Nitric oxide (NO) synthesis is induced in vascular smooth muscle cells by lipopolysaccharide (LPS) where it appears to mediate a variety of vascular dysfunctions. In some cell types tetrahydrobiopterin (BH4) synthesis has also been found to be induced by cytokines. Because BH4 is a cofactor for NO synthase, we investigated whether BH4 synthesis is required for LPS-induced NO production in rat aortic smooth muscle cells (RASMC). The total biotin content (BH4 and more oxidized states) of untreated RASMC was below our limit of detection. However, treatment with LPS caused a significant rise in biotin precursor levels and an induction of NO synthesis; both effects of LPS were markedly potentiated by interferon-γ, 2,4-Diamino-6-hydroxypyrimidine (DAHP), a selective inhibitor of GTP cyclohydrolase I, the rate-limiting enzyme for de novo BH4 synthesis, completely abolished the elevated biotin precursor levels induced by LPS. DAHP also caused a concentration-dependent inhibition of LPS-induced NO synthesis. Inhibition of NO synthesis by DAHP was reversed by sepiapterin, an agent which circumvents the inhibition of biotin precursor synthesis by DAHP by serving as a substrate for BH4 synthesis via the pterin salvage pathway. The reversal by sepiapterin was overcome by methotrexate, an inhibitor of the pterin salvage pathway. Sepiapterin, and to a lesser extent BH4, dose-dependently enhanced LPS-induced NO synthesis, indicating that BH4 concentration limits the rate of NO production by LPS-activated RASMC. Sepiapterin also caused LPS-induced NO synthesis to appear with an abbreviated lag period phase, suggesting that BH4 availability also limits the onset of NO synthesis. In contrast to the stimulation of LPS-induced NO synthesis, observed when sepiapterin was given alone, sepiapterin became a potent inhibitor of NO synthesis in the presence of methotrexate. This is attributable to a direct inhibitory action of sepiapterin on GTP cyclohydrolase I, an activity which is only revealed after blocking the metabolism of sepiapterin to BH4. Further studies with sepiapterin, methotrexate, and N-acetylserylserotonin (an inhibitor of the BH4 synthetic enzyme, sepiapterin reductase) indicated that the BH4 is synthesized in RASMC predominantly from GTP; however, a lesser amount may derive from pterin salvage. We demonstrate that BH4 synthesis is an absolute requirement for induction of NO synthesis by LPS in vascular smooth muscle. Our findings also suggest that pterin synthesis inhibitors may be useful for the therapy of endotoxin- and cytokine-induced shock.

Nitric oxide (NO) is a potent endogenous vasodilator which appears to be identical to the endothelium-derived relaxing factor originally described by Furchgott in 1980 (1–3). NO plays a pivotal role in the regulation of vascular tone and blood pressure (4, 5). In addition to its physiological function, altered rates of NO synthesis are likely to be involved in a variety of vascular pathophysiological conditions. Indeed, overproduction of NO has been implicated as the basis for hypotension caused by bacterial endotoxin (LPS; 6, 7) and the cytokines: tumor necrosis factor (8), IL-1 (9), and IL-2 (10). While the source of vasoregulatory NO in normal physiology appears to be exclusively endothelial cells, NO synthesis can also be induced by LPS in endothelial cells (11, 12) and other vascular cell types, including smooth muscle (13–16). Preliminary studies indicate that vascular smooth muscle cells may be the dominant site of NO overproduction in LPS- and cytokine-induced vascular shock (16). Thus, an understanding of the biochemical events involved in NO synthesis induction in smooth muscle and elucidation of control points for induction and expression of this pathway should provide important new insights leading to more effective therapy of cytokine-mediated shock.

NO is synthesized from L-arginine, O₂, and NADPH by an FAD- and FMN-containing enzyme, nitric oxide synthase (NOS; EC 1.14.23.–) (17, 18). This poorly understood biochemical reaction constitutes a five-electron oxidation of one of the equivalent guanidino-nitrogens of L-arginine (19). Constitutive and inducible isotypes of NOS have been distinguished on the basis of calcium/calmodulin dependence (17, 20), inhibitor specificity (12, 21), immunogenicity (22), and substrate/cofactor requirements (23, 24). A previous distinction between constitutive and inducible isotypes of NOS was...
A requirement of the inducible isotype for tetrahydrobiopterin (BH4, 6, 7, 8-tetrahydrobiopterin (BH4), 6-(L-erythro-1,2-dihydroxypropyl)-5,6,7,8-tetrahydrobiopterin) (20, 26), a characteristic which appeared not to be shared by the constitutive enzyme (21). However, this difference is no longer accepted in view of more recent findings that constitutive NOS from brain does contain a tightly bound reduced pterin cofactor and, furthermore, that exogenous BH4 potentiates brain NOS activity (27, 28). NOS is the most recent addition to a group of only four other enzymes (29) known to utilize a bipterin cofactor.

BH4 synthesis occurs via two distinct pathways: a de novo synthetic pathway which uses GTP as a precursor and a salvage pathway for preexisting dihydropyrimidines (see Fig. 1 and Ref. 29). GTP cyclohydrolase I (EC 3.5.4.16) is the first and rate-limiting enzyme for the de novo pathway leading to synthesis of dihydropterin triphosphate. GTP cyclohydrolase I is inhibited by reduced pterins (30, 31) and can be induced by interferon-γ (IFN) in a variety of cell types including macrophages, lymphocytes, and fibroblasts (32, 33). Subsequent metabolism of dihydropterin triphosphate to BH4 occurs via tetrahydrobiopterin intermediates, which have not all been unequivocally identified; required enzymes include 6-pyruvoyltetrahydropterin synthase and sepiapterin reductase. While the dihydropyrimidines, BH2 and sepiapterin, cannot be utilized by the de novo synthetic pathway for BH4 synthesis, they are converted to BH4 via the pterin salvage pathway which utilizes dihydrofolate reductase (EC 1.5.1.3) for its final step (29).

BH4 oxidation to the quinonoid isofrm of BH2 (Q-BH2) is thought to provide two of the five electrons necessary for the oxidation of arginine to NO and citrulline (25). By analogy with other bipterin-dependent enzymes, Q-BH2 would be expected to recycle to BH4 via the NAD(P)H-requiring enzyme dihydropteridine reductase (EC 1.6.99.7) (29). BH4 recycling would thus provide for sustained NO synthesis with catalytic rather than substrate quantities of BH4.

The initial reports that NOS requires a bipterin cofactor were based on findings that BH4 stimulates NOS activity in partially purified enzyme preparations from cytosol of cytokine-activated macrophages (25, 26). BH4 levels did not appear to limit or regulate NOS activity, however, because the concentration of BH4 in cytosol from nonactivated macrophages was sufficient for maximal NOS activity (25). In any event, IFN, which markedly potentiates the induction of NO synthesis by LPS in macrophages and fibroblasts (19), also induces GTP cyclohydrolase I in these cell types (32).

Activation of the BH4 synthetic pathway in man is indicated by increased levels of plasma neopterin; the extent of activation has been useful in clinical diagnosis and prediction of therapeutic outcome of a variety of pathophysiological conditions (34). Although activation of the BH4 synthetic pathway is considered to be one of the best indicators of immune cell activation in man, and is of diagnostic and prognostic value (34), its function in immunomodulated cells is obscure. The recent addition of NO to the list of known bipterin-dependent enzymes raises the possibility that the function of BH4 in immunomodulated cells is to support co-induced NOS activity. This view is consistent with findings that inhibition of BH4 synthesis blocks immunostimulant-induced NO production by fibroblasts and endothelial cells (12, 47).

The present investigation addresses the role of BH4 synthesis in LPS-activated vascular smooth muscle cells. Using selective inhibitors of various steps in each of the two known pathways of BH4 synthesis, we demonstrate that induction of vascular smooth muscle cell NOS is strictly dependent on synthesis of BH4. Smooth muscle cell NOS arises mainly from GTP via the de novo synthetic pathway and, to a lesser extent, via pterin salvage; simultaneous inhibition of both pathways completely prevents the induction of NOS activity by LPS.

**MATERIALS AND METHODS**

**Smooth Muscle Cell (SMC) Culture—** Aortic SMC were cultured by explanting segments of the medial layer of aortas from adult male Fischer 344 rats. Aortas were removed aseptically and freed of adventitial and endothelial cells by scraping both the luminal and abluminal surfaces. Media fragments (1-2 mm) were allowed to attach to dry Primaria 25-cm² tissue culture flasks (Falcon, Oxnard, CA) which were kept moist with growth medium until cells emerged. Cultures were fed twice weekly with Medium 199 containing 10% fetal bovine serum, 2.5 mM glutamine, and penicillin (80 units/ml), streptomycin (80 µg/ml), fungizone (2 µg/ml), and the desired agents for the indicated times. In studies which assessed the effect of drugs on induced nitrite production, drugs were added simultaneously with the inducing agents. Nitrite accumulation was measured after a 16-40-h incubation period. Cell viability, as assessed by measuring cell respiration with MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide] was not significantly diminished by the highest concentrations of drugs tested: 4.5-diamo-6-hydroxypyridine (3 mM), sepiapterin (300 µM), methotrexate (30 µM), and tetrahydrobiopterin (100 µM). Nitrite was measured by addition of 100 µl of cell culture medium to 100 µl of Greiss reagent (0.5% sulfanilamide and 0.05% naphthylethylenediamine dihydrochloride in 2.5% phosphoric acid), and A₅₅₀ was immediately measured using a microplate reader (Molecular Devices, Menlo Park, CA). Nitrite concentrations were determined by comparison with standard solutions of sodium nitrite prepared in culture medium. Background nitrite levels in smooth muscle cells not exposed to cytokines were subtracted from experimental values.

**Biopterin Assay—** Total cellular bipterin (bipterin plus BH2 and
were prepared from lysates by centrifugation at 100,000 N°-nitro-L-arginine, and all other chemicals were purchased from Dr. Schirks (Jonas, Switzerland). Rat recombin. The progress of heme oxidation was continuously measured in a kinetic microplate reader (Molecular Devices, Menlo Park, CA) as a function of the concentration of L-arginine in the cell culture medium (RPMI 1640 containing 10% bovine calf serum, 2.5 mM glutamine, and 80 units/ml penicillin, 80 µg/ml streptomycin, and 2 µg/ml fungizone) supplemented with 0.2 mg/ml MTT. After 90 min equilibration of cells, media was removed by suction and cells were solubilized in 100 µl of dimethyl sulfoxide. The extent of reduction of MTT to formazan within cells was quantitated by measurement of A650 and taken as an indicator of cellular respiration.

Smooth Muscle Cell Cytosol Preparation—Confluent rat smooth muscle cells in 75-cm² culture flasks were washed twice with 5 ml of HBS. Cells were then removed with a Teflon scraper in 5 ml of HBS and pelleted at 500 X g for 10 min. The supernatant was removed and the cell pellet was lysed by three cycles of freeze-thaw (freezing in liquid nitrogen, thawing in a 37 °C water bath) in 1 ml per 10 culture flasks of a protease inhibitor mixture containing: 1 mM dithiothreitol, 0.1 mM phenylmethylsulfonyl fluoride, 5 µg/ml aprotonin, 5 µg/ml pepstatin A, and 5 µg/ml chymostatin. Cell cytosols were prepared from lysates by centrifugation at 100,000 X g for 1 h at 4 °C. Cytosols were immediately aliquoted and frozen at −70 °C until used.

NOS-Synthase Assay—Nitric oxide formation by smooth muscle cell cytosol was measured by a previously described kinetic 96-well microplate assay (12). The assay is based on the capture of NO by Fe°-myoglobin (Mb) which is subsequently oxidized to Fe°-myoglobin. The progress of hemoglobin oxidation was continuously measured in a kinetic microplate reader (Molecular Devices, Menlo Park, CA) as the rate of change in A5724. Data points were collected from all 96 wells every 16 s for 20 min at 25 °C with shaking prior to each A measurement. The slope of the best fit regression line (Almin) was used to calculate the rate of NO− synthesis. All samples contained 10 µl of crude smooth muscle cell cytosol (0.7-2.5 µg protein/ml) and final concentrations of 20 µM Mb, 500 µM L-arginine, 500 µM NADPH, 10 µM FAD, 10 µM BH4, 0.1 unit/ml dihydropteridine reductase, and 80 mM Tris, pH 7.6.

Fe°-Myoglobin Preparation—2 mM myoglobin (from horse skeletal muscle, Sigma) was reduced with an excess of sodium dithionite and immediately applied to a Sephadex G-25 column, followed by elution with 50 mM TRIS buffer, pH 7.6. Fe°-Mb was aliquoted and stored at −70 °C for up to 2 months prior to use.

Protein Assay—Protein was measured by the Bio-Rad dye-binding assay (Bio-Rad) using bovine serum albumin as a standard.

Chemicals—L-N°-Methylarginine (NMA) was synthesized as previously described (4). Sepiapterin, bioppterin, and tetrahydrobiopterin were purchased from Dr. Schirks (Jonas, Switzerland). Rat recombinant interferon-γ was from Amgen (Thousand Oaks, CA). Endotoxin (Escherichia coli, 011:B4), arginase (from bovine liver), L-arginine, N°-nitro-L-arginine, and all other chemicals were purchased from Sigma.

RESULTS

Characterization of LPS Induction of the Arginine-NO Pathway—Bacterial endotoxin (LPS) activates rat aortic smooth cells (RASMC) to synthesize and release nitrite (Fig. 2). LPS-induced nitrite synthesis increased as a function of LPS concentration and the duration of LPS exposure (Fig. 24). Characteristically, a lag phase of 6-8 h preceded induction of nitrite synthesis, followed by a progressive increase in nitrite synthesis and a tapering by 48 h. As shown in Fig. 1B, LPS-induced nitrite release is markedly potentiated by IFN. In the presence of 50 ng/ml IFN, the concentration-response curve for LPS-induced nitrite release is shifted to the left by more than 2 log units. In contrast, RASMC treated with IFN alone (CONTROL) did not activate nitrite production (<0.2 nmol/24 h). All points represent the mean of four values ± S.E. In cases where error bars are not apparent they are contained within the data symbols.

Several findings suggest that LPS-induced nitrite production by RASMC originates from arginine-derived nitric oxide. As shown in Fig. 3A, the quantity of nitrite produced by RASMC during a 24-h exposure to LPS (50 µg/ml) increases as a function of the concentration of L-arginine in the cell culture medium (EC50 ≈ 20 µM). Moreover, RASMC treated for 24 h with a combination of LPS (50 µg/ml) and IFN (50 ng/ml) in arginase-containing culture medium (4.1 units/ml) were found to produce less than 2% of the nitrite observed when similarly treated in the absence of arginase. Prototypic nitric oxide synthase inhibitors, N°-methyl- and N°-nitro-L-arginine (NMA and NNA, respectively) elicit concentration-dependent inhibitions of LPS/IFN-induced nitrite synthesis (Fig. 3B); EC50 concentrations for NMA and NNA were 30 and 100 µM versus an L-arginine concentration of 1.24 mM. This rank order of potency is similar to that which we have...
previously observed for the induced form of nitric oxide synthase in macrophages and endothelial cells (12, 21). Inhibition of LPS/IFN-induced NO synthesis by NMA was reversible by high concentrations of L-arginine (Fig. 3A), suggesting that this action of NMA is mediated specifically via competitive inhibition of the arginine-NO pathway.

To test the requirement of LPS/IFN-induced nitrite synthesis on mRNA synthesis, experiments were performed with actinomycin D (ACT D). Inclusion of ACT D (0.5 µg/ml) during a 15-h pretreatment period with LPS/IFN (50 ng/ml and 50 pg/ml, respectively), followed by washout and replacement with fresh medium, resulted in a >98% reduction in nitrite synthesis during the next 20 h. In contrast, when cells were induced with LPS/IFN for 15 h in the absence of ACT D, but ACT D was continuously present during the 20-h postinduction period, nitrite synthesis during these 20 h was completely unaffected. Thus, the action of ACT D is not mediated by nonspecific cytotoxicity. Furthermore, mRNA synthesis is required for induction of nitrite synthesis by LPS/IFN, but it is not required to sustain the activity of the NO synthetic pathway, once induced.

Induction of nitrite synthesis is coincident with the release of a factor which oxidizes Fe²⁺-heme myoglobin (Mb) to met-myoglobin; Fe²⁺-heme iron oxidation is a prototypic reaction of NO and one which has served as the basis for a useful kinetic assay for NO (37, 12). As shown in Fig. 4, RASMC that had been pretreated for 16 h with LPS/IFN in L-arginine-containing culture medium oxidized Mb at a rate 7-fold greater than control cells. While the basal rate of Mb oxidation observed with untreated RASMC is unaffected by addition of 1 mM NMA, the increased rate of Mb oxidation by LPS/IFN-pretreated cells is abolished. Moreover, LPS/IFN-activated Mb oxidation by RASMC is markedly diminished (≈85%) by removal of L-arginine from the cell culture medium.

Effect of Immunostimulants on total biopterin content (oxidized plus reduced forms) of rat aortic smooth muscle cells. Biopterin was assayed in groups of confluent cells that were either untreated (basal), or treated for 12 h with LPS (30 µg/ml), interferon-γ (IFN, 50 ng/ml), the combination of LPS and IFN, or the combination of LPS, IFN, and 2,4-diamino-6-hydroxypteridine (DAHP, 3 mM). Bars indicate mean values ± S.E. of three to four replicate treatments, and data are expressed as a fraction of protein concentration. n.d. indicates no detectable biopterin (<0.2 ng/ml).

Effect of Tetrahydrobiopterin (BH4) Synthesis Modulators on Induction of Nitric Oxide Synthesis—In addition to blocking BH4 synthesis, DAHP elicited a concentration-dependent inhibition of LPS/IFN-induced nitrite synthesis (EC₅₀ = 1.6 nM; see inset to Fig. 6). The influence of DAHP (2 nM) on the time course of nitrite production by LPS/IFN-activated RASMC is shown in Fig. 6. DAHP inhibited nitrite production at all times studied. Inhibition by DAHP was completely overcome by co-administration of sepiapterin (SEP, 100 µM), an agent which is a substrate for BH4 synthesis via the dihydrofolate reductase-dependent pterin salvage pathway (see Fig. 1) and therefore would be expected to restore BH4 synthesis during GTP cyclohydrolase 1 blockade. In the pres-
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Fig. 6. 2,4-Diamino-6-hydroxypuridine (DAHP) inhibits the activation of nitrite production elicited in rat aortic smooth muscle cells by LPS (50 μg/ml) in combination with interferon-γ (50 ng/ml). Nitrite accumulation in the cell culture medium was assayed as a function of time after addition of LPS/IFN alone (CONTROL) or LPS/IFN in the presence of DAHP (3 mM), DAHP plus sepiapterin (SEP, 100 μM), or DAHP plus sepiapterin and methotrexate (MTX, 10 μM). Note that DAHP inhibits induced nitrite synthesis by a mechanism which is reversed by sepiapterin and restored by MTX. Inset, concentration-response relationship for inhibition by DAHP of 24-h nitrite accumulation elicited by LPS/IFN. All points represent mean values ± S.E. (n = 4).

Fig. 7. Concentration-response relationships showing the influence of sepiapterin and methotrexate on LPS/IFN-induced nitrate production by rat aortic smooth muscle cells. Nitrate accumulation in the cell culture medium was assayed after a 24-h exposure to the concentrations of DAHP, DAHP plus sepiapterin (SEP), 100 μM sepiapterin and methotrexate (MTX, 10 μM). Note that DAHP inhibits induced nitrite synthesis by a mechanism which is reversed by sepiapterin and restored by MTX. Inset, concentration-response relationship for inhibition by DAHP of 24-h nitrate accumulation elicited by LPS/IFN. All points represent mean values ± S.E. (n = 4).

ence of methotrexate (10 μM, MTX), a selective inhibitor of dihydrofolate reductase, SEP no longer restored nitrite synthesis to DAHP-treated, LPS/IFN-activated, RASM.

In the absence of other biopterin synthesis modulators, SEP elicited a concentration-dependent potentiation of LPS/IFN-induced NO synthesis by RASM (Fig. 7A, open circles). At a concentration of 300 μM, SEP more than doubled 24-h LPS/IFN-induced nitrite production. In contrast, when 10 μM MTX was present, SEP elicited a dose-dependent and complete inhibition of induced NO synthesis (EC50 = 30 μM; Fig. 7A, closed circles). These findings are consistent with prior reports demonstrating that in addition to sepiapterin being a substrate for BH4 synthesis via the pterin salvage pathway, it is also a potent inhibitor of GTP cyclohydrolase I (38, 39) and therefore a blocker of de novo BH4 synthesis. Thus, when the metabolism of SEP to BH4 is blocked by MTX, inhibition of de novo BH4 synthesis by SEP is revealed.

MTX, alone, diminished LPS/IFN-induced NO synthesis to a maximal extent ranging from 10 to 40% (n = 4), depending on the RASM preparation studied. The maximal inhibitory effect of MTX was observed even with 0.3 μM, the lowest concentration studied (Fig. 7B, open circles). This potency of MTX is consistent with its established Ki for inhibition of dihydrofolate reductase in the nanomolar range (40). In the presence of 100 μM SEP, MTX (0.3–30 μM) elicited a dose-dependent and complete inhibition of LPS/IFN-induced nitrite synthesis with an EC50 < 1 μM (Fig. 7B, filled circles). The finding that BH4 synthesis becomes 100% dependent on dihydrofolate reductase-dependent pterin salvage in the presence of SEP can be explained by SEP's inhibitory action on GTP cyclohydrolase I. Thus, when de novo synthesis of BH4 is blocked by SEP, MTX-inhibitable pterin salvage is the only alternative route for cellular BH4 synthesis.

Media supplementation with the combination of 100 μM hypoxanthine and 40 μM thymidine did not alter the ability of MTX to reduce LPS/IFN-induced NO synthesis, indicating that inhibition of nucleotide synthesis does not contribute to the inhibitory effect of MTX on induced NO synthesis. Moreover, inhibition of induced nitrite synthesis by 10 μM MTX did not result from a generalized cytostatic action, because 40-h exposure did not markedly reduce RASM viability as determined by MTT cell respiration assay (88.8 ± 6.2% of control, n = 4). In contrast, a 40-h exposure to LPS/IFN (50 and 30 μg/ml, respectively) was found to abolish RASM respiration, both in the absence and presence of 1 mM SEP (3.2 ± 2.2% and 5.4 ± 5.7% of control, respectively; n = 4). Consistent with the view that NO is the mediator of LPS/IFN-induced respiratory inhibition in RASM, we observed that 10 μM MTX in the absence of 100 μM SEP (which blocks LPS/IFN-induced NO synthesis, see Fig. 7B) significantly reduced LPS/IFN-induced cytotoxicity, whereas MTX alone afforded little protection (52.4 ± 3.5% versus 9.9 ± 5.7% of control, respectively).

Detailed study of the the time course of induction of NO synthesis by LPS/IFN revealed that the potentiation of NO synthesis caused by 100 μM SEP was much more marked at early time points (Fig. 8). While SEP enhanced NO production 2-fold at 24 h, production was enhanced by over 10-fold after only 6 h. Moreover, results shown in Fig. 8 demonstrate that, in the presence of SEP, induction of NO synthesis by LPS/IFN is shifted to earlier times and rises at a steeper rate. These findings suggest that BH4 limits both the onset and magnitude of LPS/IFN-induced NOS activity. Fig. 8 also shows that in the presence of 10 μM MTX, SEP does not enhance the rate of induction of NO synthesis. Indeed, consistent with results depicted in Fig. 6, the combination of MTX and SEP was found to completely abolish induction of...
NO synthetic activity by LPS/IFN.

In contrast, with SEP, BH4 elicited only a small potentiation of LPS/IFN-induced nitrite synthesis at the highest concentration studied (15% with 500 μM; see Fig. 9). It is apparent that BH4 can be utilized to support NOS activity, because BH4 elicited a concentration-dependent restoration of LPS/IFN-induced NO synthesis to cells in which de novo BH4 synthesis has been blocked with DAHP (Fig. 9). However, restoration of NO synthesis to DAHP-treated cells was not observed when BH4 was administered in combination with 10 μM MTX. This finding suggests that BH4 does not enter the cell as BH4 per se. Rather, BH4 being unstable, likely enters the cell as a dihydropterin, requiring reduction by dihydrofolate reductase back to BH4 for NOS utilization.

N-Acetylseryltonin (NAS) has been shown to block BH4 synthesis in cells by selectively inhibiting sepiapterin reductase (41), an enzyme which catalyzes two consecutive NADPH-dependent keto-oxidation reactions, the final step in BH4 synthesis from GTP (42). As shown in Fig. 10, NAS elicited a concentration-dependent inhibition of LPS/IFN-induced nitrite synthesis in RASMC; the maximal extent of inhibition was ≈ 60% with an EC50 of ≈ 20 μM. When 10 μM MTX was simultaneously present, NAS caused a near-complete inhibition of LPS/IFN-induced nitrite synthesis, also with a 20 μM EC50. This suggests that NAS is acting by reducing the cellular levels of BH4.

DISCUSSION

Recent findings suggest that overproduction of NO within the blood vessel wall is a major cause of LPS- and cytokine-induced hypotension (6–10). Since it is likely that the principal source of LPS-induced NO synthesis in the endotoxic vessel wall is smooth muscle (15, 16), we have sought to elucidate mechanisms which contribute to this induction process. Results of the present study clearly indicate that BH4 synthesis is a necessary event for induction of NO synthesis in vascular smooth muscle.

We have measured the accumulation of nitrite, a stable oxidation product of the unstable free radical nitric oxide, to quantitate induction of NO synthesis. The validity of this measurement is supported by our findings that induced nitrite synthesis is: 1) arginine-dependent (Fig. 3A), 2) potently inhibited by selective NOS inhibitors in an arginine-reversible fashion (Fig. 3, A and B), and 3) associated with the production of a factor which resembles NO, in that: (a) it oxidizes Fe2+-myoglobin, (b) its synthesis requires arginine, and (c) is blocked by NMA (Fig. 4).

LPS-induced nitrite production by vascular smooth muscle cells occurs after a lag-phase of 6–8 h, during which time mRNA synthesis is required. While NO synthase is clearly one of the genes transcribed in this interval,5,6 additional gene products may include secondary cytokines, cytokine receptors, and possibly synthetic enzymes for necessary cofactors such as BH4.

LPS-induced NO synthesis is markedly potentiated by IFN, both in terms of magnitude and LPS concentration required for half-maximal activation (see Fig. 2). Synergistic effects of IFN with IL-1α and tumor necrosis factor-α for induction of smooth muscle nitrite synthesis have also been observed (9, 16). Since sepsis is a condition in which all of these cytokines are released in concert (43), potent stimuli abound during sepsis for induction of NO synthesis in vascular smooth muscle.

Synergistic actions between IFN and other immunomodulators for induction of NOS activity have been described for various cell types including: macrophages (44), endothelial cells (11), hepatocytes (45), and fibroblasts (46). The mechanism of synergy between IFN and LPS for induction of NO synthesis is presently unknown. Previous studies demonstrating induction of GTP cyclohydrolase I by IFN in vascular and circulating blood cells (32, 33) raise the possibility that synergistic induction of NO synthesis by LPS and IFN may depend, at least in part, on the combined appearance of each of two factors which are required for NO synthesis: NOS and the enzymatic machinery for BH4 production.

In the present studies we have found that RASMC do not contain detectable quantities of BH4; however, BH4 accumulates upon treatment with LPS. IFN potentiated the LPS-induced accumulation of BH4 by ≈ 2-fold, similar to the degree of enhancement by IFN of LPS-induced NO synthesis. Interestingly, IFN on its own affected neither LPS-induced biopterin accumulation nor NO synthesis, therefore its action can be clearly attributed to synergism with LPS for both effects. The inability of IFN to enhance BH4 levels in RASMC came as a surprise in that it has been shown to do so in macrophages, lymphocytes, and fibroblasts (32, 33), where it acts by increasing activity of GTP cyclohydrolase I, the first and rate-limiting enzyme for de novo synthesis of BH4. While it is likely that induction of GTP cyclohydrolase I by LPS/IFN is also the basis for elevated levels of BH4 in vascular smooth muscle, direct assay of GTP cyclohydrolase

5 S. Gross, unpublished observation.

6 The induction of vascular smooth muscle cell NO synthesis is coincident with the appearance of a 120–125-kDa protein that is undetectable in noninduced cells and which purifies with NOS activity.
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I activity will be required to confirm this possibility. Given our finding in the present study that BH4 content of RASMC limits LPS-induced NO synthesis, one contribuant to the synergistic effect of LPS and IFN on induced NO synthesis is a synergistic elevation of BH4 levels. It should be noted, however, that additional mechanisms, at the level of NO synthase transcription, appear also to contribute to the synergism between LPS and IFN for NO synthesis induction.

Pharmacological probes were used to assess the contribution of BH4 synthetic pathways to induced NO production in LPS-activated vascular smooth muscle cells. Results strongly indicate that synthesis of BH4 is an absolute requirement for induction of NOS activity. A significant reduction in LPS/IFN-induced smooth muscle NO synthesis was observed upon inhibition of the de novo pathway for BH4 synthesis from GTP, and to a lesser extent upon inhibition of BH4 synthesis via the pterin salvage pathway. In these experiments, de novo BH4 synthesis was selectively abolished with DAHP and NAS, agents previously shown to be selective inhibitors of GTP cyclohydrolase I (47) and SEPReductase (41), respectively. These agents attenuated induced NO synthesis to a maximal extent ranging from 60 to 90% of control values (see Figs. 6 and 10). This range of inhibitor potency observed with different preparations or passages of cultured smooth muscle cells may reflect differences in cell metabolic status or, more likely, arise from lot-to-lot differences in t he pterin content of serum used for supplementation of the cell culture medium. Neither DAHP nor NAS inhibited smooth muscle cell respiration, thereby ruling out nonspecific cytotoxicity as a mechanism of inhibition of induced NO synthesis. Moreover, inhibition of NO synthesis by DAHP was circumvented by BH4 and by SEPReductase, substrates for the pterin salvage pathway (see Figs. 7 and 9). The reversal of inhibition by DAHP with BH4 and SEPReductase, an inhibitor ofthe terminal enzyme for dihydropterin salvage, dihydrofolate reductase. Thus, both SEPReductase and BH4 require metabolism by dihydrofolate reductase to a product which can override the inhibition of induced NO synthesis by DAHP. These findings strongly suggest that the mechanism of NO synthesis inhibition by DAHP is specifically via blockade of de novo BH4 synthesis.

When administered alone, MTX maximally inhibited LPS/IFN-induced NO synthesis by 10–40% of the control level. However, administration of MTX in combination with an inhibitor of de novo BH4 synthesis (DAHP, NAS, or sepiapterin) completely abolished the ability of LPS/IFN to induce smooth muscle NO synthesis. It is noteworthy that MTX, at concentrations up to 300 μM, does not attenuate NOS activity of crude LPS/IFN-induced vascular smooth muscle cytosol. Thus, the mechanism by which MTX inhibits induced NOS activity appears to be specifically via inhibition of BH4 synthesis by the dihydrofolate reductase-dependent pterin salvage pathway. These findings suggest that the BH4 which is required for induced NOS activity arises predominantly from GTP via de novo synthesis and, to a lesser extent, via pterin salvage. Whether this is also the case in vivo remains to be tested.

A major conclusion of the present studies is that LPS/IFN-induced NO synthesis by vascular smooth muscle cells is rate-limited by BH4 availability. Providing excess BH4, via administration of the BH4 precursor SEPReductase, or BH4 itself, increased LPS/IFN-induced NO synthesis to 200% and 117% of control values, respectively (see Figs. 7 and 9). While the mechanism of potentiation of LPS-induced NOS activity by SEPReductase and BH4 is most likely via enhanced cofactor availability, we currently cannot rule out the possibility that these agents potentiate induced levels of NOS. The lower efficacy of BH4, relative to SEPReductase, for potentiating induced NO synthesis probably reflects a rapid oxidation of BH4 in cell culture medium and a poor cellular uptake of the BH2 formed. It is noteworthy that the lag period which precedes the appearance of NO synthesis by LPS/IFN-treated smooth muscle cells is abbreviated in the presence of sepiapterin, indicating that NOS induction may actually occur earlier than is evident from measurement of nitrite accumulation in the cell culture medium (see Fig. 8).

The abolition of induced NO synthesis in smooth muscle cells treated with the combination of DAHP and MTX is clearly attributable to inhibition of the synthesis of biopterin cofactor required for NOS activity, rather than a diminished level of NOS enzyme. Indeed, cytosolic NOS activity in cells treated with LPS/IFN in the presence of DAHP/MTX (3 mM and 10 μM, respectively) is actually greater than that in cells treated for an equivalent time with LPS/IFN alone. Moreover, crude cytosolic NOS activity in cells induced in the presence of these pterin synthesis inhibitors showed a much more marked dependence on added BH4 (90%–dependent) than control induced cells (<20%–dependent).

Although the de novo synthetic pathway for BH4 synthesis has been shown to be inducible by immunological stimuli in various cell types, the function of immunologically induced BH4 has remained elusive. The present findings are consistent with the view that a major function for this induced BH4 synthesis may be to support co-induced NOS. This is supported by the observation that the addition of BH4 to the combination of DAHP and MTX treated cells results in an almost complete inhibition of NO synthesis. The abolition of induced NO synthesis in smooth muscle cells treated with the combination of DAHP and MTX is clearly attributable to inhibition of the synthesis of biopterin cofactor required for NOS activity, rather than a diminished level of NOS enzyme. Indeed, cytosolic NOS activity in cells treated with LPS/IFN in the presence of DAHP/MTX (3 mM and 10 μM, respectively) is actually greater than that in cells treated for an equivalent time with LPS/IFN alone. Moreover, crude cytosolic NOS activity in cells induced in the presence of these pterin synthesis inhibitors showed a much more marked dependence on added BH4 (90%–dependent) than control induced cells (<20%–dependent).

Much attention has been focused recently on the potential of arginine-based NOS inhibitors for therapy of clinical conditions which have been associated with NO overproduction, i.e. septic and cytokine-induced hypotension (6–10). Therefore, it is reasonable to consider biopterin synthesis inhibitors as a pharmacological strategy which may be employed to limit inducible NO synthesis. Since neither DAHP (300 μM), MTX (10 μM), nor SEP (300 μM) alter vasorelaxation to endothelium-dependent dilators in vitro over a period of at least 1–2 h, it is conceivable that biopterin synthesis inhibitors can be used to selectively abolish LPS- and cytokine-induced NO synthase activity. Although biopterin synthesis inhibitors appear to have no acute inhibitory effect on the constitutive NOS of endothelial cells, chronic treatment has recently been shown to block endothelial NO synthesis (48). The apparent selectivity of biopterin synthesis inhibitors for induced NOS activity may reflect a requirement of induced NOS for de novo BH4 synthesis, whereas constitutive NOS may already be replete with BH4. Thus, it is conceivable that toxicities which may arise from treatment with nonselective arginine-based NOS synthase inhibitors (which inhibit the physiological constitutive production of NO in brain and endothelium in addition to pathophysiological NO overproduction) may be avoided with biopterin synthesis inhibitors. We speculate that biopterin synthesis inhibitors will have an important place in...
the treatment of conditions arising from NO overproduction.

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