Regulation of Nitric Oxide Synthesis by Proinflammatory Cytokines in Human Umbilical Vein Endothelial Cells

Elevations in Tetrahydrobiopterin Levels Enhance Endothelial Nitric Oxide Synthase Specific Activity

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Abstract

We have examined cytokine regulation of nitric oxide synthase (NOS) in human umbilical vein endothelial cells (HUVEC). 24-h treatment with IFN-γ (200 U/ml) plus TNF (200 U/ml) or IL-1β (5 U/ml) increased NOS activity in HUVEC lysates, measured as conversion of [^{14}C]L-arginine to [^{14}C]L-citrulline. Essentially, all NOS activity in these cells was calcium dependent and membrane associated. Histamine-induced nitric oxide release, measured by chemiluminescence, was greater in cytokine-treated cells than in control cells. Paradoxically, steady-state mRNA levels of endothelial NOS fell by 94±2.0% after cytokine treatment. Supplementation of HUVEC lysates with exogenous tetrahydrobiopterin (3 μM) greatly increased total NOS activity, and under these assay conditions, cytokine treatment decreased maximal NOS activity. IFN-γ plus TNF or IL-1β increased endogenous tetrahydrobiopterin levels and GTP cyclohydrolase I activity, the rate-limiting enzyme of tetrahydrobiopterin synthesis. Intracellular tetrahydrobiopterin levels were higher in freshly isolated HUVEC than in cultured cells, but were still limiting. We conclude that inflammatory cytokines increase NOS activity in cultured human endothelial cells by increasing tetrahydrobiopterin levels in the face of falling total enzyme; similar regulation appears possible in vivo. (*J. Clin. Invest.* 1994. 93:2236–2243.) Key words: tumor necrosis factor • interleukin • interferon • GTP cyclohydrolase I • endothelium

Introduction

Proinflammatory cytokines such as TNF, IL-1β, and IFN-γ are important mediators of the host response to infection including inflammation. Vasodilation is a cardinal sign of local inflammation and systemic vasodilation is a key component of septic shock (1). There is considerable evidence that cytokines are responsible for both local and systemic vasodilation in these settings (2–4). Vasodilatory effects of proinflammatory cytokines may be mediated by host cell production of vascular smooth muscle relaxants.

Nitric oxide (NO)[1] is a potent vasodilatory autacoid that is produced by several different cell types including vascular endothelial cells (5), vascular smooth muscle cells (6), neurons (7), mononuclear phagocytes (8), and hepatocytes (9). In all of these cell types, NO is generated from the conversion of L-arginine to L-citrulline by the enzymatic action of nitric oxide synthase (NOS) (10). Tetrahydrobiopterin, heme, flavin mononucleotide, and flavin adenine dinucleotide are required as cofactors, and NADPH and molecular O_2 are used as cosubstrates for enzymatic activity (8, 11–14). Three different NOS isoforms have been cloned and characterized: neuronal (7, 15), endothelial (16, 17), and cytokine inducible. The last group includes macrophage (18, 19), hepatocyte (20, 21), and vascular smooth muscle NOS (22). These isoforms share 50% amino acid identity and constitute a gene family. Different NOS isoforms may be distinguished by their subcellular localization and by their requirement for calcium. Specifically, endothelial NOS is membrane associated (5) by virtue of *N*-myristoylation (23), whereas both neuronal and cytokine-inducible forms are cytosolic proteins (6, 8). The endothelial and neuronal isoforms are activated by calcium/calmodulin complexes and are, therefore, regulated by intracellular free calcium levels (24, 25). In contrast, the cytokine-inducible isoforms bind calmodulin in the absence of calcium and are tonically active (8, 26). The cytokine-inducible form is also capable of generating much more NO than the calcium-regulated isoforms and is sometimes referred to as "high output."

Recent studies using inhibitors of NOS have implicated NO as the principal cytokine-induced vasodilator in TNF and endotoxin-induced hypotension, as well as in clinical sepsis (27–29). Studies in humans with sepsis have also found evidence of increased plasma levels and urinary excretion of nitrates (30), the oxidation products of NO. The connection between proinflammatory cytokines, and increased NO production has generally been attributed to cytokine-induced expression of NOS. In rodents, IFN-γ alone or in combination with IL-1 or TNF cause macrophages to express the cytokine-inducible NOS isoform (8). However, it has been difficult to confirm that human macrophages respond similarly (31, 32). Human hepatocytes do express a cytokine-inducible NOS isoform (20), but it is unlikely that hepatocytes are the cellular source of NO responsible for systemic vasodilation. Therefore, attention has focused on the cytokine responses of vessel wall cells, including smooth muscle and endothelium. There have been a number of conflicting reports about cytokine effects on vascular endothelial NOS expression, involving cells isolated from a number of spe-

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1. *Abbreviations used in this paper:* GAPDH, glyceraldehyde-3 phosphate dehydrogenase; HUVEC, human umbilical vein endothelial cells; NO, nitric oxide; NOS, nitric oxide synthase.

cies and vascular beds (6, 33–39). In the present study, we have examined cytokine effects on human endothelial cells cultured from umbilical veins. We find no evidence for a cytosolic, calcium-independent NOS isoform in these cells. Instead, our findings suggest that inflammatory cytokines increase endothelial NO production by augmenting the specific activity of the endothelial NOS isoform through elevations of the limiting cofactor, tetrahydrobiopterin.

Methods

Materials. Human recombinant TNF (specific activity = 5.3×10^6 U/mg) and IFN-γ (specific activity = 2.7×10^7 U/mg) were gifts from Biogen Inc. (Cambridge, MA). Human recombinant IL-1β (specific activity = 2.7×10^6 U/mg) was obtained from Collaborative Research (Bedford, MA). Endotoxin levels in the TNF, IL-1β, and IFN-γ preparations were < 1 pg/ml, as measured by Limulus assay (Associates of Cape Cod, Inc., Woods Hole, MA). (6R,S)-5,6,7,8-Tetrahydro-L-biopterin was purchased from B. Schircks Laboratory (Jona, Switzerland). Arginine L-[guanido-^{14}C] (specific activity = 57.8 mCi/mmol) and citrulline L-[ureido-^{14}C] (specific activity = 55.6 mCi/mmol) were purchased from NEN Research Products (Boston, MA). Leupeptin was obtained from Boehringer Mannheim Corp. (Indianapolis, IN). Hepes was purchased from American Bioanalytical (Natick, MA) and sucrose from Bethesda Research Laboratories (Gaithersburg, MD). Bovine brain calmodulin and all other reagents, unless specified, were purchased from Sigma Chemical Co. (St. Louis, MO).

Endothelial cells. Endothelial cells were isolated from umbilical vein (40). Where indicated, the isolated cells were analyzed immediately, but in most cases, human umbilical vein endothelial cells (HUVEC) were pooled from three to five separate veins and serially cultured in Medium 199 (Gibco Laboratories, Grand Island, NY) containing 20% heat-inactivated fetal bovine serum supplemented with penicillin (100 U/ml), streptomycin (100 μg/ml), L-glutamine (2 mM; Gibco Laboratories), porcine heparin (100 μg/ml), and endothelial cell growth factor (50 μg/ml; Collaborative Research) (41). Cells were grown to confluence in plastic tissue culture flasks (Corning Glass Works, Corning, NY) that were precoated with gelatin (J. T. Baker Chemical Co., Phillipsburg, NJ). Cells were used at passage levels 3–6, except for one experiment (n = 3) in which cells were used in primary culture.

Measurement of NOS activity in cell lysates and membranes. Confluent HUVEC monolayers grown in 175-cm^2 culture flasks were treated with various combinations of TNF (200 U/ml), IL-1β (5 U/ml), and IFN-γ (200 U/ml) or mock treated for 24 h. These doses were selected because they have previously been shown to be optimal for modulating HUVEC gene expression (42). In a separate series of experiments, the time course of cytokine effects was examined by treating cells for 0–72 h before the assay. At the end of the indicated treatment period, cells were suspended by brief treatment with trypsin-EDTA (Gibco Laboratories), washed with Dulbecco's phosphate-buffered saline, and used to prepare total cell lysates or membrane and cytosolic fractions for measurement of NOS activity.

To prepare total cell lysates, cultured or, in some experiments, freshly isolated HUVEC were resuspended in cold lysis buffer (sucrose [0.3 M], Hepes [10 mM], 1% Nonidet P-40, EDTA [0.1 mM], dithiothreitol [1 mM], leupeptin [10 μg/ml], aprotinin [2 μg/ml], soybean trypsin inhibitor [10 μg/ml], and PMSF [50 μM], pH 7.4), vortexed, and kept at 4°C. To analyze NOS activity, cell lysates (150–250 μg) were incubated with NADPH (2 mM), $CaCl_2$ (230 μM), and [^{14}C]L-arginine (0.06 μCi; 40 μM) in the absence or presence of tetrahydrobiopterin (3 μM) for 90 min at 37°C (total vol = 31 μl). Calcium-independent NOS activity was measured in replicate samples by replacing $CaCl_2$ with EDTA (1.7 mM).

To prepare membrane and cytosolic fractions, HUVEC were resuspended in cold buffer (Tris-HCl [50 mM] pH 7.5, EGTA [0.1 mM], EDTA [0.1 mM], dithiothreitol [1 mM], leupeptin [10 μg/ml], aprotinin [2 μg/ml], soybean trypsin inhibitor [10 μg/ml], PMSF [50 μM], disrupted by three cycles of rapid freezing and thawing, and centrifuged at 10,000 g for 45 min at 4°C. The supernatant was passed through an AG50WX-8 Dowex resin (Bio-Rad Laboratories, Melville, NY) to remove endogenous arginine. Cell membranes or cytosol (50–75 μg) were then incubated with NADPH (1 mM), $CaCl_2$ (1.6 mM), calmodulin (120 nM), and [^{14}C]L-arginine (0.06 μCi; 40 μM) in the absence or presence of tetrahydrobiopterin (3 μM) for 45 min at 37°C. Calcium-independent NOS activity was measured in replicate samples by replacing $CaCl_2$ with EDTA (1.7 mM).

Incubations were terminated by adding ice-cold methanol (100 μl). The samples were centrifuged (10,000 g for 10 min), and the supernatant (45 μl) was spotted onto silica gel 20 × 20 thin-layer chromatography plates (Sigma Chemical Co.). The plates were developed sequentially in the solvent system methanol:chloroform:ammonium hydroxide/water (4.5:0.5:2:1, vol/vol) over a distance of 13 cm, dried and further developed in butanol/acetic acid/water (4:1:1, vol/vol) over an additional 4 cm. (The second step was needed to separate L-citrulline from an as yet unidentified L-arginine metabolite. Formation of this unidentified conversion product was not inhibited by N^G-nitro-L-arginine methyl ester and will not be discussed further). The Rf value for ^{14}C-labeled L-citrulline was identical to that of the authentic standard. [^{14}C]L-citrulline was localized and quantitated by use of a PhosphorImager (Molecular Dynamics, Sunnyvale, CA) or by liquid scintillation counting in Ecoscint (National Diagnostics, Manville, NJ) using a Tricarb liquid scintillation analyzer (model 1500; Packard Instrument Co., Inc., Downers Grove, IL). Protein concentration of cell lysates and membranes was determined according to Bradford (43), using bovine serum albumin as a standard.

Nitric oxide production. HUVEC were grown in 35- or 100-mm^2 dishes and treated with the combination of TNF (200 U/ml), IL-1β (5 U/ml), and IFN-γ (200 U/ml) for 24 h. The media was changed to Hanks' balanced salt solution supplemented with $CaCl_2$ (1.2 mM), $MgSO_4$ (0.6 mM), and arginine (100 μM). Histamine (10 μM) was added and after 45 min, the supernatant was collected for analysis of nitric oxide by chemiluminescence (44). Samples (100 μl) containing nitrite, the primary metabolite of NO in hemoglobin-free aqueous buffer (45), were refluxed in a deoxygenated reducing solution of H_2SO_4 (0.1 M), Na_2SO_4 (0.14 M) and KI (0.1 M). Under these conditions, nitrite and other nitrosocompounds are quantitatively converted to NO. The NO gas was then purged into a nitric oxide analyzer (Sievers Research, Inc., Boulder, CO) and quantitated using an integrator (HP 3396 Series II, Hewlett-Packard Co., Palo Alto, CA) by reference to $NaNO_2$ standards.

Quantification of mRNA levels. HUVEC were grown in 100-mm^2 dishes and treated for 24 h with TNF (200 U/ml), IL-1β (5 U/ml), and IFN-γ (200 U/ml). Total RNA was extracted from the cells with TRISOLV™ (Biotecx Laboratories, Houston, TX) according to the method of Chomczynski and Sacchi (46). RNA was denatured by heating (65°C) in formamide (50%; vol/vol), formaldehyde (2.2 M). Total RNA (10–20 μg) was electrophoresed through a 0.8% agarose gel containing formaldehyde (0.4 M) and transferred to a nylon membrane (Hybond-N; Amersham Corp., Arlington Heights, IL). Membranes were hybridized to [α-^{32}P]dCTP-labeled probe for endothelial NOS (see below) by standard methods at 43°C (47). The membranes were washed twice in 2× SSC, 0.1% SDS for 15 min at room temperature, once in 2× SSC, 0.1% SDS for 30 min at 50°C, and then washed at high stringency in 0.1× SSC, 0.1% SDS for 30 min at 65°C. Membranes were stored on phosphorscreens for 4 h and/or autoradiographed in the presence of an intensifying screen with Kodak XAR film for 3–4 d at −80°C. Membranes were then stripped and then hybridized to the cDNA probe for glyceraldehyde-3 phosphate dehydrogenase (GAPDH), washed, and analyzed as for endothelial NOS with a PhosphorImager using the IMAGEQUANT software (Molecular Dynamics). Steady-state levels of mRNA for the endothelial NOS were quantitated after normalization to GAPDH.

The probes used were a full-length EcoRI fragment of bovine endothelial cell NOS (16) and a 1.0-kb fragment of human GAPDH (Clon-

tech Laboratories, Inc., Palo Alto, CA). The cDNA probes were labeled with [α-^{32}P]dCTP (1–2 × 10^9 cpm/μg) using a random primed DNA labeling kit according to the instructions of the manufacturer (United States Biochemical Corp., Cleveland, OH).

Determination of pteridines and GTP cyclohydrolase I activity in cell extracts and supernatants. HUVEC were grown in 100 mm^2 dishes and treated with combinations of TNF (200 U/ml), IL-1β (5 U/ml), and IFN-γ (200 U/ml) for 24 h. Supernatants were collected; the cells were then suspended with trypsin and pelleted by centrifugation. Both the cell supernatants and pellets were frozen at −80°C. Measurements of total neopterin and biopterin were performed by HPLC analysis after oxidation by MnO_2 or iodine as previously described (48, 49). GTP cyclohydrolase I activity was determined by HPLC measurements of neopterin released from dihydroneopterin triphosphate after oxidation and phosphatase treatment by minor modifications of previously described methods (50).

Statistical analysis. All values are presented as mean±SEM. Comparisons of changes in normalized steady state mRNA levels and NO production between two groups were made by unpaired *t* test. Comparisons between multiple treatment groups of changes in NOS activity, pteridine levels, and GTP cyclohydrolase I activity were made by ANOVA and Newman-Keuls test. Differences were considered significant at $P < 0.05$ (51).

Results

NOS activity in cultured HUVEC. We first examined the effects of cytokines on NOS activity in HUVEC, using individual or combinations of cytokines (TNF [200 U/ml], IL-1β [5 U/ml], and IFN-γ [200 U/ml]) at concentrations known to produce optimal effects in other assays (42). Treatment of HUVEC with TNF, IL-1β, or IFN-γ for 24 h had no effect on NOS activity in cell lysates (Fig. 1) as measured by conversion of [^{14}C]L-arginine to [^{14}C]L-citrulline. However, IFN-γ in combination with either TNF or IL-1β increased calcium-dependent NOS activity in cell lysates (Fig. 1). The combination of TNF, IL-1β, and IFN-γ appeared optimal and increased NOS activity approximately sixfold. Almost all NOS activity in control and cytokine-treated cells was calcium dependent (Fig. 1) and

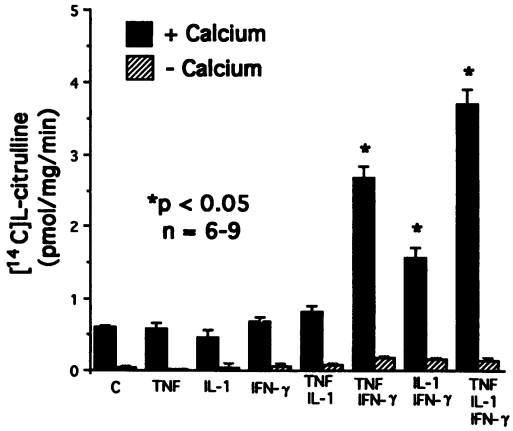

Figure 1. Effect of cytokine combinations on NOS activity in HUVEC lysates. Cultured HUVEC were treated with combinations of TNF (200 U/ml), IL-1β (5 U/ml), and IFN-γ (200 U/ml) for 24 h. Cell lysates (150–250 μg) were incubated with NADPH (2 mM), [^{14}C]L-arginine (0.06 μCi) and either $CaCl_2$ (230 μM; total activity) or EDTA (1.7 mM; calcium-independent activity) for 90 min at 37°C. The reaction was terminated by addition of methanol and [^{14}C]L-citrulline was quantitated by TLC. *C*, control.

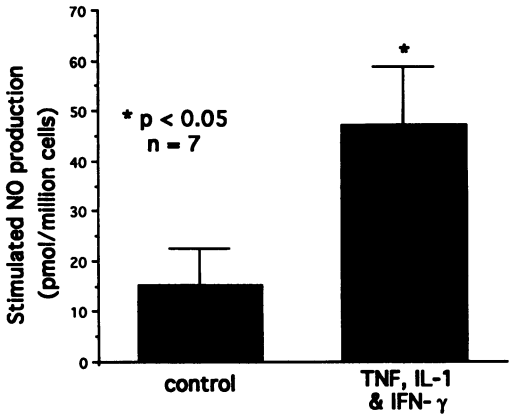

Figure 2. Effect of 24-h treatment with TNF, IL-1β, and IFN-γ on histamine-stimulated NO release from cultured HUVEC. Cultured HUVEC were treated with TNF (200 U/ml), IL-1β (5 U/ml), and IFN-γ (200 U/ml) for 24 h. After changing the media to Hanks' balanced salt solution supplemented with $CaCl_2$ (1.2 mM), $MgSO_4$ (0.6 mM), and arginine (100 μM), histamine (10 μM) was added for 45 min. NO was measured by after acidification and reduction of the supernatant with iodine using a NO analyzer. The histamine-stimulated change in NO release was calculated as the difference between stimulated and unstimulated NO levels. Unstimulated levels of NO were not significantly different between control and cytokine-treated cells.

membrane associated (see below). N^G-nitro-L-arginine methyl ester (150 μM) blocked the conversion of [^{14}C]L-arginine to [^{14}C]L-citrulline in cell lysates by > 95% (n = 3). By the criteria of calcium dependency, membrane association, and N^G-nitro-L-arginine methyl ester sensitivity, we conclude that the observed cytokine effect involves an increase in the activity of the endothelial isoform of NOS.

NO production from HUVEC. To determine whether the cytokine-induced increase in calcium-dependent NOS activity can produce an increase in NO production, we measured NO release from control and cytokine-treated intact cells after stimulation with histamine for 45 min. In control cells, histamine (10 μM) did not produce a significant increase in NO levels (Fig. 2). However, after 24 h of treatment with TNF, IL-1β, and IFN-γ, histamine was able to stimulate measurable production of NO. There was no difference between control and cytokine-treated cells in unstimulated NO levels.

NOS mRNA levels in HUVEC. To test whether the cytokine effect on endothelial NOS activity involved new synthesis of enzyme, we measured the effect of cytokines on endothelial NOS mRNA levels by Northern blotting. Surprisingly, the combination of TNF, IL-1β, and IFN-γ decreased steady-state mRNA levels of endothelial NOS normalized to GAPDH by 94±2.0% (n = 4) after 24 h (Fig. 3). The effect appeared specific for endothelial NOS since levels of GAPDH mRNA did not change significantly.

Regulation of NOS activity by tetrahydrobiopterin. The discrepancy between the observed increases in NOS activity and decreases in NOS mRNA levels suggested that enzyme specific activity rather than enzyme quantity was increased. To determine factors that regulate NOS-specific activity, we reassayed NOS activity in cell lysates in the presence of excess exogenous calmodulin or tetrahydrobiopterin. Addition of calmodulin (100 μM) had no effect on NOS activity (data not shown),

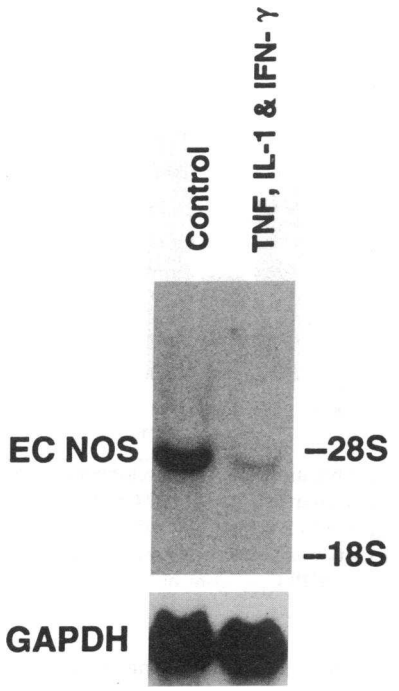

Figure 3. Effect of TNF, IL-1β, and IFN-γ on steady-state mRNA levels of endothelial cell NOS. Cultured HUVEC were treated with TNF (200 U/ml), IL-1β (5 U/ml), and IFN-γ (200 U/ml) for 24 h. 15 μg micrograms of total RNA was electrophoresed through a formaldehyde gel and transferred to a nylon membrane. The filters were hybridized to ^{32}P-labeled cDNA probe for endothelial NOS by standard methods at 43°C (47). The hybridized filters were washed twice in 2× SSC, 0.1% SDS for 15 min at room temperature, once in 2× SSC, 0.1% SDS for 30 min at 50°C, and once in 0.1× SSC, 0.1% SDS for 30 min at 65°C. Filters were then subject to autoradiography for 3–4 d at −80°C. The depicted Northern blot is representative of four separate experiments. Hybridization with GAPDH revealed no significant differences in mRNA loading between the samples.

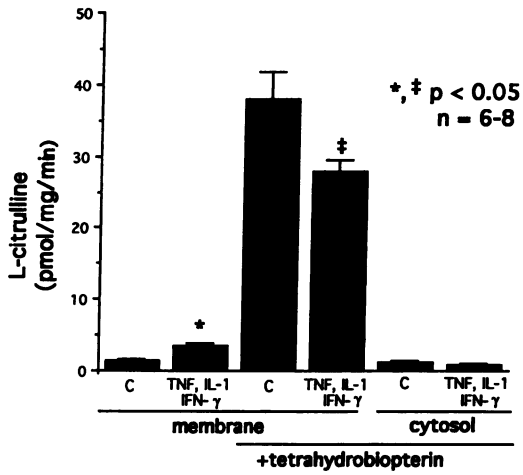

Figure 5. Effect of tetrahydrobiopterin on total NOS activity in HUVEC membranes and cytosol. Cultured HUVEC were treated with TNF (200 U/ml), IL-1β (5 U/ml), and IFN-γ (200 U/ml) for 24 h. Membrane and cytosolic fractions were incubated with NADPH (1 mM), $CaCl_2$ (1.6 mM), calmodulin (120 nM), and [^{14}C]L-arginine (0.06 μCi), in the absence or presence of tetrahydrobiopterin (3 μM) for 45 min at 37°C. In these experiments, the actual value of L-citrulline has been ascertained.

suggesting that this regulatory protein was not limiting. However, incubation of control cell lysates with tetrahydrobiopterin (3 μM) increased NOS activity 12-fold (Fig. 4). In cytokine-treated cells, exogenous tetrahydrobiopterin also increased NOS activity, but by less than twofold. Maximal NOS activity in cytokine-treated cells was actually ~ 30% less than in control cells.

Qualitatively similar results were obtained using HUVEC membranes (Fig. 5). Under all conditions, > 94% of the total NOS activity was localized to the particulate fraction in HUVEC. In control cell membranes, addition of tetrahydrobiopterin (3 μM) increased NOS activity 27-fold. However, in cytokine-treated membranes, exogenous tetrahydrobiopterin still increased activity by eightfold. Increasing the tetrahydrobiopterin to 300 μM did not further increase maximal NOS activity in either control or cytokine-treated membranes. Under these assay conditions, maximal NOS activity (in the presence of exogenous tetrahydrobiopterin) in cytokine-treated cells was also ~ 25% less than in control cells. When longer treatment periods, such as 48–72 h, were examined in cell lysates, maximal NOS activity further decreased by > 90% (Fig. 6). Thus,

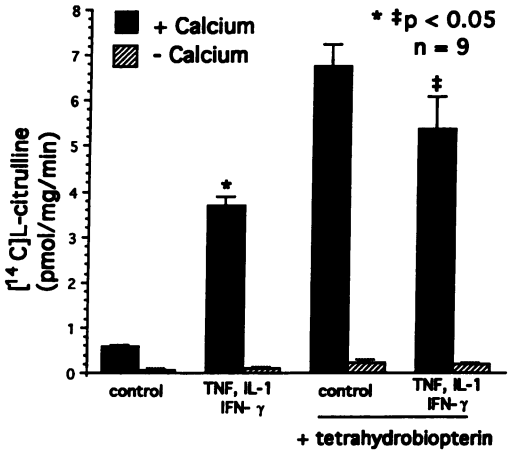

Figure 4. Effect of tetrahydrobiopterin on the cytokine-induced increase in NOS activity in HUVEC lysates. Cultured HUVEC were treated with TNF (200 U/ml), IL-1β (5 U/ml), and IFN-γ (200 U/ml) for 24 h. Cell lysates (150–250 μg) were incubated with NADPH (2 mM), [^{14}C]L-arginine (0.06 μCi), and either $CaCl_2$ (230 μM) or EDTA (1.7 mM) in the absence or presence of tetrahydrobiopterin (3 μM) for 90 min at 37°C.

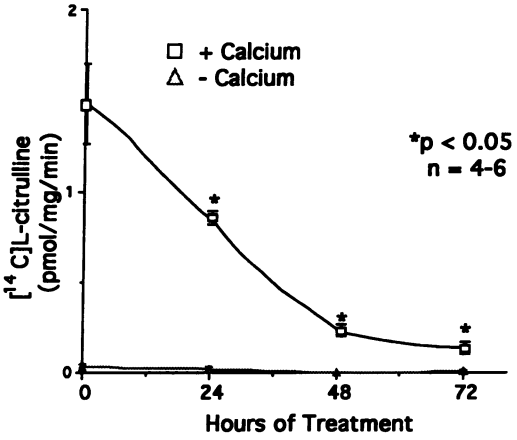

Figure 6. Time course of TNF, IL-1β and IFN-γ induced decrease in NOS activity in HUVEC lysates. Cultured HUVEC were treated with TNF (200 U/ml), IL-1β (5 U/ml), and IFN-γ (200 U/ml) for ≤ 72 h. Cell lysates (150–250 μg) were incubated with NADPH (2 mM), [^{14}C]L-arginine (0.06 μCi), and tetrahydrobiopterin (3 μM) in the presence of $CaCl_2$ (230 μM) or EDTA (1.7 mM) for 90 min at 37°C.

cytokine treatment appeared to increase the specific activity of the endothelial isoform of NOS by reducing the requirement for exogenous tetrahydrobiopterin. At no time examined did cytokine treatment induce any calcium-independent activity in HUVEC cytosol (Fig. 5) or lysates (Fig. 6).

Tetrahydrobiopterin levels in cultured HUVEC. Since tetrahydrobiopterin appears to limit NOS activity, we next investigated whether cytokines regulated the endogenous levels of pteridines (measured as biopterin and neopterin) in HUVEC. Treatment with TNF, IL-1β, or IFN-γ individually had no effect on pteridine levels (Fig. 7). However, the combinations of cytokines had a similar pattern of increasing pteridine levels as they did in increasing NOS activity. Specifically, IFN-γ in conjunction with either IL-1β or TNF increased both intracellular (Fig. 7) and extracellular (not shown) pteridine levels.

Pteridines are synthesized by a de novo pathway from guanosine triphosphate and by salvage pathways from oxidized pterins (52). In control cells, GTP cyclohydrolase I activity was undetectable. Treatment with TNF, IL-1β, or IFN-γ individually for 24 h had no effect (Fig. 8). However, IFN-γ in combination with either IL-1β or TNF increased GTP cyclohydrolase I activity. Thus, the ability of the various cytokine combinations to induce GTP cyclohydrolase I activity mirrored their effect on both pteridine levels and NOS activity.

NOS activity and tetrahydrobiopterin levels in freshly isolated HUVEC. To determine whether NOS activity is also limited in situ by low levels of tetrahydrobiopterin, we measured both intracellular pteridines and NOS activity in freshly isolated HUVEC. Biopterin levels were higher in freshly isolated cells than in control cultured cells (Table I), but were comparable to those in cytokine-treated cultured cells. The fall in pteridine levels occurs rapidly, since confluent primary cultures of HUVEC had undetectable amounts of pteridine. NOS activity in freshly isolated HUVEC lysates was higher than in cultured cell lysates (Fig. 9), but it still could be augmented twofold by incubation with exogenous tetrahydrobiopterin ($n = 4$; $P < 0.05$).

Discussion

The principal conclusions of our study are that NOS activity in cultured HUVEC is limited by tetrahydrobiopterin levels and

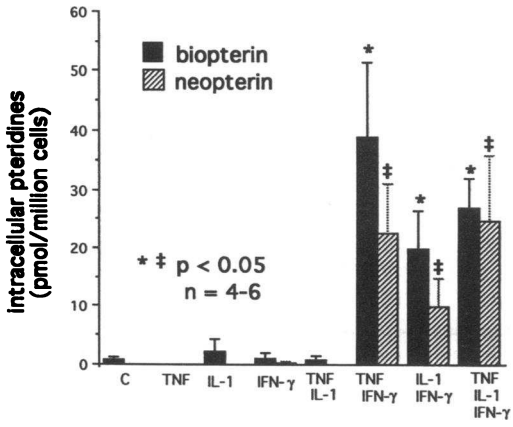

Figure 7. Effect of cytokine combinations on intracellular pteridine levels in cultured HUVEC. Cells were isolated after 24 h of treatment with TNF (200 U/ml), IL-1β (5 U/ml), and IFN-γ (200 U/ml). Pteridines were analyzed by HPLC after oxidation by iodine. *C*, control.

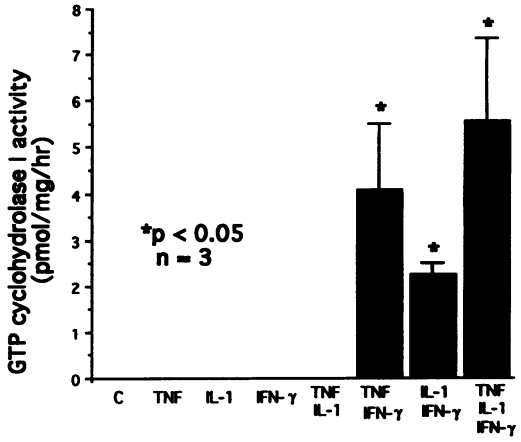

Figure 8. Effect of cytokine combinations on GTP cyclohydrolase I activity in cultured HUVEC. Cells were isolated after 24 h of treatment with TNF (200 U/ml), IL-1β (5 U/ml), and IFN-γ (200 U/ml). GTP cyclohydrolase I activity was measured by conversion of GTP to neopterin and analysis by HPLC. *C*, control.

that cytokines increase NOS activity by increasing tetrahydrobiopterin levels, probably through increased GTP cyclohydrolase I activity. Intracellular tetrahydrobiopterin levels were higher in freshly isolated HUVEC than in cultured cells, but were still limiting. Paradoxically, our data show that cytokines actually decrease NOS levels measured as steady-state mRNA and enzyme activity in the presence of exogenous tetrahydrobiopterin. However, the fall in NOS is more than compensated for by an increase in tetrahydrobiopterin at 24 h. Indeed, NO production after histamine stimulation was higher in cytokine-treated HUVEC than in control cells. In all cases examined, NOS activity in control and cytokine-treated HUVEC is membrane associated and calcium dependent and, thus, may be attributed to the endothelial isoform of NOS. We found no evidence for a high output, calcium-independent enzyme activity at any time examined, $\leq$ 72 h of cytokine treatment.

Our finding that cytokines increase NOS activity in HUVEC should be considered in light of previous reports regarding the cytokine regulation of endothelial cells. In intact cells, both Kilbourn and Belloni (27) and Lamas et al. (33) demonstrated that cytokines enhance NO production from murine brain microvascular, bovine aortic, and renal endothelial cells,

Table I. Intracellular Pteridine Levels in Cultured and Freshly Isolated HUVEC

HUVEC conditions	Pteridine levels	
	Biopterin	Neopterin
	pmol/million cells	
Cultured, passage 3–6		
Control (4)	<2	<2
TNF, IL-1β, and IFN-γ (4)	27.6±6.7*	24.6±11‡
Cultured, primary (3)	<2	<2
Freshly isolated (3)	31.7±1.3*	ND

Pteridines were analyzed by HPLC after oxidation by iodine. Values are mean±SEM. *‡ $P < 0.05$ vs control cultured HUVEC. Numbers in parentheses represent the number of samples. ND, not done.

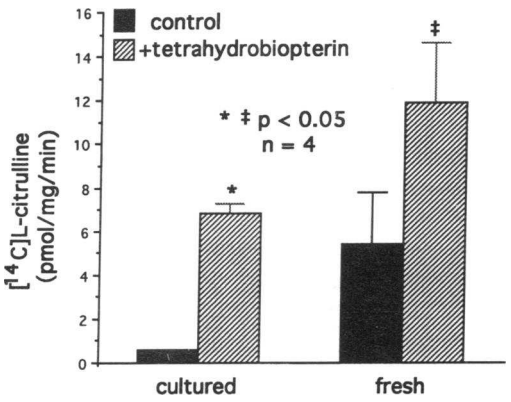

Figure 9. Effect of tetrahydrobiopterin on NOS activity in cultured and freshly isolated HUVEC. Cell lysates (150–250 μg) were incubated with NADPH (2 mM), [^{14}C]L-arginine (0.06 μCi), and $CaCl_2$ (230 μM) in the absence or presence of tetrahydrobiopterin (3 μM) for 90 min at 37°C.

respectively. Werner et al. (37) demonstrated that cytokines increase both basal and stimulated NO release from HUVEC as measured by cGMP accumulation. In contrast, Lamas et al. (33) demonstrated that TNF increased basal but not stimulated NO production, as measured by cGMP accumulation and [^{14}C]arginine to [^{14}C]L-citrulline conversion. Reports using cell lysates to characterize the induced NOS activity in endothelial cells have also yielded conflicting data. Busse and Mulsch (6) reported that TNF and IL-1β increased calcium-dependent NOS activity in porcine aortic endothelial cells. In contrast, Radomski et al. (35) reported that IFN-γ and LPS increased calcium-independent, cytosolic NOS activity in porcine aortic endothelial cells. Similarly, Gross et al. (53) also found that TNF and IFN-γ increased calcium-independent, cytosolic NOS activity in murine brain endothelial cells. Recently, Suzcheck et al. (38) demonstrated that IL-1β and IFN-γ increased steady-state mRNA level for a cytokine-inducible NOS isoform in rat aortic endothelial cells as detected by the probe for the mouse macrophage–inducible isoform, but they did not characterize the calcium dependency or subcellular localization of NOS activity. Werner et al. (37) reported that cytokines did not alter NOS activity in HUVEC lysates, while Radomski et al. (54) demonstrated that either TNF or IL-1β alone increased a calcium-independent activity in Simian virus-40–transformed HUVEC lysates. The ability of the different cytokine combinations to induce NOS activity is also in dispute. In our study, IFN-γ in conjunction with either TNF or IL-1β increased NOS activity, and no single cytokine was effective. In contrast, Lamas et al. (34) reported that TNF alone induced NOS activity, while IFN-γ inhibited it in bovine, aortic, and renal endothelial cells, and Busse and Mulsch (6) reported that TNF plus IL-1β increased NOS activity in porcine aortic endothelial cells.

The discrepancies among these studies may be attributed to four factors. First, isolation techniques for NOS are important and may alter the calcium dependence of the enzyme. Exclusion of calmodulin or addition of high concentrations of EDTA during purification or assay may strip calmodulin from the calcium-independent isoform, thus rendering it calcium sensitive (55, 56). In our studies, addition of exogenous calmodulin did not alter the results, suggesting that the observed calcium dependence was not an artifact of isolation. Second, the concentration of tetrahydrobiopterin in the assay conditions is critical. In our studies, tetrahydrobiopterin is limiting in HUVEC and the cytokine-induced increase in NOS activity may be masked by the addition of exogenous tetrahydrobiopterin. In the report by Werner-Felmayer et al. (37), NOS activity in cell lysates was measured in the presence of exogenous tetrahydrobiopterin, obscuring potential cytokine effects. Third, there may be important species differences; human mononuclear phagocytes do not demonstrate inducible NOS activity under conditions in which this enzyme is readily expressed in rodent cells (31). It is conceivable that a similar difference may underlie the divergent response of endothelial cells from human vs other species. Finally, the vascular source of endothelial cells may be important. Heterogeneity in the response of endothelial cells from different vascular beds is well documented (57). Therefore, our data on HUVEC should not be extrapolated as characteristic of all human endothelial cells. Further experiments will be required to address this issue.

Paradoxically, we demonstrated that treatment with a combination of TNF, IL-1β, and IFN-γ decrease steady state mRNA levels and enzyme activity (in the presence of exogenous tetrahydrobiopterin) of endothelial NOS after 24 h. Yoshizumi et al. (39) recently demonstrated that TNF downregulates endothelial NOS mRNA by decreasing its stability. The mechanism of the TNF effect is not known. It is unlikely that increased tetrahydrobiopterin levels directly alter transcription or mRNA stability of NOS, because TNF alone has no effect on tetrahydrobiopterin levels. In our experiments, the fall in endothelial NOS mRNA levels induced by cytokines at 24 h was much larger than the decline in NOS activity. The lag in the fall of enzyme activity may reflect greater stability of the NOS protein compared to mRNA or may involve posttranslational increases in enzyme specific activity.

Cytokine regulation of NO production by tetrahydrobiopterin synthesis has been previously documented. Werner et al. showed that IFN-γ could increase tetrahydrobiopterin levels in human tumor cell lines in human and murine fibroblasts and in macrophages (58, 59). In HUVEC, Werner-Felmayer et al. (37) reported that IFN-γ or TNF alone increased pteridine levels and that their effect was synergistic. In our study, IFN-γ in conjunction with either TNF or IL-1β, but no single cytokine, increased tetrahydrobiopterin levels in cultured HUVEC. The basis for the apparent discrepancy in the ability of the various cytokines to increase pteridine levels in HUVEC is not clear.

It is not surprising that NOS can be regulated through the availability of tetrahydrobiopterin. Werner-Felmayer et al. (60) demonstrated that the increase in tetrahydrobiopterin levels was crucial for cytokine-induced NO production in murine fibroblasts. The causal relationship between increased tetrahydrobiopterin levels and cytokine-induced increase in NO production was confirmed in rat aortic smooth muscle cells (61) and a murine macrophage cell line (62). Tetrahydrobiopterin is an important cofactor for full activity of all NOS isoforms (62–64). The EC_{50} of endothelial NOS for tetrahydrobiopterin is 0.1 μM (5). Initial reports indicated that tetrahydrobiopterin was stoichiometrically oxidized in the conversion of L-arginine to NO (65). More recent evidence contradicts these observations and suggests that tetrahydrobiopterin is an allosteric effector of NOS (12, 63) through stabilization of the dimeric, active form of the enzyme (66).

Our studies indicate that cytokines increase GTP cyclohydrolase I activity, the rate-limiting enzyme for de novo tetrahydrobiopterin synthesis. This observation is in accordance with previous observations by Werner-Felmayer et al. (37). Cytokines may increase GTP cyclohydrolase I activity in HUVEC either by increasing enzyme activity or levels. In rat vascular smooth muscle cells, LPS and IFN-γ increased steady state levels of mRNA for GTP cyclohydrolase I (67). Preliminary studies in our laboratory suggest that cytokines induce a single transcript in HUVEC that hybridizes to the cDNA probe for rat liver GTP cyclohydrolase I (68). Further characterization of this induction is in progress.

A major question posed by our study is whether cytokines increase NO production by increasing tetrahydrobiopterin levels in vivo. Both tetrahydrobiopterin and neopterin are detectable in human tissues (50) and in fluids such as urine and plasma (49). Moreover, elevated neopterin levels correlate with activation of cellular immunity (reviewed in reference 69), and increased neopterin levels have been documented in patients with overwhelming and fatal sepsis (70, 71). However, NO production has not been measured in these same patients. We found that freshly isolated HUVEC have markedly higher tetrahydrobiopterin levels than cultured cells (passages 3–6). The levels closely approximate tetrahydrobiopterin levels in cytokine-treated HUVEC, and freshly isolated HUVEC display higher NOS specific activity. However, even at these levels, tetrahydrobiopterin still appears limiting in freshly isolated cells, and excess tetrahydrobiopterin still increases NOS activity twofold. Therefore, the possibility remains that cytokines could potentially further increase tetrahydrobiopterin levels in freshly isolated cells. Furthermore, tetrahydrobiopterin levels in fresh HUVEC may have been stimulated by cytokines associated with parturition, resulting in levels that are elevated over basal values.

Why are resting levels of tetrahydrobiopterin so low in cultured HUVEC? It seems likely that this deficiency results from the undetectable levels of GTP cyclohydrolase I activity in these cells. It is possible that this low level of GTP cyclohydrolase I activity reflects the presence of an inhibitor of enzymatic activity. However, this seems unlikely because enzyme rates were directly proportional to amount of added protein and recovery of added rat liver GTP cyclohydrolase I was > 90%. Activity is not lost because of cell senescence, since GTP cyclohydrolase I activity was equally low in confluent primary cultures as in serially passaged HUVEC. Thus, it seems that enzyme synthesis is shut off in HUVEC culture. This could be caused by the presence of serum or other factors in culture medium. Also, a key difference between endothelial cells in situ and those in culture is that the former are quiescent. Even at confluence, HUVEC in culture remain in cell cycle, perhaps because of the presence of growth factors. It remains to be seen if this difference is relevant, and whether there could be similar suppression of tetrahydrobiopterin production, and concomitantly NO production, at sites of endothelial injury and regeneration in vivo.

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