Neopterin and 7,8-dihydroneopterin induce apoptosis in the rat alveolar epithelial cell line L2

Wolfgang Schobersberger[a,*], Georg Hoffmann[b], Petra Hobisch-Hagen[a], Günther Böck[c], Harald Völkl[d], Gabriele Baier-Bitterlich[e], Barbara Wirleitner[e], Helmut Wachter[e], Dietmar Fuchs[e]

[a]*Division for General and Surgical Intensive Care Medicine, Clinic for Anaesthesia and General Intensive Care Medicine, University of Innsbruck, Anichstrasse 35, A-6020 Innsbruck, Austria*
[b]*Institute of Physiology I, University of Bonn, Nussallee 11, D-53115 Bonn, Germany*
[c]*Institute for Experimental Pathology, University of Innsbruck, Fritz Pregl Strasse 3, A-6020 Innsbruck, Austria*
[d]*Institute of Physiology, University of Innsbruck, Fritz Pregl Strasse 3, A-6020 Innsbruck, Austria*
[e]*Institute of Medical Chemistry and Biochemistry, University of Innsbruck, Fritz Pregl Strasse 3, A-6020 Innsbruck, Austria*

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Abstract The neopterin derivatives, neopterin and 7,8-dihydroneopterin, modulate the cellular oxidant-antioxidant balance as well as the expression of the inducible nitric oxide synthase (iNOS) gene. Since apoptosis can be induced by reactive oxygen intermediates and nitric oxide (NO) we investigated whether these neopterin derivatives induce apoptotic cell death. As model we selected the rat alveolar epithelial cell line L2. 24 h incubation of neopterin (1–1000 μM) as well as 7,8-dihydroneopterin (1–1000 μM) resulted in a significant increase of percent apoptotic cells (measured by FACS analysis). Coincubation of both pteridines with the cytomix (interferon-γ plus tumor necrosis factor-α) led to a significantly higher apoptosis than the cytomix alone. In contrast to the cytomix, no iNOS gene expression and no NO release could be detected after incubation with neopterin or 7,8-dihydroneopterin. We conclude that neopterin and 7,8-dihydroneopterin are per se inducers of apoptosis which is not mediated by nitric oxide. This may be of importance in inflammatory pulmonary diseases associated with an activation of the cellular immune system.

Key words: Neopterin; 7,8-Dihydroneopterin; Apoptosis; Nitric oxide; Reactive oxygen intermediate; Alveolar epithelial cell L2

1. Introduction

Apoptosis is a specific mode of cell death characterized primarily by typical morphological alterations [1]. In contrast to necrosis, apoptosis is a biochemically active metabolic process [2,3] as indicated by the fact that several triggers, such as changes in the concentrations of growth factors and cytokines (e.g. tumor necrosis factor-α, TNF-α), are potent apoptotic stimuli [4,5]. TNF-α in combination with interferon-γ (IFN-γ) potently activates the gene expression of inducible nitric oxide synthase (iNOS) in many cells [6], thus producing and releasing nitric oxide (NO). Recently a link between NO formation and apoptosis was suggested. NO and NO-derived reactive oxygen species-mediated apoptosis in murine peritoneal macrophages [7] and the macrophage cell line RAW 264.7 [8]. One suggested mechanism is the rapid reaction of NO with molecular oxygen by forming nitrogen oxides and/or peroxynitrite [9]. Once protonated, peroxynitrite decays rapidly by producing hydroxyl radicals (OH^-). Since oxidative stress by the formation of reactive oxygen intermediates (ROI) is an important inducer of apoptotic cell death [10], NO-induced ROI formation could be one important pathway of apoptosis signalling.

The neopterin derivatives, neopterin and 7,8-dihydroneopterin, are produced in large amounts by human monocytes/macrophages upon stimulation with IFN-γ [11]. Neopterin derivatives may modulate the redox balance in biological systems. Depending on the culture or medium conditions they act either as oxidants or as antioxidants. For example, neopterin was shown to enhance chloramine-T- and hydrogen peroxide-mediated chemiluminescence [12]. In the absence of iron, however, neopterin scavenges oxygen radicals and quenches H_2O_2-induced chemiluminescence [12–14]. Except for very high concentrations [15], 7,8-dihydroneopterin is established to act as a potent scavenger under in vivo as well as in vitro conditions [12,14].

There are data available that high concentrations of 7,8-dihydroneopterin are capable of superinducing TNF-α-mediated apoptosis in monocytic U937 cells [15]. However, in that study no direct effect of the compound was found. Recently we could demonstrate that neopterin, but not 7,8-dihydroneopterin, is a potent iNOS activator in rat vascular smooth muscle cells (VSMC). iNOS gene expression by neopterin was followed by the release of NO [16]. Hoffmann et al. [17] showed that one intracellular mechanism for neopterin-induced iNOS gene expression in VSMC is the activation of nuclear factor-κB (NF-κB). Since ROI are potent inducers of apoptotic cell death it is conceivable that neopterin derivatives per se could mediate apoptosis.

The aim of this study was to test whether neopterin derivatives mediate apoptotic cell death in the alveolar epithelial cell line L2 (adult rat) which – upon stimulation with cytokines – was shown to express the iNOS gene and to release NO [18].

2. Materials and methods

2.1. Reagents

Dulbecco's modified Eagle's medium (DMEM) with phenol red was purchased from ccPro, Karlsruhe, Germany. Recombinant rat IFN-γ and TNF-α were from IC Chemicals, Ismaning, Germany. L-Arginine, L-glutamine, *N*-(1-naphthyl-ethylene) diamine, propidium iodide, β-NADPH, the phenol-chloroform-isoamyl alcohol mixture and DMEM without phenol red were obtained from Sigma Chemicals, Deisenhofen, Germany. Fetal calf serum (FCS), penicillin-streptomycin, trypsin-EDTA, and M-MLRV superscript reverse transcriptase

*Corresponding author. Fax: (43) (512) 504-2450.

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were purchased from Gibco Life Tech., Eggenstein, Germany. Sulfanilamide and Triton-X 100 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. Nitrate reductase was from Boehringer, Mannheim, Germany. Neopterin and 7,8-dihydroneopterin were purchased from Schircks Lab., Jona, Switzerland. Hoechst 33342 was obtained from Molecular Probes and 0.1% trisodium citrate dihydrate was purchased from Merck, Darmstadt, Germany.

2.2. Characteristics and culture of L2 cells

The alveolar epithelial cell line L2 (adult female Lewis strain rat), was purchased from American Type Culture Collection (Rockville, MD, USA). This cell line was first described by Douglas and Kaighn and characterized to be of type II pneumocyte origin because of the observed osmiophilic lamellar inclusions in early passages [19]. L2 cells were grown in 6-well plates (9 cm^2) in DMEM containing 10% FCS, L-glutamine (2 mM) and penicillin-streptomycin (100 U/ml-100 μg/ml) in a humidified atmosphere (5% CO_2 in air) at 37°C (Heraeus Incubators, Hanau, Germany). Confluent L2 cells were washed with sterile phosphate-buffered saline (PBS; 4.2 mM potassium, 153 mM sodium, 140 mM chloride, 1.47 mM dihydrogen phosphate and 8.1 mM hydrogen phosphate, pH 7.5) and incubated for 24 h in DMEM prior to the experiments. Passages 51–55 were used for the present studies.

For cell incubations following substances were used: cytomix (= C; consisting of 100 U/ml IFN-γ and 500 U/ml TNF-α), neopterin (1 μM, 10 μM, 100 μM, 1000 μM), and 7,8-dihydroneopterin (1 μM, 10 μM, 100 μM, 1000 μM). Possible cytotoxicity of the cytokines and pteridines was assessed by means of the [3-(4,5-dimethylthiazol-2-yl)-2,5-biphenyl]tetrazoline bromide assay. No cytotoxicity could be detected.

2.3. Evaluation of cellular apoptosis

2.3.1. Fluorescence activated cell sorter (FACS) analysis. Cells washed with PBS were incubated with the cytomix and/or neopterin and 7,8-dihydroneopterin for 24 h. Cells that grow as monolayers tend to become detached and to float off into the supernatant medium when they get apoptotic. Thus, following incubation, supernatants were removed and retained. The adherent cells were rinsed once in PBS, detached with a scraper and combined with the cells in the supernatant. The cells were centrifuged (1200 rpm, 4°C for 8 min) and the pellet was resuspended in propidium iodide staining solution and assayed on a FACS Scan (Becton Dickinson, Sunnyvale, CA, USA). Apoptotic cells were characterized by morphology (forward scatter versus side scatter) and by internuclear contents of fluorescing DNA as described earlier [20].

2.3.2. Fluorescence measurements. L2 cells were grown to subconfluence on glass cover slips. After incubation with cytomix and/or neopterin derivatives the fluorescence dyes Hoechst 33342 (5 μg/ml) and propidium iodide (PI; 5 μg/ml) were added. After 20 min at 37°C cells were washed with PBS and analyzed under an inverted microscope (Axiovert 35, Zeiss, Oberkochen, Germany) equipped for epifluorescence and photometry. Excitation light was produced by a monochromator (TILL Photonics, Planegg, Germany). Further details have recently been described [21]. Upon excitation at 330 nm and 505 nm, respectively, the emitted light of Hoechst 33342 and PI was measured after passing 420 nm or 520 nm cut-off filters.

2.4. Nitrite and nitrate assay

Synthesis of the stable NO metabolites nitrite (NO_2^-) and nitrate (NO_3^-) was determined in the cell-free culture supernatants incubated for 24 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 [22].

2.5. RNA isolation and polymerase chain reaction (PCR)

After 9 h stimulation cells were washed with sterile PBS and lysed 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 [23], 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 primer for reverse transcriptase. RT-generated cDNA encoding for rat iNOS and rat glyceraldehyde-3-phosphate dehydrogenase (GAPDH) genes were amplified using PCR as described previously [16]. Expression of the housekeeping gene GAPDH served as control. RNA without GAPDH bands was excluded from further investigations.

2.6. Statistics

Data shown in the Table and Figures are expressed as means ± S.E.M. Statistical analysis was made by the two-tailed Student's *t*-test. $P < 0.05$ was considered to indicate statistical significance.

3. Results

3.1. Role of neopterin and 7,8-dihydroneopterin in the induction of apoptotic cell death

Fig. 1 shows the degree of apoptosis in L2 cells evaluated by FACS analysis of propidium iodide stained isolated nuclei following incubation of cells with increasing amounts of neopterin and 7,8-dihydroneopterin. In untreated control cultures the percent apoptosis was less than 3% after 24 h incubation. In comparison with control cultures 24 h incubation with the cytomix resulted in a significant increase of apoptotic cells. Neopterin alone induced significant apoptotic cell death at all concentrations used in the experiments (Fig. 1A). At neopterin concentrations of 10 μM and 100 μM apoptosis was significantly higher than apoptosis induced by the cytomix. The effect was maximal at 100 μM neopterin and dropped markedly when cells were incubated with 1000 μM. 7,8-Dihydroneopterin had no effect on apoptosis at a concentration of 1 μM, but was able to induce apoptosis similar to that of neopterin at 10 μM, 100 μM and 1000 μM (Fig. 1B).

In Fig. 2, the results of the coincubation experiments (neopterin plus cytomix: Fig. 2A; 7,8-dihydroneopterin plus cytomix: Fig. 2B) are shown. In each experiment, coincubation of neopterin with the cytomix led to a significantly higher percent apoptosis than the cytomix alone. Maximal apoptosis was obtained with the combination of the cytomix plus 100 μM neopterin (22.7 ± 2.5%) (Fig. 2A). 7,8-Dihydroneopterin (1 μM) and cytomix resulted in apoptotic cell death which was statistically not different from that induced by the cytomix alone (Fig. 2B). In contrast, coincubation of the cytomix with 10 μM, 100 μM and 1000 μM 7,8-dihydroneopterin resulted in a significantly higher degree of apoptotic cells than the cytomix per se. Similar to the coincubation experiments with neopterin, maximal apoptotic cell death (21.9 ± 1.0%) was observed when cells were treated with the cytomix plus 100 μM 7,8-dihydroneopterin.

To evaluate morphological alterations typical for apoptosis, fluorescence measurements were performed. After 24 h incubation with the cytomix plus neopterin (100 μM) cells were incubated with Hoechst 33342 and PI. Hoechst 33342 is a fluorochrome which specifically binds to A-T base pairs. To exclude the possibility that treated cells were necrotic the same plates were stained with PI. In contrast to necrotic cells the cytoplasmic membrane of intact and apoptotic cells is impermeable for PI. Representative examples are shown in Fig. 3. As compared to untreated controls (Fig. 3A) a large amount of cells incubated with cytomix plus neopterin showed nuclear fragmentation and a higher fluorescence intensity (Fig. 3B–D). Nuclei in Fig. 3 did not show fluorescence activity following excitation with 505 nm, indicating the absence of PI within the cells.

3.2. Possible involvement of NO in apoptosis induced by neopterin derivatives

Fig. 4 summarizes the results of the qualitative analysis of iNOS mRNA expression following 9 h incubation of L2 cells. Cytomix alone as well as costimulation of cells with the cytomix and both neopterin derivatives increased iNOS mRNA expression. In contrast, no iNOS mRNA was found in untreated cells and in cells exposed to neopterin and 7,8-dihydroneopterin.

As shown in Table 1 stimulation of iNOS gene expression by the cytomix either alone or in combination with neopterin or 7,8-dihydroneopterin was accompanied by an increase in nitrite/nitrate concentrations as compared to unstimulated controls. No significant elevation of NO_2^-/NO_3^- levels was found in cells incubated with neopterin or 7,8-dihydroneopterin. In the coincubation experiments (cytomix plus neopterin and cytomix plus 7,8-dihydroneopterin, respectively) NO_2^-/NO_3^- concentrations were significantly lower than in the experiments with the cytomix alone.

4. Discussion

The heterocyclic pteridine compounds neopterin and 7,8-dihydroneopterin are produced in excess by monocytes/macrophages upon stimulation with IFN-γ, thus representing an activation of the cellular immune system [11]. Clinical conditions associated with the release of pteridines are e.g. HIV infection, certain types of cancer, and sepsis [24,25]. Due to the close connection between alveolar macrophages and alveolar epithelial cells, the latter are most likely exposed to high concentrations of macrophage-derived pteridines and cytokines during inflammatory processes. However, it remains elusive whether pteridines exhibit any biochemical functions in the whole organism. In this study we demonstrate that single treatment of the alveolar epithelial cell line L2 with either neopterin or 7,8-dihydroneopterin induces apoptosis in vitro. Moreover, both substances substantially augment apoptosis induced by the combination of IFN-γ and TNF-α in this cell type. Therefore, besides TNF-α and other cytokines, it is conceivable that pteridines are involved in apoptotic processes, too.

Findings contradictory to our results have been reported in human cells earlier [15]. Increasing concentrations of neopterin (up to 1 mM) and 7,8-dihydroneopterin (up to 300 μM) slightly decreased TNF-α-induced apoptosis in the human

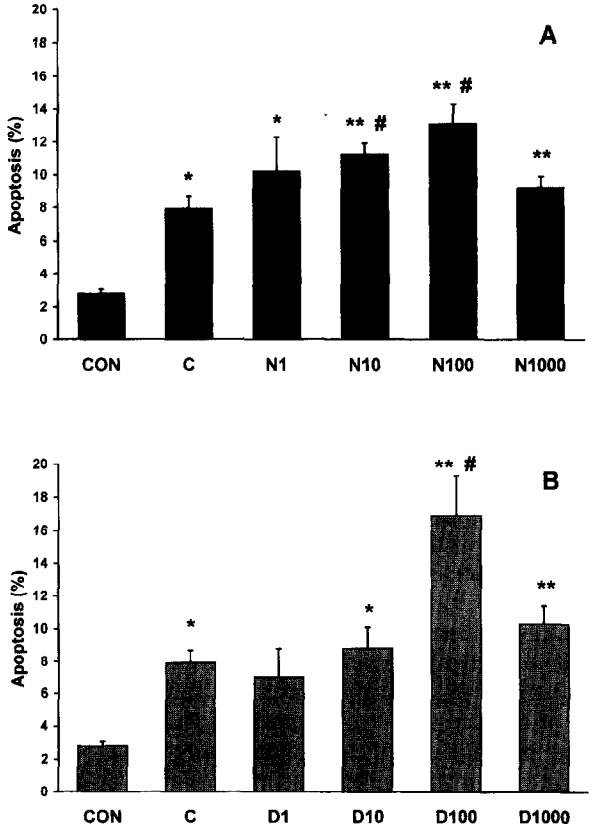

Fig. 1. Effects of cytomix, neopterin and 7,8-dihydroneopterin on apoptosis in L2 cells. L2 cells were incubated for 24 h with the cytomix (C; 100 U/ml IFN-γ plus 500 U/ml TNF-α), with neopterin (N; 1 μM, 10 μM, 100 μM, 1000 μM; A) or with 7,8-dihydroneopterin (D; 1 μM, 10 μM, 100 μM, 1000 μM; B). Apoptotic cell death is characterized by measurement of intranuclear contents of DNA by FACS analysis as described in Section 2. Data are shown as mean values ± S.E.M. ($n=7$). $^*P<0.05$ and $^{**}P<0.01$ as compared with untreated control cultures (CON), $^{\#}P<0.05$ as compared with the cytomix (C).

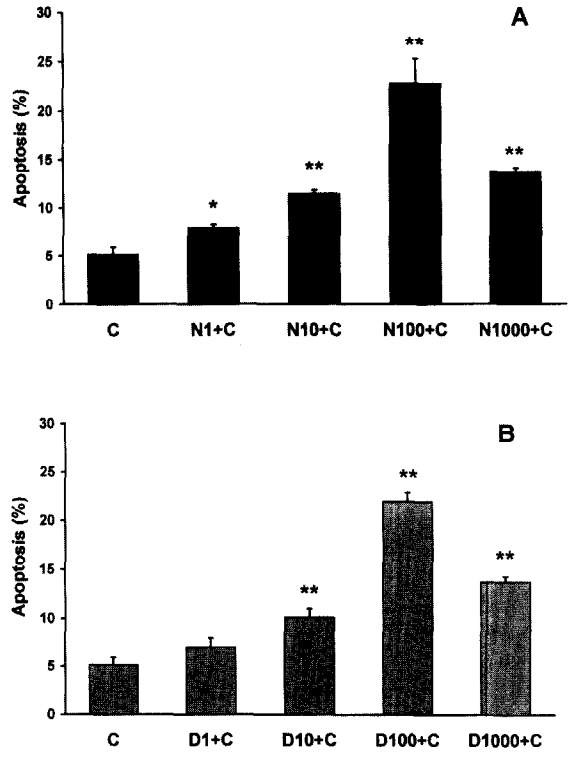

Fig. 2. Apoptotic cell death induced by the coincubation of cytomix with neopterin and cytomix with 7,8-dihydroneopterin. A: L2 cells were incubated for 24 h with the cytomix (C; 100 U/ml IFN-γ and 500 U/ml TNF-α), 1 μM neopterin plus the cytomix (N1+C), 10 μM neopterin plus cytomix (N10+C), 100 μM neopterin plus cytomix (N100+C) or 1000 μM neopterin plus cytomix (N1000+C). B: L2 cells were incubated for 24 h with the cytomix, 1 μM 7,8-dihydroneopterin plus the cytomix (D1+C), 10 μM 7,8-dihydroneopterin plus cytomix (D10+C), 100 μM 7,8-dihydroneopterin plus cytomix (D100+C) or 1000 μM 7,8-dihydroneopterin plus cytomix (D1000+C). Apoptosis is characterized by measurement of intranuclear contents of DNA by FACS analysis as described in Section 2. The percentage of apoptotic cells in untreated controls was subtracted from treated cell cultures. Data are presented as mean values ± S.E.M. ($n=7$). $^*P<0.05$ and $^{**}P<0.01$ as compared with the cytomix, $P<0.01$ as compared with the cytomix (C).

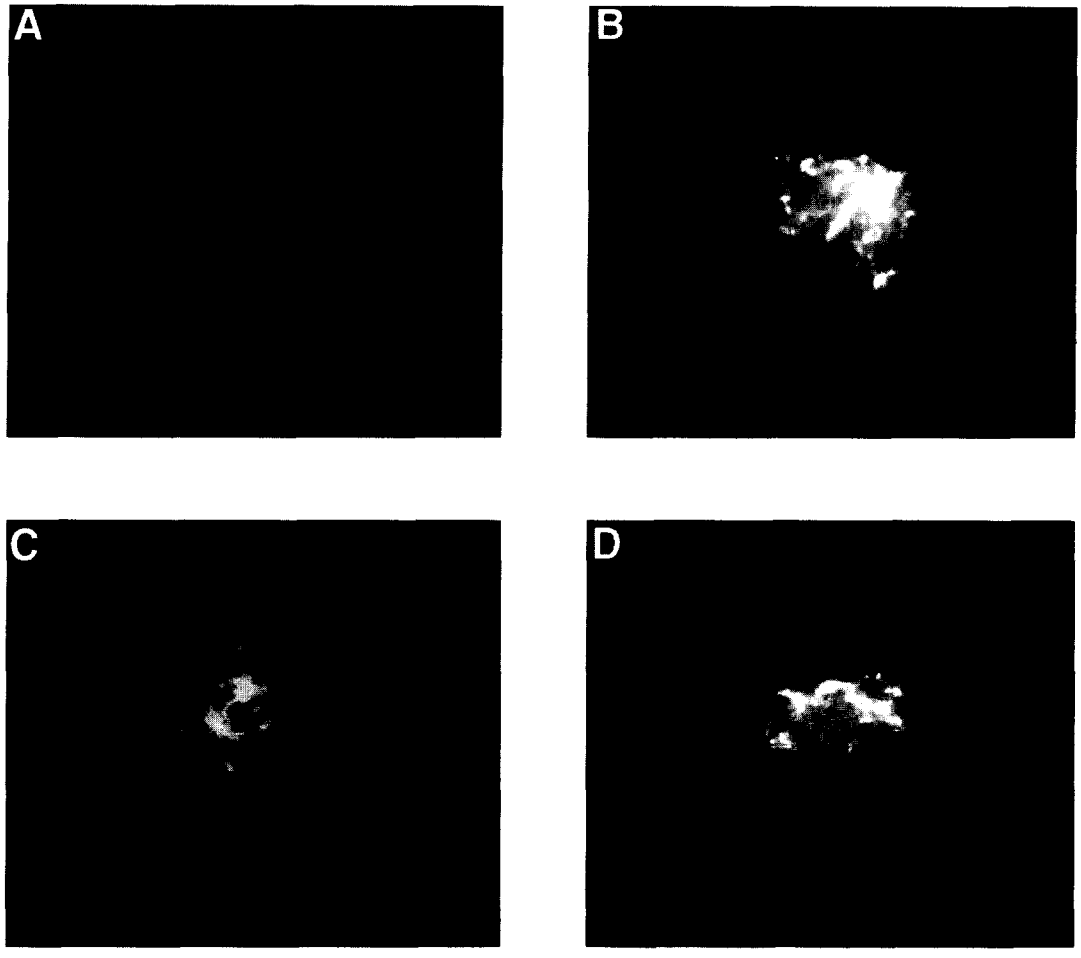

Fig. 3. Morphological characteristics of L2 cells incubated with or without neopterin (100 μM) as detected by the fluorescent dye Hoechst 33342. A: Untreated control cells. B–D: Fragmented nuclei of cells exposed to neopterin for 24 h; excitation wavelength 330 nm.

histiocytic lymphoma cell line U937, whereas only high concentrations of 7,8-dihydroneopterin (> 1 mM) superinduced TNF-α-mediated apoptosis. Species-specific as well as cell type-specific differences may account for these contradictions. On the one hand, U937 cells are monocyte-derived human cells which are able to produce and release neopterin derivatives, thus likely being less sensitive to pteridines. On the other hand, L2 cells are epithelial cells derived from a rat lung which can produce and release neither neopterin nor 7,8-dihydroneopterin. Therefore, L2 cells may be more susceptible when exposed to these compounds. In addition, U937 cells are tumor-derived cells, whereas the L2 cells are not of tumor origin. Moreover, U937 cells were preincubated with TNF-α and thereafter coincubated with both neopterin derivatives. Apoptosis in our study was even induced by the single application of neopterin and 7,8-dihydroneopterin at relatively low concentrations.

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

	Nitrite concentration (nmol/10^6 cells)
Unstimulated controls	2.8 ± 0.6
Cytomix[a]	70.5 ± 2.9[b]
Neopterin (100 μM)	1.9 ± 0.5
Neopterin (100 μM)+Cytomix[a]	56.5 ± 1.3[b,c]
7,8-Dihydroneopterin (100 μM)	3.4 ± 0.4
7,8-Dihydroneopterin (100 μM)+Cytomix[a]	47.0 ± 0.2[b,c]

Data are expressed as means ± S.E.M. ($n = 8$).
[a]Cytomix: 100 U/ml IFN-γ plus 500 U/ml TNF-α.
[b]$P < 0.05$ as compared to unstimulated controls.
[c]$P < 0.05$ as compared to the cytomix.

The biochemical mechanism(s) by which neopterin and 7,8-dihydroneopterin lead to apoptosis in L2 cells remain to be elucidated. Several data provide evidence that oxidative stress is an important mediator of apoptosis. ROI such as H_2O_2, OH^- and O_2^- were reported to be the chemical triggers for apoptotic cell death during oxidative stress [10]. However, the correlation between pteridine compounds and oxidative stress is contradictory. In the absence of iron, increasing concentrations of neopterin act as scavengers of ROI and quench hydrogen peroxide-induced chemiluminescence [12–14]. Moreover, neopterin was found to suppress superoxide-generating NADPH-oxidase in macrophages treated with phorbol myristate acetate [26]. Reduced pteridine derivatives such as 7,8-dihydroneopterin are generally reported to be potent scavengers of ROI [12,14]. In contrast to these findings, in the presence of iron, neopterin was found to enhance chloramine-T and H_2O_2-mediated chemiluminescence as well as ROI-mediated toxicity against bacteria [12]. In addition, it was shown that the amount of H_2O_2 released by human macrophages correlates with the amount of neopterin released by these cells upon stimulation with IFN-γ [27]. Neopterin and

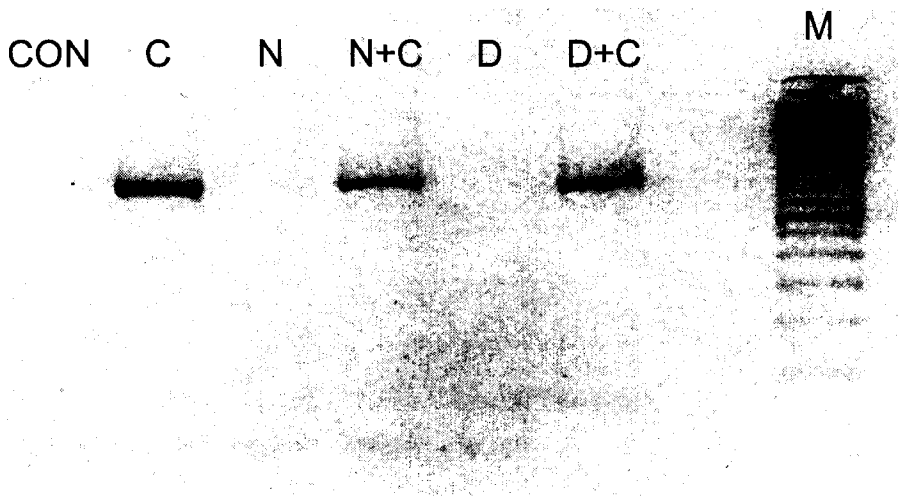

Fig. 4. Qualitative PCR analysis of iNOS mRNA expression detected as iNOS cDNA (fragment size: 498 bp) following 9 h incubation of L2 cells with the cytomix (C; 100 U/ml IFN-γ and 500 U/ml TNF-α), 100 μM neopterin (N), 100 μM neopterin plus cytomix (N+C), 100 μM 7,8-dihydroneopterin (D), and 100 μM 7,8-dihydroneopterin plus cytomix (D+C). Lane CON shows the results of a control experiment in untreated cells. Lane M indicates the molecular mass marker (50 bp ladder). Results are representative of three different experiments.

7,8-dihydroneopterin were also reported to interfere with intracellular signalling pathways known to be influenced by oxidative stress. They were found to inhibit erythropoietin gene expression [28], to induce proto-oncogene c-*fos* expression [29], and to enhance cell damage caused by UV-A irradiation [30]. Another important intracellular pathway that mediates the various effects of ROI is the activation of transcription factors, e.g. the family of NF-κB proteins [31]. In previous studies it was found that neopterin and 7,8-dihydroneopterin activate NF-κB in vascular smooth muscle cells and in Jurkat cells by modulating the production and/or the effects of ROI [17,32]. This might be one biochemical mechanism whereby neopterin influences iNOS gene expression and NO production in this cell type. In a recent study we could show that L2 cells are capable of iNOS gene expression and NO generation upon stimulation with IFN-γ and TNF-α [18]. Since NO is suggested to be a mediator of apoptotic cell death [7,8], we investigated the effects of IFN-γ, TNF-α, and both pteridine compounds on iNOS gene expression and NO release in L2 cells. In contrast to the cytomix (IFN-γ+TNF-α), neither neopterin nor 7,8-dihydroneopterin were able to activate iNOS gene expression (Fig. 4) and NO production (Table 1). In addition, treatment of L2 cells with the NO donors 3-morpholino-sydnonimine (SIN-1; 100 μM) and *S*-nitroso-*N*-acetyl-D,L-penicillamine (SNAP; 100 μM) resulted in only a slight, statistically not significant increase in apoptotic cells as detected by FACS Scan analysis (data not shown). This suggests that at least the pteridine-induced apoptosis observed in our experiments is mediated by NO-independent mechanisms that are at present unknown.

In conclusion, the present study demonstrates that both neopterin and 7,8-dihydroneopterin are inducers of apoptotic cell death in rat alveolar epithelial cells at relatively low concentrations. In addition, when L2 cells are coincubated with IFN-γ and TNF-α, these neopterin derivatives augment the cytokine-induced apoptosis. Under in vivo conditions, pteridine-mediated enhancement of cytokine-induced apoptotic cell death in alveolar epithelial cells may be relevant in inflammatory pulmonary diseases which are associated with a simultaneous release of cytokines, ROI and neopterin derivatives.

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