Neopterin activates transcription factor nuclear factor-κB in vascular smooth muscle cells

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Abstract We have previously shown that the pteridine compound neopterin stimulates inducible nitric oxide synthase (iNOS) gene expression in vascular smooth muscle cells in vitro. The mechanisms whereby neopterin exhibits these effects remained unclear. The present study demonstrates that neopterin induces the translocation of the transcription factor nuclear factor-κB (NF-κB) to the nucleus. Pretreatment of cells with the antioxidant pyrrolidine dithiocarbamate completely suppressed the effects of neopterin on NF-κB activation, iNOS gene expression, and nitric oxide release. From these data we conclude that neopterin activates the translocation of NF-κB subunits to the nucleus by modulating the intracellular redox state. This is one possible explanation for the impact of neopterin on iNOS gene expression.

Key words: Neopterin; Nuclear factor-κB; Inducible nitric oxide synthase; Nitric oxide

1. Introduction

Neopterin is a heterocyclic pteridine compound synthesized and released in excess by mononcytes/macrophages upon stimulation with interferon-γ (IFN-γ) [1]. The production of neopterin hereby closely correlates with IFN-γ serum concentration and the activation of cell-mediated immunity [2]. Therefore, determination of neopterin levels in body fluids has become a valuable diagnostic tool in conditions associated with increased activity of the cellular immune system, e.g. HIV-infection, autoimmune disease, and certain types of cancer [3,4]. Recent data suggest that neopterin itself exhibits distinct biochemical functions. It was found to enhance the effects of cytotoxic reactive oxygen species originating from chloramine T and hydrogen peroxide [5,6]. This suggests that neopterin may influence biochemical pathways of reactive oxygen intermediates, thereby modulating macrophage-induced cytotoxicity by the induction of oxidative stress. In addition, Barak and Gruener [7] described an augmentation of tumor necrosis factor-α (TNF-α)-production in peripheral blood mononuclear cells and macrophages following simultaneous exposure to neopterin and lipopolysacharide (LPS) derived from *E. coli*. In a previous study, we could demonstrate that neopterin stimulates inducible nitric oxide synthase (iNOS) gene expression at the mRNA level with a subsequent increase in nitric oxide (NO) production [8]. The biochemical mechanism(s) by which neopterin influences NO synthesis remained unclear. Oxidative stress has been described to activate the cellular transcription factor nuclear factor-κB (NF-κB) in various cell types [9–11]. Since the promotor of the iNOS gene in murine macrophages contains two putative binding sites for NF-κB [12] it is conceivable that neopterin stimulates the transcription of the iNOS gene through activation of NF-κB. In the present study, we investigated the effects of neopterin and oxidative stress on NF-κB activation and iNOS gene expression in VSMC 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, dithiothreitol (DDT), EDTA, EGTA, glycerol, HEPES, Nonidet P-40, trypsin inhibitor, *N*-(1-naphthylethylene)diamine, β-NADPH, phenol-chloroform-isoamyl alcohol mixture, phenylmethylsulfonyl fluoride (PMSF) and pyrrolidine dithiocarbamate (PDTC) 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 and Tris was from Roth, Karlsruhe, Germany; nitrate reductase was from Boehringer, Mannheim, Germany. Hydrogen peroxide (H_2O_2) was purchased from Merck, Darmstadt, Germany. Recombinant rat tumor necrosis factor-α (TNF-α) was from IC Chemicals, Ismaning, Germany, neopterin was purchased from Schircks Lab., Jona, Switzerland.

NF-κB p65 antibody (human, mouse, and rat reactive rabbit polyclonal IgG) was purchased from Santa Cruz Biotechnology, Santa Cruz, CA.

2.2. Preparation of rat aortic vascular smooth muscle cells

Preparation of VSMC from WKY rats was performed as described recently [13]. Briefly, the rats were decapitated under ether anesthesia and the thoracic aorta was cut out and dissected immediately. VSMC were isolated by enzymatic digestion using collagenase I (385 U/ml), and elastase (90 U/ml), suspended in DMEM (containing 50 μg/ml streptomycin, 50 U/ml penicillin, and 10% FCS) and grown in 75 cm^2 culture flasks (Falcon) in a humidified atmosphere of 95% air/5% CO_2. For experiments, passages 4 to 12 of subcultured cells were used. A total of 5 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 culture supernatants 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 Griess reaction [14].

2.4. RNA isolation and polymerase chain reaction

9 h after stimulation cells were washed with sterile PBS and lysed

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with 4 M guanidine isothiocyanate containing 0.1 M 2-mercaptoethanol. Total RNA was isolated by acid phenol-chloroform extraction according to the method of Chomczynski and Sacchi [15], redissolved in water and the concentration determined photometrically at a wavelength of 260 nm. 1 μg total RNA was reverse-transcribed into first strand cDNA using oligo(dT)$_{15}$ as primer for reverse transcriptase (RT). RT-generated cDNA encoding for rat iNOS was amplified using polymerase chain reaction (PCR) as described recently [8]. Expression of the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH) served as control, RNA with no GAPDH band was excluded from further investigations.

For quantitation of iNOS cDNA, a competitive PCR was performed using a DNA fragment derived from the viral oncogene V-erb B to which the iNOS primer template sequences have been added (Competitive DNA MIMIC, Clontech, Heidelberg, Germany), as described by Siebert and Larrick [16]. Increasing quantities of the standard DNA were spiked into a series of PCR reaction tubes containing equal amounts of iNOS cDNA. Following PCR, the amount of products generated from the standard DNA as well as from the iNOS cDNA were compared. The initial amounts of iNOS cDNA and competitor DNA were assumed to be equal in those reactions where the molar ratio of iNOS and standard products was judged to be equal by densitometric analysis of the scanned photographs (Imaging densitometer GS 670, BioRad GmbH, München, Germany) with regard to the different amount of ethidium bromide intercalation according to the length of the fragments. Results are expressed as amol iNOS-cDNA/μg total RNA taking into consideration that 1 μl of the 25 μl RT reaction yield was used per quantitative PCR. Oligonucleotide primers for iNOS and internal standard were: 5′ CCCTTCCG-AAGTTTCTGGCAGCAGG 3′ (upstream), and 5′ GGCTGTCA-GAGCCTTGTGCCTTTGG 3′ (downstram) corresponding to the murine macrophage iNOS [17]. Oligonucleotide primers for GAPDH were: 5′ GCAGGGGGAGCCAAAAGGG 3′ (upstream) and 5′ TGCCAGCCCCAGCGTCAAAG 3′ (downstream) corresponding to the human GAPDH gene [18].

2.5. Preparation of nuclear extracts

Nuclear extracts were prepared according to a method described by Hoppe-Seyler et al. [19]. Following a 2 h incubation, cells were rinsed with cold phosphate-buffered saline solution and lysed directly on the culture dishes in 1 ml cold lysis buffer (0.6% Nonidet P-40, 150 mM NaCl, 10 mM Tris pH 7.9, 1 mM EDTA). Lysed cells were transferred into a 2 ml Eppendorf tube and incubated for 5 min on ice. The nuclei were pelleted (1250×*g*, 4°C, 5 min), the supernatant was discarded, and nuclear proteins were extracted in 100 μl extraction buffer (420 mM NaCl, 10 mM HEPES pH 7.9, 0.1 mM EGTA, 0.1 mM EDTA, 1.5 mM $MgCl_2$, 0.5 mM DTT, 0.5 mM PMSF, 25% glycerol) on ice for 20 min. Cellular debris was removed by centrifugation (1250×*g*, 4°C, 5 min) and the supernatant containing nuclear protein was stored at −70°C.

2.6. Western blot analysis

NF-κB from nuclear extracts was detected by immunoblotting. Sample protein content was determined using bovine serum albumin as a standard according to the method of Bradford [20]. Equal protein amounts from each sample (5 μg) were separated by SDS-PAGE using an 8% acrylamide gel as described by Laemmli [21].

Separated proteins were transferred on a nitrocellulose membrane for 1 h at 250 mA by using a Bio-Rad Transblot (BioRad GmbH, München, Germany) apparatus. Membranes were then incubated for 12 h with a polyclonal rabbit anti NF-κB antibody (dilution: 1/200) corresponding to amino acids 531–550 at the carboxy terminus of the NF-κB p65 subunit. An anti-rabbit secondary antibody was used to visualize the NF-κB-antibody complex by an alkaline phosphatase reaction.

2.7. Statistical analysis

The results in Table 1 are expressed as mean values ± standard error of the mean (S.E.M.). To test for significance of differences between the mean value of a control vs. the mean value of treated cells, Student's *t*-test was used. P values < 0.05 were considered to be significant.

3. Results

Fig. 1 shows NF-κB in nuclear extracts of VSMC detected by Western blot analysis. 2 h stimulation of cells with neopterin (20 μM), TNF-α (500 U/ml), and H_2O_2 (300 μM) resulted in enhanced nuclear uptake of NF-κB whereas it was undetectable under control conditions. Pretreatment of cells with PDTC for 2 h completely suppressed the effects of neopterin and H_2O_2 on NF-κB activation but a weak NF-κB signal was still detectable in those cells exposed to PDTC and TNF-α. Fig. 2 summarizes the results of the qualitative analysis of iNOS mRNA expression following 9 h stimulation. Neopterin, TNF-α, and H_2O_2 increased iNOS mRNA expression while PDTC reduced the TNF-α-dependent stimulation. No iNOS mRNA was found in unstimulated cells (C) and in VSMC treated with PDTC prior to the addition of either neopterin or H_2O_2. The results of the quantitation of iNOS cDNA from cells incubated with neopterin, TNF-α, PDTC plus TNF-α, and H_2O_2 for 9 h are presented in Fig. 3. Neopterin enhanced iNOS mRNA expression to 62.5 amol cDNA/μg total RNA. TNF-α treatment resulted in a stronger induction of iNOS cDNA production (312.5 amol/μg total RNA), whereas H_2O_2 was a weaker stimulus of iNOS gene expression (iNOS cDNA concentration was 12.5 amol/μg total RNA). In cells preincubated with the antioxidant PDTC for 2 h, only the subsequent addition of TNF-α led to measurable amounts of iNOS cDNA, but the level (62.5 amol/μg total RNA) was considerably lower compared to TNF-α treatment alone. Nitrite/nitrate (NO_2^-/NO_3^-) determinations in cell-free culture supernatants following 24 h incubation periods are summarized in Table 1. Stimulation of iNOS gene expression by neopterin, TNF-α, and H_2O_2 was accompanied by an increase in nitrite/nitrate concentrations as compared to unstimulated controls, thus indicating that VSMC were activated to produce and release NO. In contrast, NO_2^-/NO_3^- levels were not significantly augmented in cells that had been preincubated with PDTC before the addition of neopterin or H_2O_2. Although nitrite/nitrate concentration was raised significantly

Table 1
Nitrite/nitrate levels measured as accumulated nitrite in cell-free culture supernatants following 24 h incubations

	Nitrite (nmol/10^6 cells)
Unstimulated controls	2.62 ± 0.33
Neopterin (20 μM)	9.71 ± 1.62[b]
PDTC[a] (150 μM)+neopterin (20 μM)	3.64 ± 0.90
Tumor necrosis factor-α (500 U/ml)	71.12 ± 3.82[b]
PDTC[a] (150 μM)+tumor necrosis factor-α (500 U/ml)	8.57 ± 1.24[b]
Hydrogen peroxide (300 μM)	8.99 ± 0.63[b]
PDTC[a] (150 μM)+hydrogen peroxide (300 μM)	2.55 ± 0.27

Data are expressed as means ± S.E.M. (n=8).
[a]Pyrrolidine dithiocarbamate (PDTC) was added 2 h prior to stimulation of cells with other substances.
[b]$P < 0.05$ as compared to unstimulated cells.

following coincubation of cells with PDTC plus TNF-α ($P < 0.05$ as compared to control), this increase in NO metabolite concentration was only a fraction of that measured in supernatants of cells exposed to TNF-α alone.

4. Discussion

In a recent study we could show that the heterocyclic pteridine compound neopterin stimulates iNOS gene expression and nitric oxide release in cultured vascular smooth muscle cells [8]. The biochemical mechanism(s) of these effects remained unclear. The present data demonstrate that the same amount of neopterin (20 μM) activates translocation of NF-κB to the nucleus of VSMC in vitro. Activation of NF-κB is most likely an important pathway for the transcriptional induction of iNOS by various immunostimulants. The promotor of the murine gene coding for iNOS contains at least two elements homologous with consensus sequences for the binding of NF-κB [12]. Of these two putative NF-κB binding sites the downstream oriented has been demonstrated to confer LPS inducibility of iNOS [22]. In unstimulated cells, nuclear uptake of NF-κB is suppressed by inhibitory subunits called IκB proteins. The NF-κB activation process requires the release of IκB from the cytoplasmic complex [23]. In our experiments, TNF-α which induces the production of free radicals [24–26] as well as H_2O_2 led to NF-κB activation, iNOS gene expression, and NO release in VSMC. Reactive oxygen species may modulate the cellular redox state, which has been linked to NF-κB activation [27]. It is of note that TNFα and H_2O_2 seem to induce a slower migrating form of NF-κB than neopterin which might indicate different states of NF-κB phosphorylation (Fig. 1). This observation clearly needs further investigation.

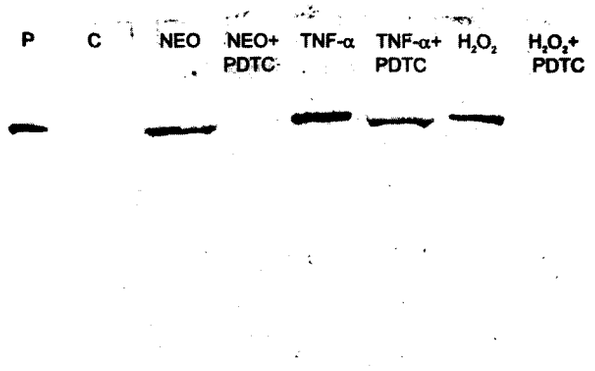

Fig. 1. Western Blot analysis of nuclear factor-κB (NF-κB) in nuclear extracts of vascular smooth muscle cells following 2 h incubation with: 20 μM neopterin (NEO), 150 μM pyrrolidine dithiocarbamate*+20 μM neopterin (PDTC+NEO), 500 U/ml tumor necrosis factor-α (TNF-α), 150 μM pyrrolidine dithiocarbamate*+500 U/ml tumor necrosis factor-α (PDTC+TNF-α), 300 μM hydrogen peroxide (H_2O_2), and 150 μM pyrrolidine dithiocarbamate*+300 μM hydrogen peroxide (PDTC+H_2O_2), respectively. Lane C shows the result of a control experiment in unstimulated cells. Lane P indicates bovine serum albumin that served as a molecular mass marker (64.2 kDa). Lane P was separately stained with Ponceau Red. Results are representative of three different experiments. *Pyrrolidine dithiocarbamate (PDTC) was added 2 h prior to stimulation of cells with other substances.

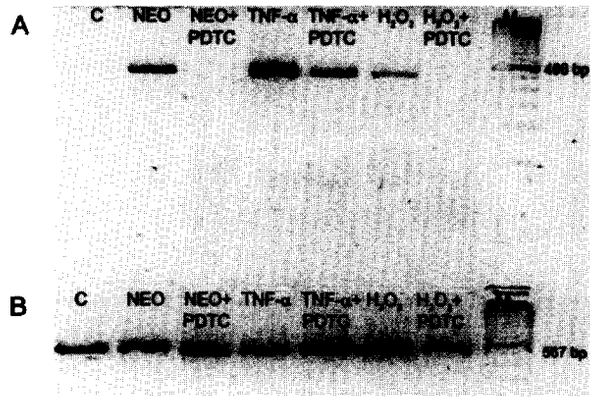

Fig. 2. (A) Qualitative PCR analysis of iNOS mRNA expression detected as iNOS cDNA (fragment size: 498 base pairs) following 9 h incubations of vascular smooth muscle cells with: 20 μM neopterin (NEO), 150 μM pyrrolidine dithiocarbamate*+20 μM neopterin (PDTC+NEO), 500 U/ml tumor necrosis factor-α (TNF-α), 150 μM pyrrolidine dithiocarbamate*+500 U/ml tumor necrosis factor-α (PDTC+TNF-α), 300 μM hydrogen peroxide (H_2O_2), and 150 μM pyrrolidine dithiocarbamate*+300 μM hydrogen peroxide (PDTC+H_2O_2), respectively. Lane C shows the result of a control experiment in unstimulated cells, lane M indicates a size standard (100 base pair ladder). (B) The lower part of the figure shows the corresponding GAPDH bands (fragment size: 567 base pairs). Results are representative of three different experiments. *Pyrrolidine dithiocarbamate (PDTC) was added 2 h prior to stimulation of cells with other substances.

Our findings imply that vascular smooth muscle cells respond to oxidative stress with an increased NO synthesis mediated by NF-κB. However, the possible connection between neopterin and oxidative stress is discussed controversially. In vitro, the amount of H_2O_2 released by murine macrophages correlates with the amount of neopterin released by the cells upon stimulation with IFN-γ [28]. On the other hand, recent data suggest that neopterin itself potentiates the effects of reactive oxygen intermediates originating from H_2O_2 and chloramine-T [5,6]. In contrast, Kojima et al. [29] found a scavenging activity by neopterin for superoxide anions. This might indicate that H_2O_2 or H_2O_2-derived reactive oxygen species are responsible for the NF-κB translocation but not superoxide anions. To evaluate our hypothesis that the effects of neopterin on NF-κB activation and iNOS gene expression are mediated by mechanisms of oxidative stress, a second set of experiments was performed in which VSMC were pretreated with PDTC for 2 h prior to the addition of neopterin, TNF-α, or H_2O_2. PDTC is a potent antioxidant that has been shown to block the NF-κB activation induced by reactive oxygen species like H_2O_2 or by TNF-α in Jurkat T cells [27]. Furthermore, PDTC was able to inhibit iNOS gene expression mediated by NF-κB activation in different cell lines [30,31] and in rat aortic strips [32]. In our study, PDTC preincubation strongly suppressed the effects of neopterin, TNF-α, and H_2O_2 on nuclear uptake of NF-κB.

This was accompanied by a comparable reduction of iNOS cDNA concentration and NO_2^-/NO_3^- release, respectively. In conclusion, our data suggest that, at least under our experimental conditions, neopterin activates NF-κB by modulating the production and/or the effects of reactive oxygen intermediates in VSMC. This might be one of the biochemical mechan-

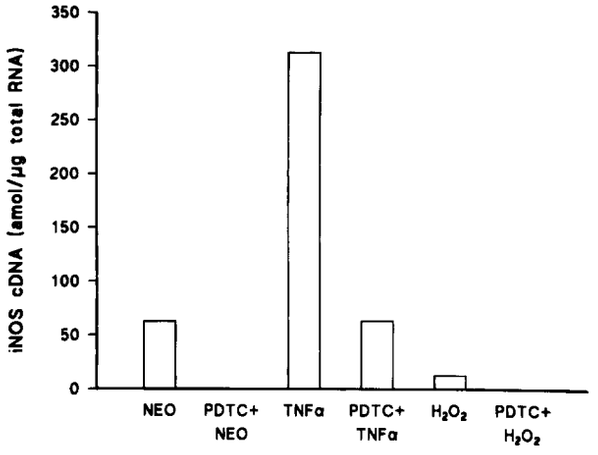

Fig. 3. Quantitative PCR analysis of iNOS mRNA expression detected as iNOS cDNA (fragment size: 498 base pairs) following 9 h incubations of vascular smooth muscle cells with: 20 μM neopterin (NEO), 150 μM pyrrolidine dithiocarbamate+20 μM neopterin (PDTC+NEO), 500 U/ml tumor necrosis factor-α (TNF-α), 150 μM pyrrolidine dithiocarbamate+500 U/ml tumor necrosis factor-α (PDTC+TNF-α), 300 μM hydrogen peroxide (H_2O_2), and 150 μM pyrrolidine dithiocarbamate+300 μM hydrogen peroxide (PDTC+H_2O_2), respectively. Samples were quantitated as described in Section 2. Results are representative of three different experiments.

ism(s) whereby neopterin influences iNOS gene expression and nitric oxide production.

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