Reduced pteridine derivatives induce apoptosis in PC12 cells

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Abstract

In cerebrospinal fluid of patients with cerebral infections, elevated concentrations of the pteridine compounds neopterin and 7,8-dihydroneopterin were detected. Here, the potential of pteridines to induce apoptosis of the rat pheochromocytoma cells (PC12) was investigated. In contrast to aromatic pteridines like neopterin, the reduced forms 7,8-dihydroneopterin, 5,6,7,8-tetrahydrobiopterin and 7,8-dihydrobiopterin led to a significant increase of apoptotic cells. After terminal differentiation, cells were less sensitive to incubation with pteridines. A noticeable augmentation of apoptosis was observed upon incubation with 7,8-dihydroneopterin and 7,8-dihydrofolic acid. Antioxidants partly protected PC12 cells from pteridine-induced apoptosis, suggesting the involvement of reactive oxygen intermediates. Exposure of cells to 7,8-dihydroneopterin led to activation of the mitogen-activated protein (MAP) kinase and to a lesser degree also of JUN/SAP kinase. Results implicate that high concentrations of reduced pteridines, might contribute to the pathogenesis involved in neurodegeneration.

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1. Introduction

Pteridines, pyrazino[2,3-d]pyrimidine compounds, may be divided into two major classes, ‘conjugated’ pteridines, which are characterized by relatively complex side chains, e.g. the vitamins folic acid, and ‘unconjugated’ pteridines, e.g. biopterin or neopterin bearing less complex side chains at the 6-position of the pterin. The first step in the biosynthesis of pteridines generally starts with the conversion of guanosine triphosphate (GTP), and is catalysed by the enzyme GTP cyclohydrolase I (Brown, 1971; Brown and Williamson, 1982; Richter et al., 1993). 7,8-Dihydrobiopterin triphosphate is the first isolable intermediate and represents the key predecessor in the biosynthesis of folate, methanopterin, 5,6,7,8-tetrahydrobiotin and neopterin (Werner et al., 1989, 1995). The most important unconjugated pteridines which are detectable in humans are biopterin and neopterin. Biopterin, in its 5,6,7,8-tetrahydro-form is a necessary cofactor of certain aromatic aminoacid monooxygenases (Kaufman, 1963) and in the nitric oxide synthase reaction converting arginine to citrulline and nitric oxide (Tayeh and Marletta, 1989; Werner-Felmayer et al., 1990). Neopterin, a chemically intimately related pteridine, is produced in increased amounts by human monocytes/macrophages upon stimulation with interferon-γ (Huber et al., 1984; Bitterlich et al., 1988). It represents a useful parameter to monitor immune activation which accompanies e.g. viral infections e.g. with human immunodeficiency virus (HBV) (Fuchs et al., 1989; Fahey et al., 1990), and parasitic infections (Reinberger et al., 1984; Brown et al., 1990), autoimmune diseases (Reinberger et al., 1986; Lohrbrun et al., 1991) or malignant cancers (for a review see Reinberger et al., 1991). Elevated neopterin excretion was also detected in sera and cerebrospinal fluids of patients with neurodegenerative diseases e.g. cerebral infections (e.g. HIV infection, AIDS dementia, Lyme neuroborreliosis, aseptic meningitis, and measles encephalomyelitis, (for a review see Hagberg et al., 1993) and also in neurodegenerative diseases such
as Alzheimer’s disease (Lübbers et al., 1998). In vitro, the excretion of neopterin was found to correlate well with hydrogen peroxide (Nathan, 1986), and may therefore serve as a marker for oxidative stress in disease (Fuchs et al., 1997). Interestingly, neopterin and 7,8-dihydropterin were found to interfere with redox-sensitive intracellular signaling pathways, and data implied a potential role of neopterin-derivatives in oxidative stress-mediated apoptosis (Baer-Bitterlich et al., 1996—for a review see Baer-Bitterlich et al., 1997). The neuronal loss in cerebral infections and pathogenesis of neurodegenerative diseases such as Parkinson’s disease, amyotrophic lateral sclerosis and Alzheimer’s disease is defined by a slow, progressive loss of specific subsets of neurons. Apoptosis has an important role in neurodegeneration, although the mechanisms and mediators in the brain are still largely unknown. Microrganisms, which play a primary role as brain resident macrophages, are involved in inflammatory and destructive processes (Gehrmann et al., 1996; Nilsson et al., 2001; Benveniste et al., 2001) may produce several neurotoxins, such as reactive oxygen species, nitric oxide, glutamate, cytokines or proteases which potentially contribute to neuronal cell death (Gehrmann et al., 1995; Giuliani et al., 1993; Giulian et al., 1995). It has been hypothesised that oxidative stress and damage by free radicals play a significant part in these diseases, e.g. a recent study with the inherited form of amyotrophic lateral sclerosis revealed mutations in the superoxide dismutase (SOD) gene, which is one of the cell’s main defence mechanisms against oxidative stress. These data prompted a direct link between oxidative stress and the development of a neurodegenerative disease (Gorman et al., 1996; Pocernich et al., 2001). In the present study we investigate the potential of various pteridines to destroy the redox balance and induce apoptosis of rat pheochromocytoma cells (PC12) which may contribute to the loss of neuronal cells.

2. Experimental procedures

2.1. Cell culture

PC12 cells (a rat pheochromocytoma cell line, ATCC, Rockville, MD, USA) were cultivated in RPMI-1640 medium (Biotechon, Berlin, Germany) complemented with 2 mM glutamine (Serva, Germany), 100 U/ml Penicillin and 0.1 mg/ml Streptomycin (Biological Industries, Israel) and 10% heat-inactivated horse serum and 5% fetal calf serum (FCS, Biochrom, Berlin, Germany). For the experiments cells were plated on collagen II coated dishes (collagen S was purchased from Boehringer Mannheim, Heidelberg, Germany) and were grown to 50% confluency. Immediately prior to experiments the medium was replaced with serum-free RPMI. For differentiation of PC12 cells into a neuronal phenotype, PC12 cells were treated with NGF-B (50 ng/ml; Sigma) for 7–8 days.

2.2. Chemicals

- Neopterin (neopterin), 7,8-dihydro-d-neopterin (7,8-dihydroneopterin), l-biopterin (biopterin), 7,8-dihydro-l-biopterin (7,8-dihydrobiopterin), 5,6,7,8-tetrahydrobiopterin, l-sepiapterin and 7,8-dihydrofolic acid were purchased from Schircks Lab., (Jona, CH). N-Acetyl-l-cysteine (NAC) was purchased from Sigma, SOD from Sigma, catalase from bovine liver from Bioproducts (Boehringer Ingelheim, Rockville, MD, USA) were cultivated in RPMI-1640 medium (Biochrom, Berlin, Germany) complemented with 2 mM glutamine (Serva, Germany), 100 U/ml Penicillin and 0.1 mg/ml Streptomycin (Bio-Rad). Immunoblotting was performed with anti-active MAP and JUN kinase antibodies (New England Biolabs, Inc., USA) and detected with the ECL system (Amersham Pharmacia Biotech, UK). Values are representative of three blots.

2.3. Apoptosis

For the evaluation of cellular apoptosis PC12 cells were grown to 50% confluence and incubated for 48 h with neopterin, 7,8-dihydroneopterin, 5,6,7,8-tetrahydrobiopterin and biopterin. Cells were stained with Hoechst 33342 (Molecular Probes, OR, USA) and analyzed on an Olympus BX50 fluorescence microscope to characterise apoptotic nuclei.

To quantify cellular apoptosis PC12 cells were grown to 50% confluency and incubated for 48 h with various concentrations of neopterin, 7,8-dihydroneopterin, biopterin, 7,8-dihydrobiopterin, sepiapterin and 7,8-dihydrofolic acid. At the end of the experiments cells were harvested and apoptotic cells were characterized by morphology: forward scatter (FSC), versus side scatter (SSC) and quantitation of the intranuclear contents of fluorescing DNA by propidium iodi staining (Nicolleti et al., 1991; Sgoen and Wick, 1994) and FACS analysis. Propidium iodi staining solution contained 50 µg/ml propidium iodi (Sigma), 0.1% Triton X-100 (Serva) and 0.1% tri-sodium-citrate-dihydrate (Merck, Darmstadt, Germany) in distilled water.

2.4. Activation of p42/p44 mitogen-activated protein kinase (MAPK) and p 46/57 JUN kinase (JNK)

Non-differentiated PC12 cells were washed two times in phosphate buffered saline (PBS). Cells (2 × 10^6 per 25 cm dish) were incubated for 5 min to 24 h with 7,8-dihydroneopterin. To prepare cell lysates, cells were centrifuged, taken up in lysis buffer (Tris pH 8.5, 50 mM, NP-40 1%, EDTA 5 mM, NaF 5 mM, NaCl 5 mM, NaVO4 50 mM, aprotinin 0.5 mg/ml, leupeptin 0.5 mg/ml) and incubated for 20 min on ice. Following centrifugation (13 000 × g, 15 min, 4 °C), supernatants were analysed for protein content (Bradford protein assay, Bio-Rad, Vienna, Austria). Cell lysates (30 µg) were separated on an SDS polyacrylamide gel (12%) and transferred to nitrocellulose membranes (Bio-Rad). Immunoblotting was performed with anti-active MAP and JUN kinase antibodies (New England Biolabs, Inc., USA) and detected with the ECL system (Amersham Pharmacia Biotech, UK). Values are representative of three blots.
3. Results

The induction of pteridine mediated apoptosis of non-differentiated and terminally differentiated PC12 cells was first analysed by propidium iodide staining and FACS analysis. In non-differentiated cells the reduced compounds 7,8-dihydronopterin, 5,6,7,8-tetrahydrobiop-
terin and 7,8-dihydrobiop-terin induced a 1.7–3.8-fold in-
crease of apoptosis, whereas aromatic pteridines neopterin, 
biop-terin and sepiapterin showed no significant effect. In-
terestingly, 7,8-dihydrofolic acid, a reduced pteridine, ap-
parently did not induce apoptosis (Fig. 1A). After terminal
differentiation cells were less sensitive. A significant increase of apoptosis was induced by 7,8-dihydroneopterin (5 mM) and 7,8-dihydrofolic acid (2.5 mM) (Fig. 1B).

Next, apoptosis was confirmed by Hoechst 33342 staining. In comparison to the control, specific characteristics which are typical for apoptosis (e.g. chromatin condensation and formation of apoptotic bodies) were observed in cells that were incubated with 7,8-dihydroneopterin, 7,8-dihydrobiopterin and 5,6,7,8-tetrahydrobiopterin. Staurosporine was used as a positive control. (Fig. 2A–F).

In the following experiments we tested the effect of antioxidants NAC (5 mM), CAT (500 U/ml), SOD (500 U/ml) and PDTC (0.1 mM) on the apoptosis of non-differentiated and differentiated PC12 cells, in order to evaluate the potential involvement of oxygen radicals on the induction of pteridine-mediated apoptosis of neuronal cells. For this study pteridines which most potently induced apoptosis were chosen. In non-differentiated cells, catalase, in comparison to the control, most effectively inhibited 7,8-dihydroneopterin (60%) and to a lesser extent also 5,6,7,8-tetrahydrobiopterin-mediated apoptosis (20%), while it did not effect 7,8-dihydrobiopterin. Pteridine-mediated apoptosis also appeared sensitive to the addition of SOD (inhibition of 7,8-dihydroneopterin and 5,6,7,8-tetrahydrobiopterin was over 50% and of 7,8-dihydrobiopterin 25%) (Fig. 3A). In our experiments basic apoptosis of control cells was also sensitive to the addition of PDTC, NAC and to a minor degree to SOD. As expected antioxidants also inhibited pteridine-mediated apoptosis in terminally differentiated cells (Fig. 3B).

**Fig. 2. Pteridine-mediated apoptosis of non-differentiated PC12 cells (Hoechst staining)** For the evaluation of cellular apoptosis PC12 cells were grown to 50% confluency and incubated for 48 h with medium (A), 1 mM staurosporine (B), 1 mM 7,8-dihydroneopterin (C), 1 mM 7,8-dihydrobiopterin (D), 1 mM 5,6,7,8-tetrahydrobiopterin (E) and 0.2 mM 5,6,7,8-tetrahydrobiopterin (F). Cells were stained with Hoechst 33342 and analysed on an Olympus BX50 fluorescence microscope to characterise apoptotic nuclei.
Fig. 3. Inhibition of apoptosis by antioxidants; non-differentiated (3A) and differentiated (3B) PC12 cells were grown to 50% confluency and incubated for 48 h with pteridines in the absence and presence of antioxidants NAC (5 mM), PDTC (0.1 mM), catalase (500 U/ml) and SOD (500 U/ml). Values are representative of the means and S.E.M. of six samples.

To further explore pteridine-mediated signaltransduc-
tion, the involvement of MAP and JUN kinase was exam-
ined. Non-differentiated PC12 cells were incubated with
7,8-dihydroneopterin (5 mM) for various time points (0 min
to 24 h). As a result we observed that p44 and to a lesser
degree also p42 MAP kinase (MAPK) were activated after
a 5 min exposure to 7,8-dihydroneopterin. The activation
was most evident at 15 min and ceased after 1 h (Fig. 4A).

Fig. 4. Activation of MAP- and JNK/SAP kinase activation of MAPK (A) and JNK/SAP kinase (B) was measured in non-differentiated PC12 cells following the treatment with 7,8-dihydroneopterin (NH₂, 5 mM) for 0, 5, 15, 30 min and 1, 2, 4, 6, and 24 h.
Neopterin did not induce MAPK activation (data not shown). To a minor degree JNK (p46) was activated following 5 min exposure to 7,8-dihydroneopterin. Interestingly the activity could still be detected at 6 h (Fig. 4B).

4. Discussion

In sera and cerebrospinal fluids of patients with cerebral infections and neurodegenerative diseases e.g. Alzheimer’s disease increased neopterin excretion was found (Leblhuber et al., 1998; for a review see Hagberg et al., 1993). Here, the potential of pteridines to contribute to neuronal apoptosis, which apparently plays an important role in neurodegeneration, was investigated. In dependence of their chemical structure pteridines mediated apoptosis of non-differentiated PC12 cells. As measured by quantitation of the intranuclear contents of fluorescing DNA by propidium iodide staining, among unconjugated pteridines the aromatic molecules neopterin and biopterin did not significantly effect cell survival, whereas the reduced dihydro-forms, 7,8-dihydroneopterin, 7,8-dihydrobiopterin, and the tetrahydroform of biopterin, induced apoptosis. This difference might probably be explained by the observation that the reduced forms of pteridines are prone to autooxidation in the presence of molecular oxygen, during which process radicals are formed primarily by electron abstraction followed by proton loss (Pearson, 1974; Blair and Pearson, 1973; Öttl et al., 1999).

Sepapterin structurally closely resembles 7,8-dihydrobiopterin since its ring is equally reduced, yet has an oxidised side-chain. These characteristics might explain the minor but insignificant increase in apoptotic cells (Rehnegger et al., 1995). Compared to earlier observations in NT2 cells (Spöttl et al., 2000), PC12 cells reacted differently to the addition of 7,8-dihydrofolic acid, a conjugated and reduced pteridine. A possible explanation might be that a certain percentage of sensitive cells is quickly destroyed leaving no apoptotic cells for detection (Spöttl et al., 2000). Similar but less remarkable effects were observed in terminally differentiated cells. These data indicate that differentiated neuronal cells too, although to a smaller degree (Spöttl et al., 2000), are sensitive to the confrontation with elevated concentrations of pteridines which may be synthesised in patients with cerebral infections (for a review see Hagberg et al., 1993) or neurodegenerative diseases e.g. Alzheimer’s disease (Leblhuber et al., 1998). Since differentiated cells react less sensitive to pteridines they are probably less efficiently killed by 7,8-dihydroneopterin which evidently led to an increase of apoptosis. Data fit well with earlier findings by Hossain et al., 1997 who found that differentiated cells reacted less sensitive to 5-azacytidine than undifferentiated PC12 cells. Also, another group (Hatayama et al., 1997) reported that the induction of HSP70 and HSP70 mRNA following stress was diminished in differentiated cells as compared to undifferentiated cells, whereas the HSF1 DNA-binding activity was enhanced in differentiated PC12 cells. Authors claim that neuronal cells apparently show an altered stress response depending on their differentiation state which according to authors opinion may thus explain the sensitivity of neuronal cells to pathophysiological stressors. Yet, contrary results have been reported by other groups who concluded from their data that differentiated neurons react more sensitive to neurotoxicity than undifferentiated cells (Clarkson et al., 1999; Oberdoerster and Rahib, 1999; Wang et al., 1998). Hence, the degree of neurotoxicity apparently depends on the specific agent and varies with different cell types.

Effective concentrations of pteridines used in our cell culture approach appear unphysiological. Yet, in contrast to an onl once added dose of stimulants in our experiments, a continuously high level of e.g. neopterin and 7,8-dihydroneopterin persists in body fluids of patients with diseases linked to immuneactivation due to continuous stimulation with IFN-γ. Direct cell-to-cell contact may further facilitate the accumulation of even higher concentrations of 7,8-dihydroneopterin in the microenvironment of cells, and its effects may be further augmented in the concert of other inflammatory cytokines which are induced upon activation of cells with IFN-γ (Billiau and Dijkmans, 1990).

The production of neopterin by monocytes/macrophages was found to correlate well with the capacity of cells to release hydroxynitroside (Nathan, 1986), and neopterin concentrations may therefore serve as a marker for oxidative stress in vivo (Fuchs et al., 1997; Murr et al., 1999). Interestingly, neopterin and 7,8-dihydroneopterin were found to interfere with redox-sensitive intracellular signaling pathways and neopterin-derivatives apparently play a potential role in oxidative stress-mediated apoptosis (Bayer-Bitterlich et al., 1997). In order to study the possible involvement of ROIs in pteridine-mediated apoptosis antioxidants NAC, CAT, SOD and PDTC were added to the experiments. Results revealed a sensitivity of pteridine-mediated apoptosis towards these antioxidants. The inhibiting effect was most obvious in differentiated PC12 cells since the basal inhibition of cells incubated with medium alone was very low.

To further characterise molecular mechanisms that regulate pteridine mediated apoptosis, the contributions to cell death of mitogen-activated protein (MAP) kinase family members, including extracellular signal-regulated kinase (ERK) and JNK (c-JUN NH2-terminal protein kinase) were examined after incubation of cells with 7,8-dihydroneopterin. The p44 and p42 ERK activities increased significantly within 5 min after incubation with 7,8-dihydroneopterin (5 mM) but not with neopterin. To a minor extent also JNK activity was detected after incubation with 7,8-dihydroneopterin. Interestingly, the MAPK activity decreased faster than JNK activity. According to the literature (Xia et al., 1995), NGF leads to proliferation, differentiation and survival via activation of ERK, while withdrawal of NGF leads to apoptosis via the activation of JNK and p38. In this view, our data appear controversial since incubation of cells with 7,8-dihydroneopterin led to...
activation of ERK rather than JNK. Yet, our results fit well with a study (Guyton et al., 1996) on signal transduction in PC12 cells incubated with hydrogen peroxide (H₂O₂), another redox-regulating molecule, which was shown to induce oxidative stress-induced apoptosis in mouse thymocytes (Forrest et al., 1994). H₂O₂ induced a potent activation of ERK2 while JNK1/SAPK was only minor activated (Guyton et al., 1996). These data provide strong support of the crucial role of the MAPK/JNK pathway in cellular regulation of oxidative stress.

Our results indicate that pteridines, especially reduced forms, potentially induce apoptosis in non-differentiated and NGF differentiated, sympathetic neuron-like PC12 cells. Recently, Foster et al., 1999 presented a model of Parkinson’s disease. They hypothesise that catecholamine or tetrahydrobiopterin metabolism contributes to apoptotic death of nigrostriatal dopamine neurons. According to our observations pteridines lead to apoptosis via the induction of reactive oxygen intermediates, including to some extent the activation of the MAPK/JNK pathway.

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References


