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Differential Inhibition of Constitutive and Inducible Nitric Oxide Synthase in Vascular Endothelial Cells by Analogues of Tetrahydrobiopterin

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Abstract

In the vasculature, a physiologic production of nitric oxide (NO) is maintained by endothelial nitric oxide synthase (eNOS). Induction of inducible nitric oxide synthase (iNOS) under inflammatory conditions (e.g. septic shock) resulting in high levels of nitric oxide (NO) is believed to be partly responsible for the pathophysiologic changes in the vascular system that occur under inflammatory conditions (e.g. septic shock). Both NOS isoforms are dependent on the obligatory cofactor tetrahydrobiopterin (BH_4). We investigated the selectivity and potency of the BH_4 analogues 4-amino-BH_4 and 5-methyl-BH_4 in inhibiting eNOS and iNOS in a murine vascular endothelial cell (MVEC) model expressing either eNOS or iNOS under physiologic and inflammatory conditions, respectively. Exogenous BH_4 and its precursor sepiapterin both enhanced physiologic eNOS activity in resting MVEC, while 4-amino-BH_4 slightly inhibited eNOS. 5-methyl-BH_4 did not have any effect on eNOS. BH_4, sepiapterin, and 5-methyl-BH_4 had no effect on iNOS in inflammatory activated MVEC. In contrast, 4-amino-BH_4 selectively inhibited iNOS with a potency comparable to the unselective NOS inhibitor Nω-monomethyl-L-arginine (L-NMMA). The present study demonstrates that 4-amino-BH_4 selectively and potently inhibits iNOS in vascular endothelial cells, while its effect on eNOS is minimal. The selective inhibition of iNOS is a promising strategy for the treatment of inflammatory conditions with high output of NO. Further in vivo studies are required to determine whether inhibition of NO production by analogues of BH_4 offers any advantage compared to inhibition by L-arginine analogues.

Key words: inflammation; vascular endothelial cell, nitric oxide synthase; pteridine; tetrahydrobiopterin; 4-amino-tetrahydrobiopterin, 5-methyl-tetrahydrobiopterin

Introduction

Recent years clearly established nitric oxide (NO) as a key mammalian metabolite for signaling in the vascular system in both physiological and pathophysiological states (1). The molecular control of NO synthases (NOS), which catalyze the five electron oxidation of L-arginine in a two-step process yielding L-citrulline and NO, has been elucidated in some detail (2). In the physiologic state, vascular endothelial cells constitutively express eNOS protein, and endothelium-derived NO diffuses into subjacent smooth muscle cells to activate soluble guanylate cyclase thereby increasing cGMP and resulting in vasorelaxation (3). However, under inflammatory conditions eNOS is downregulated whereas activating stimuli induce the expression of iNOS mRNA and subsequent "high-output" NO production (4). It is of note that the expression of iNOS has been found in murine (5), porcine (6), and rat vascular endothelial cells (7, 8), but not in human vascular endothelial cells (9, 10). In contrast, vascular smooth muscle cells from different species, including rat and human, express iNOS mRNA and functional protein upon stimulation with inflammatory stimuli (11-14).

Tetrahydrobiopterin (BH_4) is essentially required as cofactor for all NOS isoforms; for a recent review on BH_4's functions in the vascular system, see (4). The

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exact role of BH_4 in NO catalysis remains intensively debated and may include stabilization of the active conformation of the enzyme, action as an allosteric effector enhancing L-arginine binding, and coupling of L-arginine oxidation to NADPH consumption reducing the ratio of superoxide to NO production, and post-transcriptional stabilization of NO synthase mRNA. Vascular endothelial cells express all enzymes necessary for BH_4 generation. They synthesize and secrete small amounts of BH_4 even in the resting state, and inflammatory stimuli increase BH_4 synthesis manifold in these cells. iNOS in many cell types including vascular smooth muscle cells depends on exogenous BH_4 for full enzymatic activity, rendering NO production susceptible to modulation by exogenous BH_4 or analogues (4). To date, however, it is not clear whether iNOS activity in vascular endothelial cells is also prone to modulation by exogenous BH_4. Given the high rate of BH_4 synthesis and secretion in these cells, we hypothesized that iNOS in vascular endothelial cells behaves differently compared to iNOS from other cell types devoid of abundant BH_4 synthesis. We therefore investigated the effects of BH_4 and the analogues 4-amino-BH_4 and 5-methyl-BH_4 on NO production in murine vascular endothelial cells expressing both eNOS and iNOS (5).

Methods

Chemicals

5,6,7,8-tetrahydro-L-erythro-biopterin (tetrahydrobiopterin, BH_4), the analogues 4-amino-BH_4 and 5-methyl-BH_4, and sepiapterin were purchased from Dr. B. Schircks Laboratories (Jona, Switzerland). Murine recombinant interferon-γ (IFN-γ) was from Peprotech Inc. (Rocky Hill, NJ, USA). Lipopolysaccharide (LPS, E. coli O26:B6, Boivin extraction) was from Difco (Detroit, MI, USA) and Sigma (St. Louis, MO, USA). N^{ω}-nitro-L-arginine methyl ester (L-NAME) and N^{ω}-monomethyl-L-arginine (L-NMMA) were obtained from Sigma.

Cell cultures

Murine vascular endothelial cells (MVEC) (15) were maintained in a humidified 5% CO2/95% air atmosphere at 37°C in Iscove's Modified Dulbecco's Medium (Cellgro, Mediatech Inc., Herndon, VA, USA) supplemented with 10% fetal bovine serum (heat inactivated, mycoplasma/virus/endotoxin-tested, Life Technologies, Rockville, MD, USA), L-glutamine (2 mM, Cellgro) and gentamicin (50 μg ml^{-1}, Sigma). Cells were seeded in 24-well cluster plates (Corning Inc., Corning, NY, USA). Confluent cultures were used for experiments. For inflammatory stimulation, cells were treated with IFN-γ (100 U ml^{-1}) and LPS (10 μg ml^{-1}) for 24 or 48 hours as indicated. Cell viability was assessed by trypan blue exclusion and was 95-98% throughout experiments.

Determination of NOS isoform mRNA.

Expression of NOS isoforms was determined by reverse-transcriptase PCR using isoform-specific primers as described (16). eNOS, 5'TTCACTCTGCCCCCAGAGATG3' (sense) and 5'AGAGATCTTCACTGCATTGG3' (antisense), yielding a 611 bp product, located at bp 911-1522 of the murine eNOS cDNA sequence (17); iNOS, 5'ATGGCTTGCCCCTGGAAGTTTCT3' (sense) and 5'CCTCTGATGGTGCCATCGGGC3' (antisense), yielding a 817 bp product, located at bp 192-1009 of the murine iNOS cDNA sequence (18). Both iNOS and eNOS were determined in the same cDNA preparations.

Measurement of nitric oxide/nitrite

NO production was measured as nitrite (NO_2-) in culture supernatants by the Griess method as described (19) with sodium nitrite diluted in complete medium as standard. The ratio of nitrite to nitrate was assumed to remain constant during all treatment regimens used. The detection limit for NO_2- was 10 pm/well or 1.0 μmol L^{-1}.

Statistical analysis

Results are presented as mean ± SD. One-way analysis of variance (ANOVA) for repeated measures with Tukey-Kramer Multiple Comparisons Tests were performed using InStat version 3.00 (GraphPad, San Diego, CA, USA). $p<0.05$ was considered significant.

Results

Effects on eNOS and iNOS mRNA expression and NO production during inflammatory stimulation of murine vascular endothelial cells (MVEC)

In accordance with previous findings (5), resting MVEC secreted small amounts of NO, detected as nitrite in culture supernatants. After 48 hours, 15.12±4.99 μM (range: 8.17 to 29.83 μM) nitrite were produced by unstimulated MVEC (n=26; data not shown). As reported in an earlier study, incubation of these cells with IFN-γ/LPS resulted in a strong induction of NO synthesis (5). Accordingly, IFN-γ and LPS-stimulated MVEC produced 234.01±166.33 μM nitrite after 48 hours (n=19, data not shown); interexperimental variations were high, as indicated by the wide range from 62.17 to 642.00 μM nitrite. As shown in Fig. 1, reverse-transcriptase PCR experiments revealed expression of eNOS, but not of iNOS mRNA under

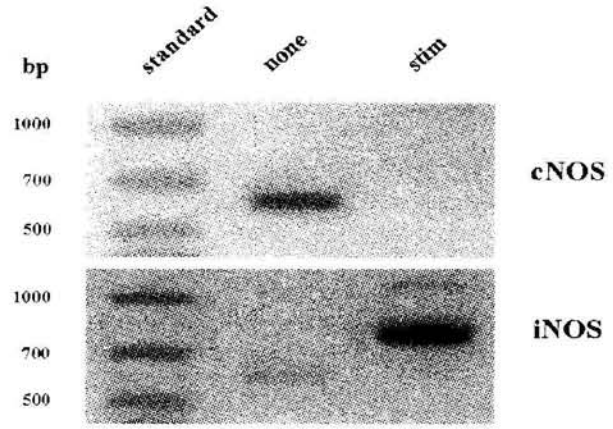

Figure 1. Differential expression of NOS isoforms in confluent MVEC. Under resting conditions, only endothelial eNOS mRNA was found by RT-PCR (upper panel). 24h after stimulation with IFN-γ (100 U ml^{-1}) and LPS (10 μg ml^{-1}), iNOS mRNA was strongly induced, whereas eNOS mRNA disappeared to undetectable levels (lower panel). Therefore, MVEC cells switch from eNOS activity under resting to iNOS activity under inflammatory condition. Both eNOS and iNOS were determined in the same cDNA preparations.

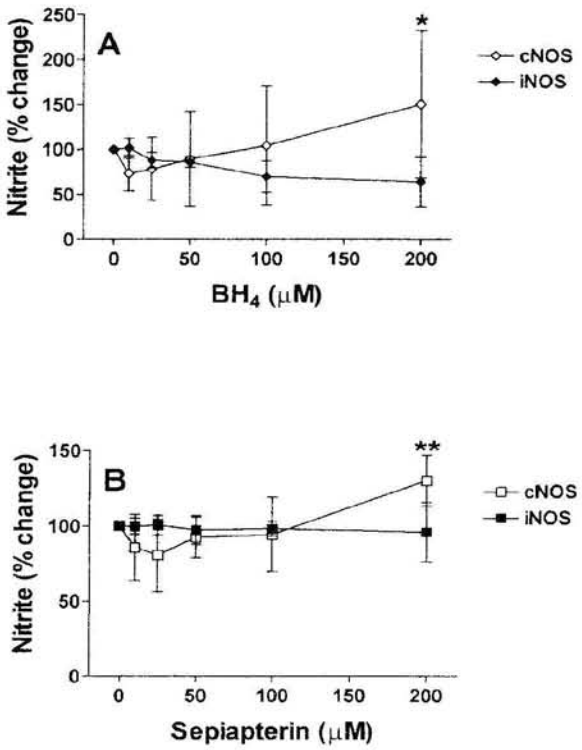

Figure 2. Effects of the natural cofactor BH4 and its substituent sepiapterin on NO production by MVEC. After achievement of confluence, MVEC were incubated without stimulus (open symbols) or treated with IFN-γ (100 U ml^{-1}) and LPS (10 μg ml^{-1}), and either BH_4 (A) or sepiapterin (B) was added at the indicated concentrations. NO production was measured as nitrite in culture supernatants after 48 hours in triplicate wells and duplicate measurements. Results are presented as % change±SD from corresponding control wells. * denotes $p<0.01$ as compared to corresponding wells treated with L-NAME 10 μM or 25 μM and $p<0.05$ as compared to 50 μM. ** $p<0.01$ compared to corresponding controls.

resting conditions. In contrast, analysis of mRNA 24h after the addition of IFN-γ and LPS showed strong expression of iNOS mRNA, whereas eNOS mRNA could no longer be detected. These basic experiments clearly confirm that unstimulated MVEC switch from low output NO production by eNOS to high output NO synthesis by iNOS after stimulation with inflammatory stimuli.

Effects of BH_4 and sepiapterin on NO production by MVEC

Fig. 2 shows the effects of increasing concentrations of the natural cofactor BH_4 and its substituent sepiapterin, which is converted intracellularly to BH_4 by the salvage pathway (4) on NO generation. Both BH_4 (Fig. 2A) and sepiapterin (Fig. 2B) slightly enhanced NO production by eNOS, e.g. in unstimulated MVEC. Nitrite in culture supernatants increased from 14.10±5.33 in controls to 18.90±7.93 for BH_4 at 200 μM, n=8, p=0.001, and from 13.79±5.12 to 17.42±4.31 for 200 μM sepiapterin, n=4, $p<0.0001$. In contrast, BH_4 and sepiapterin failed to influence NO production by iNOS in stimulated MVEC (from 190.90±179.83 to 158.33±208.97 for 200 μM BH_4, n=4, p is not significant, and from 220.33±185.46 to 203.72±162.61 for 200 μM sepiapterin, n=3, p is not significant.

Effects of the pteridine analogues 4-amino-BH_4 and 5-methyl-BH_4 on NO production by MVEC

4-amino-BH_4 slightly decreased NO production in unstimulated MVEC (Fig. 3A), as shown by a decrease of nitrite in culture supernatants from 18.42±7.84 in control wells to 11.54±4.64 for wells treated with 100 μM 4-amino-BH_4 and 12.58±1.11 for wells treated with 200 μM 4-amino-BH_4, n=4, p=0.01. In contrast to its slight effect on eNOS, 4-amino-BH_4 strongly and dose-dependently inhibited iNOS in stimulated MVEC (decrease from 167.61±20.04 in controls to 43.56±3.66 for 200 μM 4-amino-BH_4, n=3, $p<0.0001$. The other

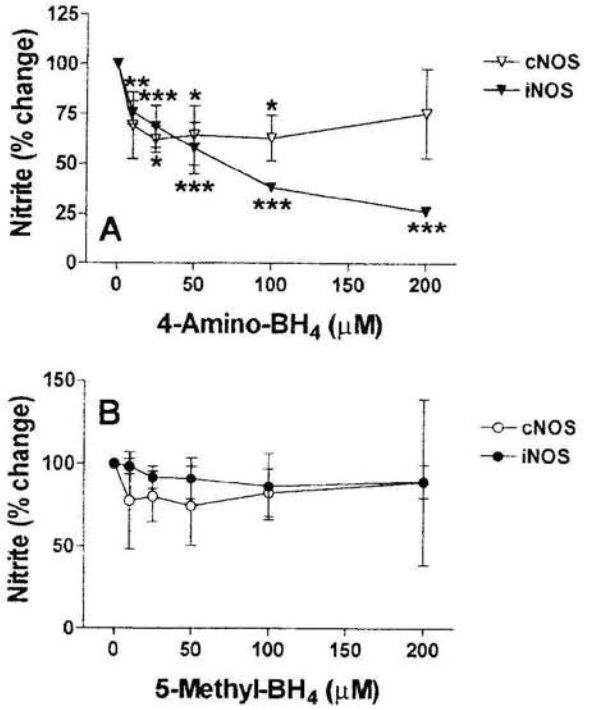

Figure 3. Effects of the pteridine analogues 4-amino-BH_4 and 5-methyl-BH_4 on NO production by MVEC. After confluence was achieved, MVEC were incubated without stimulus (open symbols) or treated with IFN-γ (100 U ml^{-1}) and LPS (10 μg ml^{-1}) and either 4-amino-BH_4 (A) or 5-methyl-BH_4 (B) was added as indicated. NO production was measured as nitrite in culture supernatants after 48 hours in triplicate wells and duplicate measurements. Results are presented as % change±SD from corresponding control wells. * $p<0.05$ compared to corresponding controls; ** $p<0.01$ compared to corresponding controls; *** $p>0.001$ compared to corresponding controls.

pteridine analogue 5-methyl-BH_4 failed to influence NO production both in unstimulated as well as in stimulated MVEC (Fig. 3B). In experiments with unstimulated cells, the amount of nitrite in supernatants was 14.89±3.43 in control wells and 13.39±7.42 in wells incubated with 200 μM 5-methyl-BH_4, n=3, p is not significant. In stimulated MVEC, supernatant nitrite was 267.39±229.91 in controls and 238.00±197.15 at 200 μM 5-methyl-BH_4, n=3, p is not significant.

Effects of the L-arginine analogues L-NAME and L-NMMA on NO production by MVEC

Both L-arginine analogues tested, L-NAME and L-NMMA, significantly inhibited NO production in unstimulated and stimulated MVEC. L-NAME (Fig. 4A) decreased NO generation by eNOS from 14.22±4.83 to 8.67±7.04 at a concentration of 200 μM L-NAME, n=3, p=0.001, and by iNOS from 344.06±270.57 to 188.50±176.46 for 200 μM L-NAME, n=3, p=0.001. L-NMMA (Fig. 4B) inhibited NO production by eNOS, as shown by a decrease of nitrite in culture supernatants from 16.04±2.87 to 10.33±4.53 for 200 μM L-NMMA, n=4, p=0.01, and it decreased NO generation by iNOS from 228.17±95.56 to 71.17±18.77 for 200 μM L-NMMA, n=3, p=0.0005.

Discussion

This study shows that highly upregulated NO production in MVEC is regulated on the NOS isoform

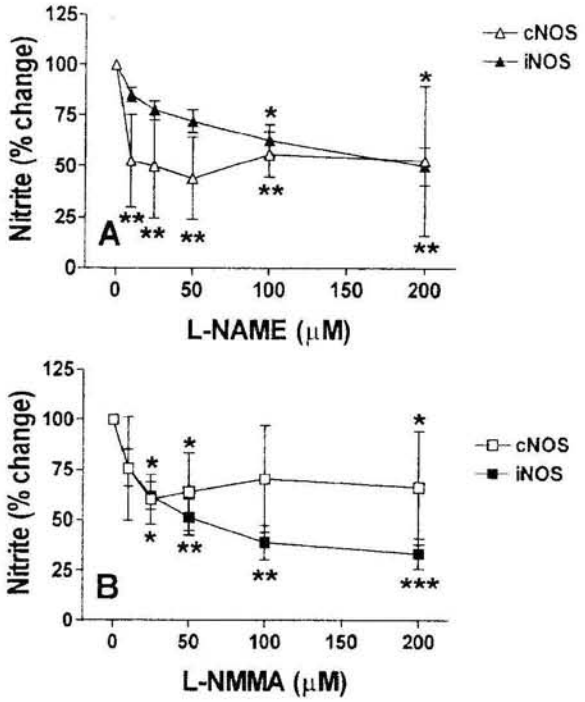

Figure 4. Effects of the L-arginine analogues L-NAME and L-NMMA on NO production by MVEC. Confluent MVEC were incubated without stimulus (open symbols) or treated with IFN-γ (100 U ml^{-1}) and LPS (10 μg ml^{-1}), and increasing concentrations of either L-NAME (A) or L-NMMA (B) were as indicated. NO production was measured as nitrite in culture supernatants after 48 hours in triplicate wells and duplicate measurements. Results are presented as % change±SD from corresponding control wells. * $p<0.05$ compared to corresponding controls; ** $p<0.01$ compared to corresponding controls; *** $p<0.001$ compared to corresponding controls.

gene transcription level, confirming the "switch model" of NOS isoforms under inflammatory conditions. Using this unique cellular model, we demonstrate the selective inhibition of iNOS by 4-amino-BH_4, and an independence of vascular endothelial iNOS from exogenous BH_4 for full enzymatic activity.

The data outlined herein unequivocally show that basal NO production of MVEC can solely be attributed to eNOS activity. However, upon stimulation with proinflammatory molecules, iNOS expression is induced while eNOS is downregulated. The reduction of eNOS mRNA and protein levels following activation by cytokines or endotoxin has also been demonstrated in cultured human and bovine endothelial cells, and this effect could at least partially be attributed to enhanced degradation rates of eNOS transcripts (5, 9, 20-23). Therefore, MVEC switch their active NOS isoform in dependence of the presence of inflammatory signals, i.e. from low-output NO production by eNOS under physiological (non-inflammatory) conditions to high-output NO generation by iNOS under inflammatory situations, referred to as "switch model" previously (4). Although eNOS mRNA is downregulated to undetectable levels within several hours after inflammatory stimulation, preexisting eNOS protein may continue to contribute to the total production of NO during the first days under inflammatory conditions (5); however, this seems to be negligible in view of the large amounts of NO generated simultaneously by iNOS.

Resting vascular endothelial cells constitutively synthesize small amounts of BH_4. Although the rate of enzyme activity and resulting BH_4 synthesis may depend on experimental conditions and probably also on the type of endothelial cell assessed, this basic characteristic has been found both in freshly isolated cells as well as in cells after prolonged culturing (4). In addition, our findings that sepiapterin slightly increased (and additional BH_4 tended to increase) the amount of NO production of vascular endothelial eNOS are in line with observations made by others and underline the regulatory capacity of the pteridine cofactor for endothelial NO production under non-inflammatory conditions. However, in vivo data suggest that under physiologic conditions levels endogenous BH_4 are nearly saturating and barely a limiting factor for optimal or near optimal endothelial eNOS activity, and only very high concentrations of BH_4 resulted in endothelium-dependent vasorelaxation in animal and human studies (24, 25).

It is generally believed that iNOS activity largely depends on exogenous BH_4, i.e. that endogenous BH_4 biosynthesis does not suffice to support iNOS for full enzymatic activity. Although BH_4 biosynthesis is induced by inflammatory stimuli in cultured cells, intrinsic BH_4 availability has nevertheless been demonstrated to be a limiting factor for iNOS activity in many cell types including macrophages, mesangial cells, vascular smooth muscle, and fibroblasts, rendering NO production susceptible to modulation of intracellular BH_4 levels by inhibition of BH_4 biosynthesis or exogenous BH_4 addition (4). However, the present study shows strikingly different characteristics for vascular endothelial cells. The vascular endothelium produces and secrets abundant amounts of BH_4 upon inflammatory stimulation, as shown for cells of murine (26) and human (27) origin. The increase in de novo BH_4 biosynthesis occurs early after inflammatory stimulation and may even lead to transiently increased NO production by eNOS despite the concomitant decrease of total eNOS protein. In view of this high level BH_4 biosynthesis in endothelial cells under inflammatory conditions, it is not surprising that endogenous BH_4 levels are saturating to fully serve endothelial iNOS protein. Moreover, since most of the newly synthesized BH_4 is secreted, probably even vectorially into the basal direction (4), endothelial BH_4 may serve underlying smooth muscle cells with additional cofactor needed for full iNOS activity, thereby accounting for an inflammatory endothelium-derived relaxing factor. On the other hand, BH_4 is a potent antioxidative and reactive oxygen scavenging agent, preventing peroxynitrite formation when present in saturating amounts (28). The high amount of BH_4 produced by endothelial cells in inflammation or sepsis could therefore be beneficial. Thus, an inhibition of iNOS and not of BH_4 synthesis seems desirable.

The 4-amino analogue of BH_4 binds with high affinity and the same potency to both recombinant eNOS and iNOS and strongly interferes with stimulation of the enzymes by exogenous BH_4, although 4-amino-BH_4 shares the same allosteric effects, namely stabilization of the active dimers and low-to-high spin transition of the heme, as the natural cofactor (29, 30). In contrast to these findings obtained with purified proteins, 4-amino-BH_4 was much less effective in inhibiting NO formation in cultured porcine endothelial cells (expressing eNOS) compared to cultured murine fibroblasts or human adenocarcinoma cells (expressing iNOS) (29). Despite the fact that the effect of 4-amino-BH_4 critically depended on intracellular BH_4 levels, the selectivity towards inhibition of iNOS could not simply be explained by different intracellular BH_4 levels, and therefore, it was suggested that the high-affinity binding of the inhibitor during protein expression may be responsible for the difference, since the turnover of iNOS protein is much more rapid than that of eNOS (half-life approximately 3 hours vs. 20 hours)

(29). Likewise, 4-amino- BH_4 was shown to be fairly selective in inhibiting iNOS in an in vivo vessel strip model of endotoxemia; in this study, the pteridine antagonist had to be added shortly after the artery strips were treated with inflammatory stimuli (31), again suggesting that the apparent selectivity of 4-amino- BH_4 may be related to high-affinity binding of the inhibitor during protein expression.

The present data are in full agreement with those obtained by (29) and (31) and show that a preferential inhibition of the iNOS protein by 4-amino-BH_4 can also be demonstrated in cells expressing both NOS isoforms, thereby excluding the possibility of cell type differences as an explanation for the observed effect. Nevertheless, different situations will be encountered in vivo in the human vasculature, where endothelial BH_4 might compete with exogenous 4-amino-BH_4 for binding on the pteridine-sites of smooth muscle cell iNOS, and in vivo experiments will ultimately be required to determine whether 4-amino-BH_4 displays a "real" selectivity for iNOS thereby offering an interesting therapeutic tool for the treatment of NO overproduction by iNOS under inflammatory conditions in humans.

The 5-methyl-analogue of BH_4 has previously been shown to substitute BH_4 as a cofactor (32); however, at high concentrations of purified NOS, the autoxidation resistant 5-methyl-BH_4 may result in fully coupled NO generation and, subsequently, NO-induced autoinhibition of NOS (33). Therefore, the effect of 5-methyl-BH_4 on net amounts of NO may be critically dependent on the experimental conditions. In the present study we did not find any significant effect of 5-methyl-BH_4 neither on eNOS nor on iNOS activity, rather excluding the use of this analogue to pharmacologically modulate NO production in the vasculature.

In summary, vascular endothelial cells switch from eNOS under resting to iNOS under inflammatory conditions; the concomitant increase in endogenous BH_4 biosynthesis make these cells independent from exogenous sources for the pteridine cofactor for full enzymatic activity. 4-amino-BH_4 was revealed as a preferential inhibitor for iNOS in vascular endothelial cells, whereas 5-methyl-BH_4 was largely ineffective. Further in vivo experiments are required to confirm these observations and to determine whether inhibition of NO production by analogues of BH_4 offers any advantage over inhibition by L-arginine-analogues in the treatment of human inflammatory diseases.

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