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Neopterin Derivatives Modulate Toxicity of Reactive Species on *Escherichia coli*

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Neopterin and 7,8-dihydroneopterin are released by human monocytes/macrophages upon stimulation with interferon-γ. In parallel, a panel of highly reactive species is produced by macrophages as part of their cytotoxic armature, which is directed against microbial and viral challenge and against malignant growth. Recently, neopterin and 7,8-dihydroneopterin were shown to modulate the action of reactive species *in vitro*. In this study we investigated the impact of neopterin and 7,8-dihydroneopterin on the toxicity of reactive species, namely chloramine-T, H_2O_2, hypochlorite, nitrite, and formaldehyde, respectively. We studied the growth inhibition of *Escherichia Coli* (*E. coli*) by these toxic agents and its modulation by neopterin and 7,8-dihydroneopterin. Bacterial growth was monitored by optical density of suspension cultures at 600 nm. Compared to control experiments, neopterin enhanced toxicity of all reactive species tested except formaldehyde, while 7,8-dihydroneopterin reduced activity of hypochlorite and chloramine-T. No significant impact of the pteridines could be established for H_2O_2-mediated and formaldehyde-mediated growth inhibition. The data support the concept that neopterin and 7,8-dihydroneopterin produced during immune response in humans could be important to modulate the action of reactive species released in parallel.

Keywords: Neopterin, 7,8-dihydroneopterin, bacterial growth, reactive species, toxicity, immune activation

INTRODUCTION

Increased concentrations of neopterin and 7,8-dihydroneopterin (Figure 1) are produced by human monocytes/macrophages upon stimulation with interferon-γ.[1] During immune activation caused by, e.g., viral infection, high concentrations of these pteridine derivatives are found *in vivo*, and measurement of neopterin concentrations in body fluids was established as a sensitive parameter to monitor cell-mediated immune activation[2,3] in, e.g., allograft recipients, HIV infections or in malignant diseases.[4,5] However, the physiological role of increased neopterin production during immune stimulation has remained unclear so far. Yet, evidence has accumulated that neopterin and 7,8-dihydroneopterin

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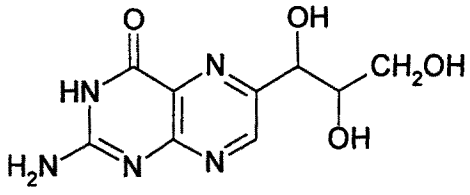
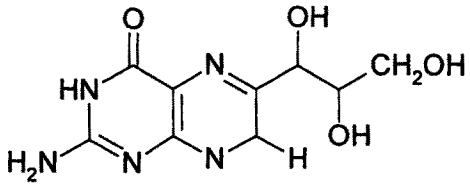


neopterin



7,8-dihydroneopterin

FIGURE 1 Neopterin and 7,8-dihydroneopterin.

influence the reactivity of endogeneously produced reactive species and hence modulate the toxicity of e.g., hydrogen peroxide (H_2O_2), superoxide anion ($O_2^{\bullet-}$), hypochlorite (OCl^-), or peroxynitrite ($ONOO^-$). These reactive species are produced by different immunocompetent cells including neutrophil granulocytes[6] and macrophages[7] under conditions, when also neopterin and 7,8-dihydroneopterin are generated. Therefore a potential interaction of these pteridines with the cytotoxic species during immune response seems possible. Earlier, neopterin was found to enhance chemiluminescence induced by chloramine-T,[8] and neopterin inhibits activity of xanthine oxidase[9] and $O_2^{\bullet-}$-generating NADPH oxidase.[10] Further, neopterin enhances tyrosine nitration by peroxynitrite,[11] and it was shown to potentiate growth inhibition of bacteria by chloramine-T.[12,13] In contrast, 7,8-dihydroneopterin was found to show a variable behavior, in concentrations $< 300\,\mu M$ mostly diminishing the effects of reactive species, but in high concentrations (5 mM) super-inducing, e.g., H_2O_2-mediated chemiluminescence.[14] *In vitro* experiments extend evidence of the modulating role of neopterin and 7,8-dihydroneopterin to the cellular level. An impact of these pteridines on redox-sensitive intracellular signal transduction pathways[15,16] was demonstrated, e.g., influencing the rate of apoptosis[14] or inducing the nuclear factor (NF)-κB.[17]

In this study, we further examined the impact of neopterin and 7,8-dihydroneopterin on cultures of *Escherichia coli* (*E. coli*) exposed to various reactive species or their precursors, respectively.

MATERIALS AND METHODS

Material

The bacterial strain *E. coli* XL1 was a gift from the Institute of Hygiene (University of Innsbruck, Austria). This strain was shown earlier to be susceptible to chloramine-T-induced toxicity.[13] The culture medium (LB Broth, Miller) was obtained from Difco Laboratories (Detroit, MI, USA) containing trypton (10 g/L), yeast extract

(5 g/L), and sodium chloride (10 g/L). D-erythro-neopterin and D-erythro-7,8-dihydroneopterin were purchased from Schircks Laboratories (Jona, Switzerland). Potassium chloride, potassium dihydrogen phosphate, sodium chloride, disodium hydrogen phosphate, sodium nitrite ($NaNO_2$), H_2O_2, formaldehyde (HCHO), and chloramine-T were from Merck (Darmstadt, Germany). Sodium hypochlorite (NaOCl) was from Sigma (Vienna. Austria).

Solutions

Saturated suspensions of *E. coli* were diluted with LB-medium by 1 : 10. A phosphate buffered saline (PBS) solution was prepared by dissolving 0.2 g KH_2PO_4, 2.9 g $Na_2HPO_4 \times 12\ H_2O$, 8 g NaCl and 0.2 g KCl in 1 L of bidistilled water. Neopterin was dissolved in PBS in concentrations of 500 μM and 1 mM, which is close to the maximum solubility of neopterin, and 7,8-dihydroneopterin was dissolved giving 500 μM, 1 and 6 mM, approaching the limit of solubility. The solutions were placed in an ultrasonic bath for 1 h and were then sterile filtered (pore size 0.2 μM). Fresh solutions of the following reactive species were prepared in PBS: NaOCl (6 mM), chloramine-T (4 mM), H_2O_2 (20 mM), $NaNO_2$ (40 mM), HCHO (20 mM). The concentrations applied had been determined in pilot experiments to achieve an approximate 60% inhibition of bacterial growth.

Method

Growth of the *E. coli* strain XL1 was monitored photometrically by measurement of the optical density of the bacteria solutions. Each 1 mL of the pre-diluted bacteria suspension was mixed with 2 mL PBS or with 2 mL PBS containing neopterin or 7,8-dihydroneopterin, respectively, in different concentrations in sterile test tubes. Then 1 mL of the toxic agents was added, so that the final incubation mixture had a 4 mL volume. In a control experiment, 1 mL PBS was supplemented instead of the toxic agents to examine a possible direct influence of neopterin and 7,8-dihydroneopterin on bacterial survival. To evaluate blank values, the absorbance of the mixtures was immediately measured with a Hitachi U-200 spectrophotometer (Tokyo, Japan) at 600 nm wavelength. Then the test tubes were shaken for 100 min at 37°C. Thereafter optical density was measured again to estimate the influence of the compounds added on bacterial growth.

Calibration

The bacterial suspensions were diluted 1 : 10 with LB medium, further diluted by 1 : 4 with PBS and incubated at 37°C. After 0, 45, 90 and 150 min, the bacterial suspensions were placed in the photometer, and bacterial growth was monitored by optical density. In parallel for every time point, samples of 100 μl of the culture were diluted 1 : 1000 with LB medium and 100 μL aliquots were further diluted by 1 : 100, yielding a final dilution factor of 10^5. From this solution, 150 μL were plated on LB agar petri dishes. After incubation for 24 h at 37°C, the numbers of colonies were counted. The original bacteria density in culture medium was back-calculated from the number of colonies and compared to the optical density measured. A close correlation existed between optical density and the number of bacteria in the culture medium evaluated by plating and counting the colonies (Figure 2). The values of optical density in the experiments ranged from 0.4 to 0.9.

Calculation

The blank values measured at the beginning of the incubation were subtracted from the values measured after 100 min incubation. The incubation mixtures containing neopterin and 7,8-dihydroneopterin were compared to the control incubations without pteridines. The deviations of bacterial growth under the influence of pteridines were expressed as percent from the control.

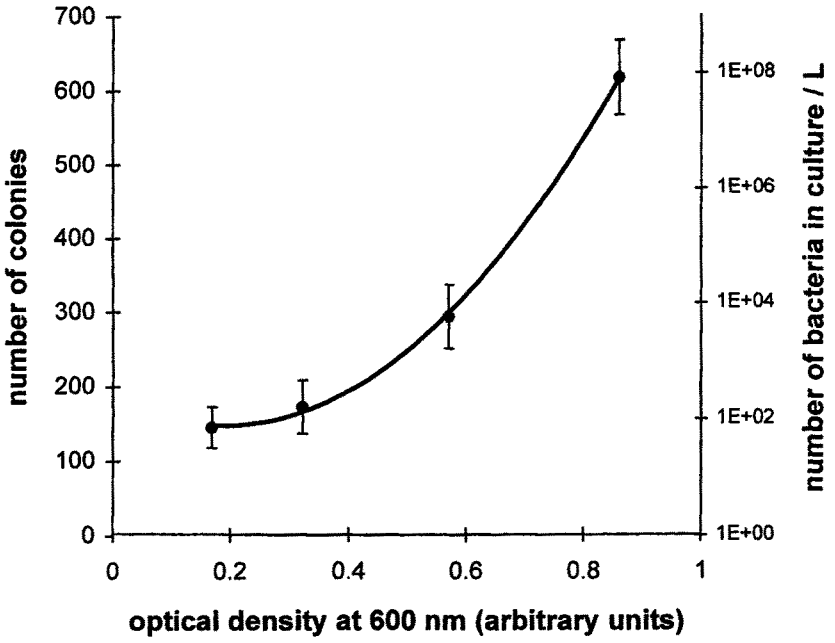


FIGURE 2 Correlation between the number of bacteria in solution and optical density. (The number of bacteria was determined by diluting the solution and plating on agar dishes, counting the colonies after 24 h incubation).

Mean values ± standard error of the mean were calculated from ten independent incubation sets. Differences between control incubations and incubations containing pteridines were computed by Students' *t*-test, and p values < 0.05 were considered for significance.

RESULTS

Neopterin and 7,8-dihydroneopterin did not exert any statistically significant influence on bacterial growth. Changes in optical density due to pteridines without microbicidal agents were $< 3\%$ (all p not significant). In contrast, the changes of optical density induced by the pteridines due to modulation of different toxic agents well exceeded this value.

Figure 3 summarizes the effects of neopterin on the inhibition of bacterial growth induced by various toxic agents. In general, neopterin enhanced the microbicidal effects of the examined reactive species in a dose-dependent manner (Figure 3). The toxicity was strongest for H_2O_2 and $NaNO_2$ followed by chloramine-T and NaOCl-mediated toxicity. Approximately two-fold growth inhibition was achieved by doubling the pteridine concentration except for $NaNO_2$ where a two-fold concentration of neopterin induces only 1.5-fold reduction of growth inhibition. Neopterin seemed to slightly reduce the toxic effect of HCHO. The effect was concentration-dependent but did not reach statistical significance.

7,8-Dihydroneopterin decreased toxicity of chloramine-T and NaOCl in a concentration-dependent manner (Figure 4). The strongest inhibition of toxicity was established for chloramine-T and it was less strong for the NaOCl incubation. Increasing concentrations of the reduced pteridine led to a more pronounced inhibition of toxicity, however, the dependence on concentration was not linear. 7,8-dihydroneopterin did not exert any significant modulation on H_2O_2-, $NaNO_2$- and HCHO-mediated toxicity.

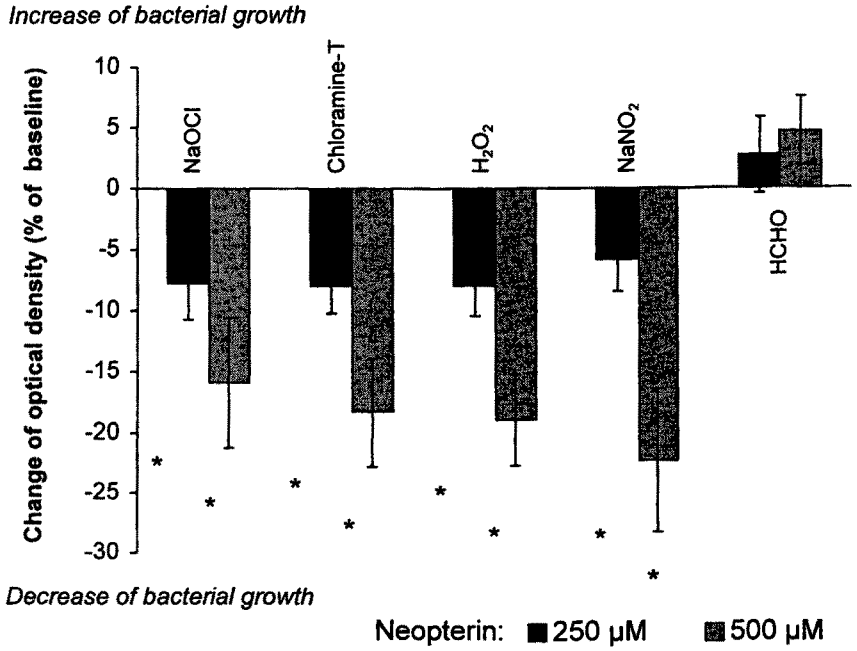


FIGURE 3 Effect of neopterin in two concentrations on the growth inhibition of bacteria as determined by optical density of cultures at 600 nm induced by different toxic species ($*p < 0.01$; mean values ± SEM are shown; $n = 10$). Growth inhibition by toxins in the absence of pteridines was achieved as follows: NaOCl (1.5 mM) 28%, chloramine-T (1 mM) 45%, H_2O_2 (5 mM) 24%, $NaNO_2$ (10 mM) 25% and HCHO (5 mM) 42%.

DISCUSSION

The modulation of toxicity of various microbicidal species on bacterial growth by neopterin and 7,8-dihydroneopterin was studied. Chloramine-T produces highly reactive oxygen and chlorine radicals, affecting important microbial structures, e.g., proteins, lipids or nucleic acids.[18] HCHO, another disinfectant, interacts with proteins.[19] Hypochlorite and H_2O_2 are produced by immunocompetent cells[6] as part of their cytotoxic armature.[20] Our study shows that neopterin enhances the microbicidal effect of all toxic agents tested except HCHO, whereas 7,8-dihydroneopterin decreases the toxicity of NaOCl and chloramine-T. The findings are similar to those found earlier in chemiluminescence experiments.[8] High concentrations of neopterin and 7,8-dihydroneopterin are released in parallel, when the Th1-type immune response is activated.[2–4] This type of immune response is preferentially activated during infections by viruses and intracellular microorganisms, malignant diseases, and during autoimmune disorders, being closely associated with enhanced neopterin production. Also in long lasting bacterial infections when the cellular immune system is involved, neopterin and 7,8-dihydroneopterin are secreted in increased amounts.[21] Recently, some evidence has accumulated that dentritic cells are particularly capable of producing neopterin.[22]

Production of high concentrations of neopterin is unique for humans and primates.[4] Despite considerable interest in neopterin as a clinical laboratory marker, no clearcut explanation was found yet regarding its biological function. However, many recent studies outlined a potential role of neopterin and 7,8-dihydroneopterin in modulating processes mediated by reactive species.[5] In addition, clinical investigations reveal a striking association between immune related tissue damage, e.g. in infections, or in rheumatoid

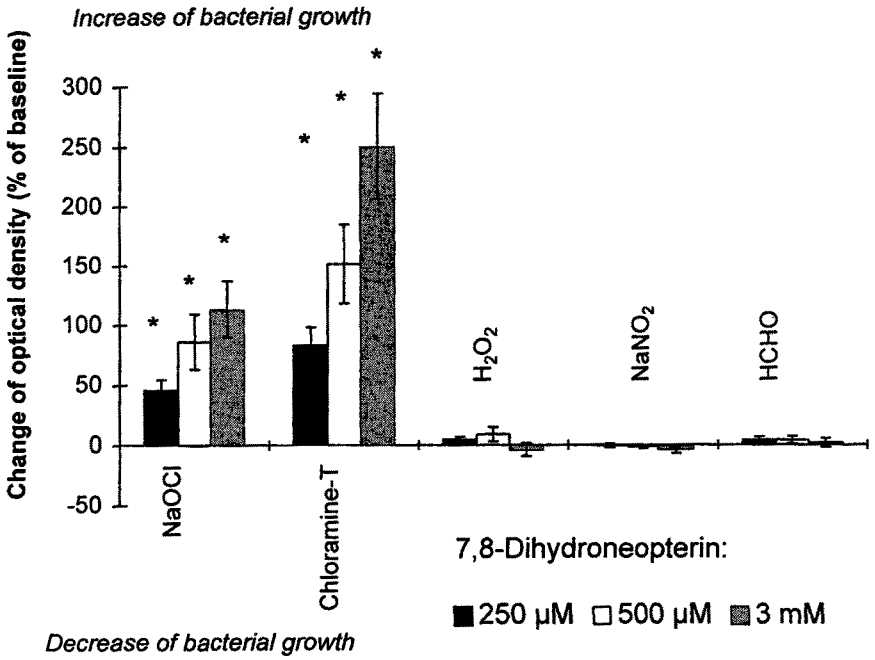


FIGURE 4 Effect of 7,8-dihydroneopterin in three concentrations on the growth inhibition of bacteria as determined by optical density of cultures at 600 nm induced by different toxic species ($*p < 0.01$; mean values ± SEM are shown; $n = 10$).

arthritis[23] and high neopterin concentrations[5,24,25] providing a basis for the hypothesis of an interaction between pteridines and cytotoxic species. However, the mechanistic aspects of these interactions remain speculative. Earlier *in vitro* observations provided evidence that neopterin is involved in cytotoxic processes in a catalytic manner since it is not chemically modified when exposed to, e.g., H_2O_2.[26] 7,8-Dihydroneopterin was shown to act as an antioxidant.[8,27] From this viewpoint it becomes evident that reactive species are likely inactivated by the reducing capacity of 7,8-dihydroneopterin which has been shown earlier to be degraded during the impact of reactive species.[11,26]

It seems that the quotient of neopterin and 7,8-dihydroneopterin crucially influences the balance between pro- and antioxidative mechanisms.[28] In addition, oxidative stress under inflammatory conditions may lead to a shift in the ratio when 7,8-dihydroneopterin is consumed due to its scavenging properties. Then the pro-oxidative effects of neopterin would become prevailing. Accordingly, new findings revealed that high neopterin concentrations *in vivo*, e.g. in CSF of patients with dementia, are associated with a decreased intrathecal antioxidant status indicated by decreased α-tocopherol concentrations.[29] Similarly, in uremic patients, neopterin was found to correlate with the amount of advanced oxidation protein products.[30] Therefore, high neopterin concentrations seem to be associated with oxidative stress *in vivo*, and our findings support the view that neopterin could even be directly involved in the generation of oxidative stress.

Additional pro-oxidative attitudes could possibly be necessary to compensate the deficiency of human monocytes/macrophages to produce sufficient amounts of NO,[28] an important mediator of the cytotoxic armature of immunocompetent cells. In contrast to rodent mononuclear

phagocytes, human monocytes/macrophages fail to produce relevant amounts of NO on cytokine- or LPS-treatment,[31] and there are only a few circumstances in which NO is released at all.[32] Thus, NO does not appear to be a common constituent of the antimicrobial armature, whereas the production of neopterin is high during immune activation. Neopterin could therefore compensate the lack of NO-derived reactive species in human monocytic cells by increasing the cytotoxic potential of available oxidants.

The concentrations of neopterin in our experiments are certainly higher than those found in serum during immune activation (magnitudes around 300 nM neopterin, 1 μM 7,8-dihydroneopterin). Yet, at the site of inflammation, the pteridine concentrations may be higher compared to those in the blood circulation, and in addition, physiological conditions could support the effects which neopterin derivatives exert on redox reactions. Pteridine concentrations in urine are usually 200- up to 500-fold higher than in serum[33] and therefore reach the magnitude of concentrations applied in our experiments.

Our study demonstrates that neopterin and 7,8-dihydroneopterin are able to interfere with toxic reactions which are directed against living microorganisms, and it extends earlier findings on the impact of pteridines on chemical model reactions which are potentially relevant under oxidative stress conditions. Thus the endogeneous formation of neopterin and 7,8-dihydroneopterin should be additionally drawn into consideration, when discussing about cytotoxicity during immune response. Moreover, the study may provide a basis for the better understanding for the increased formation of neopterin and 7,8-dihydroneopterin upon IFN-γ activation of human monocytes/macrophages in parallel to reactive species. Further studies are still necessary to elucidate the modulating role of neopterin and 7,8-dihydroneopterin in biological systems, e.g., by examining the influence of pteridines on the cytocidal potential in cultures of human mononuclear cells.

Acknowledgement

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