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Influence of neopterin on generation of reactive species by myeloperoxidase in human neutrophils

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Abstract

Increased neopterin concentrations in human serum indicate activation of cell-mediated immune response. Earlier we have shown that neopterin enhanced generation of singlet oxygen, hydroxyl radical and nitric oxide in human peripheral blood neutrophils by NADPH-independent pathways. To further investigate a participation of neopterin in reactive species production by neutrophils, we studied its influence on myeloperoxidase (MPO) activity. MPO was isolated from human peripheral blood neutrophils from healthy donors. Generation of reactive species by MPO/H_2O_2 in Earl's solution (pH = 7.2) at 37 °C was investigated by monitoring of chemiluminescence using luminol as light emitter. In the MPO/H_2O_2 system, neopterin increased singlet oxygen in a concentration-dependent manner, but it decreased formation of other oxidizing species. Comparing several oxygen scavengers, formation of reactive species was totally blocked by sodium azide (NaN_3), both in the presence and in the absence of neopterin. Superoxide dismutase (SOD) and D-mannitol insignificantly decreased chemiluminescence of this reaction, but diazabicyclo[2.2.2]octane (DABCO) strongly inhibited it. We conclude that the effects of neopterin on neutrophils' MPO are directed to increase singlet oxygen and to decrease other reactive species via inhibition of MPO and/or scavenging of reactive species.

Keywords: Neopterin; Myeloperoxidase; Superoxide dismutase

1. Introduction

An activation of the immune system is accompanied with the increased formation of the 6-D-erythro-1′,2′,3′-dihydroxypropylpterin (neopterin) by human/primate macrophages upon stimulation by cytokine interferon-γ [1,2]. Measurement of neopterin concentrations is useful to monitor cell-mediated (Th1-type) immune activation [3]. In laboratory diagnosis the determination of neopterin concentrations in serum, urine, and cerebrospinal fluid can be applied, e.g., for early detection of immunological complications after allotransplantation or to predict prognosis in HIV infection or malignant diseases [3,4].

Experiments have shown that neopterin is capable of enhancing the oxidative potential of reactive oxygen and chlorine species produced from immunocompetent cells [5–7]. In parallel, neopterin was found to also inhibit activity of xanthine oxidase and NADPH-oxidase [8–10]. Therefore, neopterin can directly participate within the cascade of events leading to oxidative stress. In clinical conditions with increased neopterin concentrations such as viral infections including HIV infection, various malignant disorders, autoimmune diseases, and allograft rejections, activation of different immunocompetent cells is obvious.

Neutrophils of human peripheral blood generate reactive oxygen and chlorine species when activated, for example, by adhesion on glass or by influence of the numbers of phagocytosis-stimulating factors [11]. At least two enzymes take part in the generation of reactive species by neutrophils, NADPH-oxidase and myeloperoxidase (MPO). MPO is a major constituent of the azurophilic cytoplasmic granules. It is a classical heme peroxidase that uses hydrogen peroxide (H_2O_2) to oxidize a variety of substrates with production of free radical intermediates. In activated neutrophils during phagocytosis, MPO has been shown to

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form hypochlorous acid (HOCl) [12] and also different types of reactive intermediates such as hydroxyl radical (OH) [13] and/or singlet oxygen (O_2') [14]. Earlier we have shown that neopterin influenced production of reactive oxygen species (ROS) by activated neutrophils [15]. To investigate a possible effect of neopterin on the different pathways generating reactive oxygen and chlorine species in neutrophils, we studied its influence on MPO activity of neutrophils.

2. Materials and methods

2.1. Reagents

Neopterin was from Dr. Schircks Laboratories (Jona, Switzerland). Superoxide dismutase (SOD), D-mannitol, luminol (5-amino-2,3-dihydro-1,4-phthalazinedione), sodium azide (NaN_3) were from Sigma (Munich, Germany), and diazabicyclo[2.2.2]octane (DABCO) was from Merck (Darmstadt, Germany). Neopterin (250 μM) was freshly dissolved in 0.15 M NaCl by incubation for 1 h at 37 °C. ROS scavengers SOD, DABCO, D-mannitol, and NaN_3 were dissolved in 0.15 M NaCl in the following concentrations, which had been determined in pilot experiments: 0.3 μM SOD, 0.5 mM D-mannitol, 0.2 mM DABCO, 0.3 mM NaN_3.

2.2. Isolation of peripheral blood neutrophils from normal human donors

Neutrophils were isolated from heparinized peripheral blood of healthy donors by centrifugation using ficoll-verografin density gradient [16]. Lysis of remaining erythrocytes was carried out with cold distilled water during 20 s. Then cells were washed twice in 0.15 M NaCl and were resuspended in Earl's balanced salt solution (pH 7.2). The cell preparations contained more than 96% of neutrophils,

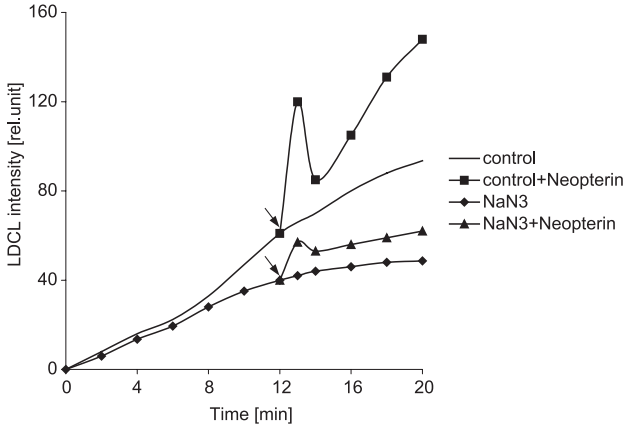

Fig. 1. Total intensity of LDCL of neutrophils in the presence of NaN_3 and in the absence (control) and after addition neopterin (50 μM) (arrows indicate the moment of neopterin addition).

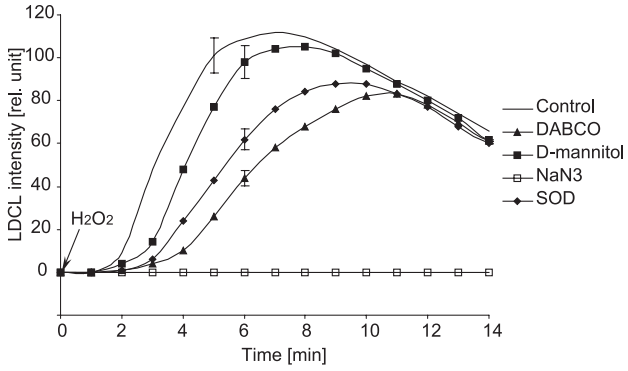

Fig. 2. The influence of various scavengers of ROS on intensity of LDCL in the MPO/H_2O_2/luminol system (50 μl H_2O_2 solution were injected, final concentration 10 μM; light output was measured for 14 min) DABCO = diazabicyclo[2.2.2]octane, SOD = superoxide dismutase.

and 10^6 cells/ml were stored at 4 °C. To extract MPO from neutrophils, 10^6 cells/ml were lysed by repeated freeze–thaw cycles in order to obtain crude lysates. The lysates were centrifuged at 2000 × *g* for 10 min. The supernatant was used for measurements of MPO activity.

2.3. Luminol-dependent chemiluminescence (LDCL) assay for MPO activity

LDCL was measured using biochemiluminometer BCL-1 (Belarus State University, Belarus) [17]. Measurements were carried out at 37 °C in a cylindrical glass cuvette (diameter 40 mm). The volume of cellular suspension was 2.4 ml. Experimental probes contained 2 ml Earl's solutions (pH 7.2), 5 mM $CaCl_2$, 10 μM luminol and 200 μl obtained supernatant. Thus, the concentration of MPO in the probe corresponded with 10^5 neutrophils/ml. Fifty microliters of hydrogen peroxide solution (50 μM) were injected (final concentration 10 μM). Light output was measured for 14 min. ROS scavengers were added in cuvette before adding supernatant. Neopterin was added 8 min after beginning of the reaction between MPO and H_2O_2. NaN_3, a non-specific inhibitor of heme enzymes, was used to inhibit MPO activity.

2.4. Chemiluminescence assay for reaction of sodium hypochlorite with luminol

Experimental probes contained 2 ml Earl's solutions (pH 7.2), 5 mM $CaCl_2$, 10 μM luminol and 0.5 μM NaOCl solution was injected in the probe. Light output was measured for 1 min. Neopterin (10, 20, 50 μM) was added into the cuvette before adding NaOCl solution.

2.5. Fluorescence assay

Natural fluorescence of neopterin in the MPO/H_2O_2 system was controlled using spectrofluorimeter LSF 1211A (Solar, Minsk, Belarus), excitation and emission wave-

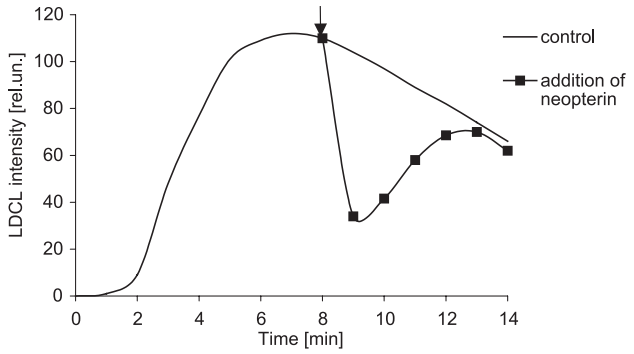

Fig. 3. Typical kinetic of LDCL in the MPO/H_2O_2/luminol system after addition of 50 μM neopterin (indicated by the arrow).

lengths were set at 353 and 444 nm. During 20 min, kinetics of fluorescence was recorded.

2.6. Calculation

The assays containing neopterin were compared to the control specimens without neopterin. Mean values ± standard errors of the mean were calculated from 10 independent sets for different donors. Statistical analysis was performed using Student's *t*-test.

3. Results

Typical LDCL kinetics of activated neutrophils at adhesion to glass surface and under exposure to NaN_3 are presented in Fig. 1, which also shows the effects of 50 μM neopterin when added to the system after 12 min. Addition of MPO inhibitor NaN_3 (0.3 mM) decreased the neopterin-induced neutrophils' response, but did not completely inhibit the additional generation of reactive species. This fact illustrates that the neopterin-induced neutrophils' response was not totally dependent on MPO activation.

Typical curves of LDCL in the MPO/H_2O_2/luminol system are presented in Fig. 2. The influence of ROS

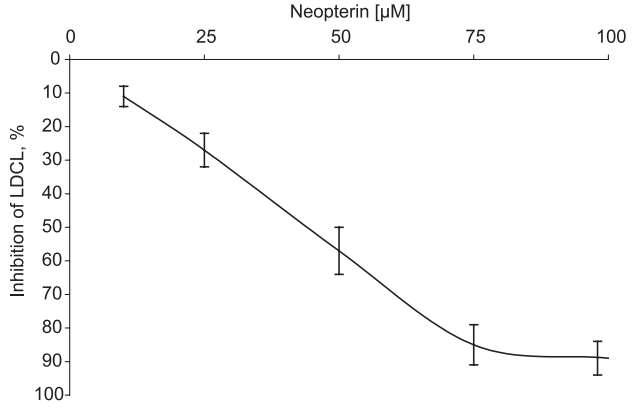

Fig. 4. Dose-dependent change of integral intensity of LDCL induced by neopterin (integral intensity compared to control without neopterin added).

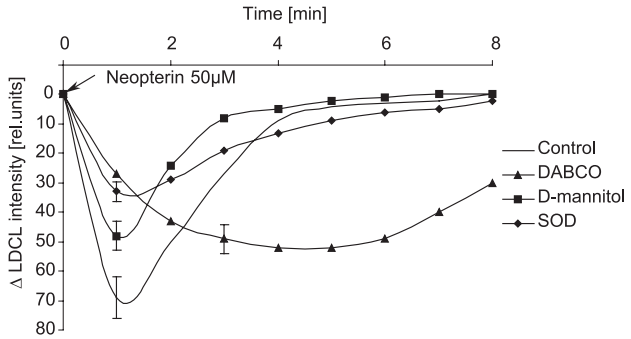

Fig. 5. The influence of various scavengers of ROS on the neopterin-induced decrease of LDCL. DABCO = diazabicyclo[2.2.2]octane, SOD = superoxide dismutase.

scavengers such as superoxide anions ($O_2^{-}\cdot$) scavenger SOD, O_2' scavenger DABCO, OH scavenger D-mannitol and NaN_3 on intensity of LDCL is shown. LDCL was completely inhibited by 0.3 mM NaN_3.

Injection of 50 μM neopterin in the analysed probe lead to an abrupt decrease of LDCL, which was followed by a return to intensity of the control within approximately 4 min (Fig. 3).

Addition of neopterin to the MPO/H_2O_2/luminol system resulted in a dose-dependent decrease of oxidizing species (Fig. 4), 100 μM led to almost total reduction of LDCL in this system. The relative magnitudes of neopterin-induced changes of chemiluminescence in the MPO/H_2O_2/luminol system in control and in the presence of ROS scavengers are presented in Fig. 5. D-Mannitol or SOD in the suspension reduced the influence of neopterin on LDCL intensity, but these scavengers did not change the kinetics of LDCL at the action of neopterin. The character of the neopterin-induced decrease of intensity of LDCL in the presence of DABCO in suspension differed from the influence of other scavengers. Injection of neopterin in probes containing NaN_3 did not lead to any changes of LDCL intensity.

Injection of NaOCl solution into the probe led to peak of LDCL within a few seconds. Neopterin in the concentrations 10, 20, and 50 μM did not inhibit LDCL in the reaction of NaOCl with luminol (data not shown).

When monitoring natural fluorescence of neopterin in the MPO/H_2O_2 system, the intensity of fluorescence did not change during 20 min (data not shown).

4. Discussion

Stimulated neutrophils of healthy donors generate various reactive oxygen and chlorine species, two main sources of these reactive compounds in neutrophils are enzymes NADPH-oxidase and MPO. Activation of the oxidative metabolism, known as the respiratory burst, firstly involves NADPH-oxidase. This enzymatic complex is able to generate O_2^{-}, which can dismutate into H_2O_2. MPO amplifies the toxic potential of H_2O_2 by producing reactive inter-

mediates. In the presence of chloride ion (Cl^-), the major product of MPO is HOCl [18].

Typical kinetic of LDCL of neutrophils activated by the attachment to glass surface has a two-stage character (Fig. 1). As discussed earlier [19,20], the first stage of this reaction is caused by activation of redox systems formed in plasma membrane. The second stage is associated with the involvement of intracellular enzymes, including MPO and/or NADPH-oxidase of secretory and specific granule membranes [19]. We have shown that neopterin induced additional chemiluminescence in suspensions of neutrophils (Fig. 1), which was not associated with the additional production of superoxide anions. To identify which enzyme can take part in neopterin-induced neutrophil response, we used NaN_3, a non-specific inhibitor of MPO and other heme enzymes. When we added NaN_3 (0.3 mM) in suspension of adhesive neutrophils (Fig. 1), we observed additional LDCL caused by neopterin (Fig. 1). At the same concentration, NaN_3 blocked production of reactive species in the MPO/H_2O_2/luminol system. Data reveal that the neopterin-induced additional production of reactive species was not associated with the activation of MPO.

Neopterin decreased formation of reactive species in the MPO/H_2O_2/luminol system in a concentration-dependent manner. Using scavengers DABCO, D-mannitol and SOD, we studied what kind of reactive species, involved in the MPO/H_2O_2/luminol system, neopterin acted on. The presence of D-mannitol or SOD in suspension did not change the curve shape of LDCL kinetics caused by neopterin. Neopterin appears to decrease $OH^{\cdot}$ and O_2^{-}, generated in the MPO/H_2O_2/luminol system. Data obtained using DABCO indicated that the injection of neopterin in the MPO/H_2O_2/luminol system resulted in an additional increase of O_2' compared with the control experiment. Thus both, the reproportioning of different types of reactive species against a background of total decreasing of ROS production, and the additional generating of O_2' are modulated by neopterin in the MPO/H_2O_2/luminol system.

The effects of neopterin were observed in neutrophils adhered to glass surfaces. This observation could be of particular interest, because neopterin derivatives have been found earlier to be capable of inducing cellular adherence via inducing the expression of intercellular adhesion molecule type 1 in the type II-like pneumocyte cell line L2 [21]. Although it is still unclear whether neopterin is also able to enhance adhesion of neutrophils, it appears to be a reasonable possibility that biochemical pathways induced in neutrophils by glass-adhesion and by neopterin are related.

It is known that activation of MPO is occurring by several simultaneous pathways with formation of an oxidized form of MPO, MPO compound 1 (MPO-1) [12]:

1. MPO + H_2O_2	→ MPO-1 + 2 H_2O	
2. MPO-1 + Cl^-	→ MPO-1-Cl	$k = 2.8 \times 10^6\ M^{-1}\ s^{-1}$
3. MPO-1-Cl + OH^-	→ MPO + HOCl	
4. MPO-1 + H_2O_2	→ MPO-2 + $O_2^{\cdot-}$	$k = 4.6 \times 10^4\ M^{-1}\ s^{-1}$

In our model system, the concentration of Cl^- is 10^{-1} M and the concentration of H_2O_2 is 10^{-5} M. We can conclude that the major product in the MPO/H_2O_2/luminol system is HOCl. Data suggest that effects of neopterin on neutrophils' MPO are associated with either inhibition of MPO (or MPO-1) or scavenging of HOCl. To verify the scavenging of HOCl by neopterin, we measured chemiluminescence in the NaOCl/luminol system. Because neopterin did not influence the intensity of LDCL, scavenging of HOCl by neopterin is improbable at component concentrations mentioned above. Natural fluorescence of neopterin in the MPO/H_2O_2 system was unchanged during the whole experiment. Data imply that neopterin took part in reaction without changes of its chemical structure.

The capacity of neopterin to modulate production and effects of specific reactive oxygen and chlorine species could be of clinical relevance, e.g., in patients after multiple trauma, activation of macrophages and of neutrophils is crucial in the pathogenesis of multiple organ failure. Interferon-γ is not only the key cytokine for the formation of neopterin by human macrophages, it also represents the key primer for ROS production in the cells [22]. Concentrations of neopterin and granulocyte elastase were found as useful parameters for the diagnosis of septicemia and monitoring the clinical course in septic patients [23]. Obviously, activation of both these cellular compartments of immune response is taking place concurrently. It appears that effects of macrophage product neopterin support effects related to ROS released from macrophages but slows down reactive chlorine species produced by MPO in neutrophils.

We conclude that there are at least two possible pathways to generate reactive species in neutrophils in which neopterin may participate: first, it induces additional generation of reactive species by adhered neutrophils, and second, it decreases the amount of reactive species generated by MPO via a direct influence on neutrophils' MPO, probably by inhibiting the enzyme. This assumption is well in line with earlier findings of Horejsi et al. [24], who demonstrated that neopterin derivative 7,8-dihydroneopterin is able to interfere with hemeproteins (metmyoglobin) and (met)hemoglobin.

Additional work is required to further specify the mechanism of MPO inhibition by neopterin.

Acknowledgements

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