0026-895X/93/010006-05$03.00/0

MOLECULAR PHARMACOLOGY, 43:6-10

ACCELERATED COMMUNICATION

Tetrahydrobiopterin Is Required for Cytokine-Induced Nitric Oxide Production in a Murine Macrophage Cell Line (RAW 264)

NAOKI SAKAI, SEYMOUR KAUFMAN, and SHELDON MILSTIEN

Laboratory of Neurochemistry, National Institute of Mental Health, Bethesda, Maryland 20892

Received September 14, 1992; Accepted October 8, 1992

SUMMARY

The murine macrophage cell line RAW 264 constitutively synthesizes tetrahydrobiopterin (BH_4), the cofactor required for the hydroxylation of the aromatic amino acids and for the production of nitric oxide. Stimulation of the cells with interferon-γ and lipopolysaccharide induced the production of nitric oxide and increased BH_4 levels further. When the cells were stimulated in the presence of 2,4-diamino-6-hydroxypyrimidine (DAHP), an inhibitor of BH_4 biosynthesis, biopterin levels decreased by 90% within 6 hr, whereas nitrite production was essentially unaffected. Pretreatment of the cells for 12 hr with DAHP decreased intracellular BH_4 concentrations by >95% yet inhibited the cytokine-stimulated production of nitric oxide by only 50%. However, pretreatment with DAHP plus *N*-acetylserotonin, an inhibitor of sepiapterin reductase, the terminal enzyme of the BH_4 biosynthetic pathway, decreased biopterin levels by >99% and inhibited nitric oxide synthesis by 90%. This inhibition could be reversed by loading the cells with dihydrobiopterin, a precursor of BH_4 via the dihydrofolate reductase salvage pathway. In addition, these studies revealed that *N*-acetylserotonin has a direct inhibitory effect on nitric oxide synthesis, acting in a BH_4-independent manner. The results presented here support previous suggestions, based on experiments with isolated enzymes, that BH_4 is absolutely required for cytokine-stimulated nitric oxide production in macrophages and they suggest that only a small fraction of the total intracellular BH_4 pool in macrophages is utilized in the production of fully active nitric oxide synthase.

NO is produced by the action of at least two different forms of NOS, which catalyze the conversion of arginine to citrulline and NO in a complex hydroxylation reaction that requires molecular oxygen and a bewildering array of redox cofactors, including NADPH, FAD, FMN, tetrahydrobiopterin (1), and even heme iron (2). One type of NOS is also Ca^{2+}/calmodulin dependent and constitutively present in a variety of different cells, including endothelial cells where NO plays a role in the control of vascular tone (3), in platelets where it regulates aggregation (4), in hippocampal neurons where it plays a role in long term potentiation (5), and in cerebellar neurons where it has been proposed to act as a neurotransmitter (6). These multiple actions of NO are thought to be mediated by cGMP produced via NO activation of guanylate cyclase (1). A cytokine-inducible form of NOS, which is Ca^{2+}/calmodulin independent, has been found in macrophages (7), vascular smooth muscle cells (8), fibroblasts (9), endothelial cells (10), renal mesangial cells (11), and hepatocytes (12). Although it was originally suggested that cytokine-induced NOS in macrophages plays an important role in tumoricidal/bactericidal activity via generation of the cytotoxic agent NO (13), the function of cytokine-induced NOS activity in other cells is still unclear.

When BH_4 was first shown to be required for the NOS reaction catalyzed by the isolated enzyme (14), it seemed logical to assume that it might participate in this reaction in the same manner as had been previously established for the aromatic amino acid hydroxylases, where its oxidation is tightly coupled to the reduction of molecular oxygen (15). However, this has remained a controversial point because stoichiometric oxidation of BH_4 does not appear to occur during the NOS reaction (16). On the other hand, there is some evidence that BH_4 is required for NO synthesis in intact cells, because inhibition of *de novo* BH_4 biosynthesis (see Fig. 1) has been shown to result in inhibition of NOS activity in cytokine-stimulated human fibroblasts (9) and in endothelial cells (17). Recent findings that complicated studies of the role of BH_4 in NO production showed that, unlike other BH_4-dependent hydroxylases, purified NOS contains tightly bound BH_4 (3, 18) and its removal leads to irreversible loss of activity (18). Although the require-

N.S. was supported by the National Institutes of Health Intramural AIDS Targeted Antiviral Program.

ABBREVIATIONS: NO, nitric oxide; BH_2, 6-(1′,2′-dihydroxypropyl-(L-*erythro*)-7,8-dihydropterin)(dihydrobiopterin); BH_4, 6(*R*)-(1′,2′-dihydroxypropyl-(L-*erythro*)-5,6,7,8-tetrahydropterin) (6(*R*)-tetrahydrobiopterin); DAHP, 2,4-diamino-6-hydroxypyrimidine; IFN-γ, interferon-γ; LPS, lipopolysaccharide; NAS, *N*-acetylserotonin; NOS, nitric oxide synthase.

ment for BH_4 was first discovered using NOS isolated from stimulated murine macrophages (14), it has not yet been demonstrated that intracellular BH_4 is required for the generation of NO and its metabolites by macrophages. In this report, we show that it is necessary to decrease intracellular BH_4 concentrations by >99% to inhibit cytokine-inducible production of NO in macrophages.

Experimental Procedures

Materials. Recombinant murine IFN-γ was obtained from R & D Systems (Minneapolis, MN). LPS from *Escherichia coli* (055:B5), DAHP, and NAS were from Sigma (St. Louis, MO). BH_2 was obtained from Dr. B. Schircks Laboratories (Jona, Switzerland). All other reagents were of the highest quality and were obtained from local sources.

Cell culture. RAW 264 cells, a mouse monocyte-macrophage cell line (obtained from Dr. Robert Aksamit, LGCB, National Institute of Mental Health), were cultured in minimum essential medium containing 10% fetal bovine serum, 4 mM L-glutamine, 100 units/ml penicillin, and 100 μg/ml streptomycin and were maintained in humidified 5% CO_2/95% air at 37°. Cells were seeded in six-well clusters (Falcon) at a cell density of 3–4 × 10^6/well and were allowed to adhere for 90 min. Culture medium was then replaced with 3 ml of fresh medium in the absence or presence of 5 mM DAHP, 5 mM NAS, or both DAHP and NAS, with cytokines as indicated. Because RAW cells are very sensitive to the cytotoxic activity of cytokines (19), preliminary experiments established that 25 units/ml IFN-γ and 1 ng/ml LPS gave maximum responses with acceptable levels of cytotoxicity. In some experiments, cells were pretreated for 12 hr with DAHP and NAS to deplete cellular BH_4 levels (see Fig. 1 and Results). Culture medium was then replaced with 3 ml of fresh medium containing the same concentrations of inhibitors plus IFN-γ and LPS where indicated in the figure legends. At various times after addition of cytokines, medium was removed and the cells were harvested by scraping followed by several washes with 10 ml of Dulbecco's phosphate-buffered saline (without calcium and magnesium). Medium and harvested cells were frozen at −70° and nitrite, protein, and pterin levels were measured as soon as possible.

Preparation of macrophage extracts. The cell pellets were resuspended in 300 μl of extract buffer (20 mM Tris·HCl, pH 7.4, 0.1 mM EDTA, 1 mM dithiothreitol, 10% glycerol, 0.1% Tween 20) and then disrupted by sonication (3 × 10 sec). After centrifugation at 16,000 × *g* for 20 min at 4°, the total pterin and protein concentrations in the supernatants were measured as described below.

Measurement of intracellular biopterin. Cell extracts (100 μl) were deproteinized by mixing with 10 μl of 30% trichloroacetic acid. After centrifugation, 75 μl of supernatants were mixed with 75 μl of 0.2 M H_3PO_4 and 10 mg of MnO_2 were added to oxidize reduced pterins to their fluorescent aromatic forms. After 10 min at room temperature, samples were filtered and analyzed by reverse phase high performance liquid chromatography with fluorometric detection, as described previously (20). The fraction of the total intracellular biopterin pool that was present as the fully reduced, tetrahydro form was determined by differential oxidation with iodine as described previously (21). The BH_4 content was determined in selected samples from each experiment and, except for treatments with BH_2 and methotrexate (see Results), was always >95%.

Nitrite assay. Nitrite levels in media, which reflect intracellular NOS activity (22), were determined by the Griess reaction using a microplate reader (Biotek EL340), essentially as described (23).

Protein determination. Protein concentrations in cell extracts were measured by the Coomassie Blue dye-binding method, with a commercial kit (Pierce).

Results

In agreement with previous studies (19), treatment of RAW cells with IFN-γ and LPS induced the synthesis of NO, as measured by nitrite production (Fig. 2B). Unlike other cell types with inducible NOS activity, murine macrophages constitutively synthesize relatively high levels of BH_4, which are further increased by cytokine treatment (Fig. 2A). Previously, to demonstrate a requirement for BH_4 in the production of cytokine-induced NO in various cell types, several laboratories have utilized DAHP, an effective competitive inhibitor of GTP cyclohydrolase (24), the rate-limiting enzyme in BH_4 biosynthesis (Fig. 1) (25). We found that treatment of RAW cells with DAHP, in the presence or absence of cytokines, resulted in 90% depletion of intracellular BH_4 levels within 6 hr (Fig. 2A). In contrast to the effects of DAHP on NOS activity in other cells, there was no corresponding inhibitory effect on the rate of the IFN-γ/LPS-induced production of nitrite (Fig. 2B). These results suggest that, although the turnover of BH_4 in macrophages is rapid and comparable to that found previously for neurons and other cells with known BH_4-dependent hydroxylation reactions (26, 27), either BH_4 is not required for NOS activity in RAW cells or the enzyme is fully active at very low concentrations.

In order to deplete intracellular BH_4 levels more completely, we examined the effect of pretreating RAW cells with DAHP before stimulation with cytokines. Treatment with 5 mM DAHP for 12 hr resulted in a decrease in BH_4 levels of 96% (Fig. 3A). In this case, IFN-γ/LPS-stimulated nitrite production was inhibited by 52%, when measured after a subsequent 12-hr treatment with the cytokines (Fig. 3B). Thus, intracellular BH_4 levels can regulate NOS activity, but even 4% of the original constitutive BH_4 concentration is still sufficient for generation of significant amounts of active NOS.

Another inhibitor of the BH_4 biosynthetic pathway that has been used to deplete BH_4 levels in several cellular systems is NAS, an inhibitor of sepiapterin reductase (see Fig. 1) (28). However, in contrast to other types of cells where NAS effectively blocks synthesis of BH_4, treatment of RAW cells with

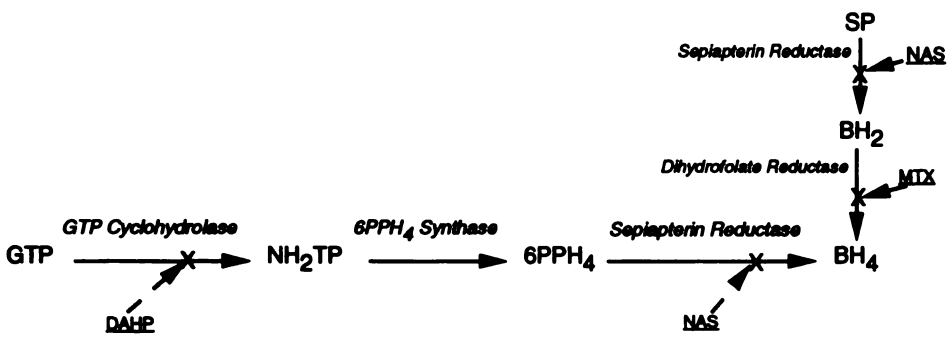

Fig. 1. Proposed pathway for biosynthesis of BH_4 in RAW cells. Although the pathway has not been completely elucidated in these cells, it is generally agreed that GTP cyclohydrolase catalyzes the initial reaction and sepiapterin reductase catalyzes the terminal reaction in the pathway in rodents. In the salvage pathway, sepiapterin is converted to BH_4 by sequential NADPH-dependent reductions catalyzed by sepiapterin reductase and dihydrofolate reductase. *NH_2TP*, dihydroneopterin triphosphate; *$6PPH_4$*, 6-pyruvoyltetrahydropterin; *SP*, sepiapterin; *MTX*, methotrexate.

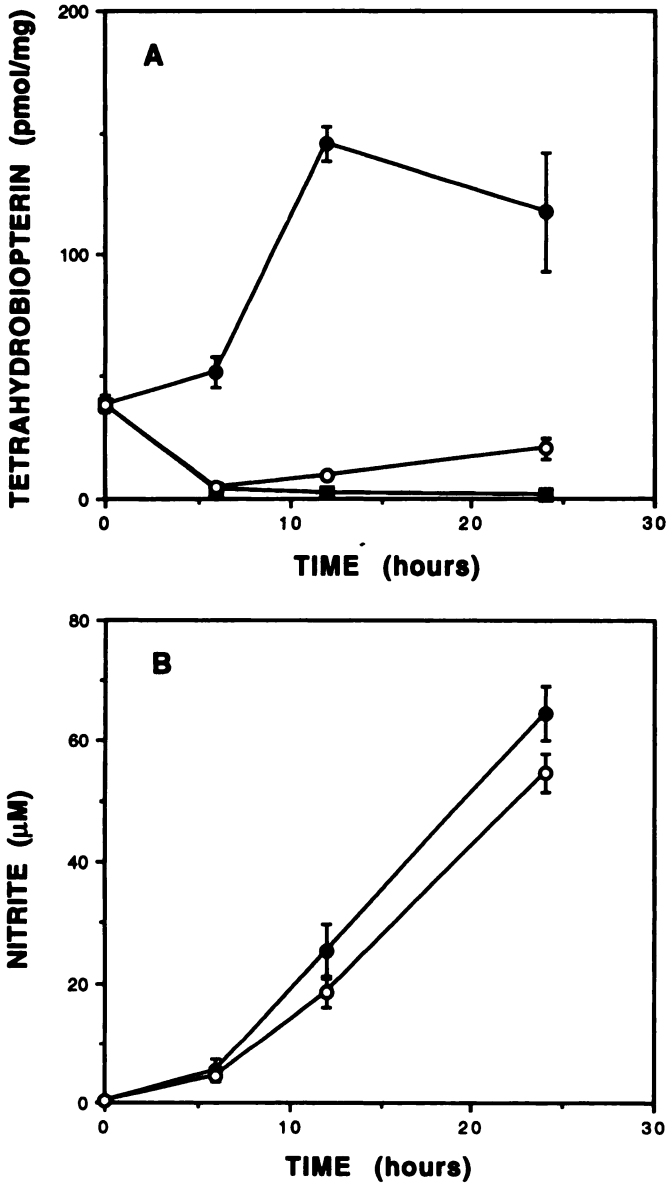

Fig. 2. Effect of DAHP on biopterin levels and nitrite production by RAW cells. RAW cells were stimulated with IFN-γ (25 units/ml) and LPS (1 ng/ml) in the absence or presence of DAHP (5 mM). Biopterin in cell extracts (A) and levels of nitrite in medium (B) were measured as described in Experimental Procedures. Data are the means ± standard deviations from two experiments carried out in triplicate (six determinations). ●, IFN-γ and LPS only; ○, IFN-γ and LPS plus DAHP; ■, DAHP only.

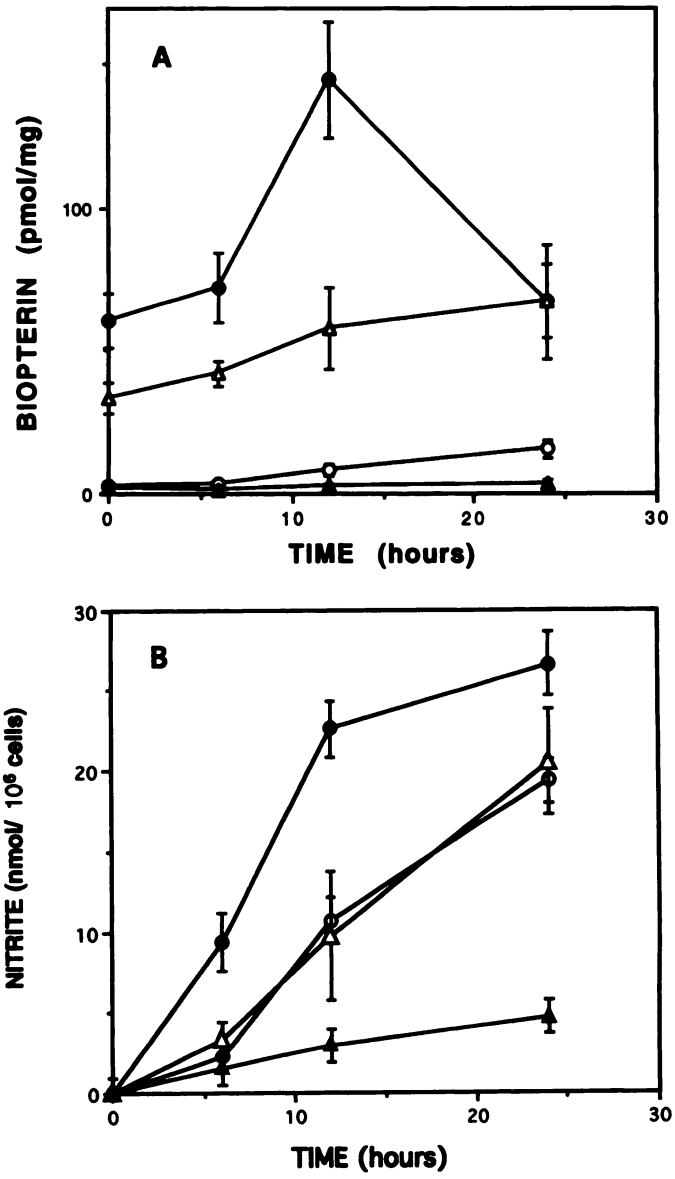

Fig. 3. Biopterin levels and nitrite production in cytokine-stimulated RAW cells pretreated with inhibitors of biopterin synthesis. Cells were treated with DAHP (5 mM), NAS (5 mM), or DAHP plus NAS for 12 hr before stimulation with IFN-γ (25 units/ml) and LPS (1 ng/ml). The inhibitors were also present at the same concentration during the stimulation period. At the indicated times, total intracellular biopterin concentration (A) and nitrite production (nmol/10^6 cells)(B) were measured as described in Experimental Procedures. Data are the means ± standard deviations from two typical experiments carried out in triplicate (six determinations) and repeated seven times. ●, IFN-γ and LPS only; ○, IFN-γ and LPS plus DAHP; △, IFN-γ and LPS plus NAS; ▲, IFN-γ and LPS plus DAHP and NAS.

NAS had very little effect on BH_4 levels (data not shown). As would be expected for inhibition of sequential reactions in a metabolic pathway, the combination of DAHP and NAS was more effective at inhibiting BH_4 synthesis than was either inhibitor alone, resulting in depletions of >99% to barely detectable levels after 12 hr (Fig. 3A). When these severely BH_4-deficient cells were treated with IFN-γ and LPS, nitrite production was dramatically inhibited (Fig. 3B). Interestingly, 5 mM NAS alone inhibited NOS activity in RAW cells, independently of any demonstrable effects on BH_4 levels. In preliminary experiments, we found that NAS and serotonin inhibited *in vitro* activity of NOS.[1]

To demonstrate that the effects of BH_4 depletion on NOS activity in RAW cells were indeed due to the measured changes in BH_4 levels, it was necessary to show that repletion with exogenous BH_4 can reverse the effects. This repletion can be accomplished in several ways, i.e., addition of BH_4 to the medium, treatment with sepiapterin, which is converted to BH_4 by the salvage pathway (29), or addition of BH_2, which can be reduced to BH_4 by the action of dihydrofolate reductase (30). Exogenous BH_4 is cytotoxic (31) and also is not stable enough to be used during the activation period. It was also not possible

[1] K. C. Campos, N. Sakai, S. Kaufman, and S. Milstien, unpublished observations.

to use sepiapterin as a source of intracellular BH_4 because in these studies we were testing the effects of NAS, a potent inhibitor of sepiapterin reductase, which is also an essential component of the salvage pathway (29). However, we found that exogenous BH_2 was effectively taken up by RAW cells and converted to BH_4. Treating the cells for 6 hr with BH_2 (100 μM) resulted in intracellular BH_4 levels of >1000 pmol/mg. As shown in Fig. 4, the inhibitory effect of 5 mM DAHP/5 mM NAS on nitrite production was reversed by exogenous BH_2. The fact that this reversal was due to the dihydrofolate reductase-catalyzed reduction of BH_2 to BH_4 was demonstrated by the finding that methotrexate, a potent dihydrofolate reductase inhibitor, blocked the stimulatory effect of BH_2 without having any effect on nitrite production by itself. As discussed above, NAS has a BH_4-independent inhibitory effect on NOS activity. Thus, the reversal of the inhibition of nitrite production by the combination of DAHP and NAS, although apparently less than 100%, was in fact equal to the stimulation expected on the basis of the BH_4-independent inhibition found with NAS alone.

Discussion

The only known role for BH_4 in macrophages is in the production of NO, where its precise mode of action in the enzymatic oxidation of arginine is still enigmatic (16). The requirement for BH_4 in the NOS reaction was discovered by Tayeh and Marletta (14), who showed that the activity of a partially purified preparation from macrophages lost its ability to oxidize arginine after removal of low molecular weight components and who found that the activity could be restored by the addition of BH_4. To date, it has not been demonstrated that intracellular BH_4 regulates NOS activity in macrophages, because murine macrophages constitutively synthesize high levels of BH_4 (Fig. 2A). BH_4-dependent synthesis of NO has been demonstrated in several systems, including cytokine-activated murine fibroblasts (9), calcium ionophore-stimulated porcine aortic endothelial cells (32), and cytokine-activated murine brain endothelial cells (17), by the use of the GTP cyclohydrolase inhibitor DAHP (see Fig. 1). However, BH_4 levels and biosynthesis rates in these cells are very low before activation, whereas RAW cells constitutively synthesize BH_4 and maintain an intracellular concentration of approximately 2 μM. Treatment of RAW cells with DAHP effectively inhibited BH_4 synthesis, resulted in 90% depletion of intracellular BH_4 levels within 6 hr and also prevented the cytokine-induced increase in BH_4 (Fig. 2A). However, the remaining BH_4 was still present at an intracellular concentration of approximately 0.2 μM, a concentration similar to the reported apparent K_m value of 0.2 μM for NOS (33). Thus, it was not surprising that, in contrast to the effects of DAHP-dependent BH_4 depletion on inhibition of NOS activity in other cells, there was not a significant inhibition of the rate of the cytokine-induced production of NO in RAW cells (Fig. 2B). However, when BH_4 levels were further decreased to 4% of control levels by preincubation of the cells for 12 hr with DAHP, cytokine-stimulated NOS activity was significantly inhibited (Fig. 3B), although NO was still produced at 50% of the control rate.

Because a significant amount of NOS activity still remained after >96% of the intracellular BH_4 was removed, we developed a different strategy for more complete inhibition of *de novo* BH_4 biosynthesis in order to determine whether BH_4 was absolutely essential for induction of NOS activity. Pretreatment of RAW cells with a combination of DAHP and NAS, inhibitors of the initial and terminal enzymes of the BH_4 pathway (Fig. 1), respectively, effectively reduced BH_4 to nearly undetectable levels and almost completely prevented the cytokine-induced increase in NOS activity. Furthermore, control experiments in which BH_4 levels were replaced by treating BH_4-depleted cells with BH_2 resulted in restoration of the NOS activity to the expected level. It should be noted that NAS alone, as well as serotonin (data not shown), inhibits NOS activity even in the presence of BH_4. Further experiments are in progress to explore this potentially important finding, because serotonin and NO have opposite effects on blood pressure.

The results presented here establish that BH_4 is required for cytokine-inducible NOS activity in macrophages. The complete removal of BH_4 from RAW cells by a combination of BH_4 biosynthesis inhibitors should allow testing of the activity of other pterin cofactors with inducible NOS and provides an additional pharmacological approach to possibly regulate overproduction of NO in various infectious diseases.

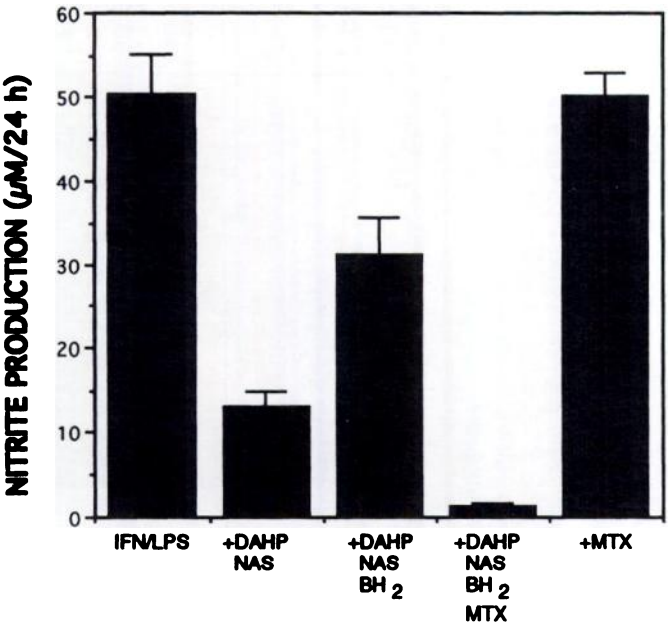

Fig. 4. Nitrite production in BH_4-depleted RAW cells is stimulated by loading with BH_4. Cells were stimulated with IFN-γ and LPS as described in the legend to Fig. 2, in the absence or presence of both DAHP (5 mM) and NAS (5 mM) and BH_2, (0.1 mM), with or without methotrexate (*MTX*) (10 μM). The medium used in the methotrexate experiments was also supplemented with thymine (40 μM) and inosine (100 μM) to prevent any effects on cell growth. Nitrite was measured in medium as described in Experimental Procedures, using the corresponding medium as blank, and the results are expressed as μM/24 hr. In the absence of methotrexate, intracellular BH_4 levels were elevated from 45 pmol/mg to approximately 1000 pmol/mg by the addition of BH_2.

References

1. Schmidt, H. H. H. W. NO, CO, and OH: endogenous soluble guanylyl cyclase-activating factors. *FEBS Lett.* **307**:102–107 (1992).
2. White, K. A., and M. A. Marletta. Nitric oxide synthase is a cytochrome P-450 type hemoprotein. *Biochemistry* **31**:6627–6631 (1992).
3. Moncada, S., R. M. J. Palmer, and E. A. Higgs. Nitric oxide: physiology, pathophysiology and pharmacology. *Pharmacol. Rev.* **43**:109–142 (1991).
4. Radomski, M. W., R. M. J. Palmer, and S. Moncada. An L-arginine/nitric oxide pathway present in human platelets regulates aggregation. *Proc. Natl. Acad. Sci. USA* **87**:5193–5197 (1990).
5. Bohme, G. A., C. Bon, J. M. Stutzmann, A. Doble, and J. C. Blanchard. Possible involvement of nitric oxide in long-term potentiation. *Eur. J. Pharmacol.* **199**:379–381 (1991).

6. Snyder, S. H. Nitric oxide: first in a new class of neurotransmitters? *Science (Washington D. C.)* **257**:494–496 (1992).
7. Marletta, M. A., P. S. Yoon, R. Iyengar, C. D. Leaf, and J. S. Wishnok. Macrophage oxidation of L-arginine to nitrite and nitrate: nitric oxide is an intermediate. *Biochemistry* **27**:8706–8711 (1988).
8. Busse, R., and A. Mulsch. Induction of nitric oxide synthase by cytokines in vascular smooth muscle cells. *FEBS Lett.* **275**:87–90 (1990).
9. Werner, E. R., G. Werner-Felmayer, D. Fuchs, A. Hausen, G. Reibnegger, J. J. Yim, and H. Wachter. Impact of tumour necrosis factor-α and interferon-γ on tetrahydrobiopterin synthesis in murine fibroblasts and macrophages. *Biochem. J.* **280**:709–714 (1991).
10. Kilbourn, R. G., and P. Belloni. Endothelial cell production of nitrogen oxides in response to interferon γ in combination with tumor necrosis factor, interleukin-1, or endotoxin. *J. Natl. Cancer Inst.* **82**:772–776 (1990).
11. Pfeilschifter, J., and K. Vosbeck. Transforming growth factor β_2 inhibits interleukin 1β- and tumour necrosis factor α-induction of nitric oxide synthase in rat renal mesangial cells. *Biochem. Biophys. Res. Commun.* **175**:372–379 (1991).
12. Billiar, T. R., R. D. Curran, D. J. Stuehr, J. Stadler, R. L. Simmons, and S. A. Murray. Inducible cytosolic enzyme activity for the production of nitrogen oxides from L-arginine in hepatocytes. *Biochem. Biophys. Res. Commun.* **168**:1034–1040 (1990).
13. Hibbs, J. B., R. R. Taintor, Z. Vavrin, and E. M. Rachlin. Nitric oxide: a cytotoxic activated macrophage effector molecule. *Biochem. Biophys. Res. Commun.* **157**:87–94 (1988).
14. Tayeh, M. A., and M. A. Marletta. Macrophage oxidation of L-arginine to nitric oxide, nitrite, and nitrate: tetrahydropbiopterin is required as a cofactor. *J. Biol. Chem.* **264**:19654–19658 (1989).
15. Kaufman, S., and D. B. Fisher. Pterin-requiring aromatic amino acid hydroxylases, in *Molecular Mechanisms of Oxygen Activation* (O. Hayaishi, ed.). Academic Press, New York, 285–369 (1974).
16. Giovanelli, J., K. L. Campos, and S. Kaufman. Tetrahydrobiopterin, a cofactor for rat cerebellar nitric oxide synthase, does not function as a reactant in the oxygenation of arginine. *Proc. Natl. Acad. Sci. USA* **88**:2091–2095 (1991).
17. Gross, S. S., E. A. Jaffe, R. Levi, and R. G. Kilbourn. Cytokine-activated endothelial cells express an isotype of nitric oxide synthase which is tetrahydrobiopterin-dependent, calmodulin-independent and inhibited by arginine analogs with a rank-order of potency characteristic of activated macrophages. *Biochem. Biophys. Res. Commun.* **178**:823–829 (1991).
18. Mayer, B., M. John, B. Heinzel, E. R. Werner, H. Wachter, G. Schultz, and E. Bohme. Brain nitric oxide synthase is a biopterin- and flavin-containing multi-functional oxido-reductase. *FEBS Lett.* **288**:187–191 (1991).
19. Stuehr, D. J., and M. A. Marletta. Induction of nitrite/nitrate synthesis in murine macrophages by BCG infection, lymphokines, or interferon-γ. *J. Immunol.* **139**:518–525 (1987).
20. Davis, M. D., S. Kaufman, and S. Milstien. Conversion of 6-substituted tetrahydropterins to 7-isomers via phenylalanine hydroxylase-generated intermediates. *Proc. Natl. Acad. Sci. USA* **88**:385–389 (1991).
21. Fukushima, T., and J. C. Nixon. Analysis of reduced forms of biopterin in biological fluids and tissues. *Anal. Biochem.* **102**:176–188 (1980).
22. Stuehr, D. J., and M. A. Marletta. Mammalian nitrate biosynthesis: mouse macrophages produce nitrite and nitrate in response to *Escherichia coli* lipopolysaccharide. *Proc. Natl. Acad. Sci. USA* **82**:7738–7742 (1985).
23. Padgett, E. L., and S. B. Pruett. Evaluation of nitrite production by human monocyte-derived macrophages. *Biochem. Biophys. Res. Commun.* **186**:775–781 (1992).
24. Gal, E. M., J. M. Nelson, and A. D. Sherman. Purification and characterization of enzymes involved in the cerebral synthesis of 7,8-dihydrobiopterin. *Neurochem. Res.* **3**:69–88 (1978).
25. Rembold, H., and W. L. Gyure. Biochemistry of the pteridines. *Angew. Chem.* **11**:1061–1072 (1972).
26. Abou-Donia, M. M., S. P. Wilson, T. P. Zimmerman, C. A. Nichol, and O. H. Viveros. Regulation of guanosine triphosphate cyclohydrolase and tetrahydrobiopterin levels and the role of the cofactor in tyrosine hydroxylation in primary cultures of adrenomedullary chromaffin cells. *J. Neurochem.* **46**:1190–1199 (1986).
27. Suzuki, S., Y. Watanbe, S. Tsubokura, H. Kagamiyama, and O. Hayaishi. Decrease in tetrahydrobiopterin content and neurotransmitter amine biosynthesis in rat brain by an inhibitor of guanosine triphosphate cyclohydrolase. *Brain Res.* **446**:1–10 (1988).
28. Katoh, S., T. Sueoka, and S. Yamada. Direct inhibition of brain sepiapterin reductase by a catecholamine and an indoleamine. *Biochem. Biophys. Res. Commun.* **105**:75–81 (1982).
29. Nichol, C. A., C. L. Lee, M. P. Edelstein, J. Y. Chao, and D. S. Duch. Biosynthesis of tetrahydrobiopterin by *de novo* and salvage pathways in adrenal medulla extracts, mammalian cell cultures and rat brain *in vivo*. *Proc. Natl. Acad. Sci. USA* **80**:1546–1550 (1983).
30. Kaufman, S. Metabolism of the phenylalanine hydroxylase cofactor. *J. Biol. Chem.* **242**:3934–3943 (1967).
31. Tanaka, K., S. Kaufman, and S. Milstien. Tetrahydrobiopterin, the cofactor for aromatic amino acid hydroxylases, is synthesized by and regulates proliferation of erythroid cells. *Proc. Natl. Acad. Sci. USA* **86**:5864–5867 (1989).
32. Schmidt, K., E. R. Werner, B. Mayer, H. Wachter, and W. R. Kukovetz. Tetrahydrobiopterin-dependent formation of endothelium-derived relaxing factor (nitric oxide) in aortic endothelial cells. *Biochem. J.* **281**:297–300 (1992).
33. Klatt, P., B. Heinzel, M. John, M. Kastner, E. Bohme, and B. Mayer. Ca^{2+}/calmodulin-dependent cytochrome *c* reductase activity of brain nitric oxide synthase. *J. Biol. Chem.* **267**:11374–11378 (1992).

Send reprint requests to: Sheldon Milstien, LNC/NIMH, Building 36, Room 3D-30, Bethesda, MD 20892.