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GTP CYCLOHYDROLASE I mRNA IS INDUCED BY LPS IN VASCULAR SMOOTH MUSCLE: CHARACTERIZATION, SEQUENCE AND RELATIONSHIP TO NITRIC OXIDE SYNTHASE

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SUMMARY: GTP cyclohydrolase I (GTPCH) is the first and rate-limiting enzyme for the synthesis of tetrahydrobiopterin (BH4), a cofactor of nitric oxide synthase (NOS). As the induction of NO synthesis by immunostimulants in vascular smooth muscle (VSM) cells requires *de novo* synthesis of BH4, we investigated whether immunostimulants enhance the expression of GTPCH mRNA. GTPCH mRNA and BH4 were measured in rat VSM cells after exposure to bacterial lipopolysaccharide (LPS) in combination with interferon-γ (IFN). Reverse transcription polymerase chain reaction (RT-PCR) was used to amplify a predicted 372 bp fragment of GTPCH mRNA, deduced from the known nucleotide sequence of rat liver GTPCH cDNA. Dideoxynucleotide sequencing of the PCR fragment revealed 100% identity between LPS/IFN-induced GTPCH mRNA of VSM and the constitutive GTPCH mRNA of liver. Although BH4 was below our limit of detection in untreated VSM, low levels of GTPCH mRNA were detectable. LPS/IFN treatment triggered the appearance of BH4 and markedly increased GTPCH mRNA. Induction of GTPCH mRNA was apparent by 2 h, peaked at 4 h, and was sustained at high levels for at least 24 h. Induction of GTPCH mRNA by LPS/IFN was substantially enhanced by cycloheximide, suggesting that mRNA levels are depressed by a labile protein. Measurement of LPS/IFN-induced NOS mRNA by RT-PCR, demonstrated a timecourse of induction which mirrors that of GTPCH. Similarly, the timecourse of appearance of cytosolic NOS activity following exposure of VSM to LPS/IFN paralleled that of the increase in BH4 content. Our studies demonstrate that immunostimulants co-induce NOS and GTPCH gene expression: both events are necessary for induction of NO synthesis by VSM.

Nitric oxide (NO) is a potent vasodilator which is produced by a family of tetrahydrobiopterin-dependent enzymes, the NO synthases (1). Bacterial lipopolysaccharide (LPS) and other immunostimulants induce an isoform of NO synthase (iNOS) in vascular smooth muscle (VSM; 2,3) which produces large quantities of NO and profound vasodilation; this process has been implicated as the cause of gram-negative septic shock (4,5). While induction of iNOS is necessary for immunostimulant-mediated NO overproduction, we questioned whether it was sufficient. Recently we found that *de novo* tetrahydrobiopterin (BH4) synthesis is elicited in VSM by LPS and is essential for iNOS activity (3). Since GTP cyclohydrolase I (GTPCH) is the

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enzyme which is normally rate-limiting for synthesis of BH4 (6), we investigated whether the combination of LPS and interferon-γ (LPS/IFN), potent inducing stimuli for iNOS, also induce GTPCH mRNA. We report here that LPS/IFN substantially induces GTPCH gene expression in VSM with a timecourse identical to that of iNOS. The induction of GTPCH by LPS/IFN is potentiated by cycloheximide, suggesting that like many other LPS-induced gene products (7,8), GTPCH mRNA may be maintained low by the action of a labile protein.

MATERIALS AND METHODS

Cell culture: Vascular smooth muscle (VSM) cells were isolated by elastase and collagenase digestion of thoracic aortae from male Wistar rats as previously described (3). Cells in passage 10-15 were seeded into 96-well plates for nitrite assay or into T175 flasks for RNA and cytosol preparations.

Analysis of mRNA levels for NOS and GTPCH by RT-PCR: RNA was extracted from VSM by a modified guanidinium isothiocyanate method (RNAzol; Cinna/Biotecx, Houston, TX). RT-PCR was performed by standard methods. Briefly, the first strand cDNA was synthetized using random primers and M-MLV reverse transcriptase (Promega, Madison, WI) followed by PCR amplification using synthetic gene specific primers for rat GTPCH or mouse iNOS. Primers used were: NOS forward 21-mer, 5'-CTGCAGGTCTTTGACGCTCGG-3'; NOS reverse 21-mer, 5'-GTGGAACACAGGGGTGATGCT-3'; GTPCH forward 21 mer, 5'-GGATACCAGGAGACCATCTCA-3'; GTPCH reverse 21 mer, 5'-TAGCATGGTGCTAGTGACAGT-3'. PCR amplification was performed using a DNA PCR kit (Perkin Ermer Cetus, Norwalk, CT) according to the following schedule: denaturation, annealing and elongation at 95°, 55° and 72°C for 30sec, 30 sec and 1 min, respectively, for 30 or 35 cycles. To ensure that equal amounts of reverse-transcribed RNA were added to the PCR reaction, the parallel amplification of glyceraldehyde-3-phosphate-dehydrogenase (GAPDH) mRNA was preformed for reference, using primers as described by Terada et al.(15). PCR products were electrophoresed on a 1.5% agarose gel containing ethidium bromide and visualized by UV-induced fluorescence. To comfirm the identity of the PCR products as GTPCH and NOS, they were each subcloned into pCRII (Invitrogen, San Diego, CA) and sequenced by the dideoxynucleotide-chain termination method (9).

Nitrite assay: Nitrite production, an indicator of NO synthesis, was measured in the VSM cell culture medium. Nitrite was quantified colorimetrically after adding 100 µl of Griess regent (1% sulfanilamide and 0.1% naphthylenediamide in 5% phosphoric acid) to 100 µl samples. OD_{550} was determined using a Molecular Devices microplate reader (Richmond, CA). Nitrite concentrations were calculated by comparison with OD_{550} of standard solutions of sodium nitrite prepared in cell culture medium.

Biopterin assay: Total cellular biopterin (BH4 and more oxidized species) was measured as previously described (10). VSM cytosol was prepared (3) and oxidized by treatment with 1% I_2 containing 2% KI in 2N HCL for 1 hr at 37°C in the dark. Ascorbate (0.1M) was added to remove residual free I_2 and extracts were neutralized with 1N NaOH followed by 200 mM TRIS, pH 7.8. Biopterin was quantitated by C_{18} reverse phase HPLC using fluoresence detection and authentic biopterin as a standard (10).

NOS assay: NO formation by the VSM cytosol was measured by a previously described kinetic 96-well microplate assay (11). The progress of NO capture by Fe^{2+}-myolobin (Mb) and subsequent oxation to Fe^{3+}-Mb was measured in a kinetic microplate reader based on the rate of increase in $OD_{405-650}$. Measurements were made every 15 sec for a period of 15 min and the slope of the best fit regression line (OD/min) was used to calculate NOS activity (11). All samples contained 10 µl of crude VSM cytosol (0.7-2.5 mg protein/ml) and final concentrations of 20 µM Mb, 500 µM L-arginine, 500 µM NADPH, 10 µM BH4 and 80 mM TRIS, pH 7.6.

RESULTS

As shown Fig. 1, biopterin levels were undetectable in the cytosol of untreated rat aortic VSM in culture. However, intracellular biopterin markedly increased by 12 hours after treatment with LPS/IFN and began to decline by 24 hours. Similarly, basal levels of NOS activity were

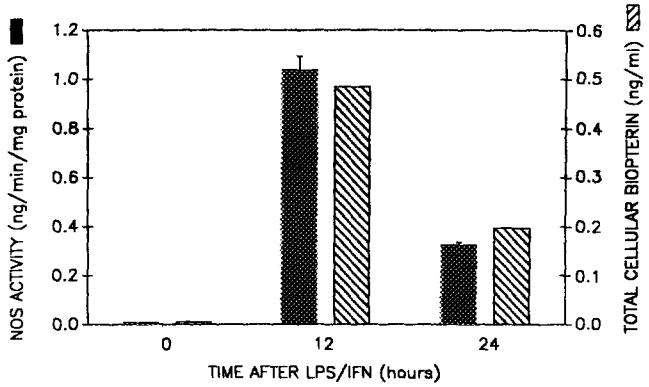

Figure 1. Influence of immunostimulants on NOS activity and total biopterin (oxidized plus reduced forms) in cytosol from rat aortic smooth muscle cells. NOS activity and total biopterin were assayed in groups of confluent cells that were either untreated or treated with a combination of LPS (30 μg/ml) and IFN (50 ng/ml) for 12 or 24 hours. Values are the mean of duplicate (biopterin) or triplicate (NOS) determinations from 3-5 culture flasks ± SEM.

undetectable in the cytosol of untreated VSM, but were induced within 12 hours after LPS/IFN treatment and fell by 24 hours.

To evaluate the basis for the increase in intracellular biopterin, we investigated whether LPS/IFN induces GTPCH mRNA. RT-PCR using specific primers for GTPCH amplified a predicted 372 bp sequence corresponding to nucleotides 295-666 of the constitutive GTPCH from rat liver (12). To confirm the PCR fragment was indeed GTPCH, it was subcloned and sequenced. The nucleotide sequence was found to be 100% identical to liver GTPCH (12). Similarly, RT-PCR for iNOS amplifed an 807 bp sequence analogous to nucleotides 607-1413 of murine macrophage iNOS (13). Subcloning and sequencing of the PCR product revealed a nucleotide sequence which was identical to the recently cloned iNOS cDNA from cytokine-activated VSM of rat (14) and 96% homologous with that from murine macrophages (13). Fig. 2 compares the timecourse for expression of GTPCH mRNA (upper panel) and iNOS mRNA (middle panel) after treatment of LPS/IFN. Although BH4 was not present in untreated VSM, low levels of GTPCH mRNA were detectable. Following treatment with LPS/IFN, GTPCH mRNA substantially increased within 2 hours, peaked by 4 hours, and was sustained at high levels for at least 24 hours. Similarly, iNOS mRNA became detectable in VSM by 2 hours after treatment with LPS/IFN. Levels of iNOS mRNA reached a maximum by approximately 4 hours and were elevated for at least 24 hours. Control PCR experiments demonstrated equivalent expression of GAPDH gene (15) in all samples (lower panel).

Fig. 3 shows the effects of dexamethasone, cycloheximide and actinomycin D on the induction of GTPCH mRNA by LPS/IFN. Induction of GTPCH mRNA by LPS/IFN was not significantly reduced by dexamethasone, but was abolished by actinomycin D. In contrast, cycloheximide caused "superinduction" of GTPCH mRNA. This suggests that not only is the synthesis of intermediary cytokines or other proteins not required for induction of GTPCH mRNA, but it appears to be inhibitory. Levels of GTPCH mRNA induced by LPS/IFN in the presence of

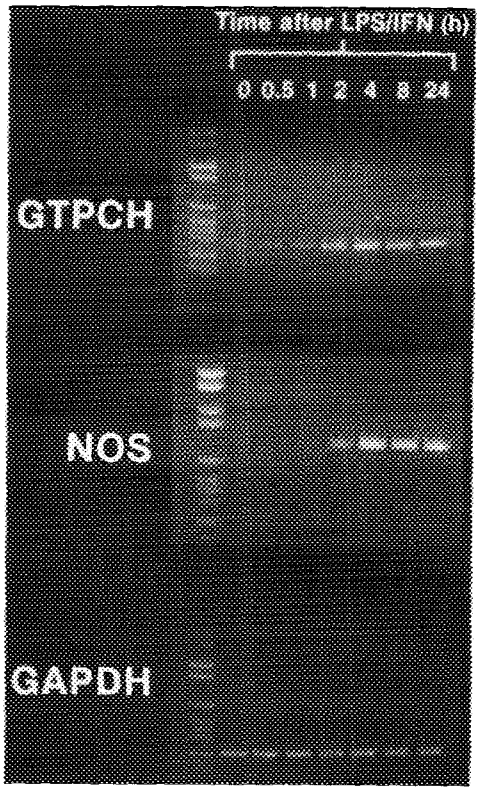

Figure 2. Timecourse of the induction of GTPCH mRNA and NOS mRNA by immunostumulants in rat aortic smooth muscle cells. Total RNA was prepared from cells after the indicated time of treatment with a combination of LPS (30 μg/ml) and IFN (50 ng/ml). RNA (1 μg) was amplified by RT-PCR using primers for GTPCH, iNOS or the house keeping gene, GAPDH. DNA size markers in the extreme left lane correspond to (bp): 2176, 1766, 1230, 1033, 653, 517, 453, 394, 298, 234, 220 and 154.

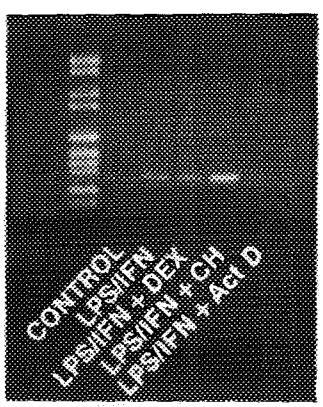

Figure 3. Effects of dexamethasone, cycloheximide and actinomycin D on the induction of GTPCH mRNA by immunostimulants in rat aortic smooth muscle cells. Total RNA was prepared from untreated cells (lane 2) or from cells 4 hrs after treatment with a combination of LPS (30 μg/ml) and IFN (50 ng/ml) alone (lane 3), or in the presence of dexamethasone (10 μM; lane 4), cycloheximide (1 μg/ml; lane 5) or actinomycin D (0.5 μg/ml; lane 6). RNA (1 μg) was amplified by RT-PCR using primers for GTPCH. Lane 1 shows DNA size markers as described in the legend to Fig. 2.

cycloheximide became higher than those elicited in the absence of cyloheximide by 4 hours, and higher levels were sustained for at least 24 hours (data not shown).

As shown previously (3), the induction of NO synthesis by LPS/IFN in VSM was inhibited by treatment with a selective inhibitor of GTPCH, 2,4-diamino-6-hydroxypyrimidine (DAHP). DAHP elicited a concentration-dependent inhibition of LPS/IFN-induced nitrite which was completely prevented by co-administration of sepiapterin (SEP; 100 μM). SEP is a pterin which is a substrate for BH4 synthesis via the pterin salvage pathway and therefore will be converted to BH4 even when *de novo* synthesis of BH4 is blocked with DAHP. The concentration dependence for DAHP-elicited inhibition of nitrite synthesis was shifted rightward by guanosine, which is converted by the purine salvage pathway to the GTPCH substrate, GTP. These findings indicate that DAHP blocks LPS/IFN-stimulated NO synthesis specifically via inhibition of GTPCH and thus, *de novo* production of BH4 (Fig. 4).

DISCUSSION

This is the first report to show that immunostimulants induce GTPCH mRNA in VSM. Immunostimulants have previously been shown to increase biopterin production by macrophages, lymphocytes, fibrobrasts and vascular smooth muscle cells (3,16). Nonetheless, the mechanism for the increase was not known. Werner et al. (17) demonstrated that immunostimulant-induced biopterin synthesis by murine macrophages and fibroblasts was not associated with an increase in GTPCH protein mass as deduced from Western blot analysis using antibodies to purified GTPCH from murine liver. This suggests that either the immuostimulant-induced biopterin synthesis arises from post-translational activation of pre-exisiting GTPCH or the inducible isoform of GTPCH is antigenically distinct from the constitutive form. Neither of these possibility are supported by our studies in VSM. The nucleotide sequence of the amplification product, representing 51% of the translated region of the mRNA, was found to be completely identical to rat liver GTPCH (8). This suggests that constitutive and inducible GTPCH are the same protein.

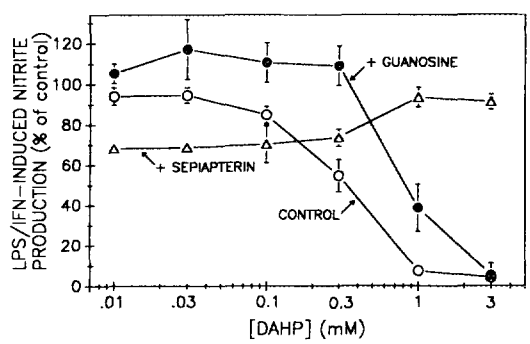

Figure 4. 2,4-diamino-6-hydroxypyrimidine (DAHP) inhibits the activation of nitrite production elicited in rat aortic smooth muscle cells by LPS (30 μg/ml) and IFN (50 ng/ml) (LPS/IFN). Nitrite accumulation in the cell culture medium was assayed 24 hrs after addition of LPS/IFN in the presence of the indicated concentrations of DAHP alone, and in the presence of sepiapterin (SEP, 100 μM) or guanosine (100 μM). Values are expressed as percent ± SEM of nitrite produced by cells treated with LPS/FN without DAHP. Nitrite production by LPS/IFN-treated cells was (μM): control 18.9±0.6; + SEP 31.6±1.4; and + guanosine 20.4±0.2.

Although low levels of GTPCH mRNA were expressed constitutively, GTPCH mRNA was substantially induced by LPS/IFN in control cells. The constitutive expression of mRNA in the absence of detectable protein activity, suggests that additional factors could be involved in the regulation of GTPCH protein translation and/or activity.

To examine whether induction of GTPCH mRNA by LPS/IFN requires protein synthesis, we assessed the influence of cycloheximide. Surprisingly, cycloheximide caused superinduction of GTPCH mRNA. Moreover, incubation of VSM with cycloheximide in the absence of LPS/IFN also elicited a significant increase in GTPCH mRNA levels (although to a lesser extent than that observed in the presence of LPS/IFN, data not shown). This suggests that GTPCH gene expression may be under the control of a labile repressor protein.

Caput et al. identified a common nucleotide sequence (UUAUUUAU) in the 3'-untranslated region of mRNA molecules specifying a variety of inflammatory mediators (7). Indeed, this conserved sequence is present in the 3'-untranslated region of mRNA for tumor necrosis factor α, interleukin-1, and urokinase-type plasminogen activator; each of these proteins are induced by treatment with cycloheximide (8). Recent reports suggest that this sequence confers a high degree of instability to mRNAs which possess it; this instability may be diminished by cycloheximide (18,19). It is interesting that two respeats of this sequence are present in the 3'-untranslated region of the DNA specifying GTPCH (8). In this context, GTPCH appears to be functionally related to inflammatory processes, including NO synthesis. Further experiments will be required to determine whether the superinduction of GTPCH mRNA by cycloheximide arises from enhanced GTPCH gene transcription or specific stabilization of mRNA.

We have previously shown that LPS induces the *de novo* synthesis of BH4 in VSM and that this event is essential for the induction of NO release (3). Indeed, blocking BH4 synthesis abolishes the induction of NO synthesis by cytokines, and conversely, providing excess BH4 enhances NO production. Taken together with the present findings, we conclude that activation of NO production by LPS in VSM requires at least two distinct transcriptional events: induction of NOS itself and induction of GTPCH, the first and rate-limiting enzyme for induction *de novo* BH4 synthesis. Measurement of LPS/IFN-induced NOS mRNA by RT-PCR demonstrated a timecourse of induction which mirrors that of GTPCH. Similarly, the timecourse of appearance of cytosolic NOS activity following treatment of VSM with LPS/IFN paralleled that for changes in BH4 content. Thus, the present study demonstrates that immunostimulants co-induce NOS and GTPCH gene expression and protein synthesis, both events are necessary for activation of cellular NO synthesis.

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