Induction of inducible nitric oxide synthase expression by neopterin in vascular smooth muscle cells

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Abstract The pteridine compounds neopterin and 7,8-dihydroneopterin serve as valuable indicators for the stimulation of the cellular immune system. Whether they exhibit distinct biochemical functions in the immunological process is at present under discussion. We show that neopterin, but not 7,8-dihydroneopterin, is a stimulus for iNOS gene expression in rat vascular smooth muscle cells in vitro. At a concentration of 20 μM, neopterin leads to an iNOS mRNA expression of 2.5 amol iNOS cDNA/μg total RNA. When cells were coincubated with 20 μM neopterin and 5 μg/ml lipopolysaccharide derived from *Escherichia coli*, at least an additive effect on iNOS mRNA expression could be detected (iNOS cDNA concentration was 5.0 amol/μg total RNA). We speculate that neopterin enhances the macrophage-induced extracellular toxicity. This might be of relevance in situations associated with excessive release of cytokines, neopterin, and nitric oxide, as observed in septic shock.

Key words: Neopterin; 7,8-Dihydroneopterin; Inducible nitric oxide synthase; Nitric oxide; Sepsis

1. Introduction

Neopterin and 7,8-dihydroneopterin are heterocyclic pteridine compounds which are synthesized and released in excess by monocytes/macrophages upon stimulation with interferon-γ [1]. Therefore, neopterin has become a valuable diagnostic marker in a number of conditions associated with increased activity of the cellular immune system, e.g. AIDS or hepatits [2]. In spite of this, it remains elusive whether pteridines per se exhibit any biochemical functions. Recent data suggest that neopterin and 7,8-dihydroneopterin may interfere with the cytotoxic mechanisms of activated macrophages since neopterin was found to potentiate the effects of reactive oxygen species whereas lower doses of 7,8-dihydroneopterin appeared to be an oxygen radical scavenger [3,4]. On the other hand, a high concentration of 7,8-dihydroneopterin in combination with tumor necrosis factor-α (TNF-α) was demonstrated to enhance apoptosis due to oxidative stress [5]. In addition, Barak and Gruener [6] described an augmentation of TNF-α-production in peripheral blood mononuclear cells and macrophages following simultaneous stimulation with neopterin and either lipopolysaccharide (LPS) from *Escherichia coli* or interferon-γ (IFN-γ). Therefore, one may speculate that pteridines play a role as modulators in situations associated with Gram-negative endotoxemia and with increased activity of several inflammatory cytokines.

It is well known that bacterial endotoxins as well as cytokines like TNF-α and IFN-γ are potent stimulators of inducible nitric oxide synthase (iNOS) gene expression in various cell types, e.g. vascular smooth muscle cells (VSMC). iNOS stimulation leads to an excessive formation of the vasodilating compound nitric oxide [7–9] which is assumed to be the final pathway of the pathophysiological hypotensive reaction characteristic of septic shock. Since neopterin levels were found to be increased in patients with sepsis [10], the present experiments were carried out to investigate whether neopterin and 7,8-dihydroneopterin interfere with iNOS gene expression in vitro.

2. Materials and methods

2.1. Reagents

Dulbecco's modified Eagle's medium (DMEM) with phenol red was purchased from ccPro, Karlsruhe, Germany. DMEM without phenol red, L-arginine, collagenase I, trypsin inhibitor, *N*-(1-naphthylethylene)diamine, β-NADPH, lipopolysacharide (LPS) derived from *E. coli* (serotype 0111:B4), and the phenol-chloroform-isoamyl alcohol mixture were from Sigma Chemicals, Deisenhofen, Germany. Fetal calf serum (FCS), penicillin-streptomycin, trypsin-EDTA, and M-MLRV superscript reverse transcriptase were purchased from Gibco Life Tech., Eggenstein, Germany. Elastase and sulfanilamide were from Serva, Heidelberg, Germany. Oligo(dT) 15 and dNTP-mix were from Amersham Buchler, Braunschweig, Germany. Taq polymerase and primer sets were obtained from Biometra, Göttingen, Germany; guanidine isothiocyanate was from Roth, Karlsruhe, Germany; and nitrate reductase was from Boehringer, Mannheim, Germany. Recombinant rat interferon-γ (IFN-γ) was from IC Chemicals, Ismaning, Germany; neopterin and 7,8-dihydroneopterin were purchased from Schircks Lab., Jona, Switzerland.

Possible cytotoxicity of the pteridines was assessed by means of the [3-(4,5-dimethylthiazol-2-yl)-2,5-piphenyl]tetrazolin bromide assay. No cytotoxicity was observed.

2.2. Preparation of rat aortic vascular smooth muscle cells

VSMC from WKY rats were isolated using a modified method originally described by Chamley et al. [11]. The rats were decapitated under ether anesthesia and the thoracic aorta was dissected out immediately. The surrounding adipose and connective tissue was removed and the aorta was incubated at 37°C for 30 min in DMEM containing collagenase I (385 U/ml), elastase (90 U/ml), trypsin inhibitor (inhibiting capacity 0.85 mg trypsin/ml), streptomycin (50 μg/ml), penicillin (50 U/ml), and 10% FCS in a humidified atmosphere of 95% air/5% CO_2. Afterwards, the adventitia was stripped off, the aorta was minced with scalpels and again incubated at 37°C for 2 h under continuous stirring in DMEM containing the supplements mentioned above. The resulting VSMC suspension was centrifuged for 5 min at 400 × *g*.

The pellet was resuspended in fresh DMEM and the cells were plated on 35 × 10 mm culture dishes and grown at 37°C in a humidified atmosphere of 95% air/5% CO_2. After 5–7 days, confluent cultures were passaged by incubation (10 min at 37°C) in phosphate-buffered saline solution (PBS; 137 mM NaCl, 2.7 mM KCl, 8.1 mM Na_2HPO_4, 1.5 mM

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KH_2PO_4, pH 7.4) containing 0.05% trypsin and 0.02% EDTA. Subcultured cells were grown in 75 cm^2 culture flasks (Falcon). For experiments, passages 4–12 were used. A total of five different cell preparations from WKY was used in the present study.

2.3. Nitrite and nitrate assay

Synthesis of the stable NO metabolites nitrite and nitrate was determined in the cell-free supernatant cultures incubated for 48 h in L-arginine enriched medium without phenol red. Nitrate was reduced to nitrite by nitrate reductase (0.4 U/ml), in the presence of 10 mM β-NADPH. Total nitrite accumulation was assayed by the Greiss reaction [12].

2.4. RNA isolation and polymerase chain reaction

At the end of the 9 h experiments cells were washed with phosphate-buffered saline and lysed with 4 M guanidinium isothiocyanate containing 0.1 M 2-mercaptoethanol. Total RNA was isolated by acid phenol-chloroform extraction according to the method by Chomczynski and Sacchi [13], redissolved in water and determined photometrically at a wavelength of 260 nm. 1 μg total RNA was reverse-transcribed into first strand cDNA using oligo(dT)15 as a primer for reverse transcriptase. RT-generated cDNA encoding for rat inducible NOS and rat glyceraldehyde-3-phosphate dehydrogenase (GAPDH) genes were amplified using PCR. Expression of the housekeeping gene GAPDH served as control; RNA with no GAPDH band was excluded from further investigations. PCR was performed in PCR buffer (50 mM Tris-HCl, pH 8.3, 50 mM KCl, 1.5 mM $MgCl_2$, 0.001% w/v gelatine), 0.2 mM of each dNTP, 300 nM of each 5′ and 3′ primer and 2 U/ml of Taq polymerase in a 50 μl reaction volume. PCR was run for 30 cycles after an initial denaturation step at 94°C for 3 min with an amplification profile of each cycle consisting of denaturation for 1 min at 94°C, primer annealing for 1.5 min at 58°C and elongation for 3 min at 73°C.

For quantitative analysis of iNOS cDNA, a competitive PCR was performed using a neutral non-homologous DNA fragment derived from the viral oncogene V-erb B to which the iNOS primer template sequences had been added (Competitive DNA MIMIC, Clontech, as described by Siebert and Larrick [14]). The use of this internal standard allows one to determine the absolute amount of target iNOS cDNA. Known molecular quantities of the standard cDNA were spiked into a series of PCR reaction tubes containing equal amounts of the iNOS cDNA. The PCR setup was as described above, with one exception: for competitive analyses, only 25 cycled PCR were performed.

Following PCR, the amount of products generated by the internal standard as well as by the iNOS target were compared. The initial amounts of iNOS cDNA and competititor cDNA were assumed to be equal in those reactions where the molar ratio of iNOS and standard products was jugded to be equal.

Oligonucleotide primers for iNOS and internal standard were: 5′ CCCTTCCGAAGTTTCTGGCAGCAGG 3′ (upstream), and 5′ GGCTGTCAGAGCCTTGTGCCTTTGG 3′ (downstream) corresponding to the murine macrophage iNOS [15]. Oligonucleotide primers for GAPDH were: 5′ GCAGGGGGAGCCAAAAGGG 3′ (upstream) and 5′ TGCCAGCCCCAGCGTCAAAG 3′(downstream) corresponding to the human GAPDH gene [16].

2.5. Statistical analysis

Results are expressed as mean values ± standard error of the mean (SEM). To test for significance of differences between the mean value of a control vs. the mean value of treated cells, Tukey's *t*-test was used. *P* values <0.05 were considered to be significant.

3. Results

Fig. 1 summarizes the results of the qualitative analysis of iNOS mRNA expression measured as iNOS cDNA following the different 9 h incubation protocols. IFN-γ (100 U/ml), LPS (5 μg/ml), neopterin (20 μM) as well as the combination of neopterin/IFN-γ, 7,8-dihydroneopterin (20 μM)/IFN-γ, neopterin/LPS, and 7,8-dihydroneopterin/LPS increased iNOS mRNA expression. In contrast, no iNOS cDNA could be detected in unstimulated cells (C) and in cells treated with 7,8-dihydroneopterin alone. Fig. 2 shows the results of a comparative quantification of iNOS cDNA following 9 h incubations with neopterin, LPS, and coincubation of cells with neopterin and LPS, respectively. Neopterin enhanced iNOS mRNA expression (2.5 amol/μg cDNA), while LPS treatment resulted in an iNOS cDNA concentration of 1.0 amol/μg total RNA. When VSMC were coincubated with neopterin and LPS, at least an additive effect on iNOS mRNA expression (iNOS cDNA concentration was 5.0 amol/μg total RNA) could be found. Further results of iNOS cDNA quantification analyses are given in Fig. 3. No difference in iNOS gene expression could be detected when cells were coincubated with 7,8-dihydroneopterin and LPS as compared to cells treated with LPS alone. In both experiments iNOS cDNA concentration was 1 amol/μg total RNA. IFN-γ strongly enhanced iNOS mRNA production. During 9 h incubation the iNOS cDNA concentration increased to 50 amol/μg total RNA. This effect was not influenced by coincubation of cells with either neopterin/IFN-γ or 7,8-dihydroneopterin/IFN-γ, both resulting in identical iNOS cDNA levels of 50 amol/μg total RNA.

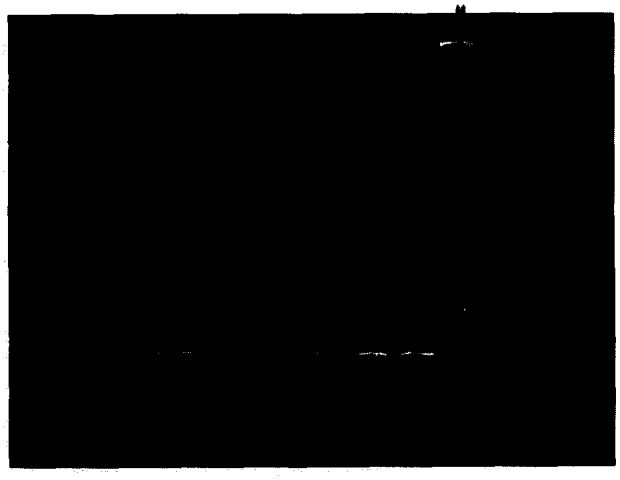

Fig. 1. Qualitative analysis of iNOS mRNA expression detected as iNOS cDNA (length: 498 base pairs) following 9 h incubations of vascular smooth muscle cells with: 100 U/ml interferon-γ (IFN-γ), 5 μg/ml lipopolysaccharide (LPS), 20 μM neopterin (N20) 20 μM 7,8-dihydroneopterin (NH20), 20 μM neopterin + 100 U/ml interferon-γ (N20 + IFN-γ), 20 μM 7,8-dihydroneopterin + 100 U/ml interferon-γ (NH20 + IFN-γ), 20 μM neopterin + 5 μg/ml lipopolysaccharide (N20 + LPS), and 20 μM 7,8-dihydroneopterin + 5 μg/ml lipopolysaccharide (NH20 + LPS), respectively. Lane C shows the control experiments in unstimulated cells, lane M indicates the molecular marker. The lower part of the figure shows the corresponding GAPDH bands (length: 567 base pairs). Expressionof the housekeeping GAPDH gene served as internal control. Results are representative of three different experiments.

Table 1 summarizes the results of the nitrite/nitrate determination in cell-free culture supernatants following 24 and 48 h incubation. Stimulation of iNOS at the mRNA level (Fig. 1) was accompanied by an accumulation of nitrite/nitrate in the supernatants, thus indicating that the VSMC were activated to produce nitric oxide. Neopterin enhanced the formation of nitrite compared to controls and was also effective in LPS-treated cultures. In contrast, 7,8-dihydroneopterin decreased nitrite formation in the cell cultures stimulated with IFN-γ or LPS. The absolute amount of nitrite produced was greater in cultures incubated for 48 h compared to 24 h. However, the

influence of incubations with IFN-γ, LPS and neopterin derivatives showed the same characteristics (Table 1).

Using higher concentrations of neopterin derivatives, e.g. 200 μM, also only marginally affected the results: after 24 h nitrite production was 6.38 ± 0.31 nmol nitrite/10^6 cells when neopterin was added, being 2.40 ± 0.12 nmol/10^6 cells in the case of 7,8-dihydroneopterin. Coincubation of 200 μM neopterin with IFN-γ (25.73 ± 0.35 nmol nitrite/10^6 cells) or LPS (12.75 ± 0.30) induced slightly higher nitrite concentrations compared to 20 μM neopterin (Table 1). 200 μM 7,8-dihydroneopterin decreased nitrite production to an extent similar to that with 20 μM 7,8-dihydroneopterin (4.45 ± 0.34; Table 1) but the nitrite production induced by IFN-γ was more decreased with the 200 μM dose (15.81 ± 0.35) compared to the 20 μM dose (Table 1).

4. Discussion

Our data demonstrate that neopterin stimulates iNOS gene expression at the mRNA level with a concomitant increase in nitric oxide production. The expression induced by neopterin was even greater than that induced by LPS and seemed to be additive to that of LPS. The biochemical mechanisms whereby neopterin modulates NO production remain to be elucidated. Neopterin is known to enhance the effects exhibited by cytotoxic reactive oxygen species originating from chloramine-T and hydrogen peroxide [3,4]. In a study performed at our laboratory, we found an increase in iNOS gene expression in VSMC following incubation with 300 μM hydrogen peroxide (unpublished observation). In addition, Adcock et al. [17] demonstrated a stimulation of iNOS gene expression induced by the superoxide anion generating compound pyrogallol, most likely via activation of the cellular transcription factor NF-κB. One may speculate that neopterin, immediately released into the circulation following macrophage stimulation in humans, is likely to play a role in amplifying macrophage-induced extracellular toxicity by potentiating the initial response to oxidative stress. This includes enhanced production of NO which is known to exhibit distinct cytotoxic effects, e.g. microbicidial and tumoricidial activities [18,19].

Another possible mechanism could be that a neopterin-induced multiplication of the effects of reactive oxygen intermediates may result in programmed cell death including an increase in iNOS gene expression. In our study, less than 1% of the cells treated with neopterin or 7,8-dihydroneopterin ± IFN-γ and LPS stained with trypan blue. Furthermore, replating of cells following a 48 h incubation period did not result in different plating efficiency between VSMC exposed to the above-mentioned substances and untreated cells. We conclude that, concerning our experiments, neither neopterin- nor neopterin-induced nitric oxide generation exhibited any cytotoxic effects on vascular smooth muscle cells.

At present it remains speculative whether our in vitro observations on rat VSMC are of in vivo relevance in conditions that are associated with increased iNOS activity in men. In this context, it is of special importance that human macrophages contain a particularly low activity of 6-pyruvoyl tetrahydropterin synthase, the second enzyme in the tetrahydrobiopterin (BH4) generating pathway. Its lower activity leads (a) to minor synthesis of BH4, an essential cofactor for iNOS activity, and (b) to the predominant production of 7,8-dihydroneopterin due to the major cleaving of 7,8-dihydroneopterin trisphosphate by phosphatases. 7,8-Dihydroneopterin is then partially oxidized to neopterin [2,20]. Consequently, the potential of human macrophages to produce NO is reduced. However, activation of human macrophages by interferon-γ results in the release of large quantities of neopterin, thereby possibly counterbalancing the limited macrophages dependent NO-induced cytotoxicity [20].

In our study 7,8-dihydroneopterin did not affect iNOS gene expression but suppressed the IFN-γ-induced augmentation of nitrite levels in the culture supernatants. Therefore, 7,8-dihydroneopterin may act as a nitric oxide suppressor at the posttranscriptional level, most likely due to its recently de-

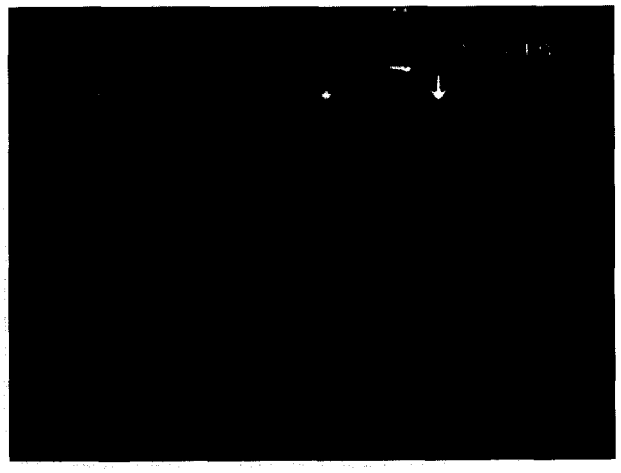

Fig. 2. Quantitative analysis of iNOS mRNA expression detected as iNOS cDNA (length: 498 base pairs) following 9 h incubations of vascular smooth muscle cells with: 20 μM neopterin (N20), 5 μg/ml lipopolysaccharide (LPS), and 20 μM neopterin + 5 μg/ml lipopolysaccharide (N20 + LPS), respectively. Lane M indicates the molecular marker. As described in detail in section 2, known molecular quantities of the standard cDNA (length: 400 base pairs) were spiked into a series of PCR reaction tubes containing equal concentrations of iNOS cDNA. Following a 25 cycled PCR the initial amount of iNOS and standard cDNA were assumed to be equal in those reactions where the molar ratio of iNOS and standard products were jugded to be equal (as indicated by ⟩). Results are representative of three different experiments.

Table 1
Nitrite levels measured in cell culture supernatants following 24 h and 48 h incubation protocol

	Nitrite (nmol/10^6 cells)	
	24 h	48 h
C	2.76 ± 0.53	3.68 ± 0.59
IFN-γ (100 U/ml)	22.30 ± 1.52[a]	82.48 ± 0.85[a]
LPS (5 μg/ml)	6.78 ± 0.39[a]	10.18 ± 0.31[a]
Neopterin (20 μM)	5.58 ± 0.40[a]	12.80 ± 0.26[a]
7,8-dihydroneopterin (20 μM)	2.05 ± 0.11	3.31 ± 0.03
Neopterin (20 μM) + IFN-γ (100 U/ml)	25.08 ± 1.00[a]	88.50 ± 2.00[a]
Neopterin (20 μM) + LPS (5 μg/ml)	10.13 ± 0.71[a]	18.37 ± 1.77[a]
7,8-Dihydroneopterin (20 μM) + IFN-γ (100 U/ml)	18.28 ± 0.25[a]	48.66 ± 1.12[a]
7,8-Dihydroneopterin (20 μM) + LPS (5 μg/ml)	3.15 ± 0.46	9.75 ± 0.46[a]

Data are expressed as means ± SEM (n = 6).
[a] $P < 0.05$ as compared to unstimulated cells (C).

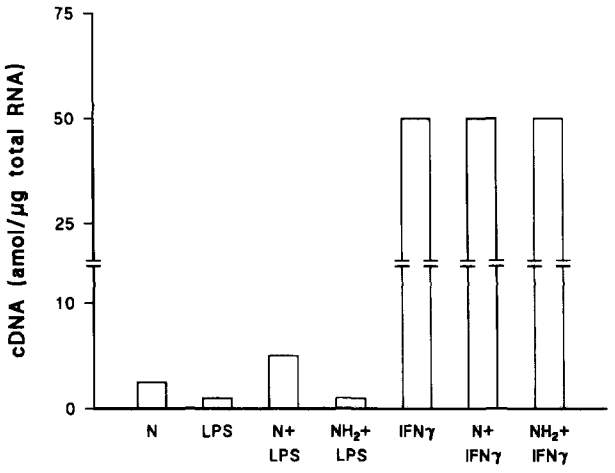

Fig. 3. Quantitative analysis of iNOS mRNA expression detected as iNOS cDNA (length: 498 base pairs) following 9 h incubations of vascular smooth muscle cells with: 20 μM neopterin (N20), 5 μg/ml lipopolysaccharide (LPS), and 20 μM neopterin + 5 μg/ml lipopolysaccharide (N20 + LPS), 20 μM 7,8-dihydroneopterin + lipopolysaccharide (NH20 + LPS), 100 U/ml interferon-γ (IFN-γ), 20 μM neopterin + 100 U/ml interferon-γ (N20 + IFN-γ), and 20 μM 7,8-dihydroneopterin + 100 U/ml interferon-γ (NH20 + IFN-γ), respectively. Results are representative of three different experiments.

scribed potential to act as a scavenger of reactive oxygen species [3].

Activation of macrophages with the subsequent release of neopterin is a common finding in several diseases characterized by an activated cellular immune system [2]. Therefore, the determination of neopterin represents a usefool tool for clinical monitoring. It is well established that septic patients, especially those with fatal outcome, have increased plasma concentrations of neopterin [10]. Moreover, patients with sepsis regularly have high concentrations of nitrite in plasma, indicating a pronounced release of NO [21,22]. It is likely that the induction of NO-synthase during sepsis is the final common pathway for vasodilatation in septic shock. Since we could demonstrate an iNOS gene expressing the effect of neopterin in VSMC, it is possible that neopterin is involved in the modulation of iNOS gene expression in septic patients. One might argue that the pronounced stimulatory effect of IFN-γ on iNOS gene expression in VSMC could potentially superimpose any neopterin-induced NO release. This is not in contradiction to our concept, since plasma IFN-γ-levels could not be detected after *E. coli* endotoxin infusion in healthy volunteers [23] nor did they correlate with the prognosis in septic patients [24]. Our findings suggest that neopterin stimulates iNOS expression at the mRNA level. Thus, it might be involved in macrophage-induced extracellular toxicity. The in vivo relevance of neopterin in conditions associated with excessive nitric oxide release, e.g., septic shock, remains to be addressed in further clinical studies.

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