Divergence in Regulation of Nitric-oxide Synthase and Its Cofactor Tetrahydrobiopterin by Tumor Necrosis Factor-α

SYNTHESIS OF 6(R)-5,6,7,8-tetrahydrobiopterin (BH4), a required cofactor for inducible nitric-oxide synthase (iNOS) activity, is usually coordinately regulated with iNOS expression. In C6 glioma cells, tumor necrosis factor-α (TNF-α) concomitantly potentiated the stimulation of nitric oxide (NO) and BH4 production induced by IFN-γ and interleukin-1β. Expression of both iNOS and GTP cyclohydrolase I (GTPCH), the rate-limiting enzyme in the BH4 biosynthetic pathway, was also markedly increased, as were their activities and protein levels. Ceramide, a sphingolipid metabolite, may mediate some of the actions of TNF-α. Indeed, we found that bacterial sphingomyelinase, which hydrolyzes sphingomyelin and increases endogenous ceramide, or the cell permeable ceramide analogue, C2-ceramide, but not C2-dihydroceramide (N-acetylsphinganine), significantly mimicked the effects of TNF-α on NO production and iNOS expression and activity in C6 cells. Surprisingly, although TNF-α increased BH4 synthesis and GTPCH activity, neither BH4 nor GTPCH expression was affected by C2-ceramide or sphingomyelinase in IFN-γ- and interleukin-1β-stimulated cells. It is likely that increased BH4 levels results from increased GTPCH protein and activity in vivo rather than from reduced turnover of BH4, because the GTPCH inhibitor, 2,4-diamino-6-hydroxypyrimidine, blocked cytokine-stimulated BH4 accumulation. Moreover, expression of the GTPCH feedback regulatory protein, which if decreased might increase GTPCH activity, was not affected by TNF-α or ceramide. Treatment with the antioxidant pyrrolidine dithiocarbamate, which is known to inhibit NF-κB and sphingomyelinase in C6 cells, or with the peptide SN-50, which blocks translocation of NF-κB to the nucleus, inhibited TNF-α-dependent iNOS mRNA expression without affecting GTPCH mRNA levels. This is the first demonstration that cytokine-stimulated iNOS and GTPCH expression, and therefore NO and BH4 biosynthesis, may be regulated by discrete pathways. As BH4 is also a cofactor for the aromatic amino acid hydroxylases, discovery of distinct mechanisms for regulation of BH4 and NO has important implications for its specific functions.

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Proinflammatory cytokines, such as IFN-γ, IL-1β, and TNF-α, as well as a bacterial endotoxin (lipopolysaccharide (LPS)), stimulate the production of nitric oxide (NO) by increasing expression of the inducible form of nitric-oxide synthase (iNOS) in several types of cells, including macrophages (1), microglia, and astrocytes (2). Synthesis of this free radical gas is primarily a protective mechanism utilized by the host against invading organisms (reviewed in Ref. 3). On the other hand, it has been suggested that overproduction of NO in the central nervous system may mediate some of the pathological sequelae of neuroinflammatory disorders, such as multiple sclerosis (4) and neuronal death following acute injury (5). iNOS is active as a homodimer of 130-kDa subunits and requires five cofactors to catalyze the conversion of L-arginine to L-citrulline, a reaction that liberates NO (reviewed in Ref. 6). Three of the cofactors, NADPH, FAD, and FMN, are usually present in cells at concentrations that are not limiting for enzyme activity. Calmodulin, the fourth cofactor, is constitutively bound to iNOS in a manner that, unlike its function with the two constitutive isoforms of NOS, makes iNOS activity calcium-independent (7). However, the intracellular level of the cofactor 6(R)-5,6,7,8-tetrahydrobiopterin (BH4) is rate-limiting for NO generation, and its synthesis is usually co-induced by cytokines (8–10). The exact role that BH4 plays in iNOS catalysis is still equivocal, but it has been shown to bind to iNOS monomers, promoting their dimerization and subsequent activation (11), and recently has been proposed to play a role in the enzymatic reaction in a radical form (12).

The cellular level of BH4 is largely regulated by the activity of GTP cyclohydrolase I (GTPCH), the first and rate-limiting enzyme in the BH4 biosynthetic pathway (6). GTPCH, a homodimer of 80-kDa subunits that are arranged as two pentamers facing one another (13), catalyzes the rearrangement of GTP to dihydronoriprotein triphosphate. This intermediate is then converted to BH4 in two subsequent reactions catalyzed by 6-pyrrolyltetrahydropterin synthase and sepiapterin reductase, respectively, neither of which is rate-limiting. GTPCH mRNA expression can be induced by the same proinflammatory stimuli that induce iNOS mRNA (14). Interestingly, in human umbilical vein endothelial cells, cytokine-stimulated NO production is predominantly regulated by increased
GTPCH mRNA expression (15, 16) and not by changes in expression of endothelial NOS.

Ceramide, formed by the sphingomyelinase (SMase)-mediated hydrolysis of sphingomyelin, is now emerging as a lipid second messenger that mediates some of the biological effects of TNF-α, IL-1β, and LPS in differentiation, apoptosis, and cell growth arrest (reviewed in Refs. 17–19). Recently, LPS and SMase-mediated elevations in ceramide have been demonstrated to potentiate NO formation and iNOS expression in rat primary astrocytes and C6 glioblastoma cells (20). The signaling pathways involved in NO production have not yet been fully elucidated, although it appears that activation of the redox-sensitive transcription factor, NF-xB, is essential for iNOS induction (21). To study the potential role of ceramide in cytokine-stimulated BH₄ production, we used C6 rat astroglialoma cells, a convenient model astrocyte cell line. Furthermore, in this cell line, as in primary astrocytes, TNF-α has been shown to stimulate degradation of sphingomyelin to ceramide (22). Although ceramide generation, similar to TNF-α treatment potentiated NO and iNOS expression induced by IFN-γ plus IL-1β, surprisingly, we found that it did not mimic the effects of TNF-α on BH₄ or GTPCH expression. Our results thus suggest that BH₄ and NO biosynthesis can be differentially regulated in C6 cells.

**EXPERIMENTAL PROCEDURES**

**Reagents**—L-[2,3,4,5-³H]arginine was supplied by Amersham Pharmacia Biotech. Murine recombinant IL-1β was purchased from Life Technologies, Inc. Recombinant rat IFN-γ and TNF-α were purchased from R&D Systems (Minneapolis, MN). N-acetyl-b-sphingosine (C₄-ceramide), C₆-dihydroceramide, sphingosine, and SN-50 and mutant SN-50 peptides were from Bionomol Research Laboratories Inc. (Plymouth Meeting, PA). *Staphylococcus aureus* SMase was purchased from Sigma. Bovine intestinal alkaline phosphatase and pyrophospholipid dioxygenase-carbamate (PDDC) were from Calbiochem (La Jolla, CA). Mouse macrophage polyinosinic polyinosine antibody and a mouse macrophage control were purchased from Transduction Laboratories (Ann Arbor, MI). Peroxidase-labeled goat anti-rabbit IgG was from KPL (Gaithersburg, MD). All Western blot materials were from NOVEX (San Diego, CA). Peroxidase-labeled goat anti-rabbit IgG was from KPL (Gaithersburg, MD). All Western blot materials were from NOVEX (San Diego, CA). Dowex 50W-X8 was from Bio-Rad and was used in the sodium direction. RNA (1 μg) was converted to cDNA with random hexamers and reverse PCR primers were used (predicted product size): iNOS, 5′-GGGACGATATGG-3′ and 5′-GGATACCAGGAGACCATCTCA-3′; GTPCH, 5′-GGTC-3′ and 5′-GATGCT-3′ (741 base pairs); GFRP, 5′-GATGCT-3′ and 5′-GAGGGCGTCATGCTG-3′ (741 base pairs). PCR products were resolved on 2% agarose gels containing ethidium bromide and visualized with UV fluorescence and a benchtop microcentrifuge for 5 min. Supernatants were removed and analyzed by reverse phase high performance liquid chromatography with fluorescence detection as described previously (23).

**Determination of BH₄ Activity—**GTPCH activity was measured essentially as described previously (24). In brief, to 30 μl of lysate were added 5 μl of 0.5 μM Tris-HCl (pH 7.4), 5 μl of 10 m M diethiothreitol, 5 μl of 10 mg/ml bovine serum albumin, and 5 μl of 10 mM GTP. Samples were incubated for 2 h at 37 °C and placed on ice, and the reaction was terminated by the addition of 5 μl of 1 M HCl, followed by 5 μl of iodine reagent (1% I₂/2% KI (1:1, v/v)). Approximately 10 mg of MnO₂ was added to oxidize reduced pterins to their fluorescent aromatic forms. After 20 min at room temperature, samples were centrifuged at maximum speed in a benchtop microcentrifuge for 5 min. Supernatants were removed and analyzed by reverse phase high performance liquid chromatography with fluorescence detection as described previously (23).

**Determination of GTPCH Activity—**GTPCH activity was measured essentially as described previously (24). In brief, to 30 μl of lysate were added 5 μl of 0.5 μM Tris-HCl (pH 7.4), 5 μl of 10 m M diethiothreitol, 5 μl of 10 mg/ml bovine serum albumin, and 5 μl of 10 mM GTP. Samples were incubated for 2 h at 37 °C and placed on ice, and the reaction was terminated by the addition of 5 μl of 1 M HCl, followed by 5 μl of iodine reagent (1% I₂/2% KI (1:1, v/v)). Approximately 10 mg of MnO₂ was added to oxidize reduced pterins to their fluorescent aromatic forms. After 20 min at room temperature, samples were centrifuged at maximum speed in a benchtop microcentrifuge for 5 min. Supernatants were removed and analyzed by reverse phase high performance liquid chromatography with fluorescence detection as described previously (23).

**Determination of iNOS Activity—**Aliquots of cell lysates (50 μl) were added to 50 μl of assay buffer containing 50 mM Tris-HCl, pH 7.4, 500 μM NADPH, 10 μM BH₄, 500 μM diethiothreitol, 10 μM l-arginine, 2 μCi of l-[2,3,4,5-³H]arginine. NOS assays were carried out at 37 °C for 45 min and terminated on ice by the addition of 400 μl of stop buffer (20 mM Tris, pH 5.5, 2 mM EDTA, 1 mM l-citrulline). [³H]Citrulline was collected by passing the samples through 1.0 ml Dowex AG 50W-X8 (Na⁺) columns, preequilibrated with stop buffer. The columns were washed twice with 0.5 ml of stop buffer. [³H]Citrulline in the combined run-through and washes was quantified by liquid scintillation counting.

**RT-PCR**—Total RNA was isolated from confluent cultures with Trizol reagent (Life Technologies, Inc.) according to the manufacturer’s directions. RNA (1 μg) was converted to cDNA with random hexamers and Thermoscript reverse transcriptase according to the manufacturer’s directions (Life Technologies, Inc.). cDNA was amplified by PCR in a Perkin-Elmer 2400 thermal cycler using the following conditions: initial denaturation at 94 °C for 3 min, followed by 30 cycles for iNOS and GTPCH feedback regulatory protein (GFRP), 32 cycles for GTPCH, and 25 cycles for actin (94 °C for 45 s, 55 °C for 45 s, and 72 °C for 1 min). Final extension was at 72 °C for 10 min. The following forward and reverse PCR primers were used (predicted product size): iNOS, 5′-GGGCATCTGTTCAGGATCT-3′ and 5′-CATACGGCTGTCAGTC-3′ (743 base pairs); GFRP, 5′-CAGATCCGTTAAGGAAGTGTTGTC-3′ and 5′-CACCCTGGTGTAGTAAAACCC-3′ (195 base pairs); GTPCH, 5′-GGATACCCAGGAGACATCTCA-3′ and 5′-TACGGCTTGATGCTAG-3′ (372 base pairs); and actin, 5′-TTGATAAACACTTGGGCAATG-3′ and 5′-TCAATTTGCATCTTCAGTCTGA-3′ (743 base pairs). PCR products were resolved on 2% agarose gels containing ethidium bromide and visualized with UV fluorescence and a video camera, and bands were quantified with the National Institutes of Health Image program. Reaction conditions were optimized in preliminary experiments so that amplifications were within the logarithmic phase and yields were approximately linear with input cDNA concentration. To ensure that contaminating genomic DNA was not being amplified, PCR was also performed without reverse transcriptase treatment.

**Western Analysis**—Aliquots of cell lysates containing 20 μg of protein were concentrated using chloroform:methanol:H₂O phase fraction. In brief, lysates were diluted to 450 μl with H₂O and mixed with 1 ml of chloroform:methanol (1:1) to give a final ratio of 1:9. The mixture was vortexed and then centrifuged in a benchtop microcentrifuge at maximum speed for 3 min to separate the phases. With this solvent combination, the proteins aggregate at the interphase. 700 μl of the upper phase was removed without disturbing the interphase and discarded, and an equivalent volume of methanol was added back to the lower phase. The protein aggregates were pelleted at maximum speed for 10 min. Supernatants were carefully aspirated, and the pellets were dried at 50 °C. Pellets were resuspended in 25 μl of 1× LDS NuPAGE...
Recently, it was shown that ceramide potentiated also coordinately increase the synthesis of the NOS cofactor, together with LPS, which induce NO production in astrocytes, inflammatory cytokines, such as IFN-γ.

Combinations of proinflammatory cytokines, such as TNF-α, IL-1β, and IFN-γ, together with LPS, induce NO production in astrocytes (20). Because TNF-α has been shown to stimulate hydrolysis of sphingomyelin to ceramide in many types of cells, including C6 cells (22), it was of interest to analyze the involvement of ceramide in TNF-α-induced NO and BH4 biosynthesis in these cells, as they express many of the properties of astrocytes.

In agreement with previous studies (26), the combination of IFN-γ, IL-1β, and TNF-α evoked a marked stimulation of NO production as measured by nitrite accumulation to a level of 36 μM (Fig. 1A). TNF-α was unable to induce NO production by itself. Furthermore, in the absence of TNF-α, the amount of nitrite produced by IFN-γ/IL-1β was dramatically lower (2.1 μM). The cell permeable ceramide analogue, C2-ceramide, in a dose-dependent manner, or exogenous bacterial SMase, which hydrolyzes sphingomyelin to generate endogenous ceramide, mimicked the effect of TNF-α, and potentiated IFN-γ/IL-1β-induced NO production (Fig. 1). This appears to be a specific ceramide effect because other related sphingolipid metabolites, including sphingosine and the inactive ceramide analogue, C2-dihydroceramide, which has the same structure as C2-ceramide but lacks the double bond, did not replicate the effects of C2-ceramide or SMase (data not shown).

In agreement with its more potent ability to potentiate NO production, TNF-α notably enhanced IFN-γ/IL-1β-induced NO and BH4 biosynthesis in these cells, as they express many of the properties of astrocytes.

In vitro iNOS activity was measured. Asterisks indicate statistically significant differences compared with IFN-γ/IL-1β-treated cells as determined by Student’s t test (p = 0.05).

A, C6 cells were stimulated for 16 h with IFN-γ (40 units/ml) and IL-1β (8 units/ml) in the absence or presence of TNF-α (4000 units/ml) or the indicated concentrations of C2-ceramide, C2-dihydroceramide, sphingosine, or sphingosine for 16 h, and in vitro iNOS activity was determined by measurement of the conversion of L-[3H]arginine to L-[3H]citrulline as described under “Experimental Procedures.” Values are expressed as pmol of citrulline formed/mg/min and are means ± S.D. of two independent experiments carried out in triplicate. B, cells were stimulated with IFN-γ and IL-1β in the absence or presence of 10 μM C2-ceramide, C2-dihydroceramide, or sphingosine for 16 h, and in vitro iNOS activity was measured. Asterisks in both panels indicate statistically significant differences compared with IFN-γ/IL-1β-treated cells as determined by Student’s t test (p = 0.05).

FIG. 1. Ceramide and TNF-α potentiate NO production induced by IFN-γ and IL-1β. C6 cells were treated for 24 h without or with IFN-γ (40 units/ml) and IL-1β (8 units/ml) in the absence or presence of the indicated concentrations of C2-ceramide, bacterial SMase (100 milliunits/ml), or TNF-α (4000 units/ml). Nitrite in the medium was measured as described under “Experimental Procedures.” Results are expressed as μM and are means ± S.D. of three independent experiments carried out in triplicate. Asterisks indicate statistically significant differences compared with IFN-γ/IL-1β-treated cells as determined by Student’s t test (p = 0.05).

FIG. 2. Ceramide specifically potentiates iNOS activity induced by IFN-γ and IL-1β. A, C6 cells were stimulated for 16 h with IFN-γ (40 units/ml) and IL-1β (8 units/ml) in the absence or presence of TNF-α (4000 units/ml) or the indicated concentrations of C2-ceramide and in vitro iNOS activity was determined by measurement of the conversion of L-[3H]arginine to L-[3H]citrulline as described under “Experimental Procedures.” Values are expressed as pmol of citrulline formed/mg/min and are means ± S.D. of two independent experiments carried out in triplicate. B, cells were stimulated with IFN-γ and IL-1β in the absence or presence of 10 μM C2-ceramide, C2-dihydroceramide, or sphingosine for 16 h, and in vitro iNOS activity was measured. Asterisks indicate statistically significant differences compared with IFN-γ/IL-1β-treated cells as determined by Student’s t test (p = 0.05).
C2-ceramide Potentiates IFN-γ/IL-1β-induced iNOS Expression and Protein—It was of interest to determine whether the stimulatory effect of TNF-α and ceramide on NO production and iNOS activity was due to an increase in iNOS expression. In agreement with previous reports, iNOS mRNA was not detectable by RT-PCR in untreated C6 cells (20, 27) but was induced by IFN-γ/IL-1β, and its expression was further enhanced by addition of TNF-α or C2-ceramide (Fig. 3A). The same pattern of responses was observed when iNOS protein levels were examined by immunoblotting (Fig. 3B). Furthermore, C2-ceramide dose-dependently increased iNOS mRNA and protein expression (Fig. 3, C and D). Thus, C2-ceramide is able to mimic the effects of TNF-α on potentiating IFN-γ/IL-1β-induced iNOS transcription, translation, and enzyme activity, albeit with less efficiency than TNF-α.

As C2-ceramide potentiated IFN-γ/IL-1β-induced iNOS activity and protein by 8–10-fold yet increased NO production to a much smaller extent, it was possible that in vivo iNOS activity might be limited by the availability of its cofactor, BH4. In order to establish whether BH4 levels were limiting for NO production, C6 cells were treated with 5 μM sepiapterin, which is readily taken up and converted to BH4 (28). Sepiapterin did not increase NO production in C6 cells treated with C2-ceramide and IFN-γ/IL-1β (data not shown). Thus, it is unlikely that iNOS activity in this case is limited by the intracellular concentration of BH4.

TNF-α, but not Ceramide, Up-regulates BH4 Synthesis and GTPCH Activity—Previously, we showed that cytokines and LPS induce both production of NO and de novo biosynthesis of BH4 in astrocytes (25). TNF-α in the presence of IFN-γ/IL-1β markedly stimulated BH4 biosynthesis in C6 cells by more than 5-fold over the effect of IFN-γ/IL-1β alone (Fig. 4). Changes in GTPCH activity mirrored the BH4 increases (Fig. 4), in agreement with its role as the rate-limiting enzyme in BH4 biosynthesis. Indeed, it is likely that the increased BH4 results from increased de novo synthesis, rather than decreased catabolism, because the GTPCH inhibitor, 2,4-diamino-6-hydroxypyrimidine, blocked the cytokine-induced BH4 increase (data not shown). Thus, it was of interest to determine whether ceramide, in a manner similar to its effects on NO production, mimicked the effects of TNF-α and mediated an increase in BH4 synthesis in these cells. However, increasing ceramide levels by treatment of C6 cells with C2-ceramide or SMase did not potentiate the effects of IFN-γ/IL-1β on BH4 biosynthesis or on GTPCH activity (Fig. 4).

It has previously been reported that proinflammatory cytokines in combination with LPS stimulate GTPCH mRNA expression in mouse osteoblasts (27) and in C6 cells (27, 29). We next determined whether the stimulatory effect of TNF-α on BH4 production and GTPCH activity in IFN-γ/IL-1β-treated C6 cells was due to an increase in GTPCH mRNA expression. We found that GTPCH mRNA is constitutively expressed at low levels in C6 cells and that its expression is increased by IFN-γ/IL-1β and further enhanced by the addition of TNF-α (Fig. 5A). In agreement with their lack of effects on BH4 and GTPCH activity (Fig. 4), neither C2-ceramide nor SMase had any significant effects on GTPCH mRNA expression (Fig. 5B). This is the first demonstration that GTPCH and iNOS expression can be differentially regulated.

As it was possible that there might have been a rapid and transient increase in GTPCH mRNA expression evoked by the addition of C2-ceramide or SMase to IFN-γ/IL-1β-treated cells that might not be obvious after 16 h (Fig. 5), we examined a more complete time course for induction of both GTPCH and iNOS expression by semi-quantitative RT-PCR. TNF-α significantly increased GTPCH mRNA expression (normalized to actin expression) within 8 h in cells treated with IFN-γ/IL-1β (Fig. 6B). Expression then increased nearly linearly for at least another 8 h. Addition of TNF-α also increased the intracellular concentration of BH4 in a time-dependent manner with a detectable increase as early as 8 h and increasing thereafter, whereas ceramide elevation did not result in BH4 increases at any time point examined (data not shown). In agreement with their lack of effect on BH4 levels and GTPCH activity (Fig. 4),
treatment with SMase (Fig. 6B) or with C2-ceramide (data not shown) did not enhance GTPCH expression in cells treated with IFN-γ and IL-1β at any time point (Fig. 6A). In contrast, iNOS expression was rapidly increased by either TNF-α, SMase or C2-ceramide (data not shown) in IFN-γ/IL-1β-treated cells, and a near maximal stimulatory effect was observed within 8 h (Fig. 6A).

A potential mechanism of regulating de novo BH4 biosynthesis independently of GTPCH expression that could result in increased BH4 levels, is a decrease in BH4 end product feedback inhibition. In some cell types, BH4 inhibits GTPCH activity through the action of the GFRP, which forms a complex with GTPCH (24, 30). Thus, cytokine-induced decreases in GFRP activity or expression might lead to an increase in GTPCH activity, and result in higher levels of BH4. However, although GFRP is expressed constitutively in C6 cells (Fig. 6C), its expression was not altered by cytokines, in the presence or absence of TNF-α, throughout the 16 h time course, and it thus does not appear to be involved in the stimulation of GTPCH activity induced by TNF-α.

**TNF-α Regulates iNOS and GTPCH Expression by Distinct Signaling Pathways**—Recently, antioxidants were shown to be potent inhibitors of cytokine-induced degradation of sphingomyelin to ceramide, suggesting that sphingomyelinase activation is redox-sensitive (31). To examine the role of ceramide generation in iNOS expression, we utilized the antioxidant PDTC, which has been shown to inhibit ceramide generation in C6 cells induced by TNF-α (22). In agreement with previous results (32), PDTC completely blocked iNOS expression induced by TNF-α. However, it had no effect on TNF-α-stimulated GTPCH expression (Fig. 7B). PDTC also inhibits the release of the inhibitory IκB subunit from the latent cytoplasmic form of NF-κB, thereby blocking its transcriptional activity (33). Because expression of iNOS is regulated, at least in part, by NF-κB (34), and because the stimulatory effect of ceramide on induction of iNOS is dependent on NF-κB activation in astrocytes (20), we also examined the effects of the more specific NF-κB inhibitor, SN-50. This peptide, which possesses a nuclear localization sequence that competes for the cellular machinery required for NF-κB nuclear translocation (35), al-

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**Fig. 5.** TNF-α but not ceramide stimulates GTPCH mRNA expression induced by IFN-γ and IL-1β. A, C6 cells were stimulated with IFN-γ (40 units/ml) and IL-1β (8 units/ml) in the absence or presence of TNF-α (4000 units/ml) or SMase (100 millunits/ml). After 16 h, RNA was isolated, and expression of GTPCH and actin mRNAs was measured by RT-PCR. B, cells were treated as indicated, and after 16 h, GTPCH, iNOS, and actin mRNAs were measured by RT-PCR and quantified by analysis of the fluorescent bands using the National Institutes of Health Image program. Data are expressed as average fold changes in mRNA, normalized to actin expression, when compared with samples treated with IFN-γ and IL-1β. A and B are from separate experiments. Similar results were obtained in two independent experiments.

**Fig. 6.** TNF-α potentiates iNOS expression in a time-dependent manner without affecting GTPCH mRNA expression. A, C6 cells were stimulated with IFN-γ (40 units/ml) and IL-1β (8 units/ml) in the absence (open squares) or presence of TNF-α (4000 units/ml) (open circles) or SMase (100 millunits/ml) (filled squares) for the indicated times, and iNOS mRNA expression was determined by RT-PCR and expressed as arbitrary units, normalized to actin expression. Results are expressed as arbitrary density units from National Institutes of Health Image integration of ethidium bromide-stained gels, as iNOS expression was below detection limits until 4 h. B, GTPCH mRNA levels were determined by RT-PCR. GTPCH mRNA expression is expressed as fold increase, normalized to actin expression, over the constitutive expression level at t = 0. C, cytokines have no effect on expression of GFRP. C6 cells were treated with IFN-γ (40 units/ml), IL-1β (8 units/ml), and TNF-α (4000 units/ml) for the indicated time periods. RNA was isolated, and GFRP and actin mRNAs were determined by RT-PCR.
and iNOS levels by TNF-α-synthesis, by discrete pathways. The stimulatory effects on NO subunit family, IκB/p50/p65 form is associated with a member of the inhibitory Bκ) or 16 h (PDTC) (A) as indicated. After 8 h (SN-50) (B, DNA), whereas a control mutant SN-50 peptide had no effect. In sharp contrast, GTPCH mRNA levels in cytokine-stimulated cells were unaffected by SN-50. Thus, TNF-α stimulates iNOS expression by a NF-κB-dependent mechanism and GTPCH expression by a pathway that does not require activation of this transcription factor.

**DISCUSSION**

In this study, we have demonstrated that TNF-α stimulates iNOS and GTPCH expression, and therefore NO and BH₄ biosynthesis, by discrete pathways. The stimulatory effects on NO and iNOS levels by TNF-α, which has previously been shown to increase ceramide levels in C6 cells (22), was mimicked by the short chain ceramide analogue, C₂-ceramide, as well as bacterial SMase. Conversely, neither C₂-ceramide nor bacterial SMase further enhanced IL-1β/IFN-γ-induced BH₄ levels, even though TNF-α increased its levels by 5-fold. Furthermore, ceramide elevations, in contrast to TNF-α, also had no effect on the GTPCH activity of IFN-γ/IL-1β-stimulated C6 cells. Hence, ceramide can up-regulate iNOS without significantly affecting the levels of its cofactor BH₄. To our knowledge, this is the first time that regulation of NO and BH₄ synthesis has been shown to diverge, as in many previous studies, elevations of BH₄ always mirrored induction of NO, suggesting common regulatory pathways (6).

The rat iNOS promoter contains consensus binding sites for numerous transcription factors (36). However, transcriptional regulation of iNOS is largely governed by the nuclear activity of the potent transcription factor NF-κB, a DNA-binding protein that is activated by TNF-α and IL-1β in diverse types of cells (21, 27, 37). The iNOS promoter has two NF-κB binding sites, a proximal site approximately 90 bases upstream of the initiation codon and a distal site located 980 bases upstream. The relative importance of these sites in regulating iNOS expression is still unclear because the NF-κB-dependent pathways may vary depending on cell type and the particular combination of cytokines (38). Cytoplasmic NF-κB can exist as either a p50/p65 heterodimer or as a p105/p50 heterodimer (39). The p50/p65 form is associated with a member of the inhibitory subunit family, IκB, which is subject to cytokine-induced proteosomal degradation (40), allowing the p50/p65 heterodimer to translocate to the nucleus and bind to promoter target sequences. Alternatively, direct processing of a p105/p65 heterodimer to p50/p65 would also allow it to translocate into the nucleus.

In contrast, the GTPCH promoter has not been well charac-
other types of cells (54, 55). In addition, other lipid metabolism has been shown to be BH$_4$-dependent (56). Thus, in some tissues and cells, parallel regulation of NO formation and BH$_4$ synthesis may not be required or desirable. Moreover, our results may have important implications for the development of novel, specific therapeutic approaches to specifically decrease aberrant levels of NO without affecting BH$_4$.

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