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Modulation of Inducible Nitric Oxide Synthase mRNA Stability by Tetrahydrobiopterin in Vascular Smooth Muscle Cells

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Tetrahydrobiopterin (BH4) regulates inducible nitric oxide synthase (iNOS) as cofactor and allosteric effector. The present paper describes a novel function of BH4 in vascular smooth muscle cells (SMC). By varying BH4 levels with dicumarol (an inhibitor of BH4 synthesis) and sepiapterin (an exogenous source of cofactor), we investigated iNOS expression in activated rat aortic SMC. In sepiapterin-supplemented cells, iNOS protein levels were increased while in dicumarol-treated cells, iNOS levels were diminished. Time-kinetic experiments revealed that inhibition or supplementation of BH4 synthesis had no effects on iNOS induction or transcription rate. However, iNOS mRNA was present over a prolonged time in sepiapterin-supplemented SMC. Analysis of iNOS mRNA levels showed stable iNOS mRNA in sepiapterin-treated cells 8 hours after transcription inhibition, while in dicumarol-treated cells iNOS mRNA disappeared. The decrease of iNOS mRNA by dicumarol was abolished by sepiapterin. These data indicate that BH4 post-transcriptionally stabilizes iNOS mRNA in SMC. By this way BH4 modulates iNOS expression in the vascular system.

Nitric oxide (NO), a radicalic gas, has strong vasodilating properties through activation of the cGMP-dependent protein kinase system in smooth muscle cells (1). Endogenously synthesized NO is an important factor for the control of the vascular tone in health and disease (2–4). In the vascular system two types of NO synthase (NOS) isoenzymes produce NO by converting L-arginine to L-citrulline. The constitutive isoform (cNOS) regulates the vascular tone and normal blood pressure under physiologic conditions (1, 5). The inducible isoform of NOS (iNOS) is responsible for the generation of NO in pathologic states such as septic shock and is trancriptionally regulated by pro- and anti-inflammatory cytokines as well as endotoxin (LPS) (4,6). cNOS is found exclusively in endothelial cells (7, 8), while iNOS is abundant in smooth muscle cells (SMC) after induction (4, 7). The expression of vascular cNOS is downregulated by inflammatory stimuli, while iNOS is concomitantly induced (7, 9).

The regulation of iNOS activity by tetrahydrobiopterin (BH4) as a cofactor and allosteric dimerization factor is well established (10-13). Other functions of BH4 in the L-arginine/NO pathway remain to be elucidated. In this regard it is noteworthy that NO synthesis and BH4 synthesis are induced by the same inflammatory signals (11, 14-17), but BH4 synthesis precedes NO production (18, 19). In SMC endogenous BH4 is not sufficient for maximal cytokine-induced NO generation (20, 21). Furthermore, in the vascular system, inflammatory activated endothelial cells synthesize large amounts of BH4 (16, 22, 23) that is basally secreted, thereby enhancing NO production of underlining vascular SMC (24).

Since vascular SMC are the main source of the prolonged NO production leading to hypotonic vasodilation in septic shock, and because BH4 seems to be an important regulatory factor of these events, we investigated the effect of BH4 on iNOS expression in rat aortic SMC. Using dicumarol, an inhibitor of endogenous BH4 production (18), and sepiapterin which is converted to BH4 through a salvage pathway (11, 20), as exogenous source of cofactor we show in the present study that BH4 modulates iNOS expression in SMC at the post-transcriptional level.

MATERIALS AND METHODS

Materials. Rat recombinant IFN-γ was from R&D systems (R&D Europe, Abingdon, UK). Sepiapterin was from Dr. B. Schircks Laboratories (Jona, Switzerland). LPS (E. coli O26:B6, Boivin extraction) was from Difco (Detroit, MI, USA). Actinomycin D and dicu-

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marol were purchased from Sigma (St. Louis, MO, USA). All other chemicals were of analytical grade from commercial sources.

Cell cultures. Primary cultures of rat aortic SMC (25) were maintained in a humidified 5% CO_2/95% air atmosphere at 37°C in IMDM (Gibco Europe, Basel, Switzerland) supplemented with 10% FCS (low tox, PAA, Linz, Austria), 4 mM L-glutamine and 50 μg/ml gentamycin (both tissue culture grade, Sigma), referred to as complete medium. Cells in passage 10-15 were seeded in 100-mm tissue culture dishes (Falcon, Oxnard, CA, USA) and used for experiments when confluence was achieved. Cultured cells were shown to be smooth muscle cells by positive immunoreactivity for smooth muscle α-actin and desmin. For induction of iNOS SMC were activated with a combination of IFNγ, 100 U/ml and LPS, 10 μg/ml, throughout experiments (21). Activated SMC were supplemented with 100μM sepiapterin or 100μM dicumarol as indicated.

Determination of iNOS activity. Confluent cells on 100 mm tissue culture dishes were incubated with the indicated agents. At the times indicated, cells were washed three times with 0.9% NaCl and scraped in 500 μl assay buffer consisting of 25 mM Tris-HCl, pH 7.5, and 10μg/ml α-macroglobulin (universal protease inhibitor, Boehringer Schweiz AG, Rotkreutz, Switzerland). Cells were disrupted by four cycles of freeze/thaw in liquid nitrogen and centrifuged at 15000 rpm for 30 minutes at 4°C. NO synthase activity was measured as described previously (11), with the following modifications: The reaction mixture contained 16 mM Tris-HCl, pH 7.5, 0.1 mM NADPH, 1 mM L-arginine, 1.0 mM Mg^{2+}, 1 mM dithioerythritol, 60 μM BH_4, and cell lysate in a total volume of 300 μl. The mixture was incubated in the dark at 37°C for 6 h. The reaction was stopped by shock freezing. Nitrates which could possibly be formed by oxidation of nitrites were reduced by 2 hours incubation at room temperature with 60 mU nitrate reductase (Boehringer) and 50 μM NADPH. Nitrite was detected in a microplate assay using Griess reagent and sodium nitrate in assay buffer as standard (11).

Western blot analysis. Cell lysates were obtained as described above. Aliquots containing 10 μg protein were electrophoresed on 4-20% gradient minigels (Bio-Rad, Hercules, CA, USA) and transferred to nitrocellulose membranes (Schleicher & Schuell, Dassel, Germany) using a mini transblot tank system (Bio-Rad). Blots were probed with anti iNOS mouse monoclonal antibody (Affinity Research Products, Nottingham, U.K.) followed by incubation with a goat anti-mouse-Ig horseradish peroxidase conjugate (Bio-Rad). iNOS protein was visualized using an enhanced chemiluminescence detection system (ECL, Amersham International, Little Chalfond, Buckinghamshire, UK).

Dot blot analysis. Aliquots of the above mentioned cell lysates containing 1μg protein were 2-fold gradually diluted onto nitrocellulose membranes using a dot-blot microfiltration unit (Bio-Rad) . Dot blots were probed for iNOS as described above.

mRNA analysis. Total cellular RNA was isolated using the guanidinium thiocyanate-phenol-chloroform method (26) with chemicals from Stratagene, La Jolla, CA, USA. RNA extracts were visualized by ethidium bromide in 1% agarose gels and quantified by OD measurements at 260 nm and 280 nm. Aliquots containing 10μg RNA were reverse transcribed using oligo(dT) primer (Boehringer) and M-MLV reverse transcriptase (Stratagene) followed by PCR using Taq DNA polymerase (Stratagene) according to the following schedule: denaturation, annealing, elongation at 94, 55 and 72°C for 1, 1, and 2 minutes, respectively, for 30 cycles. The following primers were used: rat iNOS specific primers (27), (forward: 5′-ATGGCTTGCCCCTGGAAGTTTCTC-3′ reverse: 5′-CCTCTGATGGTGCCATCGGGCATCTG-3′), and rat specific GAPDH primers (28) (forward: 5′-TCCCTCAAGATTGTCAGCAA-3′, 5′-AGATCCACAACGGATACATT-3′). PCR products were electrophoresed on ethidium bromide containing agarose gels (Visigel matrix, Stratagene). Bands were visualized by UV-induced fluorescence and analyzed with the BioRad Gel Doc 1000 System (Bio Rad).

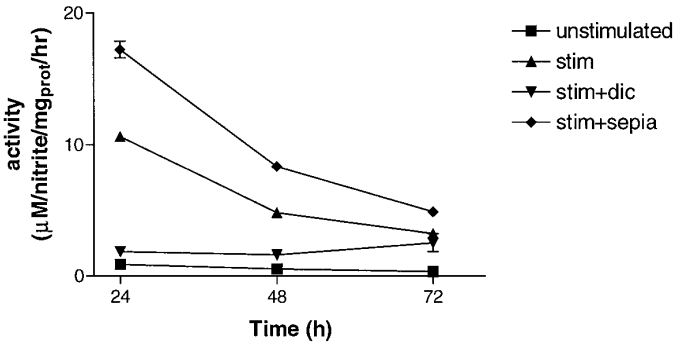

FIG. 1. Effects of sepiapterin (exogenous source of BH4), and dicumarol (inhibitor of BH4 biosynthesis) on cytosolic iNOS activity. Cells were stimulated with IFNγ, 100 U/ml and LPS, 10 μg/ml, in the presence of the indicated agents. At the times indicated, cells were lyzed and iNOS activity measured as described in the method section. Data are mean ± SD of one representative out of three identical experiments.

Determination of mRNA stability. Transcription was stopped with actinomycin D (10μg/ml) 24 hours after incubation of cells with the agents indicated. Cells were harvested at 0, 4, 6, and 8 hours after incubation with actinomycin D. Total cellular RNA isolation and RT-PCR were carried out as described above. Gels were analyzed using the Gel Doc system as described above.

Limiting-dilution PCRs. For comparative quantitation of mRNA levels limiting-dilution PCRs of cDNA were performed (29, 30). After reverse transcription as described above, cDNA was 2-fold serially diluted up to 256-fold and cycled with the same primers and conditions as described above. Gels were analyzed with the Gel doc 1000 system equipped with photoshop software (Adobe Systems Inc., Mountain View, CA, USA) in the threshold mode.

RESULTS

To induce iNOS expression in vascular SMC, cells were activated with IFNγ and LPS as indicated. Measurement of cytosolic iNOS activity (in the presence of saturating amounts of BH4 in the assay buffer) revealed enhanced enzymic activity in SMC activated in the presence of sepiapterin, that has been intracellularly converted to BH4. However, only poor iNOS activity was present in activated SMC treated with dicumarol, an inhibitor of endogenous BH4 synthesis (Figure 1). Variation of BH4 concentrations from 5-60 μM in the iNOS assay of dicumarol-treated SMC did not enhance iNOS activity, indicating that BH4 does not directly affect iNOS activity under the assay conditions applied (not shown). Likewise, addition of 10-100 μM dicumarol to the assay mixture did not lower iNOS activity (not shown), excluding a direct inhibitory effect of dicumarol on iNOS activity. These results indicated that, besides its function as a cofactor, BH4 might particularly influence the expression of iNOS in vascular SMC. In a next series of experiments we therefore investigated iNOS protein expression under conditions of BH4 supplementation or inhibition.

Western blot analysis of iNOS protein in SMC stimu-

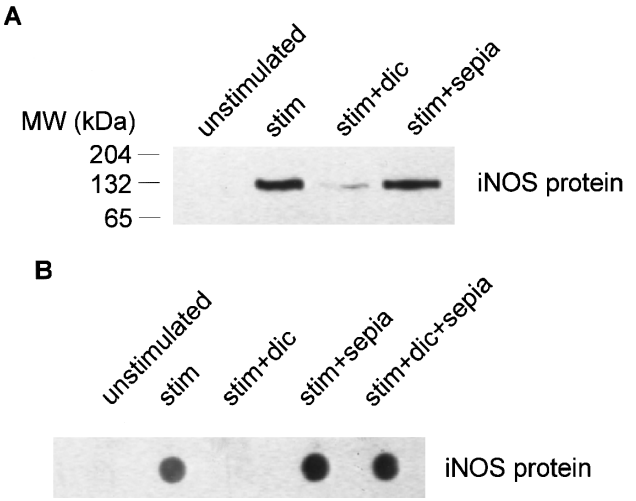

FIG. 2. Effects of sepiapterin and dicumarol on iNOS protein expression in inflammatory stimulated SMC. Cells were treated with the indicated agents for 48 hours. Protein in cell lysates was analyzed under denaturing and native conditions using Western and Dot blot analysis, respectively. Blots were probed with iNOS specific antibodies as described in the methods. (A) Western blot analysis. (B) Dot blot analysis of native iNOS protein. Results are from one representative of at least three independent experiments.

lated with IFN-γ/LPS revealed a single band at 130kD that disappeard upon exposure of the cells to dicumarol (Figure 2A). Treatment of SMC with sepiapterin during stimulation enhanced iNOS protein expression. This was clearly demonstrated when cell lysates containing equal amounts of total protein were stepwise diluted and dottet onto nitrocellulose membranes. Analysis of dot blots showed that iNOS protein disappeared after 48 hours in dicumarol-treated cells. In sepiapterin-supplemented cells however, iNOS protein was strongly augmented (Figure 2B). The suppressing effect of dicumarol on iNOS protein expression was completely reversed when sepiapterin was additionally present during the incubation time (Figure 2B), indicating a regulatory role of BH4 on iNOS expression.

To see whether this regulation takes place already at the transcriptional level, we analyzed iNOS expression at the level of mRNA. We found comparable effects of dicumarol and sepiapterin on iNOS mRNA expression as observed at the protein level. Analysis of iNOS mRNA levels at different incubation times revealed that in the presence of sepiapterin iNOS mRNA levels of activated vascular SMC were not only enhanced, but were also present over prolonged time (72 hours, Figure 3A). However, iNOS mRNA levels in dicumarol-treated SMC were diminished and disappeared already after 48 hours (Figure 3A)

The questions arose whether dicumarol and sepiapterin influenced iNOS expression either at the transcriptional or the post-transcriptional level, and whether BH4 itself could be the modulating agent. Time-kinetic analysis of iNOS induction showed no differences in iNOS mRNA levels from 30 min up to 10 hours after SMC activation, regardless of the presence of dicumarol or sepiapterin (Figure 3B), suggesting that there was no direct influence of these agents on the induction and the transcription rate of iNOS. The modulating effects of dicumarol or sepiapterin on iNOS mRNA levels observed in SMC that had been incubated for longer times (24-72 hours, Figure 3A) rather suggest an influence on the iNOS mRNA life-time. Therefore, effects on iNOS mRNA stability were tested by inhibiting the transcription with actinomycin D 24 hours after activation of SMC, either without or with the precence of dicumarol or sepiapterin. In activated SMC, iNOS mRNA was present up to 6 hours after transcription, at 8 hours however, most of the iNOS mRNA disappeared (Fig. 4A). In cells treated with the

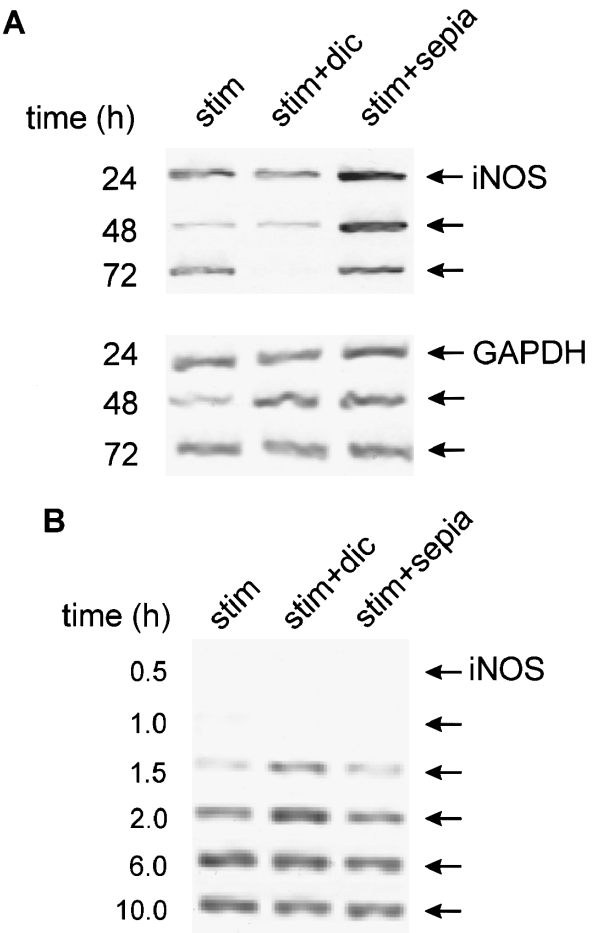

FIG. 3. Effects of sepiapterin and dicumarol on iNOS mRNA expression in inflammatory stimulated SMC. Cells were treated with the indicated agents for the indicated times, followed by RNA isolation and analysis by RT-PCR as described in the methods section. (A) Long-term effects of sepiapterin and dicumarol on iNOS mRNA expression. (B) Effects of sepiapterin and dicumarol on iNOS mRNA induction. Results are representative of at least three independent experiments.

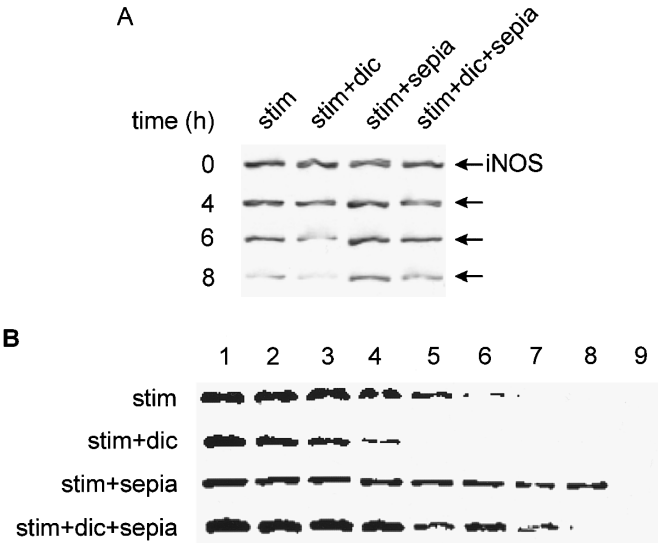

FIG. 4. Effects of sepiapterin and dicumarol on iNOS mRNA stability. 24 hours after incubation with the indicated agents transcription in SMC was blocked with actinomycin D. Cells were further incubated and total RNA was isolated at the indicated time points. (A) iNOS mRNA levels after various incubation times with actinomycin D. (B) Comparative quantification of iNOS mRNA levels by limiting-dilution analysis at 6 hours after incubation with actinomycin D. Limiting-dilution PCRs of cDNA was performed as described in the method section. The lanes represent cDNA at the following dilutions: undiluted (lane 1), 1/2 (lane 2), 1/4 (lane3), 1/8 (lane 4), 1/16 (lane 5), 1/32 (lane 6), 1/64 (lane 7), 1/128 (lane 8), 1/256 (lane 9).

BH4 synthesis inhibitor dicumarol, a decline of iNOS mRNA was evident already 4 hours after transcription, with complete disappearence at 8 hours. In cells substituted with sepiapterin, iNOS mRNA was persistently present also 8 hours after transcription. Addition of sepiapterin together with dicumarol completely reversed the effect of the BH4 synthesis inhibitor to the mRNA levels present in stimulated SMC (Fig. 4A).

To better quantify the extent of the modulation of iNOS mRNA stability by BH4, limiting-dilution PCR using stepwise 2-fold dilutions of cDNA was performed (29, 30). At 6 hours after transcription stop with actinomycin D, 4-fold less iNOS mRNA was present in stimulated SMC when their BH4 synthesis was inhibited with dicumarol (Fig. 4B), compared to cells stimulated in the absence of dicumarol. On the other hand, 4-fold more iNOS mRNA was present in sepiapterin-substituted SMC, compared to non-substituted cells. The reduction of iNOS mRNA caused by inhibition of the endogenous supply of BH4 was completely reversed by exogenous BH4 in form of sepiapterin clearly showing that the stabilizing effect was by BH4 itself (Fig. 4B).

DISCUSSION

Our study indicates that BH4 might regulate iNOS expression in vascular smooth muscle cells by affecting iNOS mRNA stability. BH4 is therefore not only a cofactor or allosteric effector of iNOS. This is of particular interest as we have previously shown that BH4 is a secretory product of activated vascular endothelial cells (23), and that BH4 is vectorially secreted into the direction of underlining SMC (24). Thus, vascular endothelial cells take the part of the "exogenous" source of BH4 for vascular SMC. We have proposed that BH4 is an endothelium-derived messenger mediating vasodilation (4, 24) presumably by regulating the activity of iNOS as a cofactor as well as an allosteric effector molecule (13, 20). The novel mechanism of stabilization of iNOS mRNA in SMC described herein represents an extension of the so far known functions of BH4. Limiting-dilution analysis of iNOS mRNA present at 6 hours after transcription stop clearly documents a 4-fold up- or down-regulation of iNOS mRNA by supplementation or inhibition of the BH4 cofactor, respectively. The prolonged presence of iNOS mRNA was paralleled by the expression of iNOS protein in corresponding amounts, indicating that the mRNA was intact and could be translated (see Fig. 2). Recently, prolonged expression of iNOS mRNA upon sepiapterin-substitution has been reported in renal mesangial cells activated with IL-1 and TNF-α (31). Our results, while confirming the results described in a more specialized SMC population restricted to the renal circulation, however, extend the implications of this novel finding to the whole vascular system. Our finding could provide an explanation not only for the enhanced, but also for the prolonged production of NO in inflammation and sepsis, leading to the uncontrollable hypotonic vasodilation in septic shock. Our previous findings that BH4 is released in large amounts by inflammatory activated vascular endothelial cells but not the SMC itself (20, 23) are greatly substantiated by the discovery of the novel function of BH4 described herein. In consequence, BH4 appears not only to be a first messenger by rapidly controlling NOS activity, but also by affecting persistently the expression of the enzyme. Accordingly, BH4 produced by endothelial cells under septic conditions has the potential to influence NO production over extended time periods through the post-transcriptional prolongation of iNOS mRNA half-life in the vascular SMC.

The mechanism by which iNOS mRNA is stabilized by BH4 remains yet to be defined. That a cofactor of an enzyme controls the enzyme's mRNA and thereby its expression, is an hitherto unknown feature. But such a mechanism would also explain the recent observation that, in addition to their reduced activities, the absolute amounts of the BH4-dependent aromatic amino acid hydroxylases, phenylalanine, tyrosine, and tryptophane hydroxylase, are decreased in BH4 deficient mice (32).

Taken together, the novel finding that BH4 can mod-

ulate iNOS expression by stabilization of its mRNA extends the functions of this intriguing molecule. Pharmacological modulation of BH4 levels is possible (11, 18), and appears a promising approach for the treatment of pathophysiological processes dependent on NO.

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