 min during its development and was dried overnight in vacuum desiccator. Sphingomyelin was quantitated by densitometric scanning using Imaging Densitometer (model GS-670; Bio-Rad), and software was provided with the instrument by the manufacturer.

Quantification of Ceramide Levels by Diacylglycerol Kinase Assay—Ceramide content was quantified essentially according to Priess et al. (24) using diacylglycerol (DAG) kinase and [32P]ATP. Briefly, dried lipids were solubilized in 20 μl of an octyl β-D-glucoside/cardiolipin solution (7.5% octyl β-D-glucoside, 5 mM cardiolipin in 1 mM DTPA) by sonication in a sonicator bath. The reaction was then carried out in a final volume of 100 μl containing the 20 μl solution sample, 50 mM imidazole HCl, pH 6.6, 50 mM NaCl, 12.5 mM MgCl2, 1 mM EGTA, 2 mM dithiothreitol, 6.6 μg of DAG kinase, and 1 μl [γ-32P]ATP (specific activity of 1.5 × 106 cpm/nmol) for 30 min at room temperature. The labeled ceramide-1-phosphate was resolved with a solvent system consisting of methyl acetate-propyl alcohol:chloroform:methanol, 0.25% KCl in water:acetic acid (100:100:40:36:2). A standard sample of ceramide was phosphorylated under identical conditions and developed in parallel. Both standard and samples had identical Rf values (0.46). Quantification of ceramide-1-phosphate was carried out by autoradiography and densitometric scanning using Imaging Densitometer (model GS-670; Bio-Rad). Values are expressed either as arbitrary units (absorbance) or as percent change.

Measurement of GSH (Reduced Glutathione) and GSSG (Oxidized Glutathione)—Concentration of intracellular reduced GSH was measured using a colorimetric assay kit for GSH from R & D Systems. Briefly, 2 × 106 cells were homogenized in 500 μl of ice-cold 5% metaphosphoric acid and centrifuged at 3000 g for 10 min. Supernatants were used to assay GSH using 4-chloro-1-methyl-7-trifluoromethyl-quinolinium methylsulfate and 50 mM NaOH at 400 nm. Concentration of GSSG was determined according to the method of Griffith (25) after derivatization with 2-vinylpyridine for 30 min at room temperature.

Detection of DNA Fragmentation—Cells (1 × 106) were pelleted in an Eppendorf tube by centrifugation at 1000 rpm for 5 min, washed with phosphate-buffered saline, pH 7.4, resuspended gently in 50 μl of a lysis buffer (200 mM NaCl, 10 mM Tris-HCl, pH 8.0, 40 mM EDTA, pH 8.0, 0.5% SDS, 125 ng of proteinase K/μl). The lysate received 200 μl of the digestion buffer (200 mM NaCl, 10 mM Tris-HCl, pH 8.0, 0.5% SDS, 125 ng of proteinase K/μl). The contents were mixed by inversion several times and then incubated at 50°C for 2 h. An equal volume of a mixture of phenol, pH 8.0, chloroform, and isomyl alcohol (25:24:1, v/v) was added, gently mixed for 10 min, and stored at room temperature for 2 min. The two phases were separated by centrifugation at 10,000 rpm for 10 min. The viscous aqueous phase was transferred to a fresh tube, and the phenol/chloroform extraction was repeated. The aqueous phase was extracted with an equal volume of chloroform, and 1.0 mM MgCl2 was added to the aqueous phase to a final concentration of 10 mM. The total DNA was precipitated by the addition of 2 volumes of absolute ethanol with several inversions. DNA was pelleted by centrifugation at 3000 rpm for 15 min, washed with 70% ethanol, and air-dried. The pellet was dissolved in 25 μl of 10 mM Tris-HCl containing 1.0 mM EDTA, pH 8.0, and electrophoresed in 1.8% agarose gel at 4°C. The gel was stained with ethidium bromide, and DNA-intercalated ethidium fluorescence was photographed on Polaroid film 665 (P/N) using an orange filter. To study DNA fragmentation, sections were lightly counterstained with methyl green.

Fragment End Labeling of DNA on Paraffin-embedded Tissue Sections—Sections of MS and X-ALD Brains—Fragmented DNA was detected in situ by the terminal deoxynucleotidyltransferase-mediated binding of 3′-OH ends of DNA fragments generated in response to apoptotic signals, using a commercially available kit (TdT FragEL™) from Calbiochem. Briefly, paraffin-embedded tissue slides were deparaffinized, rehydrated in graded ethanol, treated with 20 μg/ml proteinase K for 15 min at room temperature, and washed prior to terminal deoxynucleotidyltransferase staining. After terminal deoxynucleotidyltransferase staining, sections were lightly counterstained with methyl green.

RESULTS

NAC and PDTC Block TNF-α- and IL-1β-induced Degradation of Sphingomyelin to Ceramide in Primary Rat Astrocytes—Rat primary astrocytes were cultured in serum-free media with TNF-α or IL-1β for different times to quantify the production of ceramide using diacylglycerol (DAG) kinase. Since DAG kinase...
phosphorylates both DAG and ceramide using \( [\gamma^{32}\text{P}]{\text{ATP}} \) as substrate, both lipids can be quantified in the same assay. It was found that in astrocytes, the DAG content was much higher than the ceramide content (Fig. 1). Stimulation of cells with TNF-\( \alpha \) resulted in a time-dependent increase in the production of ceramide (about 3-fold after 45 min). In contrast to induction of ceramide production, the level of DAG, an activator of protein kinase C and acidic sphingomyelinase, was unchanged at different time points of stimulation (Fig. 1). Similar to TNF-\( \alpha \) (Figs. 1 and 2), stimulation of astrocytes with IL-1\( \beta \) for different times also induced a significant increase in the ceramide content (Fig. 3). Almost 3–4-fold increase in ceramide production was found in astrocytes after 30 or 45 min of exposure with TNF-\( \alpha \) or IL-1\( \beta \). This increase in ceramide was paralleled by TNF-\( \alpha \)- and IL-1\( \beta \)-induced decrease in sphingomyelin (Figs. 2 and 3). Sphingomyelin turnover of approximately 18–25% could be observed as early as 15 min following treatment of astrocytes (Figs. 2 and 3), and maximal effects of up to 45–50% SM hydrolysis were observed after 30–45 min of treatment with TNF-\( \alpha \) or IL-1\( \beta \). These experiments suggest that both TNF-\( \alpha \) and IL-1\( \beta \) can modulate the degradation of sphingomyelin to produce ceramide, the putative second messenger of the sphingomyelin signal transduction pathway, in rat primary astrocytes within a short time. Interestingly, we found that treatment of astrocytes with antioxidants like NAC (10 mM) 1 h before the addition of TNF-\( \alpha \) or IL-1\( \beta \) potentially blocked the decrease in sphingomyelin as well as the increase in ceramide (Figs. 2 and 3), whereas 10 mM acetate had no effect on the degradation of SM to ceramide (data not shown). Similar to NAC, another antioxidant PDTC also inhibited cytokine-mediated degradation of SM to ceramide (Figs. 2 and 3). These experiments suggest that reactive oxygen species (ROS) are possibly involved in cytokine-induced degradation of SM to ceramide.

**TNF-\( \alpha \) and IL-1\( \beta \) Decrease Intracellular Level of Reduced Glutathione (GSH) in Rat Primary Astrocytes and NAC Blocks This Decrease**—Since the intracellular level of GSH is an important regulator of the redox state of a cell, to understand the relationship between induction of ceramide production and intracellular level of GSH in cytokine-stimulated astrocytes, cells were stimulated with TNF-\( \alpha \) or IL-1\( \beta \), and the level of GSH was measured at different times. Fig. 4 shows that stimulation of cells with TNF-\( \alpha \) or IL-1\( \beta \) resulted in an immediate decrease in intracellular level of GSH with the maximal decrease (66–70% of control) found within 15–30 min of initiation of stimulation, and with a further increase in time of incubation, the level of GSH was found to be almost normal (88–95% of control at 90 min). These experiments suggest that cytokine stimulation apparently induces rapid, short term production of oxidants which transiently deplete GSH. However, the lack of decrease of GSH (Fig. 4) and lack of hydrolysis of SM (Fig. 2 and 3) in the presence of NAC in the cytokine-treated cells suggest that NAC inhibited the cytokine-induced degradation of SM to ceramide by maintaining the normal levels of GSH (Fig. 4).

**Thiol-depleting Agents Induce the Production of Ceramide in Rat Primary Astrocytes**—Since NAC, a thiol antioxidant, blocked cytokine-mediated depletion of intracellular levels of GSH and breakdown of SM to ceramide, we investigated the effect of thiol-depleting agents (diamide and buthione-(SR)-sulfoximine) on ceramide production. Diamide reduces the intracellular level of GSH by its oxidation to GSSG, whereas buthione-(SR)-sulfoximine does so by blocking the synthesis of GSH (27, 28). Stimulating rat primary astrocytes with diamide resulted in an immediate decrease in intracellular level of GSH due to rapid consumption of intracellular GSH through its nonenzymatic conversion to the oxidized dimer, GSSG (27), and marked induction of ceramide production (about 7-fold after 30 min of stimulation) (Fig. 5) suggesting that intracellular level of GSH is the important regulator of degradation of SM to ceramide. Consistent with this hypothesis, treatment of cells with NAC blocked diamide-mediated decrease in GSH level and induction of ceramide production (Fig. 5). Similar to diamide, buthione-(SR)-sulfoximine also decreased the level of GSH and induced the production of ceramide (data not shown). In light of the recent report that GSH and similar molecules inhibit the activity of neutral SMase *in vitro* and GSSG has higher inhibitory effect than GSH (18), we investigated the
intracellular level of GSSG in astrocytes treated with TNF-\(\alpha\) and diamide. In contrast to the decrease in intracellular level of GSH (Figs. 4 and 5), both TNF-\(\alpha\) and diamide increased the intracellular level of GSSG (Fig. 6). Thus it appears that the low GSH and/or high intracellular oxidant (ROS) levels induced by cytokines and thiol-depleting agents facilitated the induction of ceramide production, whereas the normal levels of GSH and/or low ROS induced or maintained by the addition of NAC under these conditions blocked the hydrolysis of sphingomyelin to ceramide. Taken together, these results demonstrate that the intracellular levels of GSH and/or ROS regulate the extent to which sphingomyelin is degraded to ceramide, and ceramide-mediated signaling cascades are transduced.
Aminotriazole and Hydrogen Peroxide Induce the Production of Ceramide in Rat Primary Astrocytes—
Inhibition of cytokine-mediated induction of ceramide production by antioxidants and induction of ceramide production by thiol-depleting agents alone suggest the possible involvement of ROS in the induction of ceramide production. Therefore, we examined the effect of exogenous addition of an oxidant like H$_2$O$_2$ or endogenously produced H$_2$O$_2$ by inhibition of catalase with aminotriazole (ATZ), which inhibits endogenous catalase to increase the level of H$_2$O$_2$, on the induction of ceramide production. Fig. 7 depicts the time course of ceramide production in rat primary astrocytes following the addition of ATZ. Approximately 45 min following the addition of ATZ, ceramide generation increased more than 5-fold over baseline (Fig. 7). However, pretreatment of cells with NAC blocked the ATZ-mediated increase in ceramide production. Consistent with the increase in ceramide production by ATZ, addition of exogenous H$_2$O$_2$ to astrocytes also induced the production of ceramide with the maximum increase of about 7-fold after 15 min. These results clearly indicate that intracellular levels of ROS regulate the production of ceramide.

**FIG. 7.** Effect of aminotriazole and H$_2$O$_2$ on the induction of ceramide production in rat primary astrocytes. Cells were incubated with 5 mM aminotriazole (ATZ) (A) or 0.5 mM H$_2$O$_2$ (B) in presence or absence of 10 mM NAC. At different time intervals, cells were washed with HBSS and scraped off. Lipids were extracted, and the level of ceramide (100% value is 4.51 ± 0.1 nmol/mg protein) was measured as described under "Materials and Methods." Results are mean ± S.D. of three different experiments.

**FIG. 8.** NAC inhibits TNF-α-mediated ceramide production in rat primary microglia (A), oligodendrocytes (B), and C$_6$ glial cells (C). Cells preincubated with 10 mM NAC for 1 h in serum-free DMEM/F-12 received TNF-α (50 ng/ml). Cells were washed with HBSS and scraped off at different intervals. Lipids were extracted, and ceramide content (100% value for microglia, oligodendrocytes, and C$_6$ glial cells are 2.72 ± 0.53, 3.37 ± 0.32, 4.73 ± 0.21 nmol/mg protein, respectively) was measured as described under "Materials and Methods." Results are mean ± S.D. of three different experiments.

Aminotriazole and Hydrogen Peroxide Induce the Production of Ceramide in Rat Primary Astrocytes—Inhibition of cytokine-mediated induction of ceramide production by antioxidants and induction of ceramide production by thiol-depleting agents alone suggest the possible involvement of ROS in the induction of ceramide production. Therefore, we examined the effect of exogenous addition of an oxidant like H$_2$O$_2$ or endogenously produced H$_2$O$_2$ by inhibition of catalase with aminotriazole (ATZ), which inhibits endogenous catalase to increase the level of H$_2$O$_2$, on the induction of ceramide production. Fig. 7 depicts the time course of ceramide production in rat primary astrocytes following the addition of ATZ. Approximately 45 min following the addition of ATZ, ceramide generation increased more than 5-fold over base line (Fig. 7). However, pretreatment of cells with NAC blocked the ATZ-mediated increase in ceramide production. Consistent with the increase in ceramide production by ATZ, addition of exogenous H$_2$O$_2$ to astrocytes also induced the production of ceramide with the maximum increase of about 7-fold after 15 min. These results clearly indicate that intracellular levels of ROS regulate the production of ceramide.

Inhibition of Cytokine-mediated Production of Ceramide in Rat Primary Microglia, Oligodendrocytes, and C$_6$ Glial Cells by NAC—Since NAC inhibited the cytokine-mediated production of ceramide in rat primary astrocytes, we examined the effect of NAC on cytokine-mediated induction of ceramide production in rat primary oligodendrocytes, microglia, and C$_6$ glial cells. Fig. 8 shows that addition of TNF-α to microglia (A), oligodendrocytes (B), or C$_6$ glial cells (C) induced the production of ceramide. The increase in ceramide in these cells ranges from 2.5- to 4-fold with highest increase in glial cells and lowest in oligodendrocytes. The ceramide levels peaked in glial cells at 30 min following stimulation and 45 min of stimulation in oligodendrocytes and C$_6$ glial cells. These observations show that similar to astrocytes, the SM cycle is also present in microglia, oligodendrocytes, and C$_6$ glial cells. Consistent with the effect of NAC on the production of ceramides in astrocytes, this antioxidant also potently blocked the TNF-α-induced production of ceramide in microglia, oligodendrocytes, and C$_6$ glial cells indicating that ROS are also involved in cytokine-mediated ceramide production in these cells (Fig. 8).

NAC Inhibits TNF-α- and Diamide-mediated Apoptosis in Rat Primary Oligodendrocytes by Increasing the Intracellular Level of GSH and Decreasing the Production of Ceramide—Since cytokine-mediated ceramide production is implicated in apoptosis of different cells including brain cells (29, 30), we investigated the effect of NAC on TNF-α as well as diamide-mediated apoptosis in rat primary oligodendrocytes as evidenced by electrophoretical detection of hydrolyzed DNA fragments (“laddering”). To understand the role of the intracellular level of GSH in inducing apoptosis, we treated oligodendrocytes with TNF-α or with diamide, a thiol-depleting agent. Both TNF-α and diamide decreased the intracellular level of GSH, increased the level of ceramide, and induced internucleosomal fragments.
DNA fragmentation as evident from the typical ladder pattern (Fig. 9). Interestingly, blocking of the diamide- and TNF-α-mediated decrease in intracellular levels of GSH by pretreatment with NAC inhibited the induction of ceramide formation and DNA fragmentation suggesting that intracellular levels of GSH may regulate apoptosis in oligodendrocytes through ceramide formation. To prove this hypothesis further, oligodendrocytes were treated with C2-ceramide (a cell-permeable ceramide analog) in the presence or absence of NAC. In contrast to the inhibitory effect of NAC on TNF-α-mediated apoptosis, NAC had no effect on C2-ceramide-mediated apoptosis in oligodendrocytes (Fig. 10).

FIG. 9. NAC blocks diamide- and TNF-α-mediated DNA fragmentation in rat primary oligodendrocytes by modulating the levels of GSH and ceramide. Cells preincubated with 10 mM NAC for 1 h received either diamide (0.5 mM) or TNF-α (50 ng/ml). After 12 h of incubation, cells were harvested and washed with phosphate-buffered saline, and genomic DNA was extracted and run on agarose gels (A) as mentioned under “Materials and Methods.” Ten micrograms of DNA was loaded in each lane. This is the representative of three different experiments. Levels of ceramide (B) (100% value is 3.37 ± 0.32 nmol/mg protein) and GSH (C) were measured in homogenates as mentioned under “Materials and Methods.” Results are mean ± S.D. of three different experiments.

FIG. 10. Effect of NAC on C2-ceramide-mediated DNA fragmentation in rat primary oligodendrocytes. Cells preincubated with 10 mM NAC for 1 h received C2-ceramide. After 12 h of incubation, cells were harvested and washed with phosphate-buffered saline, and genomic DNA was extracted and run on agarose gels as mentioned under “Materials and Methods.” Ten micrograms of DNA was loaded in each lane. This is the representative of three different experiments.

DNA Fragmentation in Banked Human Brains with X-ALD and MS—In the central nervous system, apoptosis may play an important pathogenetic role in neurodegenerative diseases such as ischemic injury and white matter diseases (31, 32). Both X-ALD and MS are demyelinating diseases with the involvement of proinflammatory cytokines in the manifestation of white matter inflammation. Several studies demonstrating the induction of proinflammatory cytokines at the protein or mRNA level in cerebrospinal fluid and brain tissue of MS patients have established an association of proinflammatory cytokines (TNF-α, IL-1β, IL-2, IL-6, and IFN-γ) with the inflammatory loci in MS (33–35). Recent documentation of the presence of TNF-α, IL-1β, and IFN-γ in X-ALD brain has revealed the neuroinflammatory character of this disease (36, 37). Therefore, to understand the underlying relationship among intracellular levels of GSH, levels of ceramide, and DNA fragmentation in cytokine-inflamed central nervous system of X-ALD and MS, we measured the levels of GSH and ceramide in homogenates and also studied the DNA fragmentation in nuclei from brains of patients with X-ALD and MS. Regions surrounding the plaques in white matter were used for these studies. In contrast to white matters of control brains, white matters of both X-ALD and MS brains had several plaque regions. Control 1 is the age- and sex-matched control for X-ALD and Control 2 is the age- and sex-matched control for MS (Fig. 11). In both X-ALD and MS brain homogenates, the level of GSH was lower (55–70% of control), and the level of ceramide was higher (2–3-fold) compared with those found in control brains (Fig. 11). Consistent with a lower level of GSH and a higher level of ceramide, genomic DNA isolated from nuclei of X-ALD and MS brains when run on agarose gels formed the typical ladder pattern, an indicator of apoptosis, which was absent in both of the normal brains (Fig. 11). To confirm apoptosis in regions surrounding the plaques of white matters of X-ALD and MS, we observed increased DNA fragmentation in isolated nuclei of X-ALD and MS brains when run on agarose gels compared with those of controls (Fig. 12). These biochemical and morphological observations indicate that intracellular level of GSH may be an important factor in cytokine-mediated degradation of SM to ceramide and apoptosis in inflammatory demyelinating diseases like X-ALD and MS.
DISCUSSION

Changes in the cellular redox state toward either prooxidant or antioxidant conditions have profound effects on cellular functions. Several lines of evidence presented in this work suggest that the first step of cytokine-induced sphingomyelin signal transduction pathway (i.e. breakdown of sphingomyelin to ceramide and phosphocholine) is redox-sensitive. First, cytokines like TNF-α and IL-1β decreased intracellular GSH and induced the degradation of sphingomyelin to ceramide in rat primary astrocytes, oligodendrocytes, microglia, and rat C6 glial cells, and pretreatment of the cells with antioxidants like NAC restored the levels of GSH and blocked the degradation of sphingomyelin to ceramide. Second, depletion of endogenous glutathione by diamide or buthione sulfoximine alone induces the degradation of sphingomyelin to ceramide which is blocked by NAC. Third, the increase in intracellular H₂O₂ by the addition of exogenous H₂O₂ or by the inhibition of endogenous catalase by aminotriazole induced the degradation of sphingomyelin to ceramide which is also blocked by NAC. Fourth, besides NAC, PDTC, an antioxidant but not the precursor of GSH (38), also inhibited the TNF-α- and IL-1β-induced hydrolysis of sphingomyelin to ceramide.

The signaling events in cytokine-mediated activation of sphingomyelin degradation to ceramide are poorly understood. Since the discovery of the sphingomyelin cycle, several inducers have been shown to be coupled to sphingomyelin-ceramide signaling events, including 1α,25-dihydroxyvitamin D₃, radiation, antibody cross-linking, TNF-α, IFN-γ, IL-1β, nerve growth factor, and brefeldin A (1–5, 39). In the case of TNF-α, the pathway is initiated by the action of TNF-α on its 55-kDa

FIG. 11. Levels of GSH and ceramide and DNA fragmentation in human brains with X-ALD and MS. Regions surrounding plaques of brain white matter were used for DNA laddering and to measure the levels of ceramide and GSH. Control 1 and control 2 are age- and sex-matched controls for X-ALD and MS, respectively. Since there was no plaque in control brains, we used white matter of control brain for this study. A, genomic DNA isolated from nuclei of banked human brains was run on agarose gel and photographed as mentioned under “Materials and Methods.” Ten micrograms of DNA was loaded in each lane. This is the representative of three different experiments. B, same amount of brain material (based on protein concentration) was used to measure the level of ceramide as mentioned under “Materials and Methods.” Results are mean ± S.D. of three different experiments. Concentrations of ceramide in control-1 and control-2 were 46.6 ± 2.56 and 61.6 ± 6.69 nmol/mg protein, respectively. C, concentration of GSH was measured in homogenates as mentioned under “Materials and Methods.” Results are mean ± S.D. of three different experiments.

FIG. 12. Fragment end labeling of DNA on tissue sections of X-ALD and MS brains. Terminal deoxynucleotidyltransferase-mediated end labeling of 3’-OH ends of DNA fragments on paraffin-embedded tissue sections (A, control; B, X-ALD; C, MS) was carried out using a commercially available kit from Calbiochem. Arrows indicate apoptotic bodies. Regions surrounding plaques were used for this study.
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