Role of Tetrahydrobiopterin Availability in the Regulation of Nitric-oxide Synthase Expression in Human Mesangial Cells

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Human mesangial cells express an inducible form of nitric-oxide synthase (iNOS) after treatment with cytokines. Tetrahydrobiopterin (BH₄), an essential cofactor for NOS, is required for cytokine-induced NO generation. We report here that BH₄ is necessary not only for the activity but also for the expression of iNOS in human mesangial cells. Inhibition of de novo BH₄ synthesis with 2,4-diamino-6-hydroxypteridimine (DAHP) significantly attenuated iNOS activity as well as mRNA and protein expression in response to interleukin 1β plus tumor necrosis factor α (IL-1β/TNF-α). In contrast, sepiapterin, which provides BH₄ through the pterin salvage pathway, strongly potentiated IL-1β/TNF-α-induced iNOS expression and abrogated the inhibitory effect of DAHP. Inhibition of the pterin salvage pathway with methotrexate abolished sepiapterin potentiation of iNOS induction but did not alter the effect of IL-1β/TNF-α. Determination of intracellular pteridines confirmed that sepiapterin markedly raised BH₄ content, an effect that was blocked by methotrexate. These results suggest that BH₄ availability plays an important role in the regulation of iNOS expression. The effect of BH₄ appears to be mediated, at least in part, by an increase in mRNA stability, as indicated by the observation that DAHP shortened, whereas sepiapterin prolonged the half-life of IL-1β/TNF-α-induced iNOS mRNA. Taken together, our results suggest that the biosynthesis of BH₄ contributes to cytokine induction of iNOS expression in human mesangial cells through the stabilization of iNOS mRNA.

Nitric oxide (NO) is a biological messenger that is involved in many physiological processes such as the regulation of vascular tone, neurotransmission, and cell-mediated cytotoxicity (1, 2). In addition, an altered NO generation is involved in several pathophysiological situations, including chronic and acute inflammation, atherosclerosis, glomerulonephritis, and renal failure (3–5). The synthesis of NO is accomplished by the NO synthases (NOS), a family of enzymes from which three isoforms are presently known. The neuronal and endothelial NOSs are considered constitutive, and their activity is regulated mainly postranslationally. The inducible isoform (iNOS) is expressed in various cell types, including macrophages and vascular smooth muscle cells, in response to certain stimuli, such as cytokines or bacterial cell products, and its regulation occurs mostly at the level of transcription (6, 7). NOS require several cofactors for activity, namely, FAD, FMN, heme, and tetrahydrobiopterin (BH₄) (8, 9). Heme and BH₄ have been shown to be required for dimerization and acquisition of NO-generating capacity by macrophage iNOS (8). In addition, BH₄ availability has been demonstrated to be a limiting factor for iNOS activity in many cell types (10–13). The intracellular levels of BH₄ are determined by the activity of two different biosynthetic pathways: the de novo synthesis from GTP and the regeneration of BH₄ from dihydropteridines through a pterin salvage pathway (14). The first enzyme in the de novo pathway is GTP cyclohydrolase I. Cytokines have been reported to stimulate potently the de novo synthesis of BH₄ in several cell types through the induction of GTP cyclohydrolase I (15–17). In fact, expression of GTP cyclohydrolase I and iNOS appears to be regulated coordinately (16, 18). This phenomenon has been interpreted previously as a mechanism to ensure an adequate supply of BH₄ for the activity of cytokine-induced NOS (10–13, 18). In addition to its catalytic role, BH₄ has been reported to protect NO from feedback inhibition by NO in vitro (19, 20), and to stabilize the structure of both the macrophage and the neuronal NOS proteins (8, 21). We were interested in studying the possibility that BH₄ could also be modulating iNOS protein or mRNA expression. To test this hypothesis we have used human mesangial cells (HMC) as an experimental model. Mesangial cells are specialized vascular smooth muscle cells that are disposed around the capillaries of the renal glomerulus serving functions of structural support and regulation of glomerular filtration rate (22). These cells express iNOS in response to cytokines (23–25). In contrast to rat mesangial cells, which can be induced with single stimuli (24, 25), HMC require multiple cytokines to produce NO (26). In this article, we have studied the effect of BH₄ availability on the expression of iNOS by HMC elicited by a combination of interleukin 1β plus tumor necrosis factor α (IL-1β/TNF-α). The results herein reported suggest that BH₄ modulates iNOS mRNA expression, thus identifying a new role for this cofactor in the complex regulation of cytokine-induced NO generation.

EXPERIMENTAL PROCEDURES

Materials—Recombinant human TNF-α (9.8 × 10⁶ units/mg) was a gift from Knoll Pharmaceuticals (Whippany, NJ). Recombinant human IL-1β (5 × 10⁶ units/mg) was from Boehringer Mannheim. Cell culture media and fetal calf serum were from Life Technologies, Inc. [α-³²P]dCTP (3,000 Ci/mmol) was from Amersham Corp. Antibodies for cell characterization were obtained from local sources. l-Sepiapterin was from Alexis Co. (Laufelfingen, Switzerland). All other reagents used were of the highest purity available from Sigma.

1 The abbreviations used are: NO, nitric oxide; NOS, nitric-oxide synthase(s); iNOS, inducible nitric-oxide synthase(s); BH₄, 2-amino-4,6-dihydroxypteridin; DAHP, 2,4-diamino-6-hydroxypteridimine; HMC, human mesangial cells; HPLC, high performance liquid chromatography; IL, interleukin; TNF-α, tumor necrosis factor α; L-NAME, L-nitroarginine methyl ester.
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Cell Culture—HMC were obtained from normal cadaver kidneys that were unsuitable for transplantation. Primary cultures were obtained using a technique of graded sieving. Groups of cells with mesangial morphology were subcloned and expanded. Mesangial cells displayed positive staining with anti-vimentin, anti-Thy 1.1, and anti-myosin and absence of staining with anti-factor VIII. The cells were grown in RPMI 1640 supplemented with 10% fetal calf serum, 2 mM glutamine, 100 units/ml penicillin, and 24 μg/ml gentamicin. For experiments, passages 7–12 were used. Confluent HMC were washed twice with RPMI without phenol red and incubated in this medium with the indicated agents in the absence of serum. TNF-α was used at 100 ng/ml, and IL-1β was used at 2 ng/ml, final concentrations. Several doses of sepiapterin (10−7−10−3 M), DAHP (0.5−2 mM), and methotrexate (5−20 μM) were tested, and those found optimal for potentiation or inhibition of iNOS activity in HMC, respectively (100 μM sepiapterin, 1.5 mM DAHP, 10 μM methotrexate), were used for further experimentation. Potential toxicity of the reagents used on HMC was evaluated by trypan blue exclusion. According to these criteria, cell viability was above 90% under all experimental conditions studied.

Nitrite Determination—The accumulation of nitrite in the cell culture supernatant of HMC was taken as an index of iNOS activity. After treatment with the various agents for 24 h, nitrite was determined in the supernatants of HMC by the Griess reaction as described previously (27), using sodium nitrite as a standard.

RNA Isolation and Northern Blot Analysis—Total cellular RNA was isolated from HMC using the guanidinium thiocyanate-phenol-chloroform method (28). 10 μg of total RNA was separated on 1% agarose, 0.66 M formaldehyde gels, transferred to Hybond hybridization transfer membranes (Amersham), and UV cross-linked before hybridization as described previously (27). A 1.8-kilobase fragment of the human hepatic inducible NOS isoform cDNA, a gift of Dr. D. Geller (29), was labeled with [α-32P]dCTP using a commercial kit for random hexamer labeling (Boehringer Mannheim) and used as probe for Northern analysis. Hybridization was performed at 42 °C for 12–16 h. Membranes were then washed at final stringency conditions of 55 °C, 0.2 × SSC, 0.1% SDS, and exposed to XAR Kodak film at −80 °C using an intensifying screen. To identify differences in mRNA expression specific for iNOS, blots were prehybridized and then hybridized with a judiciously chosen probe (30). Autoradiographic analysis was performed on a computing densitometer or on a PhosphorImager (Molecular Dynamics, Sunnyvale, CA). Results were expressed in arbitrary units as the ratio of iNOS to β-actin expression.

Determination of mRNA Half-life—HMC were stimulated with IL-1β/TNF-α for 24 h in the presence or absence of DAHP or sepiapterin. For determination of mRNA half-life, actinomycin D (10 μg/ml) was added subsequently, and cells were then harvested for RNA isolation and Northern blot analysis every 2 h for 8 h as described above.

Determination of Intracellular Biopterin—HMC treated for 24 h with the indicated agents were harvested with a cell scraper and washed twice with phosphate-buffered saline. Pellets were resuspended in 250 μl of distilled water and homogenized by sonication with an ultrasonic cell disrupter. Cell extracts were centrifuged at 14,000 × g for 3 min, and the supernatants were split into two aliquots that were subjected to oxidation with iodine at alkaline or at acidic pH, essentially as described (31). During alkaline oxidation 7,8-dihydrobiopterin is converted into biopterin (90% conversion), and BH4 is converted into pterin by oxidation with iodine at alkaline or at acidic pH, essentially as described (31). During alkaline oxidation 7,8-dihydrobiopterin is converted into biopterin (90% conversion), and BH4 is converted into pterin by oxidation with iodine at alkaline or at acidic pH, essentially as described (31). During alkaline oxidation 7,8-dihydrobiopterin is converted into biopterin (90% conversion), and BH4 is converted into pterin by oxidation with iodine at alkaline or at acidic pH, essentially as described (31). During alkaline oxidation 7,8-dihydrobiopterin is converted into biopterin (90% conversion), and BH4 is converted into pterin by oxidation with iodine at alkaline or at acidic pH, essentially as described (31). During alkaline oxidation 7,8-dihydrobiopterin is converted into biopterin (90% conversion), and BH4 is converted into pterin by oxidation with iodine at alkaline or at acidic pH, essentially as described (31).

BH4 Synthesis Is Required for Cytokine-induced NO Generation by HMC—Treatment of HMC with IL-1β/TNF-α for 24 h resulted in the induction of NO generation as indicated by the accumulation of nitrite in the incubation medium (Fig. 1). NO generation in response to cytokines was l-arginine-dependent since it was inhibited potently by the NOS antagonist l-nitroarginine methyl ester (L-NAME). The addition of 500 μM L-NAME during cytokine stimulation produced an 80–85% inhibition of NO synthesis. To determine the importance of BH4 supply for cytokine-induced NOS activity, we explored the effect of both donors and inhibitors of BH4 generation on nitrite formation by IL-1β/TNF-α-stimulated HMC. Inhibition of the de novo synthesis of BH4 with DAHP, an inhibitor of GTP cyclohydrolase I (33), significantly reduced IL-1β/TNF-α-induced nitrite production. In contrast, addition of sepiapterin, which provides BH4 via the dihydrofolate reductase-dependent pterin salvage pathway (14), produced a strong potentiation of cytokine-induced NO generation and circumvented the inhibitory effect of DAHP, although it did not elicit nitrite formation by unincubated cells (Fig. 1). Sepiapterin potentiation of cytokine-induced nitrite accumulation was dose-dependent, 1 μM being the lowest concentration that induced a significant (2-fold) increase over the values obtained by stimulation with IL-1β/TNF-α alone (results not shown). At 100 μM, sepiapterin produced a 6–8-fold potentiation of cytokine-elicted NO generation (Fig. 1). This effect, which was also l-arginine-dependent, was unaffected by DAHP. However, inhibition of dihydrofolate reductase with methotrexate (14) completely prevented sepiapterin potentiation of cytokine-induced NO generation. In
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**Fig. 2.** Effect of donors and inhibitors of BH4 generation on the levels of iNOS mRNA in IL-1β/TNF-α-treated HMC. HMC were treated with the indicated agents for 24 h as in Fig. 1, and the levels of iNOS and β-actin mRNA were analyzed by Northern blot as detailed under “Experimental Procedures.” Panel A, autoradiographic exposures from a representative experiment. Kb, kilobases. Panel B, densitometric quantitation of iNOS mRNA expressed in arbitrary units as the ratio of iNOS to β-actin mRNA levels. Results are average values from three independent experiments ± S.E. *p < 0.05 versus control; ‡p < 0.05 versus cytokine combination.

In contrast, the addition of methotrexate to IL-1β/TNF-α-treated HMC did not affect the accumulation of nitrite in the incubation medium of HMC (Fig. 1), suggesting that generation of BH4 through the salvage pathway is quantitatively less important during cytokine stimulation of HMC. Taken together, these results indicate that BH4 biosynthesis is a necessary event during cytokine-induced NO generation by HMC.

BH4 Modulates the Steady-state mRNA and Protein Levels of iNOS—The potentiating of cytokine-induced nitrate accumulation described above could be due to an enhancement of the catalytic activity of iNOS by BH4. In addition, BH4 could play a regulatory role at the level of protein or mRNA expression. To test this hypothesis, we examined the levels of iNOS mRNA in HMC in the conditions under which we had observed changes in iNOS activity. Treatment of HMC with IL-1β/TNF-α alone or in combination with methotrexate for 24 h induced the appearance of the iNOS transcript, which was undetectable in untreated cells as well as in cells treated with sepiapterin alone (Fig. 2). The addition of DAHP significantly diminished the levels of iNOS mRNA in response to IL-1β/TNF-α. Conversely, supplementing the incubation medium with sepiapterin resulted in a 6–8-fold potentiation of cytokine-induced iNOS mRNA expression. Sepiapterin potentiation of iNOS mRNA expression was unaffected by DAHP, but it was completely prevented by methotrexate. Quantitation of these results by densitometric analysis revealed the existence of a close correlation between iNOS activity (Fig. 1) and mRNA levels (Fig. 2B) under the various conditions studied. This correlation could also be evidenced when iNOS expression was studied at the protein level by Western blot analysis (Fig. 3).

These results suggested that BH4 biosynthesis was required not only for iNOS activity but also for its expression. To study the relationship between BH4 availability and the observed effects on iNOS activity and expression, changes in BH4 levels in response to the various agents used were monitored. BH4 levels in control HMC were below 0.5 pmol/mg protein. Although no significant changes in intracellular BH4 levels could be evidenced after 24-h treatment with either IL-1β/TNF-α or IL-1β/TNF-α plus DAHP, the addition of sepiapterin to the incubation medium did result in a potent increase in total intracellular bipterin levels (sum of bipterin plus 7,8-dihydrobiopterin plus BH2) up to 53.5 ± 8.5 pmol/mg protein. Approximately 65% of this increase was due to the generation of BH4. The conversion of sepiapterin into BH4 was totally and selectively prevented by methotrexate, without significantly reducing total bipterin levels (45.4 ± 9.6 pmol/mg protein). This indicates that sepiapterin potentiation of iNOS expression requires its conversion into BH4.

Effect of BH4 Availability on iNOS mRNA Half-life—To gain insight into the mechanism of the effect of BH4 on iNOS expression, we evaluated its potential contribution to iNOS mRNA stability. For this, the half-life of iNOS mRNA from HMC that had been treated with IL-1β/TNF-α or in combination with sepiapterin or DAHP as in Fig. 2 was estimated after the addition of actinomycin D (Fig. 4). We observed that DAHP significantly shortened iNOS mRNA half-life, from 6.1 ± 1.0 to 2.8 ± 0.1 h (p < 0.05), whereas the addition of sepiapterin to cytokine-treated HMC resulted in a marked stabilization of iNOS mRNA, the half-life of which increased to 14.4 ± 3.2 h (p < 0.05). These effects contribute to generate the differences in iNOS mRNA levels illustrated in Fig. 2.

**Fig. 3.** Effect of DAHP, sepiapterin, and methotrexate on cytokine-induced iNOS protein expression. HMC were treated with the indicated agents for 24 h as in Fig. 1, and the levels of iNOS protein were estimated by Western blot analysis as detailed under “Experimental Procedures.” 2 μg of a lysate of the macrophage cell line RAW 264.7 stimulated with 1 μg/ml lipopolysaccharide plus 10 ng/ml interferon-γ (LPS+IFN-γ) for 12 h was used as positive control. Results are representative of four independent experiments. The position of the iNOS band is indicated on the right and that of molecular weight markers on the left of the figure.
treatment with cytokines did not induce any further increase in iNOS mRNA levels.

**DISCUSSION**

BH4 biosynthesis appears to be an essential requirement for the induction of NOS activity in a variety of experimental models (11, 13, 35). Evidence for this has arisen mainly from the use of inhibitors of the BH4 biosynthetic pathway, including inhibitors of GTP cyclohydrolase I, such as DAHP (33), or of sepiapterin reductase, such as N-acetylserotonin, phenprocoumon, or dicumarol (36, 37), which cause a depletion of intracellular BH4 levels, both in resting and in stimulated cells (15, 17, 38). Since the amount of NO generated seems to be limited by the amount of BH4 present in cells, the inhibition of BH4 biosynthesis has been envisaged as a target for the design of therapeutic tools to be used in the pathological conditions as-sociated with an abnormally increased NO production (12, 13, 35). It is assumed that the main role of the increased pteridine synthesis observed during cytokine stimulation in a variety of cell types is to provide a cofactor for the BH4-dependent generation of NO by the cytokine-induced NOS. Our results indicate, however, that BH4 biosynthesis also contributes to the regulation of the expression of iNOS mRNA and protein in response to cytokine stimulation of HMC. We have observed that the GTP cyclohydrolase I inhibitor DAHP notably diminishes cytokine-induced iNOS mRNA accumulation. In contrast, increasing BH4 availability via the salvage pathway by the addition of sepiapterin not only circumvents the inhibitory effect of DAHP, but it strongly potentiates the effect of cytokines on iNOS mRNA and protein induction. Thus, in HMC, the mechanism of DAHP inhibition of iNOS expression appears to be due to the limitation of BH4 biosynthesis, although a BH4-independent effect of DAHP on iNOS expression, analogous to that reported in primary murine macrophages (39), cannot be ruled out. Therefore, our results reinforce the significance of the frequently observed coinciduous of GTP cyclohydrolase and iNOS by inflammatory stimuli (16, 17). Although cytokines have been reported to stimulate the activity of GTP cyclohydrolase I strongly in a variety of systems, the overall stimulation of BH4 biosynthesis and its impact on iNOS induction appear to be highly variable depending on the cell type, the stimuli employed, and the basal BH4 levels (18, 39). The BH4 content of untreated HMC is remarkably low. The cytokine combination used throughout this study, IL-1β/TNF-α, elicited a submaximal induction of iNOS in these cells, which was not paralleled by a significant increase in intracellular BH4 levels, but it was nevertheless susceptible to modulation by the inhi-
tion of BH₄ biosynthesis or by the addition of an exogenous BH₄ source. This suggests that BH₄ availability is indeed playing a role in the regulation of iNOS expression in HMC. On the other hand, discrepancies between nitrite accumulation and cytosolic BH₄ concentrations during iNOS induction have been documented and interpreted previously on the basis that the newly synthesized iNOS protein, which has high affinity for BH₄ (40), depletes BH₄ from the cytosol rapidly (12). In this way, BH₄ would become a limiting factor for full iNOS activity probably because of a relatively lesser induction of BH₄ biosynthesis (12).

The effect of BH₄ on iNOS expression in HMC appears to be mediated, at least in part, by a contribution to mRNA stabilization since DAHP significantly reduced iNOS mRNA half-life, whereas sepiapterin prolonged it. This implies that in HMC, iNOS expression can be subjected to regulation at the posttranscriptional level. In addition, preliminary results from our laboratory suggest that increasing BH₄ availability with sepiapterin could also have stimulatory effects on the transcription rate of iNOS mRNA in nuclear runoff assays (results not shown). However, the intrinsic biochemical mechanisms of these phenomena remain to be elucidated. It should be noted that an increment in BH₄ levels per se, like that brought about by sepiapterin supplementation, was not able to induce iNOS activity or mRNA expression in HMC. In addition, the existence of BH₄-dependent proteins involved in mRNA stabilization and/or transcription factors has not been documented. Thus, a direct effect of BH₄ on iNOS mRNA expression seems unlikely. Clearly, other possibilities of interaction between BH₄ availability and iNOS induction need to be considered, including the potential contribution of the NO generated as a consequence of iNOS activity. NO has been reported to exert both negative and positive feedback on iNOS activity and expression (41, 42). In astroglial cells, the expression of iNOS mRNA elicited by a combination of IL-1γ and IFN-γ has been found to be amplified in the presence of NO inhibitors or NO-trapping agents and to be reduced by NO donors (41). Interestingly, BH₄ has been reported to inactivate NO by inducing its oxidation in a 1:1 stoichiometry (43). Therefore, BH₄ could be acting as a NO scavenging agent in HMC, thus attenuating NO negative feedback on iNOS expression. In contrast, in rat mesangial cells, NO has been described to potentiate IL-1β-induced iNOS expression (42). In this context, BH₄ availability would influence iNOS activity and consequently the amount of NO generated, which in turn would be responsible for the potentiation of iNOS induction (42). However, in neither of these experimental systems did NO appear to influence iNOS mRNA stability (41, 42). Under our experimental conditions, the manipulation of the NO generated by means of the NO donor sodium nitroprusside and the NO inhibitor l-NMMA did not result in substantial changes in cytokine-induced iNOS expression or in its potentiation by sepiapterin. Although detailed studies would be needed to clarify completely the effects of NO on iNOS expression, our results do not support a major role for NO in the amplification of iNOS induction observed in HMC.

The results herein reported suggest that the induction of iNOS in HMC results from the integration of multiple signals. On one hand, cytokines would directly stimulate responsive elements on the iNOS gene. At the same time, they would indirectly potentiate the activity and expression of iNOS by stimulating the synthesis of BH₄, which would contribute to the catalytic activity of iNOS and to iNOS mRNA expression. In vivo, the supply of BH₄ to perform these functions could come from several sources: from the activation of the BH₄ biosynthetic pathway in the mesangial cells and/or in the endothelial cells that line the capillaries of the glomerulus. In human endothelial cells, interferon-γ, bacterial lipopolysaccharide, IL-1, and TNF-α have been reported to stimulate the synthesis of BH₄ (15, 35, 44, 45). Interestingly, up to 90% of the newly synthesized BH₄ is released into the culture medium (45). This release is not random, but vectorially directed into the basal compartment, thereby providing underlying smooth muscle cells with the cofactor needed for NO production (46).

Our results suggest that the secreted BH₄ can also potentiate iNOS mRNA expression in HMC. Importantly, some of the so-called deactivating cytokines, including IL-4 and IL-10, have been reported to suppress profoundly the BH₄ synthesis elicited by inflammatory stimuli in endothelial cells, leading to the complete disappearance of extracellular BH₄ (45). These deactivating cytokines are also known to inhibit iNOS activity and expression in various cell types (47, 48). It would be interesting to determine whether the inhibitory effect of these cytokines on iNOS expression can be mediated, at least in part, by the down-regulation of BH₄ biosynthesis, and if so, whether GTP cyclohydrolase I can be a target for their action.

In conclusion, our observation that the availability of BH₄ can modulate the expression of iNOS in HMC strengthens the hypothesis that this cofactor can play an important role in the regulation of NO generation and consequently of vascular tone. Therefore, the pharmacological manipulation of BH₄ levels emerges as a valuable approach for the management of NO-related pathophysiological processes.
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