Pteridine Biosynthesis in Human Endothelial Cells

IMPACT ON NITRIC OXIDE-MEDIATED FORMATION OF CYCLIC GMP

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Stimulation of nitric oxide (NO) synthase in endothelial cells by Ca2+ influx leads to increased intracellular levels of cGMP. NO synthase from various sources is known to use tetrahydrobiopterin, flavins, and NADPH as cofactors. We studied the effect of interferon-γ, tumor necrosis factor-α, and lipopolysaccharide on tetrahydrobiopterin biosynthetic activities in human umbilical vein endothelial cells (HUVEC). These stimuli led to an up to 40-fold increase of GTP cyclohydrolase I (EC 3.5.4.16) activity and to increased accumulation of neopterin and tetrahydrobiopterin in HUVEC. Further enzyme activities of tetrahydrobiopterin biosynthesis, i.e. 6-pyruvoyltetrahydrobiopterin synthase and sepiapterin reductase (EC 1.1.1.53), remained unchanged. NO synthase activity in protein fractions from homogenates of cells treated with interferon-γ plus tumor necrosis factor-α was not influenced as compared with untreated controls. However, interferon-γ alone or in combination with tumor necrosis factor-α significantly increased intracellular cGMP formation in intact HUVEC by 50 and 80%, respectively. These stimuli increased intracellular tetrahydrobiopterin concentrations up to 14-fold. NO-triggered cGMP formation was similarly increased by incubation of otherwise untreated cells with sepiapterin, leading to elevated intracellular tetrahydrobiopterin levels. Thus, cytokines indirectly stimulate the activity of constitutive NO synthase in HUVEC by up-regulating production of the cofactor tetrahydrobiopterin.

Nitrergic activation of smooth muscle leads to increased accumulation of neopterin and tetrahydrobiopterin in human umbilical vein endothelial cells (HUVEC). These stimulations led to an increased intracellular tetrahydrobiopterin formation by 14-fold. NO synthase activity in protein fractions from homogenates of cells treated with interneron-γ plus tumor necrosis factor-α was not influenced as compared with untreated controls. However, interferon-γ alone or in combination with tumor necrosis factor-α significantly increased intracellular cGMP formation in intact HUVEC by 50 and 80%, respectively. These stimuli increased intracellular tetrahydrobiopterin concentrations up to 14-fold. NO-triggered cGMP formation was similarly increased by incubation of otherwise untreated cells with sepiapterin, leading to elevated intracellular tetrahydrobiopterin levels. Thus, cytokines indirectly stimulate the activity of constitutive NO synthase in HUVEC by up-regulating production of the cofactor tetrahydrobiopterin.

Nitric oxide (NO) synthase converts L-arginine to L-citrulline and NO in an NADPH-dependent reaction (1). This enzyme occurs in different isoforms, and the most prominent effects of NO are relaxation of smooth muscle, inhibition of platelet aggregation, cytotoxicity, and neurotransmission (for review, see Ref. 2). NO released from endothelial cells has been shown to account for the biological activity of NO synthase in endothe-

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1The abbreviations used are: NO, nitric oxide; HUVEC, human umbilical vein endothelial cells; NMMA, Nω-monomethyl-L-arginine; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonic acid; DAHP, 2,4-diamino-6-hydroxypteridine; SNP, sodium nitroprusside; HPLC, high performance liquid chromatography.

EXPERIMENTAL PROCEDURES

Materials—Human recombinant interferon-γ had a specific activity of 2 × 106 units/mg of protein and was generously provided by Bioferon (Laupheim, Germany). Human recombinant tumor necrosis factor-α with a specific activity of 2 × 106 units/mg of protein, as assayed on actinomycin D-treated L929 cells, lipopolysaccharide (phenolic extract) from Escherichia coli 055:B5, endothelial cell
growth supplement from bovine pituitary, gelatin from bovine skin, l-glutamine, N<sub>2</sub>-monomethyl-L- arginine (NMMA), CHAPS, 3-isobutyl-1-methyl-xanthine, Dowex 50W, and 2,4-diamino-6-hydroxy-pyrimidine (DAHP) were obtained from Sigma (St. Louis, Mo.). Sephadex G-25 was from Merck (Darmstadt, Germany). Pteridines were purchased from Dr. B. Schürcks Laboratories (Jona, Switzerland). L-[2,3,4,5-<sup>3</sup>H]Arginine monohydrochloride (35–70 Ci/mmol) and the radioimmunoassay for cGMP determination were from Amersham Corp. L-[2,3,4,5-<sup>3</sup>H]Arginine was purified by HPLC over a Nucleosil 10 SA column from Macherey-Nagel (Düren, Germany) in 100 mM sodium acetate buffer, pH 4.5. Fetal calf serum and Medium 199 with Earle’s salts were from Biochrome (Berlin, Germany). The protein dye reagent was from Bio-Rad. GTP cyclohydrolase I, purified from Drosophila (strain Oregon R) (28), were a gift of Dr. J. J. Yin (University of Seoul, Korea).

**HPLC System**—For quantitation of pteridines, an HPLC apparatus consisting of a liquid chromatograph (LC 5500) (Varian Associates, Inc., Palo Alto, CA), an LS 4 fluorescence detector (Perkin-Elmer, Beaconsfield, United Kingdom), and an AAS module (Varian) for direct insertion of solid-phase cation-exchange cartridges (Varian) was used. Fluid connections of the AAS instrument were modified as described (33). The reversed phase columns used were Lichrosorb RP 18 columns with 7 µm particle size (Merck).

**Cell Culture—HUVEC** were obtained from Technoclone (Vienna, Austria). Cells were cultured on polystyrene surfaces coated with 0.1% (w/v) gelatin, using Medium 199 with Earle’s salts, supplemented with 20% (v/v) heat-inactivated fetal calf serum, 2 mM l-glutamine, 100 units/ml penicillin, 100 µg/ml streptomycin, and 50 µg/ml endothelial cell growth supplement. For experiments, cells of passages 3–8 were used, and stimulations of cellular pteridine concentrations were determined. Concentrations are given as pmol/10<sup>6</sup> cells, corresponding to about 300 pg of total pteridines.

**Enzyme Activities**—GTP cyclohydrolase I activity was measured after incubation of 7-dihydroneopterin triphosphate, freshly prepared by GTP cyclohydrolase I purified from E. coli, in the presence of excess sepiapterin reductase, NADPH, and Mg<sup>2+</sup>. Thus, the resulting 6-pyruvoyl tetrahydropterin is converted into tetrahydrobiopterin, which is then determined as the fluorescent biopterin by HPLC after iodine oxidation at acidic pH. 6-Pyruvoyl tetrahydropterin synthesis is given as pmol of biopterin formed per mg of protein/min. Sepiapterin was used as substrate for sepiapterin reductase determination, and the resulting tetrahydrobiopterin was oxidized to biopterin. Sepiapterin reductase activity is given as pmol of biopterin formed per mg of protein/min.

**Protein Determination** was done according to Bradford (37), using bovine serum albumin as a standard.

**Determination of Pteridines and Nitrite in Supernatants—HUVEC** were grown in 24-well plates and treated with interferon-γ, tumor necrosis factor-α, or lipopolysaccharide for 24 h. Supernatants were then collected and assayed for total neopterin and biopterin (after iodine oxidation at acidic pH), using HPLC reversed phase chromatography (33). Concentrations given are nmol/liter accumulated in the supernatant after 24 h. Due to supplementation with fetal calf serum, culture medium contained 17.3 ± 2.9 nmol/liter biopterin (mean of six determinations ± S.D.). This blank value was subtracted for calculation of biopterin accumulation in the supernatants.

**Nitrite** was determined in these supernatants by the Griess reaction (38) using the stable Griess-Illesvay’s reagent from Merck. The detection limit is 1 µmol/liter.

**Determination of Intracellular GMP Levels—Determination of NO-triggered intracellular cGMP was modified from Schmidt et al. (39). Briefly, HUVEC (untreated or treated with interferon-γ) were grown in 24-well plates and treated with interferon-γ plus tumor necrosis factor-α, 25 units/ml each, for 24 h. Incubation was then carried out in 40 mM Tris-HCl, pH 8.0, containing 100 µM phenylmethylsulfonyl fluoride. The pellet was washed twice with phosphate buffer, pH 7.4 (see Materials). The supernatant was collected and Sephacryl S-300 (2.5–3.0 cm) was equilibrated with 40 mM Tris-HCl, pH 8.0, with phenylmethylsulfonyl fluoride for 20 min on ice with gentle shaking in order to solubilize the particulate NO synthase from endothelial cells. After centrifugation at 10,000 × g for 10 min, the supernatant was purified from low molecular mass compounds by passing Sephadex G-25 eluted with 40 mM Tris-HCl, pH 8.0, containing 100 µM L-arginine, 25 µM FAD, 25 µM FMN, 2 mM NADPH, 5 µM (6R)-tetrahydrobiopterin, 100 µM phenylmethylsulfonyl fluoride, 60,000–80,000 cpm of purified L-[2,3,4,5-<sup>3</sup>H]arginine (see "Materials"), and 100 µL of cell extract or CHAPS extract, containing about 300 µg of total protein. The free Ca<sup>2+</sup> concentration was adjusted in these incubation mixtures using 0.15 mM EGTA, 0.9 mM EDTA, 2.05 mM MgCl<sub>2</sub> without CaCl<sub>2</sub> for Ca<sup>2+</sup>-free conditions, and then incubated for 15 min with 1 mM 5-isobutyl-1-methylxanthine at 37 °C and then treated with 1 µM A23187 or vehicle in a final volume of 1 ml for 6 min. SNP (1 µM) was used for directly stimulating guanylyl cyclase without involvement of endogenous NO synthase.

**Determination of NO Synthase Activity in Cell Extracts—**NO synthase was assayed as modified by Stuehr et al. (14) and Mayer et al. (16). Cell extracts from HUVEC (untreated or treated with interferon-γ) plus tumor necrosis factor-α, 250 units/ml each, for 24 h) were prepared by rapid freezing of cells in distilled water. After thawing and centrifugation at 10,000 × g for 10 min, the supernatant was collected and Sephadex G-25 eluates were prepared using 40 mM Tris-HCl, pH 8.0, containing 100 µM phenylmethylsulfonyl fluoride. The pellet was washed twice with phosphate buffer, pH 7.4 (see Materials). The supernatant was then incubated for 15 min with 1 mM 5-isobutyl-1-methylxanthine at 37 °C and then treated with 1 µM A23187 or vehicle in a final volume of 1 ml for 6 min. SNP (1 µM) was used for directly stimulating guanylyl cyclase without involvement of endogenous NO synthase.

**Cell Viability Assay—**This assay is based on staining adherent cells after incubation with various stimuli. Since anchorage-dependent cells do not survive or proliferate after they have detached from the surface, it is generally agreed that such an assay reasonably estimates the amount of viable cells. Briefly, HUVEC were grown in 96-well plates and treated with interferon-γ, DAHP, cytokines, or lipopolysaccharide for 24 h. Supernatants were then removed, and cells were fixed with 5% (v/v) formaldehyde for 10 min. After washing with tap water, cells were stained with crystal violet (33).
RESULTS

Table I summarizes the activities of the three enzymes involved in the biosynthesis of tetrahydrobiopterin from GTP, as well as the intracellular concentrations of pteridines following the treatment of HUVEC with different activators. As shown in Table I, 250 units/ml interferon-γ led to a 20-fold increase of GTP cyclohydrolase I activity, when applied as a single stimulus, and to a 46-fold increase when applied in combination with 250 units/ml tumor necrosis factor-α. This was not further enhanced by higher doses (up to 1000 units/ml) of interferon-γ. Tumor necrosis factor-α alone (250 units/ml) induced GTP cyclohydrolase activity 5-fold (p < 0.001, Student's t test). Lipopolysaccharide (1 µg/ml) stimulated GTP cyclohydrolase I activity about 13-fold. Lipopolysaccharide applied in combination with interferon-γ had only an additive effect but did not potentiate the interferon-γ stimulus like tumor necrosis factor-α. Further enzyme activities of tetrahydrobiopterin synthesis, i.e. 6-pyruvoyl tetrahydropterin synthase and sepiapterin reductase, were not significantly (Student's t test) changed upon treatment with these stimuli. In parallel to the stimulation of GTP cyclohydrolase I activity, neopterin and biopterin derivatives accumulated in the cells. At least 80% of biopterin occurred in its tetrahydro form, as was calculated from the difference of biopterin detected after oxidation with iodine in alkaline or acidic media (35).

Neopterin and biopterin were also released from the cells but were not detectable in supernatants of untreated controls or of cells treated with tumor necrosis factor-α alone (see Table I). No nitrite accumulation (<1 µmol/liter) could be observed in supernatants of these long term treated cultures indicating the lack of a macrophage type, Ca²⁺-independent, high output NO synthase activity.

We then investigated the effect of manipulating intracellular levels of tetrahydrobiopterin by incubating otherwise untreated cells with various concentrations of sepiapterin or DAHP for 24 h. Sepiapterin is intracellularly converted into tetrahydrobiopterin via a salvage pathway (31). The GTP cyclohydrolase I inhibitor DAHP inhibits de novo synthesis of tetrahydrobiopterin by acting as an analogue of the first pyrimidine intermediate formed in the GTP cyclohydrolase I reaction subsequent to opening the GTP ring structure in position 8 (41). The correlation between cGMP levels accumulated after stimulation of Ca²⁺ influx by A23187 and intracellular tetrahydrobiopterin levels is shown in Fig. 1. The amount of tetrahydrobiopterin found in untreated control cells (C in Fig. 1) was 3.9 ± 0.5 pmol/10⁶ cells. No biopterin was detectable with 5 mM DAHP, and in this case cGMP formation was also reduced to undetectable levels. A 50% inhibition of tetrahydrobiopterin biosynthesis was achieved by about 0.3 mM DAHP. Intracellular tetrahydrobiopterin concentrations were strictly correlated to the sepiapterin dose applied (linear correlation coefficient 0.999). As little as 0.96 ± 0.15 pmol of tetrahydrobiopterin/10⁶ cells, achieved by 1 mM DAHP, were still sufficient to detect 0.57 ± 0.08 pmol of cGMP/10⁶ cells. Like the Ca²⁺ ionophore-stimulated cGMP formation (Fig. 1), basal NO-triggered cGMP formation was also increased from 0.52 ± 0.02 to 0.96 ± 0.09 pmol/10⁶ cells (mean ± S.D. from triplicate cultures, p < 0.002, Student's t test) upon treatment with 250 µM sepiapterin and decreased to 0.06 ± 0.02 pmol/10⁶ cells by 0.5 mM DAHP.

Table II shows that DAHP and sepiapterin specifically influenced NO synthesis rather than guanylyl cyclase itself, since SNP-stimulated guanylyl cyclase activity was not affected by these components. Further, the effect of DAHP could be restored by concurrent application of sepiapterin. The arginine analogue NMMA decreased NO-triggered cGMP formation, and this inhibition could not be overcome by sepiapterin. Interferon-γ increased basal and A23187-stimulated NO-triggered cGMP formation by 50% (p < 0.01, Student's t test) but did not alter guanylyl cyclase activity itself. The action of DAHP, sepiapterin, and NMMA was not influenced by interferon-γ treatment. Interferon-γ in combination with tumor necrosis factor-α increased NO-triggered cGMP formation by about 80% (p < 0.002 as compared with untreated controls, p < 0.05 as compared with interferon-γ alone, Student's t test) (Table II). As compared with untreated control cells, a 24-h treatment of HUVEC with 100 µM sepiapterin, 5 mM DAHP, 100 µM sepiapterin plus 5 mM DAHP, 250 units/ml interferon-γ, 250 units/ml tumor necrosis factor-α, or 1 µg/ml lipopolysaccharide did not affect cell viability. In contrast, a combination of

Table I

| Enzyme activities | Intracellular pteridines | Pteridines in supernatants |
|-------------------|-------------------------|---------------------------|
| **Treatment** | **GTP cyclohydrolase I** | **6-Pyruvoyl tetrahydropterin synthase** | **Sepiapterin reductase** | **Neopterin** | **Tetrahydrobiopterin** | **Neopterin** | **Bioterin** |
| | pmol·mg⁻¹·min⁻¹ | pmol/10⁶ cells | pmol/liter | |
| None | 0.08 ± 0.02 | 0.52 ± 0.08 | 379 ± 124 | 1.0 ± 0.3 | 2.6 ± 0.6 | <1.0 | <1.0 |
| IFN-γ | 1.85 ± 0.07 | 0.65 ± 0.18 | 384 ± 65 | 1.9 ± 1.1 | 11.5 ± 1.8 | <1.0 | <1.0 |
| TNF-α | 0.41 ± 0.02 | 0.62 ± 0.08 | 290 ± 51 | 0.8 ± 0.6 | 5.2 ± 1.6 | <1.0 | <1.0 |
| LPS | 1.05 ± 0.02 | 0.50 ± 0.10 | 349 ± 132 | 2.8 ± 0.9 | 28.9 ± 11.4 | <1.0 | 11.7 ± 1.3 |
| IFN-γ | 3.69 ± 0.14 | 0.85 ± 0.20 | 256 ± 25 | 5.0 ± 1.7 | 37.5 ± 3.9 | 6.3 ± 0.7 | 64.9 ± 3.0 |
| Plus TNF-α | 2.20 ± 0.15 | 0.63 ± 0.13 | 323 ± 92 | 10.2 ± 4.9 | 71.3 ± 32.5 | 10.2 ± 0.6 | 77.6 ± 5.0 |
| IFN-γ | 2.20 ± 0.15 | 0.63 ± 0.13 | 323 ± 92 | 10.2 ± 4.9 | 71.3 ± 32.5 | 10.2 ± 0.6 | 77.6 ± 5.0 |
| Plus LPS | 2.20 ± 0.15 | 0.63 ± 0.13 | 323 ± 92 | 10.2 ± 4.9 | 71.3 ± 32.5 | 10.2 ± 0.6 | 77.6 ± 5.0 |
FIG. 1. Dependence of cGMP formation on intracellular tetrahydrobiopterin levels. Confluent monolayers of HUVEC were grown in 6-well plates for cGMP determination and pteridine determination in the case of sepiapterin treatment and in 75-cm² culture flasks for pteridine determination in the case of DAHP treatment. Cells were treated with various doses of either DAHP or sepiapterin for 24 h. Formation of cGMP was determined upon stimulation of NO synthase with 1 μM A23187 for 6 min (see “Experimental Procedures”). Cells of the same passage treated in parallel in the same experiment were collected by trypsinization, and intracellular bioterin levels were assessed in cell extracts by HPLC after oxidation in acidic or alkaline media as described under "Experimental Procedures." Levels of intracellular cGMP were plotted against the logarithm of intracellular tetrahydrobiopterin levels (means ± S.D. of triplicate cultures). DAHP: A, 1 mM; B, 0.5 mM; C, untreated control. Sepiapterin: D, 1 μM; E, 5 μM; F, 25 μM; G, 100 μM; H, 250 μM.

TABLE II
Intracellular cGMP levels in HUVEC: the effect of interferon-γ, DAHP, sepiapterin, and NMMA
Confluent monolayers of HUVEC grown in 6-well plates were treated with various additives for 24 h. Supernatants were then removed, and basal cGMP levels as well as cGMP accumulated after treatment with 1 μM A23187 or 1 μM SNP for 6 min were determined as described under "Experimental Procedures." Values are means ± S.D. from triplicate cultures from one of two similar experiments. IFN-γ, interferon-γ (250 units/ml); TNF-α, tumor necrosis factor-α (250 units/ml); nt, not tested.

| Additive | Control A23187 | SNP |
|----------|----------------|-----|
| None     | 0.60 ± 0.09    | 5.51 ± 0.22  |
| 5 mM DAHP| 0.30 ± 0.03    | 0.65 ± 0.06  |
| 100 μM sepiapterin | 1.80 ± 0.49 | 11.97 ± 1.27 |
| 5 mM DAHP + 100 μM sepiapterin | 1.35 ± 0.09 | 11.49 ± 2.71 |
| 250 μM NMMA | 0.29 ± 0.05 | 1.50 ± 0.13 |
| 250 μM NMMA + 100 μM sepiapterin | 0.68 ± 0.13 | 2.67 ± 0.31 |

interferon-γ with tumor necrosis factor-α and lipopolysaccharide reduced cell viability by 25 ± 5% and by 32 ± 7%, respectively (mean ± S.D. of 8 wells from two experiments). Unlike in murine fibroblasts (24), concurrent treatment with 5 mM DAHP, 100 μM sepiapterin, or 250 μM NMMA did not influence the cytotoxic effect of these combined stimuli, thus excluding involvement of NO synthase in the observed cytotoxicity.

NO synthase activity in protein fractions from homogenates of cells treated with interferon-γ plus tumor necrosis factor-α (250 units/ml each) was not higher than in those of untreated control cells. NO synthase activities detected were

(14H)citrulline, pmol·mg⁻¹·min⁻¹, mean ± S.D. of triplicate incubations; for cell extracts in the presence of 3 μM Ca²⁺ (untreated, 10.0 ± 0.5; treated, 11.6 ± 1.0), for cell extracts in the absence of Ca²⁺ (untreated, 1.7 ± 0.8; treated, 1.3 ± 0.6), for CHAPS extracts in the presence of 3 μM Ca²⁺ (untreated, 49.3 ± 0.2; treated, 44.9 ± 5.3), and for CHAPS extracts in the absence of Ca²⁺ (untreated, 5.7 ± 0.4; treated, 5.4 ± 0.1).

DISCUSSION
In the present investigation, we describe the effects of interferon-γ, tumor necrosis factor-α, and lipopolysaccharide on pteridine biosynthesis and the resulting accumulation of neopterin and tetrahydrobiopterin in HUVEC with respect to NO-mediated cGMP formation in intact cells. We found that interferon-γ strongly stimulated the biosynthesis of tetrahydrobiopterin in HUVEC by increasing the activity of the key enzyme GTP cyclohydrolase I. Parallel accumulation of neopterin can be explained by the comparatively low 6-pyruvoyl tetrahydrobiopterin synthase activity typical for human cells (28,30). Pteridine synthesis is also stimulated by tumor necrosis factor-α and lipopolysaccharide, and tumor necrosis factor-α potentiates the effect of interferon-γ. Both neopterin and bipterin derivatives leak from cells treated with these cytokines or lipopolysaccharide and are detectable in supernatants. Thus, the previously reported increased supernatant concentrations of neopterin (42) in HUVEC cultures treated by cytokines are an indicator of increased intracellular de novo synthesis of tetrahydrobiopterin in these cells.

Regarding endothelial cells, high output NO synthase leading to accumulation of nitrogen oxides in supernatants has been demonstrated thus far only for murine endothelial cells from brain (43). A cytokine-inducible, Ca²⁺-independent NO synthase was also reported for porcine aortic endothelial cells, as determined by inhibition of platelet aggregation and by spectrophotometric detection of NO (44). In tumor necrosis factor-α-treated bovine aortic endothelial cells, an up to 5-fold increase of intracellular cGMP levels was observed, suggesting induction of NO synthesis (45). As expected from work with other human cells in vitro (for discussion of this phenomenon, see Refs. 46 and 47), high output NO synthase leading to accumulation of nitrite in supernatants could not be induced in HUVEC. NO-mediated cGMP formation in intact HUVEC, however, is significantly increased by interferon-γ alone or in combination with tumor necrosis factor-α. Furthermore, our results obtained by manipulating otherwise untreated cells with drugs altering the intracellular tetrahydrobiopterin concentrations (Fig. 1) show that the amount of cGMP formed can be varied about 10-fold by decreasing and increasing the intracellular tetrahydrobiopterin concentration. In particular, increasing intracellular tetrahydrobiopterin levels with sepiapterin to levels comparable with cells treated with interferon-γ alone or together with tumor necrosis factor-α also leads to a comparable increase in cGMP formation. Under these conditions, activity, Ca²⁺ dependence, and intracellular localization of NO synthase in cell homogenates remain unchanged. We conclude, therefore, that the increase of cGMP formation in HUVEC following cytokine treatment is due to the increased endogenous synthesis of tetrahydrobiopterin, one of the cofactors of NO synthase.

Using N-15-labeled arginine, an up to 10-fold increase in turnover from arginine to nitrogen oxides, indicating induction of NO synthase activity, has been demonstrated in humans receiving interleukin 2 therapy (47). In these patients interleukin-2 causes NO production most likely due to induction of cytokines such as interferon-γ or tumor necrosis...
factor-α (for discussion, see Ref. 47). Interleukin-2 therapy is also known to lead to increased pteridine synthesis in humans, as measured by increased concentrations of neopterin in body fluids (49). Highly elevated formation of pteridines in humans has also been observed in a number of clinical situations (for review, see Ref. 49) including septic complications (50). Some extent to the observed stimulation of NO synthesis by cytokines in humans in vivo (47).

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