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Neopterin derivatives together with cyclic guanosine monophosphate induce c-*fos* gene expression

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Abstract We have previously shown that neopterin enhances hydrogen peroxide and chloramine T activity in a luminol-dependent chemiluminescence assay and strengthens toxicity of these agents against bacteria at slightly alkaline pH (pH 7.5), while 7,8-dihydroneopterin was shown to be a scavenger independent of the pH value. Besides various oxidants, phenolic antioxidants were shown to specifically induce expression of the c-*fos* and c-*jun* mRNAs. Using an inducible cfosCAT reporter transactivation system we studied the function of the pteridine derivatives on c-*fos* transactivation. For the first time, we demonstrate that neopterin and 7,8-dihydroneopterin, particularly together with cyclic guanosine monophosphate, induce c-*fos* gene expression. In humans, interferon-γ induces the release of neopterin and 7,8-dihydroneopterin and also the synthesis of nitric oxide radical which in turn stimulate the formation of cGMP. Thus, in certain situations all three substances, namely neopterin, 7,8-dihydroneopterin and cGMP, may be present locally and even in the circulation at the same time. Based on our findings this constellation would significantly enhance the risk of c-*fos* gene expression and therefore promote tumour growth and development.

Key words: Neopterin; 7,8-Dihydroneopterin; Cyclic GMP; c-*fos*; NIH3T3 fibroblast

1. Introduction

In humans, activation of the cellular immune system is associated with increased neopterin concentrations [1] because large amounts of neopterin are released from human monocytes/macrophages upon stimulation with interferon-γ. A concomitant production of 7,8-dihydroneopterin occurs in parallel. Recent data suggest that the formation of neopterin and 7,8-dihydroneopterin is linked with the cytotoxic repertoire of activated macrophages because neopterin was found to be an enhancer for mechanisms mediated by reactive oxygen and chloride species [2,3] whereas 7,8-dihydroneopterin is a scavenger [2–4]. Both substances together may contribute to an endogenous redox equilibrium [5], the direction of its biological effect strongly depending on the pH and the presence of chelated iron [3]. In addition, neopterin and 7,8-dihydroneopterin and other pteridine derivatives were shown to increase intracellular calcium in monocytic cells [6].

Among its pleiotropic activities, interferon-γ induces the formation of nitric oxide in a variety of mammalian cells [7]. The thereby enhanced 5,6,7,8-tetrahydrobiopterin up-regulates the activity of inducible nitric oxide synthase [8]. Nitric oxide is a free radical which mediates some of its effects via the formation of cyclic guanosine monophosphate (cGMP) because NO activates soluble guanylyl cyclase, most probably by binding to its heme group [9]. In mammalian cardiac myocytes it was shown that a cGMP-dependent protein kinase regulates intracellular Ca^{2+}-levels [10]. Reactive oxygen species have been frequently implicated in the initiation and promotion of carcinogenesis. Direct effects on growth factors and other signalling pathways of both antioxidants and oxidants have recently been shown [11]. Besides various oxidants, phenolic antioxidants were shown to specifically induce expression of c-*fos* and c-*jun* mRNAs [12]. In this study, we explored the potential of neopterin, 7,8-dihydroneopterin and cGMP on c-*fos* gene expression.

2. Materials and methods

2.1. Reagents

Dulbecco's modified Eagle's medium (DMEM), fetal calf serum (FCS), L-glutamine, geneticin (G418) and acetyl-coenzyme A were obtained from Boehringer-Mannheim, Mannheim, Germany; RPMI 1640 medium and FCS were purchased from Schoeller Pharma, Vienna, Austria. Neopterin and 7,8-dihydroneopterin were obtained from Dr. Schircks Laboratories, Jona, Switzerland and dissolved in Dulbecco's phosphate-buffered saline (PBS) in glassware. Lysine-rich histone H1 (calf thymus-type III), 1,2-dioctanoyl-rac-glycerol (DAG), L-α-phosphatidyl-L-serine (PS), phorbol 12,13-dibutyrate (PDBu), phorbol 12-myristate 13-acetate (TPA), guanosine 3′,5′-cyclic monophosphate (cGMP), leupeptin, and aprotinin were purchased from Sigma Chemicals, Munich, Germany. Silica gel-coated high performance thin layer chromatography plates (20 × 20 cm, No. 13181) were from Eastman Kodak Co., Rochester, NY, USA. [γ-^{32}P]ATP (30 Ci/mmol) was obtained from New England Nuclear (NEN, Vienna, Austria), and D-threo [dichloroacetyl-1-^{14}C]chloramphenicol (57 mCi/mmol) was supplied by Amersham, Little Chalfort, UK. Ethylacetate was obtained from Fluka Chemicals, Buchs, Switzerland. Multi-screen-HA 96-well filtration plates were from Millipore, Vienna, Austria. DEAE-cellulose (DE-52) was obtained from Whatman, Clifton, NJ, USA.

2.2. Cell culture

NIH3T3 fibroblasts were maintained in Dulbecco's modified Eagle's medium supplemented with 2 mM L-glutamine, 10% fetal calf serum (FCS), 50 μg/ml gentamycin and incubated in a humidified atmosphere of 95% air with 5% CO_2. When confluent, the cells were passaged with a 1:3 dilution procedure. Before treatment with various compounds, cells were made quiescent by incubation in DMEM medium containing 0.5% FCS for 48 h.

2.3. Plasmid construct: c-fos *CAT promoter construct*

The c-fos(−711)CAT construct was obtained from P. Herrlich, Karlsruhe, Germany [13]. In the plasmid pcfos(−711)CAT the human c-*fos* sequences from position −711 (*Xho*I site) to position +45 (*Xba*I site) are derived from the plasmid p(−750)fos and inserted into the *Sal*I and *Xba*I sites of the plasmid pBLCAT3 [14] (Fig. 1).

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2.4. Cell transfection procedure

NIH3T3 fibroblasts were grown at +37°C and 5% CO_2 in DMEM supplemented with 10% FCS. Stabile transfections of plasmid DNA were performed using the calcium phosphate co-precipitation technique. To obtain stable transfectants, NIH3T3 fibroblasts were co-transfected with the appropriate plasmid constructs pcfos(−711)CAT (5 μg), and the pSV2 neo plasmid (1 μg) and selected for geneticin (G418) resistance in DMEM medium containing 10% FCS and 700 μg/ml G418. G418-resistant single colonies were isolated with trypsin-soaked filter discs. Cells derived from TPA-responsive colonies were propagated in selection medium to $1–10 \times 10^6$ cells and then used for further experiments.

2.5. CAT assay

Cellular extracts for determination of CAT activity in single clone cultures were made by freeze–thawing of cells that had been harvested with a plastic rubber in a solution of 0.25 M Tris-HCl, pH 7.8. After the cell extracts had been centrifuged at $500 \times g$ for 2 min (+4°C) in a microfuge (Sigma, 2K 15), the supernatants were analyzed for CAT activity [14]. The extracted materials were analyzed on silica gel thin-layer chromatography plates (20 × 20 cm, No. 13181; Eastman Kodak Company, Rochester, NY, USA) as described [15]. The protein concentration of each supernatant was determined by the method of Bradford [16]. Quantitation of CAT assays was performed by scintillation counting.

2.6. Preparation of protein kinase C

PKC was partially purified from NIH3T3 cells as described [17] with minor modifications. Cell extracts were prepared from logarithmically growing NIH3T3 cells (10 cm cell culture dishes). The medium was discarded, and the cell monolayer was rapidly washed twice with ice-cold Dulbecco's PBS. All subsequent steps were carried out +4°C. The PBS solution was removed and the cells were collected and resuspended in homogenization buffer (50 mM Tris-HCl, pH 7.5, 50 mM mercaptoethanol, 1 mM phenylmethylsulfonyl fluoride, 2 mM EGTA, 0.1% Triton X-100 (w/v), 20 μg/ml leupeptin, and 2 μg/ml aprotinin). The cells were disrupted by sonication (MSE sonifier at 60 W) and centrifuged at $15{,}000 \times g$ for 15 min, yielding crude nuclear pellets and post-nuclear supernatants. The supernatants were pooled and centrifuged at $110{,}000 \times g$ for 1 h and the resulting soluble membrane extracts were applied onto DEAE-52 columns (2.5 × 8 cm; Whatman). Protein was eluted with a gradient from 0.0 to 0.17 M NaCl in elution buffer A (20 mM Tris-HCl, pH 7.5, 20 mM mercaptoethanol, 0.1 mM EGTA, 0.1 mM EDTA, 2% glycerol).

2.7. Determination of protein kinase C activity

PKC (4.25 μg protein/25 μl) was assayed by detecting the incorporation of ^{32}P from [γ-^{32}P]ATP into histone H1 according to Aftab et al. [18]. Reactions (total volume of 125 μl, containing 0.132 μM [γ-^{32}P]ATP, (30 Ci/mmol), 50 μM Na_2ATP, 20 mM Tris-HCl, pH 7.4, 1 mM $CaCl_2$, 700 μM EGTA, 50 μg histone H1 type III, 1 μM PS and 1.8 μM DAG were set up in a 96-well plate. Non-specific phosphotransferase activity was determined by using a lipid-free reaction mixture in the presence of EGTA. The assays were started by the addition of [γ-^{32}P]ATP with an eight-channel pipettor. After incubation for 15 min at +32°C the reactions were terminated by the addition of 50 μl 20% trichloroacetic acid. The reaction mixtures were transferred to 96-well filter plates and filtered using a semiautomatic 96-well assay system. The filter disks (type HA, Millipore) were collected by using a filter punch apparatus (Millipore) and counted in a liquid scintillation counter. PKC activity was calculated as the difference of specific Ca^{2+}- and phospholipid-dependent PKC activity and non-specific phosphotransferase activity.

3. Results

NIH3T3 mouse fibroblasts were stably transfected with a cfosCAT construct containing the 5′-flanking sequence of the c-*fos* gene up to position −711 (Fig. 1). Expression of the cfosCAT construct was stimulated by the known inducer of c-*fos* transcription phorbol 12,13-dibutyrate (PDBu) (Fig. 2). As shown in Fig. 2, the simultanious application of 7,8-dihydroneopterin (400 nM) and cGMP (20 μM) led to a significant transcriptional activation of the cfosCAT reporter construct. A similar but less pronounced effect could be achieved by using neopterin and 7,8-dihydroneopterin. The direct effect of neopterin and 7,8-dihydroneopterin on c-*fos* transactivation was tested in the same experiment. As pointed out in Fig. 2, the direct transactivation of the cfosCAT reporter construct was marginal in comparison to the influence of the combination of neopterin derivatives with cGMP.

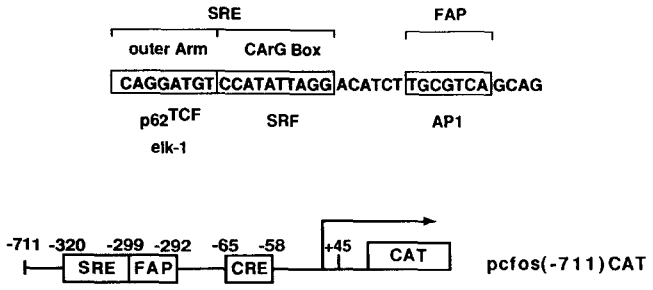

Fig. 1. Diagram of the plasmid vector used. The SRE contains a palindromic sequence known as the dyad symmetry element (DSE) and is located between position −321 to −299 (composed of the outer arm and the CArG box of the c-*fos* promoter [31]. In many systems activation through the SRE is mediated by a ternary complex assembled on the SRE consisting of the serum response factor (SRF) and a ternary complex factor (TCF/elk-1) [32]. Another sequence, the c-*fos* AP-1 site (FAP), is localized immediately adjacent to the SRE (from −299 to −292). The FAP site (5′-TGCGTCA-3′) is closely related to the AP-1 consensus binding site (5′-TGAG/CTCA-3′). The FAP site is also implicated in the c-*fos* induction by cAMP via protein kinase A. Another element which confers a strong cAMP response, is the cAMP responsive element (CRE) located between positions −71 to −59 of the c-*fos* promoter [13].

In order to investigate whether the induction of c-*fos* by neopterin or 7,8-dihydroneopterin alone or in combination with cGMP was mediated by PKC, the effects of the pterin derivatives were studied in cell-free extracts. As shown in Fig. 3 neither 7,8-dihydroneopterin and neopterin nor cGMP alone or in combination with the pterines modulate PKC activity in vitro.

4. Discussion

In our study we show that 7,8-dihydroneopterin in the presence of cGMP strongly induces c-*fos* gene expression in NIH3T3 fibroblasts. A similar but less pronounced effect was achieved by using neopterin instead of 7,8-dihydroneopterin. There was also a direct effect of neopterin and 7,8-dihydroneopterin on c-*fos* transactivation, however, it was diminished compared to the influence of the combinations of neopterin derivatives with cGMP. The effect induced by the combination of 7,8-dihydroneopterin and cGMP was similar to that which was observed when phorbol-esters like PDBu or a selected growth factor such as PDGF (data not shown), which are classical inducers of c-*fos* via activation of protein kinase C, were used.

We have found that neither 7,8-dihydroneopterin and neopterin nor cGMP modulate activity of protein kinase C in cell-free extracts from NIH3T3 fibroblasts. Thus from this in vitro data, we conclude that these substances affect c-*fos* gene induction downstream of the protein kinase C activation pathway, and have no direct effect on protein kinase C.

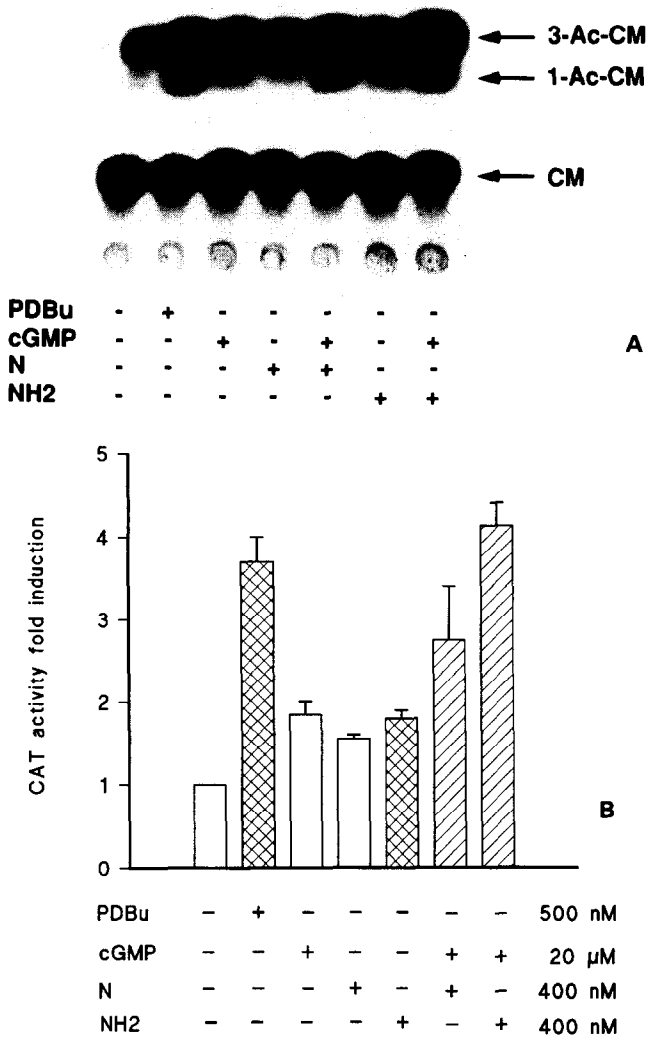

Fig. 2. Activation of cfosCAT expression by neopterin and 7,8-dihydroneopterin together with cGMP. Stably cfosCAT-transfected NIH3T3 fibroblasts were growth arrested by incubation in 0.5% FCS for 48 h and CAT activity was induced by the addition of either 500 nM PDBu or 20 μM cGMP, respectively. Where indicated, 400 nM neopterin and 7,8-dihydroneopterin alone or together with cGMP were added 18 h prior to cell harvesting. CAT activity was determined after administration of the agonists. (A) An autoradiograph of a representative CAT assay (N represents neopterin and NH2 7,8-dihydroneopterin, respectively. (B) Mean CAT activities were calculated from two independent experiments performed in duplicate and expressed as fold induction of uninduced controls (328 ± 68 pmol/min/mg protein).

It is not clear at present what the biochemical background of our observations is. It is possible that the effects are mediated via modulation of the Ca^{2+} current, which is known to strongly influence pathways of protein kinases [10], but the antioxidant activity of 7,8-dihydroneopterin and other pterin derivatives [3–5] could also be important. Recently, induction of c-*fos* gene expression mediated by the serum responsive element (SRE) was demonstrated by various phenolic antioxidants such as butylated hydroxyanisole (BHA) or butylated hydroxytoluene (BHT) [12]. Promoter mapping results demonstrated that the c-*fos* promoter requires the SRE but not the adjacent AP-1 site for antioxidant-specific transactivation [12]. In contrast, a recent report demonstrated that, under certain circumstances, AP-1 sites can be involved in the response to phenolic antioxidants [19]. The consensus sequence of the antioxidant response element (ARE), 5′-ggTGACaaaGC-3′, of genes which are controlled by phenolic antioxidants, such as the NADP(H)-quinone reductase [19] and the Ya-subunit of glutathione *S*-transferase [20], is similar to the AP-1 consensus site, 5′-TGACTCA-3′. Although the biochemical basis for the activation of c-*fos* by antioxidants is still obscure, variations in redox parameters have been directly implicated in controlling the activation of several transcription factors, including c-Jun and c-Fos as well as NFκB and the glucocorticoid receptor [21]. If the antioxidant activity of 7,8-dihydroneopterin is important for the induction of c-*fos* gene expression, this could explain why neopterin is less active, because neopterin is much less an antioxidant than 7,8-dihydroneopterin; neopterin may even act as a prooxidant depending on the pH value [2,3].

The finding that neopterin derivatives directly support oncogene expression may have some pathogenetic relevance with regard to human malignant diseases. Increased neopterin concentrations correlate to the extent and activity of the disease in infections with viruses and intracellular bacteria, in autoimmune disorders and in malignancies [1]. Moreover, neopterin concentrations were shown to bear predictive information, higher concentrations being associated with more rapid disease progression in patients with human immunodeficiency virus (HIV) infection [22,23] and in certain types of malignancies [24–26]. This increase in neopterin concentrations appears to be invariably associated with a parallel rise in concentrations of a reduced derivative of neopterin, namely 7,8-dihydroneopterin, and concentrations of neopterin and 7,8-dihydroneopterin are closely related in humans. An approximate 2-fold excess of 7,8-dihydroneopterin was found when compared to neopterin in healthy individuals [27,28]. A similar relationship seems to exist during the status of immune activation, e.g. in homosexual men with HIV infection, concentrations of neopterin and of total neopterins (the sum of neopterin and its 7,8-dihydro form) were of equal value in predicting the onset of AIDS [29].

Increased concentrations of cGMP are found in patients with cardiac disorders but also in malignant diseases [30]. However, in cancer patients the frequency of elevated concentrations of cGMP appears to be higher in urine than in plasma. In humans, interferon-γ induces the release of neopterin and 7,8-dihydroneopterin [1,29] and also the synthesis of the nitric oxide radical which in turn stimulates formation of cGMP [9]. Thus, in certain situations all three substances, namely neopterin, 7,8-dihydroneopterin and cGMP, may be present locally and even in the circulation at the same time. This constellation would significantly enhance the risk of c-*fos* gene expression and therefore promote tumour growth and development. The prognostic value of higher neopterin concentrations to predict disease progression and death in malignant diseases could be related to the capacity of neopterin derivatives to induce oncogene expression. The endogenous formation of cytokines at local sites of inflammation could be of importance within this context.

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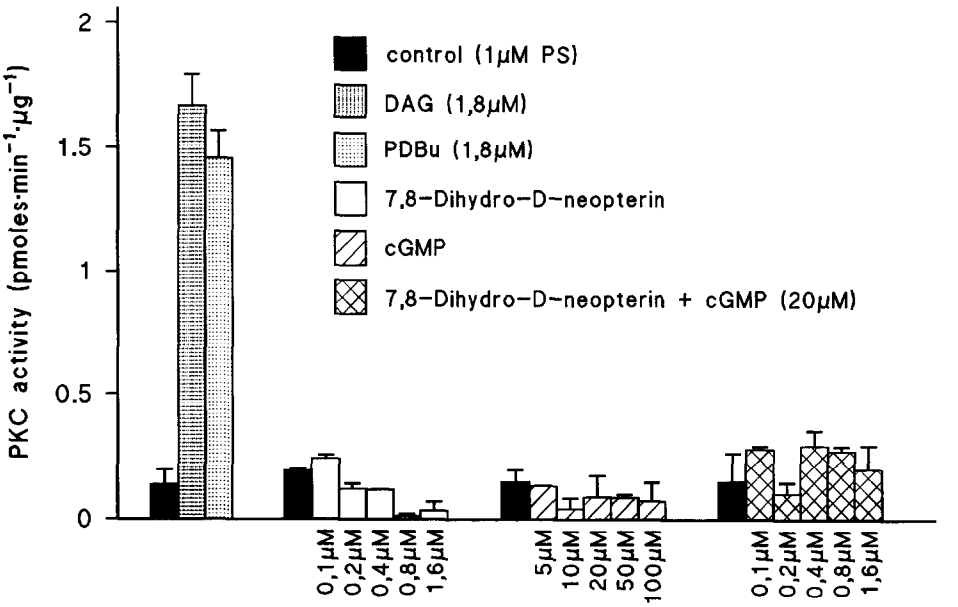

Fig. 3. Effect of 7,8-dihydroneopterin and cGMP alone, or in combination, on PKC activity. PKC partially purified from NIH3T3 fibroblasts was measured under standard conditions described in section 2. The enzyme (4.25 µg protein/25 µl) was incubated for 15 min in the presence of 1 µM phosphatidylserine (PS) and 1 mM $CaCl_2$ (control, bar 1) or fully activated by the addition of 1,2-dioctanoyl-rac-glycerol (1.8 µM DAG, bar 2). In bars 5–9, 11–15, as well as 17–21, increasing concentrations (0.1, 0.2, 0.4, 0.8, 1.6 µM) of 7,8-dihydroneopterin, cGMP (5, 10, 20, 50, 100 µM) alone or in combination with cGMP (20 µM) were added in the absence of DAG. As a positive control the tumour promoter PDBu (1.8 µM), which can fully activate nPKC isoforms by competing with the endogenous PKC activator DAG, was added to the assay (bar 3). Each bar represents mean values from two independent experiments, in which triplicate determinations were taken.

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