2,4-Diamino-6-hydroxypyrimidine, an inhibitor of tetrahydrobiopterin synthesis, downregulates the expression of iNOS protein and mRNA in primary murine macrophages

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Received 23 February 1995

Abstract 2,4-diamino-6-hydroxy-pyrimidine (DAHP), an inhibitor of GTP cyclohydrolase I, blocks the synthesis of tetrahydrobiopterin (BH_4), which is a known cofactor of inducible nitric oxide synthase (iNOS). Previously, DAHP was shown to suppress the production of nitric oxide by cytokine-activated fibroblasts, smooth muscle cells or endothelial cells which could be attributed to its function as a cofactor antagonist. Here, we demonstrate that in interferon-γ-activated murine peritoneal macrophages DAHP suppresses the expression of iNOS mRNA and protein in a BH_4-independent manner and, thus, acts by a novel mechanism.

Key words: 2,4-Diamino-6-hydroxy-pyrimidine; Inducible nitric oxide synthase; Nitric oxide; Tetrahydrobiopterin; GTP cyclohydrolase I

1. Introduction

Nitric oxide (NO) is now recognized as a potent inter- and intracellular effector molecule which is implicated in a great variety of physiological and pathological processes including the regulation of vascular tone, the secretion of hormones, the contractility of cardiomyocytes, the killing of microbes and tumor cells, the induction of immunosuppression and the destruction of tissue in autoimmune processes. Inducible nitric oxide synthase (iNOS), the enzyme responsible for high output-production of NO from L-arginine and molecular oxygen in cytokine-activated cells (reviewed in [1–4]), requires tetrahydrobiopterin (BH_4) as a cofactor for activity [5,6]. In mouse fibroblasts, murine brain endothelial cells, rat vascular smooth muscle cells and the mouse macrophage cell line RAW264.7, the induction of NO release by interferon-γ (IFN-γ), tumor necrosis factor-α and/or lipopolysaccharide (LPS) is paralleled by an enhanced formation of BH_4 and an up-regulation of GTP-cyclohydrolase I [7–15], the rate-limiting enzyme for the de novo-synthesis of BH_4 (reviewed in [16,17]). Conversely, 2,4-diamino-6-hydroxy-pyrimidine (DAHP), an inhibitor of GTP-cyclohydrolase I, was found to suppress the accumulation of NO_2^- in these cells [8, 10–14]. In all these studies the decreased generation of NO after DAHP treatment was attributed to a lack of BH_4 and subsequent reduced activity of iNOS. More recent studies suggest that BH_4, in addition to its presumed activity as a redox-active cofactor, is also required for the dimerization and stability of the iNOS protein [18–20]. Deactivating cytokines which cause suppression of iNOS [21, 22] might therefore act via inhibition of BH_4 synthesis. In order to test the hypothesis that a lack of BH_4 directly affects the expression of iNOS protein, we utilized DAHP as an inhibitor of biopterin synthesis and investigated its effect on iNOS in primary murine macrophages. Unexpectedly, we found that DAHP down-regulates the expression of iNOS protein *and* mRNA in a BH_4-independent manner.

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Abbreviations: BH_4, 2-amino-4-hydroxy-6-(L-erythro-1,2-dihydroxypropyl)-5,6,7,8-tetrahydropteridin; DAHP, 2,4-diamino-6-hydroxy-pyrimidine; iNOS, inducible nitric oxide synthase (NOS whose activity is independent of exogenous calmodulin and elevation of intracellular $[Ca^{2+}]$ above the level in resting cells; IFN-γ, interferon-γ; LPS, bacterial lipopolysaccharide; MTT, 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide; NO, nitric oxide; L-Sep, L-sepiapterin; TGF-β, transforming growth factor-β.

2. Experimental

2.1. Materials

Recombinant murine IFN-γ (batch M3RD48; protein concentration 1.0 mg/ml; specific activity 5.2×10^6 U/mg; LPS content < 10 pg/ml) was a kind gift of Dr. G. Adolf (Ernst Boehringer Institut für Arzneimittelforschung, Vienna, Austria). The hemisulfate salt of 2,4-diamino-6-hydroxy-pyrimidine (DAHP; lot 13H3437), 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide (MTT) and LPS O111:B4 were obtained from Sigma Chemie (Deisenhofen, Germany). L-sepiapterin and (6R-) tetrahydrobiopterin were purchased from Dr. Schircks Co. (Jona, Switzerland).

2.2. Macrophage cultures

Thioglycolate-elicited peritoneal macrophages were prepared from female CD1 mice (8–12 weeks old; Charles River Breeding Laboratories, Sulzfeld, Germany). The cells were cultured with RPMI 1640 medium plus 5% fetal bovine serum (Sigma) (final LPS-content: <10 pg/ml as determined by a colorimetric limulus amebocyte lysate assay; Whittaker Bioproducts, Walkersville, MD) in 96-well plates (2×10^5 macrophages in 100 μl), 24-well plates (10^6 macrophages/well in a volume of 500 μl), or 78 cm^2 tissue culture dishes ($12–20 \times 10^6$/dish; 500 μl medium per 10^6 macrophages) at 37 °C in 5% CO_2/95% air and enriched for macrophages by a 1–2 h adherence step as described [22,23]. Subsequently, macrophages were stimulated with IFN-γ (20 ng/ml) ± LPS (20 ng/ml) in the absence or presence of DAHP (± L-sepiapterin).

2.3. MTT transformation assay for assessment of cellular toxicity

Macrophage monolayers were stimulated in 96-well plates with IFN-γ (20 ng/ml) in the presence or absence of 10 mM DAHP. After 48 h MTT was added to a final concentration of 5 μg/ml for 4 h. The

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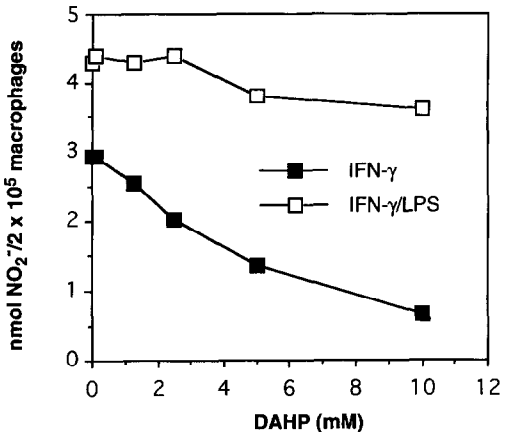

Fig. 1. Effect of DAHP on NO production by macrophages stimulated with IFN-γ ± LPS (20 ng/ml each) for 48 h. NO_2^- accumulation in unstimulated cultures was 0.003 nmol/2 × 10^5 macrophages. One of 4 similar experiments.

resulting formazan crystals were dissolved by the addition of 10% SDS/0.01-N HCl for 16 h and the absorbance was read at a test wavelength of 570 nm (reference wave length 630 nm) in a MR5000 plate reader (Dynatech, Denkendorf, Germany) as described in detail elsewhere [24].

2.4. Determination of NO_2^- accumulation in culture supernatants, iNOS enzyme assay, SDS-PAGE, and Western blot analysis

For measurement of NO_2^- accumulation in culture supernatants as an indicator of NO production, the preparation of macrophage lysates, determination of iNOS enzyme activity and detection of iNOS protein by SDS-PAGE and Western blot with a polyclonal anti-iNOS antibody, we exactly followed previously published methods [22].

2.5. Determination of intracellular pteridines and GTP cyclohydrolase I activity in macrophage extracts

Macrophage monolayers were washed with PBS, lysed in sterile distilled water, flash frozen in liquid nitrogen and kept at −70°C until oxidation. Lysates were centrifuged at 10,000 × g and the supernatants were immediately used for measurement of intracellular levels of total biopterin and BH_4 as well as for GTP cyclohydrolase I activity assays. Pteridine measurements and enzyme assays were carried out as detailed previously [7]. Briefly, lysate samples were oxidized at acidic or alkaline pH and biopterin was detected by HPLC with fluorescent detection. The amount of tetrahydrobiopterin was calculated from the difference of total biopterin (acidic oxidation) to 7,8-dihydrobiopterin plus biopterin (alkaline oxidation). GTP cyclohydrolase I activity was determined using 2 mM GTP as a substrate in the presence of 5 mM EDTA. After treatment with 8 U/ml of alkaline phosphatase (from calf intestine, 3077 U/mg protein; Serva, Heidelberg, Germany) and oxidation with iodine at acidic pH, the resulting neopterin was quantified by HPLC.

2.6. Determination of extracellular pteridines

Supernatants from macrophage monolayers were flash frozen in liquid nitrogen and assayed for total biopterin after iodine oxidation in acidic media, using HPLC with fluorescent detection as detailed elsewhere [7].

2.7. RNA preparation, Northern blot and hybridization

Total RNA was isolated from macrophage monolayers by a rapid guanidinium isothiocyanate method [25], electrophoresed and transferred to reinforced nitrocellulose membranes, which were UV-crosslinked, prehybridized, hybridized and exposed exactly as previously described [22,23]. The probe specific for murine iNOS was an 814-bp *Eco*RI/*Acc*I fragment of iNOS cDNA clone B2 [26], which was random-primer labelled with 50 μCi of [α-^{32}P]dCTP (3,000 Ci/mmol, 10 mCi/ml; NEN Dupont, Bad Homburg, Germany) according to the manufacturer's protocol (Boehringer, Mannheim, Germany). Membranes were subsequently hybridized with a random-primer radiolabelled mouse β-actin DNA fragment (corresponding to bp 62–635 of the murine β-actin sequence) or an oligoprobe for murine 16S mitochondrial rRNA [27] which was 5′ end-radiolabelled as described [22].

3. Results

3.1. DAHP inhibits the accumulation of NO_2^- in the supernatant of IFN-γ activated primary murine macrophages

When macrophages were stimulated with IFN-γ in the presence of 5 or 10 mM DAHP, the accumulation of NO_2^- in the culture supernatants was reduced by 75 ± 2.5% (mean ± S.E.M., n = 33). The concentration of DAHP causing 50% inhibition (IC_{50}) was 3.4 ± 0.5 mM (mean ± S.E.M., n = 9) and, thus, similar to values reported for other cell types [8,11]. A representative dose-curve experiment is shown in Fig. 1. In contrast, induction of NO production by IFN-γ (20 ng/ml) plus LPS (20 ng/ml) was only weakly inhibited by 10 mM DAHP (34 ± 3.5% in 5 experiments). If DAHP was added at 24 h after initiation of IFN-γ stimulation, it failed to inhibit and slightly enhanced further accumulation of NO_2^- during a subsequent 24–48 h culture period (not shown).

In accordance with previous studies [8,11,14], DAHP at the concentrations used did not decrease the viability of the macrophages as indicated by three different sets of experimental evidence. First, the total yield of protein from DAHP-treated

Table 1
Effect of DAHP (± L-sepiapterin) on iNOS enzyme activity and the BH_4 content of macrophages activated by IFN-γ (20 ng/ml) for 48 h (Exp. 1) or 35 h (Exp. 2)

Exp.	IFNγ	DAHP (mM)	L-Sepiapterin (μM)	iNOS activity (pmol/mg/min)	Total biopterin (nmol/l supernatant)	Total biopterin (pmol/mg homogenate)	BH_4 (pmol/mg homogenate)
1	−	−	−	0	42.9	485	454
	+	−	−	545.1	102.9	438	416
	+	10	−	140.8	29.4	23	18
	+	2	−	312.3	29.4	41	37
	+	0.2	−	347.1	41.2	265	238
2	−	−	−	0	38.1	393	369
	+	−	−	484.2	73.2	481	442
	+	10	−	31.2	19.5	35	22
	+	10	100	93.5	1,524.4	3,472	2,986

Duplicate cultures were processed for the determination of iNOS activity or intra- and extracellular biopterin levels.

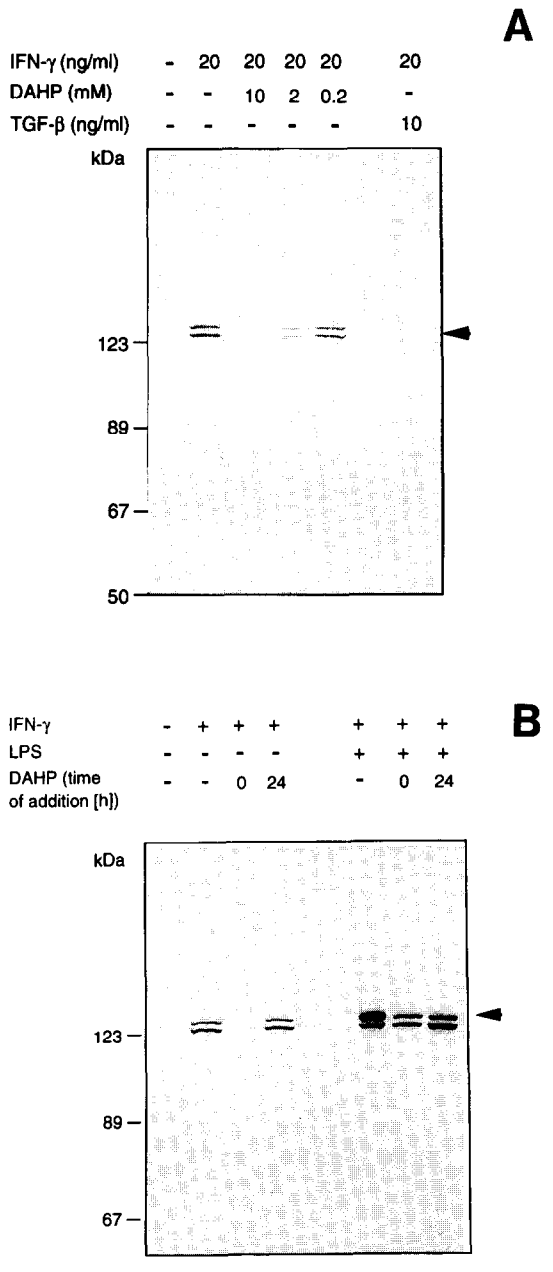

Fig. 2. Western blot analysis (20 μg lysate/lane) of the expression of iNOS protein in macrophages activated by IFN-γ ± LPS (20 ng/ml each) for 48 h. Panel A: DAHP (0.2–10 mM) was added together with the stimulus. Panel B: DAHP (10 mM) was added together with or 24 h after the stimulus. The enzyme activities of the lysates in panel A are shown in Table 1 (Exp. 1). (→) Position of the typical iNOS doublet (ca. 130 kDa). One of 14 (panel A) or 3 (panel B) similar experiments.

macrophages was not significantly different from 48 h control cultures (428 ± 43 μg/ml vs. 456 ± 42 μg/ml, $n = 17$). Second, DAHP treatment did not affect mitochondrial activity as determined by the transformation of MTT into formazan (OD_{570} nm was 0.338 ± 0.008 in DAHP-treated vs. 0.358 ± 0.01 in control cultures after IFN-γ stimulation; one of 3 experiments). Finally, expression of β-actin mRNA (not shown) or 16S ribosomal RNA (see below) was unaltered in the presence of DAHP.

3.2. DAHP leads to a decrease of the intra- and extracellular biopterin levels

As a known competitive inhibitor of GTP-cyclohydrolase I [28] DAHP was previously shown to diminish the concentrations of intracellular biopterin in fibroblasts, vascular smooth muscle cells and the RAW 264.7 cell line [8,11,13]. We found that DAHP also concentration-dependently depleted IFN-γ stimulated primary macrophages of intracellular BH_4 and total biopterin (Table 1). In the presence of 10 mM DAHP, the intracellular BH_4 content was reduced by 95 ± 0.4% (mean ± S.E.M. of 4 experiments) compared to untreated controls. Furthermore, the IFN-γ induced up-regulation of extracellular biopterin levels was antagonized by DAHP. Thus, the suppressive effect of DAHP on macrophage NO production is paralleled by decreased formation of BH_4.

3.3. BH_4-independent down-regulation of iNOS enzyme activity and protein expression by DAHP

In order to further analyse the functional relationship between DAHP-mediated NO suppression and BH_4-depletion, we determined the activity of iNOS in lysates from DAHP-treated and control macrophages. Despite the presence of excess amounts of BH_4 (4 μM) in the assay system [22] the enzymatic activity was strikingly reduced in lysates from macrophages stimulated with IFN-γ in the presence of DAHP (Table 1). The mean (±S.E.M.) suppression of enzyme activity afforded by 10 mM DAHP was 82 ± 4.3% ($n = 11$). This result indicated that DAHP-mediated suppression of iNOS activity is not due to a lack of intracellular BH_4.

Subsequent Western blot analysis revealed that DAHP down-regulates the IFN-γ-induced expression of iNOS protein at 18–66 h of stimulation to a similar extent as transforming growth factor-β (TGF-β; [22]) (Fig. 2A and data not shown). In contrast, DAHP caused only weak suppression of iNOS enzyme activity (35 ± 3.8%; mean ± S.E.M. of 3 experiments) and protein expression if the macrophages were stimulated with IFN-γ plus LPS instead of IFN-γ alone (Fig. 2B). When DAHP was added to the macrophages at 24 h of stimulation, i.e. after full induction of iNOS, no suppression of iNOS protein (Fig. 2B) or activity (not shown) was observed during the following 24–30 h of culture.

Finally, and most importantly, the suppressive effect of DAHP on NO_2^- accumulation, iNOS enzyme activity and protein expression was not reversed by L-sepiapterin (Fig. 3A,B; Table 1). Once taken up by cells, L-sepiapterin yields BH_4 via a sepiapterin reductase-dihydrofolate reductase salvage pathway [16]. Although the intra- and extracellular biopterin levels rose dramatically in the presence of L-sepiapterin (Table 1), the suppression of iNOS enzyme activity achieved by DAHP only slightly decreased from 87 ± 6.7% to 75 ± 4% (mean ± S.E.M.) in a series of 4 experiments.

3.4. DAHP down-regulates the expression of iNOS mRNA

Whereas the results of the Western blot analysis are amenable to the hypothesis that DAHP down-regulates the expression of iNOS protein via depletion of intracellular BH_4, the inability of L-sepiapterin to counteract the effect of DAHP strongly argues against this possibility. We therefore tested whether DAHP affects the expression of iNOS mRNA. As illustrated in Fig. 4A, DAHP decreased the levels of iNOS mRNA in IFN-γ activated macrophages. The extent of suppression

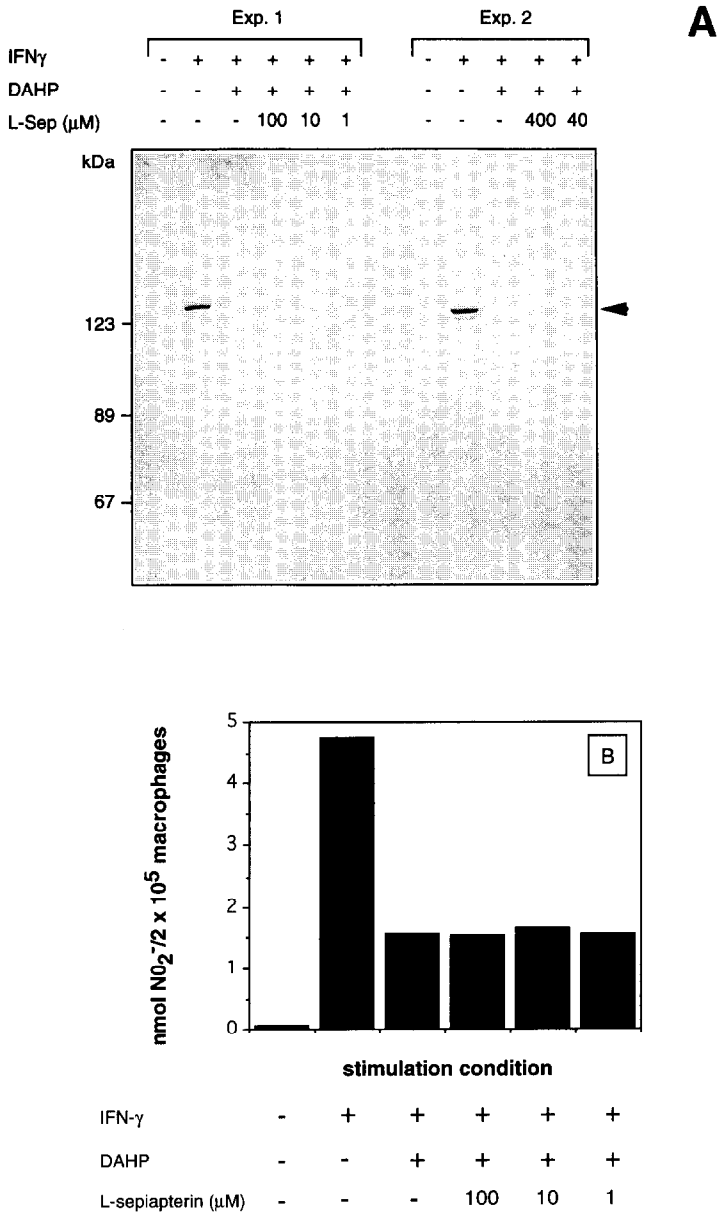

Fig. 3. L-sepiapterin is unable to reverse the down-regulation of iNOS protein by DAHP. A: Macrophages were stimulated with IFN-γ (20 ng/ml) ± DAHP (10 mM) for 72 h (Exp. 1) or 66 h (Exp. 2). Gels used for the Western blot were loaded with 15 μg (Exp. 1) or 20 μg protein per lane (Exp. 2). B: NO_2^- accumulation in the supernatant of the cultures of Exp. 1. One of 5 similar experiments.

appeared to be comparable at 24, 48 and 72 h of stimulation (Fig. 4 A,B). These results demonstrate that the marked suppression of iNOS protein after DAHP treatment results from a strongly reduced iNOS mRNA expression.

4. Discussion

Previous studies have demonstrated that high-output generation of NO by iNOS and synthesis of BH_4 are coinduced in mouse fibroblasts [8], murine brain endothelial cells [10], and rat vascular smooth muscle cells [11]. Conversely, DAHP reduced both the intracellular BH_4 content and the accumulation of NO_2^- in the respective cultures. As the DAHP effect was at least partially reversed by precursors of BH_4 (e.g. L-sepiapterin), it was concluded that DAHP downregulates the production of NO via reduced formation of BH_4 [8, 10, 11]. The experiments presented here demonstrate that the mechanism of NO suppression by DAHP is different in primary murine macrophages. Besides NO_2^- measurements we performed iNOS enzyme activity assays, Western and Northern blot analysis, which revealed that DAHP down-regulates iNOS mRNA and protein expression in addition to its suppression of BH_4 synthesis. The effect of DAHP on iNOS was dependent on the stimulus inasmuch as it was largely abolished in the presence of LPS, but it was not reversed by L-sepiapterin and, thus, independent of intracellular BH_4 levels.

In the past DAHP was shown to inhibit cytokine-induced NO_2^- accumulation in a number of different macrophage popu-

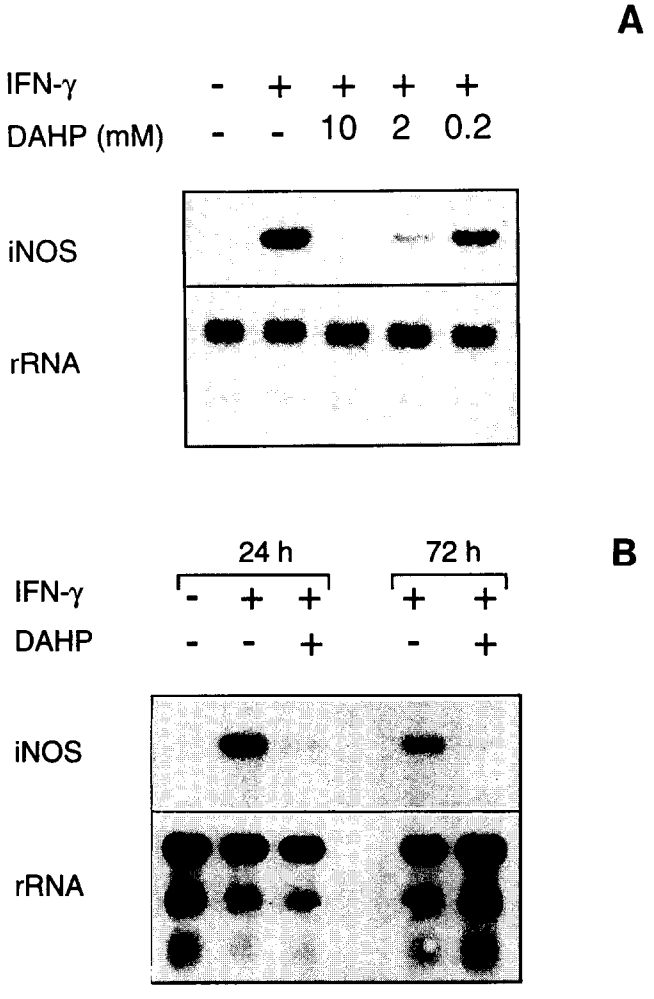

Fig. 4. Northern blot analysis of the effect of DAHP on the expression of iNOS mRNA in macrophages stimulated with (panel A) IFN-γ ± DAHP (0.2–10 mM) for 48 h or with (panel B) IFN-γ ± DAHP (10 mM) for 24 or 72 h. Blots (10 μg (A) or 5 μg (B) of total RNA/lane) were exposed to X-ray film for 48 h (rRNA) or 4 days (iNOS).

lations, but no detailed studies on its mechanism of action were provided [12–14]. The results reported, however, are compatible with ours. In IFN-γ activated rat alveolar macrophages L-sepiapterin also failed to reverse the effect of DAHP and turned out to be inhibitory by itself [12]. The NO production of RAW 264.7 cells stimulated with IFN-γ plus LPS was not suppressed by coincubation with DAHP, and even after pretreatment of the cells with DAHP the inhibition was weak [13]. Finally, DAHP down-regulated the release of NO by IFN-γ/LPS-activated murine peritoneal exudate macrophages, but data which would allow to ascribe the effect to inhibition of BH_4 synthesis were not presented [14].

The question arises why DAHP apparently suppresses iNOS by two different mechanisms depending on the cell type. Proliferating cells such as fibroblasts, endothelial cells or vascular smooth muscle cells have very low or even undetectable basal biopterin levels [8,10,11]. Upon cytokine stimulation, their biopterin content rises quite dramatically, which is a prerequisite for the induction of iNOS activity. In such a situation, inhibition of BH_4 synthesis by DAHP will suffice to down-regulate the release of NO, and addition of an exogenous BH_4 source (e.g. L-sepiapterin) restores the production of NO, even if iNOS mRNA and protein were somewhat reduced by DAHP. At least in rat vascular smooth muscle cells the inhibitory effect of DAHP was observed in the absence of altered iNOS mRNA levels, and the cytosol of treated cells even exhibited a 5-fold *higher* activity of iNOS if tested in the presence of exogenous BH_4 [29]. In macrophages, in contrast, basal BH_4 levels are already high and cytokine induction of iNOS is paralleled by only marginal increases of the intracellular BH_4 content [1,13] (Table 1). Unless the cells are completely depleted of BH_4 [13], the activity of iNOS will primarily depend on the iNOS mRNA and protein levels. In this case, the inhibitory effect of DAHP results from reduced iNOS mRNA expression rather than from decreased intracellular BH_4 concentrations, which makes it largely insensitive to L-sepiapterin.

So far we can only speculate on the mechanism by which DAHP down-regulates iNOS mRNA expression. It is conceivable that the inhibition of GTP-cyclohydrolase I by DAHP leads to accumulation of intracellular GTP. This might affect the activity of G-proteins and, thus, alter intracellular signalling [30,31] . In fact, there is recent evidence that G-proteins are involved in the induction of iNOS [32]. Alternatively, it is possible that DAHP down-regulates the activity of a so far unidentified enzyme involved in the activation/translocation of transcription factors or the transcription process itself. Finally, DAHP is a pyrimidine and might therefore directly interfere with mRNA synthesis.

In summary, we have identified primary murine macrophages as a cell type in which the suppression of NO production by DAHP is due to a decreased expression of iNOS protein and mRNA. Such a mechanism should be taken into consideration in future studies whenever DAHP is used for analysing the BH_4 dependency of iNOS in a certain type of cell.

Acknowledgements: We are grateful to Drs. Qiao-wen Xie and Carl Nathan (New York City, NY), Jeffrey Weidner and Rick Mumford (Rahway, NJ), as well as Georg Adolf (Vienna, Austria), for generous supply of antibodies or cytokines. We also wish to thank Heike Thüring for help with the preparation of the micrographs and Daniela Gmehling and Petra Höfler for excellent technical assistance. This study was supported by grants to C.B. from the Deutsche Forschungsgemeinschaft (Bo 996/2–1, SFB 263 A5) and the Austrian Research Funds 'Zur Förderung der wissenschaftlichen Forschung' (Project P9685).

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