Cytokines, including tumor necrosis factor-α (TNF-α), may elicit cytotoxic response through the sphingomyelin-ceramide signal transduction pathway by activation of sphingomyelinases and the subsequent release of ceramide: the universal lipid second messenger. Treatment of bovine cerebral endothelial cells (BCECs) with TNF-α for 16 h followed by cycloheximide (CHX) for 6 h resulted in an increase in ceramide accumulation, DNA fragmentation, and cell death. Application of a cell permeable ceramide analogue C2 ceramide, but not the biologically inactive C3 dihydroceramide, also induced DNA laddering and BCEC death in a concentration- and time-dependent manner. TNF-α/CHX-mediated ceramide production apparently is not a result of sphingomyelin hydrolysis because sphingomyelin content does not decrease in this death paradigm. In addition, an acidic sphingomyelinase inhibitor, desipramine, had no effect on TNF-α/CHX-induced cell death. However, addition of fumonisin B1, a selective ceramide synthase inhibitor, attenuated TNF-α/CHX-induced intracellular ceramide elevation and BCEC death. Together, these findings suggest that ceramide plays at least a partial role in this paradigm of BCEC death. Our results show, for the first time, that ceramide derived from de novo synthesis is an alternative mechanism to sphingomyelin hydrolysis in the BCEC death process initiated by TNF-α/CHX.

Cytokines are key mediators in inflammatory and immune disorders of the central nervous system injury (1–5). Among these, tumor necrosis factor-α (TNF-α) has been shown to play a pivotal role in the inflammatory responses following secondary brain injury after ischemic and traumatic insults to the brain (6–10). A major target of TNF-α action is cerebral endothelial cells, which, once activated by TNF-α, can trigger a series of inflammatory reactions ultimately leading to neuronal cell death (11). Accordingly, exploration of the mechanisms by which TNF-α exerts neurodegenerative effects through cerebral endothelial cells has assumed increasing importance in the attempt to attenuate secondary brain injury.

Binding of TNF-α to the TNF-α receptors on endothelial cells could initiate proliferative, differentiative, or apoptotic signaling pathway depending on the experimental conditions. The signaling cascades that couple TNF-α receptor activation to specific biological responses, particularly apoptosis, have been extensively studied and molecularly dissected in transformed cells (12, 13). However, most untransformed mammalian cells are inherently resistant to the cytotoxic effects of TNF-α. A number of compelling results now reveal that this resistance can be reversed by the presence of a protein or RNA synthesis inhibitor (14, 15), providing an important paradigm in normal cells for investigating TNF-α-mediated cell death mechanism. The death induced by TNF-α in the presence of cycloheximide (CHX), a protein synthesis inhibitor, showed typical morphological and biochemical features of apoptosis and is blocked by Bcl-2 and CrmA expression (16, 17). The sensitizing effect of CHX is believed to result from the inhibition of synthesis of certain general survival factor(s) and, thus, potentiates the killing activity of TNF-α (17, 18).

Ceramide, a lipid second messenger, has recently emerged as an important mediator in transmitting the death signal following TNF-α receptor activation (19). Ligation of TNF-α and TNF-α receptor activates a neutral membrane-associated sphingomyelinase as well as an acidic lysosome-localized sphingomyelinase, both liberate ceramide from sphingomyelin (20). Interestingly, only the activation of acidic sphingomyelinsase was found to be death domain-dependent (21). It is believed that ceramide, which mediates TNF-α cytotoxic effects, is resulted from sphingomyelin hydrolysis by the acidic form of sphingomyelinase. Although ceramide generated in response to extracellular signals appears to be derived from the hydrolysis of sphingomyelin in most cases, it may also be produced from de novo synthesis via ceramide synthase (19). For instance, retinoic acid (22) and daunorubicin have been shown to stimulate de novo ceramide synthesis (23).

Cultured bovine cerebral endothelial cells (BCECs) provide an excellent model system for understanding TNF-α actions on the secondary injury processes. We report here that sequential TNF-α and CHX treatment induces a progressive elevation of intracellular ceramide level, which is followed by DNA fragmentation and cell death in BCECs. More importantly, ceramide in this BCEC death paradigm is derived from de novo synthesis, but not the conventional pathway, namely the hydrolysis of sphingomyelin. Our findings provide evidence for a crucial role of ceramide in the TNF-α/CHX-induced apoptotic process and indicate that de novo ceramide synthesis is a unique signaling mechanism in this cell death paradigm in BCECs involving TNF-α.
EXPERIMENTAL PROCEDURES

Materials—All chemicals and reagents were purchased from Sigma, and all cell culture supplies were from Life Technologies, Inc. unless specified.

Bovine Cerebral Endothelial Cell Culture—Bovine cerebral endothelial cells (BCECs) were prepared and characterized as described previously (24, 25). Briefly, bovine brains from freshly slaughtered adult animals were immediately placed in ice-cold Hank's balanced salt solution containing appropriate antibiotics. Meninges and superficial blood vessels were removed, homogenized, and filtered, and the resulting microvessel fraction was then sequentially digested with collagenase B (4 mg/ml) for 2 h and collagenase/dispase (1 mg/ml) for 8 h followed by centrifugation in 40% Percoll solution. The band containing microvessels was collected and washed prior to plating onto collagen-coated dishes. BCECs migrating from vessels were pooled to form a culture of proliferating endothelial cells and were maintained in Dulbecco's modified Eagle's medium containing 10% fetal calf serum, heparin (0.5 mg/ml), and endothelial growth supplements (75 µg/ml). BCECs of passages 4–15, which were uniformly positive for factor VIII experiments.

Quantitative and Qualitative Assessment of TNF-α/CHX Cytopathic Effects—BCECs were exposed to TNF-α (20 ng/ml) for 16 h followed by CHX (10 µg/ml) for 6 h and then maintained for various periods (up to 24 h) or to the appropriate concentrations of C2 ceramide (10–100 µM) (Calbiochem, La Jolla, CA) for 24 h before cytopathic assessment. Cell death was measured at various time points after treatments by lactate dehydrogenase (LDH) release (27), 3-(4,5-dimethylthiazol-2-yl)-2,5-di-phenyl-tetrazolium bromide (MTT) assay (28), and trypan blue exclusion. In addition, the levels of histone-associated DNA fragments released into cytoplasm after the induction of cell death was quantitatively determined by a Cell Death Detection kit (Boehringer Mannheim). The assay utilizes mouse monoclonal antibodies directed against DNA and histones, and allows the specific determination of mono- and oligonucleosome levels in the cell lysate (25). Apoptotic cell death was further confirmed by Hoechst 33422 (Molecular Probes, Eugene, OR) staining and fluorescence microscopy. TUNEL staining of BCECs after treatments to induce cell death was performed using an ApopTag in situ apoptosis detection kit (Oncor, Inc., Gaithersburg, MD) (25). For detection of DNA ladders, BCECs were treated with TNF-α/CHX (16 h/6 h) or C2 ceramide (50 µM) for 24 h. After washing with phosphate-buffered saline, cells were harvested and lysed by addition of cell lysis solution in the presence of RNase A, and then the high molecular weight chromosomes and proteins were removed by the precipitation solution according to the supplier (Promega, Madison, WI). The extracted DNA samples (10 µg/lane) were electrophoresed in Tris acetate buffer (0.4 M Tris, 0.25 M sodium acetate, 0.22 mM EDTA, pH 7.8) containing 0.4 µg/ml ethidium bromide on a 1.5% agarose gel. DNA was visualized through ultraviolet light and photographed (25).

Measurement of Endogenous Sphingomyelin/Ceramide Content—Determination and quantification of intracellular sphingomyelin/ceramide content were performed as described (29) with some modifications. This method is comparable to the diacylglycerol kinase assay, and both give quantitatively similar data (29). BCECs (1 × 10⁵ cells) were cultured in Dulbecco's modified Eagle's medium containing 10 µCi of [³H]palmitate (1 mCi/ml, Amersham Pharmacia Biotech) for 16–24 h before treatment with TNF-α/CHX or vehicle. Labeled cells were collected and washed with 2 ml of ice-cold phosphate-buffered saline three times to remove free isotope. For lipid extraction, the cell pellet was resuspended in 400 µl of methanol: H₂O: n HCl (100:5:1, v/v) followed by adding 800 µl of chloroform and 240 µl of water. The mixture was then vortex-mixed and centrifuged at 1,000 × g for 5 min, and the upper, aqueous phase was discarded. The lipid fraction recovered from the lower phase was extracted again with 1 ml of chloroform:methanol (2:1, v/v) and dried under nitrogen. The final samples were resuspended in chloroform:methanol (2:1, v/v) and analyzed by thin layer chromatography (TLC) using chloroform:methanol:acetic acid:water (85:5:5:5, 0.5, 0.5, 0.5, v/v) system as resolving system, and chloroform: methanol:acetic acid:water (50:30:8:4, v/v) for sphingomyelin. Standard lipids were separated from or in combination with the samples and visualized in iodine vapor. The radioactive spots corresponding to ceramide and sphingomyelin were scraped off and quantitated by liquid scintillation counting. Radioactivity recovered from the ceramide or sphingomyelin fraction was converted to picomoles of product by using the specific activity of the substrate palmitate.

RESULTS

Sequential Treatment of TNF-α- and CHX-induced Cell Death in BCECs—BCECs, like most untransformed cells in cultures, are resistant to the cytotoxic effects of TNF-α. There was no sign of BCEC death up to 48 h after 20 ng/ml TNF-α exposure. CHX (10 µg/ml) alone also did not cause cell death. However, death of BCECs was noted with sequential exposure to TNF-α (20 ng/ml, 16 h) and CHX (10 µg/ml, 6 h). Cell death in these cultures was asynchronous and reached approximately 30% at 22 h following TNF-α/CHX treatment as measured by trypan blue exclusion, MTT assay, and release of intracellular LDH (Fig. 1). Dying cells developed progressive shrinkage and detached from the substrate. BCECs exposed to TNF-α/CHX
treatment displayed the typical morphology characteristic of apoptosis, e.g., cell shrinkage, nuclear condensation, and chromosomal and internucleosomal DNA fragmentations (Fig. 2, A and B). The amount of histone/DNA complexes liberated into cytoplasm after TNF-α/CHX exposure was also elevated compared with that of cells treated by TNF-α or CHX alone (Fig. 2C). It is noteworthy that signs of cell death (e.g., LDH release) induced by TNF-α/CHX were preceded by DNA fragmentation, which began as early as 1 h after CHX incubation and persisted to reach a maximum at 8 h (data not shown). These results are consistent with the notion that TNF-α/CHX treatment results in BCEC apoptosis.

TNF-α/CHX Treatment Increased Intracellular Ceramide Accumulation—Ceramide has been shown as a key second messenger for TNF-α action, especially in apoptotic paradigm, in a number of cell systems (19, 30). We thus measured the intracellular level of ceramide in BCECs after TNF-α/CHX treatment. As shown in Fig. 3A, TNF-α/CHX treatment resulted in a 5–6-fold increase in intracellular ceramide level as measured by [3H]palmitate incorporation. In contrast, treatment with either TNF-α or CHX alone for the same period of time did not show significant change in ceramide content as compared with control cells. More importantly, the increase in cellular ceramide level in response to TNF-α/CHX treatment apparently did not result from sphingomyelinase activation. The sphingomyelin content did not decline significantly over a 6-h period following addition of CHX (Fig. 3B). However, ceramide induction started within the first hour (~2-fold), progressively increased and reached its peak at 3 h after CHX challenge (~4-fold), after which both ceramide and sphingomyelin levels slightly declined. It appears that ceramide accumulation in BCECs following TNF-α/CHX stimulation was not associated with sphingomyelin hydrolysis.

C2 Ceramide Caused BCEC Apoptosis in a Dose- and Time-dependent Manner.—To test further if ceramide could serve as a mediator of TNF-α/CHX-induced BCEC death, we tested the effect of a cell-permeable ceramide analogue, namely C2 ceramide. Incubation of C2 ceramide at concentrations ranging from 10 to 100 μM for 24 h caused BCEC death in a concentration-dependent manner with an EC50 of approximately 50 μM (Fig. 4A). At a concentration of 50 μM, C2 ceramide-induced cell death began at ~6 h and proceeded progressively until 24 h after treatment when the maximal effect was achieved (Fig. 4B). A biologically inactive ceramide analogue, dihydroceramide, was not cytotoxic to BCECs at concentrations up to 50 μM, demonstrating that the effect was C2-specific (Fig. 4C). The characteristic changes in BCECs following C2 treatment were similar to those shown following TNF-α/CHX exposure and included positive TUNEL staining and internucleosomal DNA fragmentation revealed by agarose gel electrophoresis (Fig. 5). Our results indicate that C2 ceramide, like TNF-α/CHX, has the potential to elicit apoptosis in BCECs, and raise the possibility that ceramide production may mediate cytotoxic effects of TNF-α/CHX in BCECs.

Ceramide Synthase Inhibitor Attenuated TNF-α/CHX-induced BCEC Death—Because the previous results revealed that ceramide generation by TNF-α/CHX treatment was not from sphingomyelin hydrolysis, we considered the possibility of de novo synthesis of ceramide as an alternative mechanism.
Ceramide synthase is the key enzyme in de novo ceramide biosynthesis (31) and thus is a good candidate for studying its role in TNF-α/CHX-triggered BCEC death. We first studied the effect of a natural specific inhibitor of ceramide synthase, fumonisin B1. Fumonisin B1 is a fungal metabolite with substantial structural similarities to sphinganine and sphingosine (23). It has been well documented that fumonisin B1 is a potent and specific inhibitor of ceramide synthase with no obvious effects on other major enzymes of sphingolipid metabolism (32). As shown in Fig. 6A, preincubation of BCECs with increasing concentrations of fumonisin B1 (0.01–1 μM) for 2 h prior to TNF-α/CHX treatment displayed a dose-dependent attenuation of cell death. At 1 μM, fumonisin B1 also significantly reduced the intracellular ceramide level (Fig. 6B). At concentrations higher than 10 μM, fumonisin B1 was toxic to BCECs.

In contrast to fumonisin B1, an acidic sphingomyelinase inhibitor, desipramine, at same concentrations showed no protective effect against TNF-α/CHX action. These findings further support our previous results that ceramide accumulation in BCECs following TNF-α/CHX stimulation was not caused by sphingomyelinase activation. Instead, ceramide production by TNF-α/CHX treatment could be a result of de novo ceramide synthesis via up-regulation of ceramide synthase activity.
DISCUSSION

Results from the present study provide evidence that ceramide contributes at least partially to TNF-α/CHX-induced BCEC death. Ceramide production preceded the onset of DNA fragmentation and cell death. The time courses of ceramide generation and BCEC death in response to TNF-α/CHX are consistent with the contention that ceramide is an early messenger for this process. The mechanism of ceramide accumulation following sequential TNF-α/CHX treatment does not appear to be secondary to the activation of sphingomyelinase pathways, which are usually responsive to TNF-α, Fas-, and γ-irradiation-initiated apoptosis (33, 34). In these paradigms, ceramide generation was concurrent with sphingomyelin hydrolysis by activated sphingomyelinases. In the TNF-α cascade, both the membrane-associated neutral and the acidic forms of sphingomyelinases are activated by TNF-α receptors through different cytoplasmic domains. Each domain was specifically coupled to selective pathways of TNF-α signaling; ceramide generated by the neutral sphingomyelinase activates phospholipase A₂ and proline-directed protein kinases, whereas ceramide generated by the acidic form has been linked to the cytotoxic response (12, 13, 20, 21). In this study, we found that TNF-α or CHX alone did not activate any ceramide cascade. TNF-α/CHX-induced BCEC death, however, may involve a distinct ceramide pathway, which apparently results from de novo synthesis via ceramide synthase. Lack of sphingomyelin degradation following TNF-α/CHX treatment indicates that sphingomyelinases are not activated in this BCEC death paradigm. Furthermore, acidic sphingomyelinase may not be involved in this apoptotic pathway because application of an acidic sphingomyelinase inhibitor failed to attenuate TNF-α/CHX-mediated cell death.

There were a number of studies describing apoptosis in human and bovine endothelial cells mediated by TNF-α alone or TNF-α plus CHX (35–37). Reactive oxygen species, Bcl-2, and caspases have been implicated in this process. However, the exact mechanisms of apoptosis in these paradigms have not been fully delineated. In addition, although ceramide is proposed to be an active messenger in the signaling pathway, a causal role for ceramide in TNF-α-mediated endothelial cell apoptosis has not been established. Our results suggest that ceramide could be an important mediator in BCEC death in response to TNF-α/CHX treatment. First, either TNF-α or CHX alone, which has no apparent cytotoxic effects on BCECs, could not induce ceramide production. Second, intracellular ceramide accumulation in response to TNF-α/CHX occurred at a very early stage. Third, exogenous application of a ceramide analogue (C₂ ceramide) mimicked the TNF-α/CHX effect in induc...
ing BCEC death. Finally, fumonisins B1, a specific ceramide synthase inhibitor, attenuated both ceramide accumulation and TNF-α/CHX-induced cell death.

TNF-α in combination with a protein or RNA synthesis inhibitor, such as CHX, is a well established apoptotic paradigm in a number of cell types in vitro (14–17). The cytotoxic effect of TNF-α unmasked by CHX has led to the hypothesis that CHX blocks TNF-α-induced expression of survival factors. This hypothesis was supported by the following findings. The mRNA level of manganese-dependent superoxide dismutase, a mitochondrial reactive oxygen species scavenging enzyme, was increased in response to TNF-α treatment (38). TNF-α has recently been shown to induce expression of a novel Bcl-2-related protein called A1 in human umbilical vein endothelial cells (36). In human umbilical vein endothelial cells, TNF-α treatment also enhanced the expression of a zinc finger protein A20, which functions as an anti-apoptotic factor in certain cell types (39). In addition, TNF-α is a potent activator of NF-κB, a transcription factor with anti-apoptotic potential (40, 41). On the other hand, CHX at low concentrations has been shown to alter Bcl-2 expression related to its neuroprotective effect (42). However, this latter observation may not be applicable to our paradigm because the concentration of CHX we used is at least 2 orders of magnitude higher. In addition, we have found that the effect of cycloheximide and TNF-α is concentration-dependent, e.g., lower concentrations of cycloheximide (1–5 μM) in combination with TNF-α also causes apoptosis, but at a slower rate and of lesser magnitude. The combined effects of TNF-α and CHX may also exist in vivo in certain pathological states. Protein synthesis is inhibited for several hours following ischemia in vivo (43); in addition, exogenous administration of protein synthesis inhibitor has been explored as a treatment intervention capable of reducing cell death in the ischemic brain or traumatized spinal cord (44, 45), a setting where, as noted above, TNF-α induced expression is induced.

So far, the mechanism underlying TNF-α/CHX regulation of ceramide synthase in BCECs is not clear. Nevertheless, BCECs may contain an unique ceramide synthesis cascade which can be activated by TNF-α/CHX to cause cell death. This novel mechanism of cell death has not been demonstrated in any cell of neural origin or any endothelial cell preparation. The only known example in which ceramide biosynthesis serves as a mechanism for generating death signal was daunorubicin-induced apoptosis in both human and murine leukemia cells (23). Our data here have unveiled, for the first time, a possible novel mechanism of TNF-α/CHX-induced BCEC death, which is mediated by ceramide but is independent of sphingomyelinase cascade. Further exploration of this unique cerebral endothelial cell death pathway involving ceramide synthase may broaden our insight into therapeutic interventions directed at the secondary inflammatory reaction following brain injury, which is associated with progressive endothelial injury (5, 6).

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