Influence of neopterin on the generation of reactive oxygen species in human neutrophils

Julia A. Razumovitch[a,*], Galina N. Semenkova[a], Dietmar Fuchs[b], Sergey N. Cherenkevich[a]

[a] *Department of Biophysics, Physics Faculty, Belarus State University, Scoryny avenue 4, 220050 Minsk, Belarus*
[b] *Institute of Medical Chemistry and Biochemistry, University of Innsbruck, and Ludwig Boltzmann Institute of AIDS Research, Fritz Pregl Straße 3, A-6020 Innsbruck, Austria*

Received 15 April 2003; revised 27 June 2003; accepted 4 July 2003

First published online 22 July 2003

Edited by Vladimir Skulachev

Abstract Neopterin is synthesized by human monocyte-derived macrophages primarily upon stimulation with the cytokine interferon-γ. We studied the influence of neopterin on the generation of reactive oxygen species (ROS) in human peripheral blood neutrophils. Radical formation was measured using a biochemiluminometer. Neutrophils were isolated from peripheral blood of healthy donors. The generation of ROS by neutrophils suspended in Earl's solution (pH = 7.4) at 37°C was investigated by monitoring of chemiluminescence using luminol and lucigenin as light emitters. Neopterin induced chemiluminescence in suspensions of neutrophils in the presence of luminol, but not of lucigenin. Neopterin affected only adhesive cells. Addition of neopterin into the suspension of the cells involving D-mannitol, L-histidine and diazabicyclo[2.2.2]octane (DABCO) decreased luminol-dependent chemiluminescence (LDCL) of the neutrophils. The action of superoxide dismutase (SOD) and 2-phenyl-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide (PTIO) reduced neopterin-induced LDCL of neutrophils. Data suggest that neutrophils respond on exposure to neopterin with additional generation of singlet oxygen, hydroxyl radical and nitric oxide by nicotinamide adenine dinucleotide phosphate (NADPH)-independent pathways.

Key words: Neopterin; Neutrophil; Chemiluminescence; Luminol; Lucigenin; Singlet oxygen

1. Introduction

Neopterin is a pyrazino-pyrimidine derivative, namely 2-amino-4-hydroxy-(1′,2′,3′-trihydroxypropyl)-pteridine, biosynthetically derived from guanosine triphosphate (GTP). Enzyme GTP-cyclohydrolase I, which is inducible by cytokine interferon-γ, converts GTP to 7,8-dihydroneopterin triphosphate, which in turn is transformed to 7,8-dihydroneopterin and neopterin in human/primate macrophages and dendritic cells [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 human immunodeficiency virus (HIV) infection or malignant diseases [3,4].

Experiments have shown that neopterin is capable to enhance the oxidative potential of reactive oxygen species (ROS) produced from immunocompetent cells [5–7]. In parallel, neopterin was found to also inhibit activity of xanthine oxidase and reduced nicotinamide adenine dinucleotide phosphate (NADPH)-oxidase [8–10]. Therefore neopterin can be a direct participant 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. It is know that neutrophils of human peripheral blood generate ROS when activated, for example, by adhesion on glass or influence of a number of phagocytosis-stimulating factors [11]. This study provides first results on the influence of neopterin to interfere with neutrophils to generate ROS.

2. Materials and methods

2.1. Chemicals

Superoxide dismutase (SOD), 2-phenyl-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide (PTIO), D-mannitol, luminol (5-amino-2,3-dihydro-1,4-phthalazinedione), and lucigenin (bis-*N*-methylacridinium nitrate) were from Sigma (Munich, Germany). L-histidine (L-α-amino-β-[4-imidazolyl]propionic acid) was from Reanal (Budapest, Hungary). Diazabicyclo[2.2.2]octane (DABCO) was from Merck (Darmstadt, Germany). Neopterin was from Dr. Schircks Laboratories (Jona, Switzerland).

2.2. Experimental procedure

Neutrophils were isolated from heparinized peripheral blood of healthy donors by centrifugation using Ficoll-verografin density gradient [12]. 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, and 10^6 cells/ml were stored at 4°C.

The generation of ROS in neutrophils was studied by a luminol- and lucigenin-dependent chemiluminescence method with use of biochemiluminometer BCL-1 (Belarus State University, Belarus) [13]. Luminol-dependent chemiluminescence (LDCL) was studied under adhesion of cells to the bottom of a cylindrical glass cuvette (diameter 40 mm). The volume of the cellular suspension was 2.4 ml, the concentration of neutrophils in the cuvette was 10^5 cells/ml.

Neopterin (250 μM) was freshly dissolved in 0.15 M NaCl by incubation for 1 h at 37°C. Measurements were carried out in Earl's

*Corresponding author.
E-mail address: julikby@yahoo.com (J.A. Razumovitch).

Abbreviations: DABCO, diazabicyclo[2.2.2]octane; GTP, guanosine triphosphate; LDCL, luminol-dependent chemiluminescence; PTIO, 2-phenyl-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide; ROS, reactive oxygen species; SOD, superoxide dismutase

0014-5793/03/$22.00 © 2003 Published by Elsevier B.V. on behalf of the Federation of European Biochemical Societies.
doi:10.1016/S0014-5793(03)00796-8

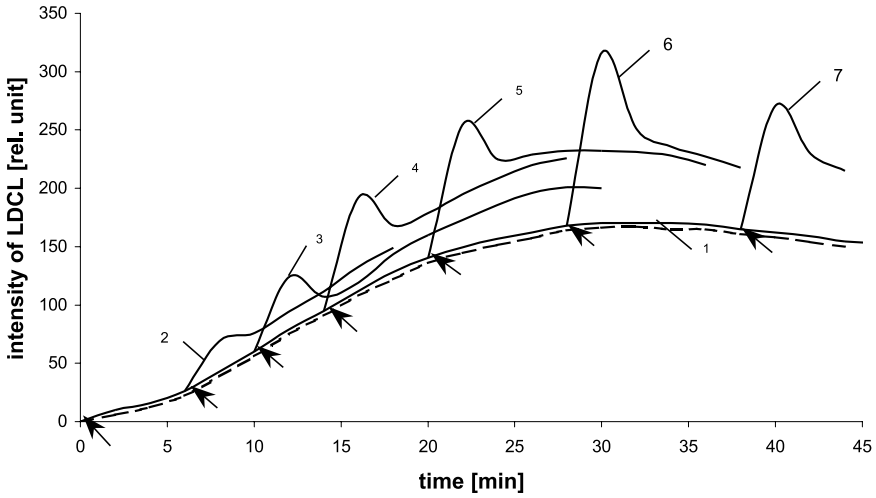

Fig. 1. Total intensity of luminol-induced chemiluminescence in control neutrophils and after addition of neopterin (arrows indicate the moment of neopterin addition).

solutions (pH = 7.2) by addition of 5 mM $CaCl_2$ at 37°C. Concentration of luminol and lucigenin was 0.5 μM. The bottom of the cuvette was covered by a layer of 20% Ficoll solution in 0.15 M NaCl (thickness of layer 2 mm) in the experiments with impeding the adhesion of cells. The ROS scavengers SOD, PTIO, DABCO, D-mannitol, L-histidine and their combinations were used. All scavengers were dissolved in the 0.15 M NaCl in the following concentrations, which had been determined in pilot experiments: 0.3 μM SOD, 32 μM PTIO, 0.5 mM D-mannitol, 0.2 mM DABCO, 0.3 mM L-histidine.

2.3. Calculation

The cell mixtures 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 are presented in Fig. 1 (curve 1). Fig. 1 also summarizes the effects of neopterin on LDCL of neutrophils during their adhesion to glass (curves 2–7). The kinetics of neopterin-induced LDCL responses in cells depended on the time after beginning of neutrophil adhesion and had two phases. The first maximum light output was achieved 2–3 min after addition of neopterin to the cells. The second phase appeared after 4–5 min from the time when neopterin was added. The appearance of the second phase depended on the concentration of neopterin (see below) and the time from addition of neopterin in the cuvette. The second phase was characterized by a lower rate of ROS generation and longer duration as compared with the first phase. Neopterin affected only adhesive cells and/or cells generating ROS. In conditions impeding the adhesion of cells (neutrophils stratified on 20% Ficoll solution), generation of ROS did not take place (data not shown).

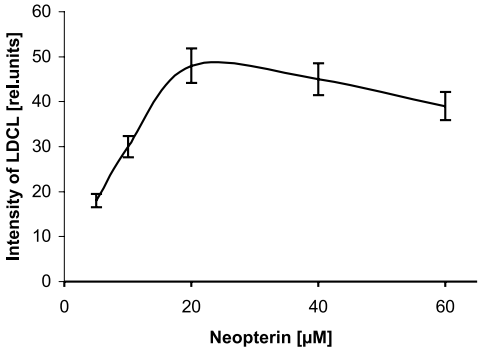

Fig. 2. Dose-dependent change of maximal intensity of luminol-induced chemiluminescence (first phase) in neutrophils (increasing compared to controls without neopterin added).

Neopterin enhanced ROS formation in a concentration- and time-dependent manner (Fig. 2). 20 μM concentration was selected as working solution, concentrations lower than 5 μM of neopterin had no significant effect.

Changes in chemiluminescence intensity of neutrophil suspensions upon addition of neopterin were observed in the presence of luminol, but not of lucigenin.

Superoxide scavenger SOD and nitric oxide scavenger PTIO decreased LDCL. However, only the second phase of neutrophil responses was inhibited whereas the first phase remained unchanged (Fig. 3). Singlet oxygen scavenger DABCO in cellular suspensions led to noticeable increase of LDCL intensity during neutrophil adhesion. Hydroxyl radical scavenger D-mannitol and L-histidine (scavenger of hydroxyl radical and singlet oxygen) insignificantly decreased this readout (data not shown). Addition of neopterin into the suspension of the cells containing D-mannitol, L-histidine and DABCO decreased the intensity of LDCL of neutrophils (Fig. 4).

4. Discussion

Stimulated neutrophils of healthy donors are known to generate ROS. This effect can be studied by monitoring of LDCL. Typical kinetic curves of LDCL of neutrophils activated by the attachment to glass surface had a two-stage character. As discussed earlier [14,15] the first stage was caused by activation of redox systems formed in plasma membrane. NADPH-oxidase localized in the plasma membrane reduced oxygen to superoxide anion radicals, which spontaneously dismuted to hydrogen peroxide and oxygen [16]. Superoxide anion and hydrogen peroxide were precursors for many other reactive species such as singlet oxygen, hydroxyl radicals, hypochlorous acid which could interact with

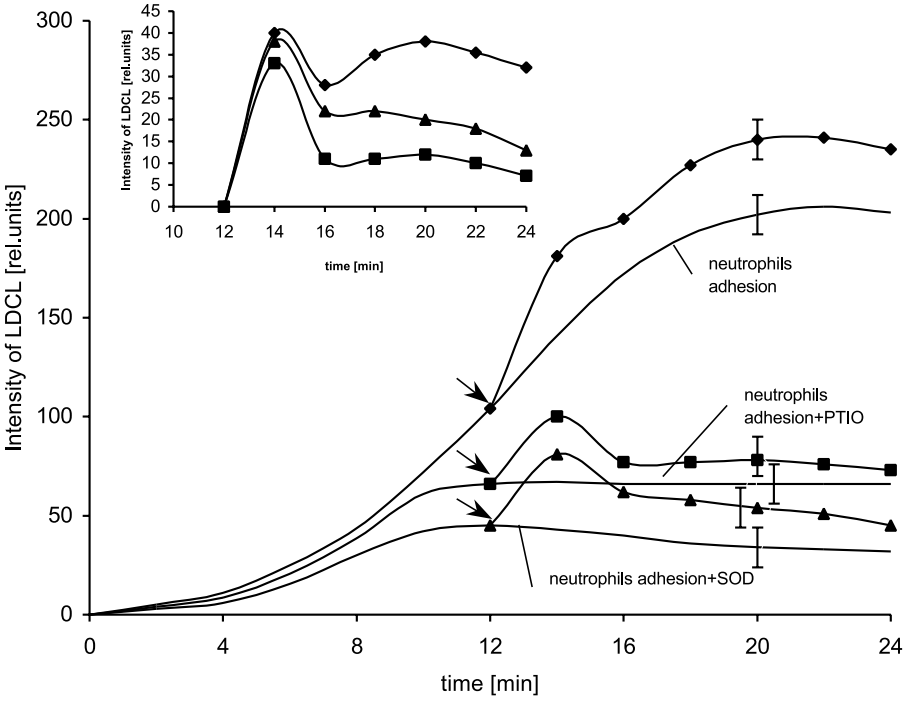

Fig. 3. Typical kinetics of luminol-induced chemiluminescence of neutrophils under adhesion to glass (neutrophil adhesion) and addition of neopterin (diamonds), influence of SOD on neutrophil adhesion (neutrophil adhesion+SOD) and on addition of neopterin (triangles) and influence of PTIO on neutrophil adhesion (neutrophil adhesion+PTIO) and on addition of neopterin (squares). Arrows indicate the moment of neopterin addition. The insert depicts the relative magnitude of neopterin-induced changes of luminol-induced chemiluminescence in neutrophils.

each other and with cell components [17]. The second stage is associated with the involvement of intracellular enzymes, including myeloperoxidase system or NADPH-oxidase of secretory and specific granule membranes [14]. The first stage continued for about 6 min and we could not observe any influence of neopterin at this stage. The second stage reached a maximum from 30–40 min after the beginning of adhesion. The ratio of these stages could change in different donors due to individual properties. Our data demonstrate that neopterin influences the generation of ROS by neutrophils only at the second stage (Fig. 1). The greatest response was obtained at the maximum of the second stage, i.e. at maximal ROS generation of neutrophils.

Lucigenin is a specific indicator for the production of superoxide anions. Lucigenin-dependent chemiluminescence was absent in neutrophils at the action of neopterin (data not shown), which excludes any participation of NADPH-oxidase of plasma membrane in the response of neutrophils. This is confirmed also by the finding that SOD did not inhibit the first phase of neopterin-induced neutrophil response. SOD cannot penetrate through plasma membranes and therefore can interact only with superoxide which is produced by enzymes at the cell surface or which penetrates through cell membranes. Lack of inhibitory action of SOD on the first phase of neopterin-induced neutrophil LDCL could indicate that NADPH-oxidase localized in the plasma membrane did not participate in neopterin-induced ROS generation in this period. Considerable reduction of LDCL intensity at the second phase of response rather is associated with superoxide production by intracellular enzymatic systems.

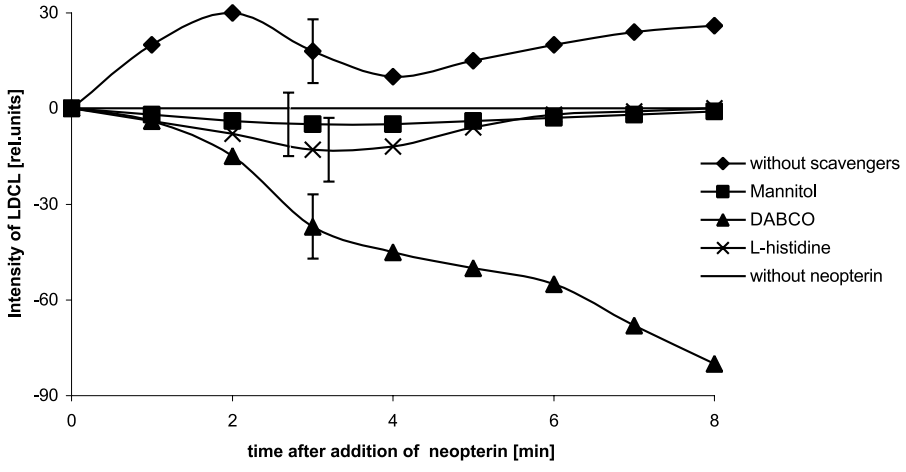

Fig. 4. Effects of neopterin on luminol-induced chemiluminescence in the absence and in the presence of scavengers of ROS.

PTIO significantly decreased LDCL intensity of neutrophils. Data indicated the participation of nitric oxide in neopterin-induced cell response. Still PTIO did not influence neopterin-induced lucigenin-dependent chemiluminescence. With this respect it might be of relevance that neopterin was found earlier to induce nitric oxide synthase gene expression and nitric oxide production in rat vascular smooth muscle cells [18]. Peroxynitrite is the product of the reaction of nitric oxide with superoxide and it can serve as the important mediator of tissue injury during conditions associated with enhanced nitric oxide and superoxide production [19]. If superoxide, acquired by neopterin action, had been completely transformed into peroxynitrite, lucigenin-dependent chemiluminescence would have been increased by PTIO. The absent influence of PTIO on neopterin-induced lucigenin-dependent chemiluminescence proved that neopterin did not activate superoxide-generating system in neutrophils.

The neutrophil response on neopterin was greatly decreased in the presence of D-mannitol. This fact suggests a contribution of hydroxyl radical in neopterin-induced LDCL in neutrophils. Similar results were received in the presence of L-histidine, scavenger of hydroxyl radical and singlet oxygen. It was known that superoxide anion is both a source and an efficient quencher of singlet oxygen [20]. Therefore an increase of neutrophil LDCL intensity during adhesion in the presence of DABCO could explain enlarged superoxide amount in a cuvette. Decrease of LDCL intensity of neutrophil suspension containing DABCO after the addition of neopterin could be explained by neopterin activation of singlet generation pathway. The influence of scavengers shows that neopterin induces singlet oxygen and hydroxyl radical generation in neutrophils. Interestingly spontaneous formation of hydroxyl radicals in aqueous solutions of neopterin derivatives has been demonstrated earlier also in cell-free experiments using, e.g. electron spin resonance [21,22]. Recently, 6-substituted pteridines including neopterin were found to induce singlet oxygen in also aqueous solutions [23].

The observation that neopterin, a product of monocyte-derived macrophages upon stimulation with interferon-γ, is able to induce singlet oxygen in granulocytes is of special interest. It is well in line with the observation that Th1-type cytokine interferon-γ enhances antifungal activity of granulocytes by means of enhancing oxidative burst [23]. Data add also to the observation that neopterin is able to enhance the production and efficacy of toxic compounds during immune response [5–10]. Likewise, neopterin concentrations have been found to be associated with measures of oxidative stress in various diseases such as infection, autoimmunity and malignancy [24], and neopterin concentrations correlate with the course of such diseases and predict prognosis [3,4,25].

In summary, the results of this study suggest that neopterin does not activate NADPH-oxidase of plasma membrane in the neutrophils and that the neutrophils respond on exposure to neopterin with additional generation of singlet oxygen, hydroxyl radical and nitric oxide by NADPH-independent pathways.

References

[1] Huber, C., Batchelor, J.R., Fuchs, D., Hausen, A., Lang, A., Niederwieser, D., Reibnegger, G., Swetly, P. and Troppmair, J. (1984) J. Exp. Med. 160, 310–316.
[2] Wirleitner, B., Reider, D., Ebner, S., Böck, G., Widner, B., Jaeger, M., Schennach, H., Romani, N. and Fuchs, D. (2002) J. Leukocyte Biol. 72, 1148–1153.
[3] Fuchs, D., Hausen, A., Reibnegger, G., Werner, E., Dierich, M. and Wachter, H. (1988) Immunol. Today 9, 150–154.
[4] Fuchs, D., Weiss, G. and Wachter, H. (1992) Crit. Rev. Clin. Lab. Sci. 29, 307–341.
[5] Weiss, G., Fuchs, D., Hausen, A., Reibnegger, G., Werner, E.R., Werner-Felmayer, G., Semenitz, E., Dierich, M.P. and Wachter, H. (1993) FEBS Lett. 321, 89–92.
[6] Murr, C., Fuchs, D., Gössler, W., Hausen, A., Reibnegger, G., Werner, E.R., Werner-Felmayer, G., Esterbauer, H. and Wachter, H. (1994) FEBS Lett. 338, 223–226.
[7] Widner, B., Baier-Bitterlich, G., Wede, I., Wirleitner, B. and Fuchs, D. (1998) Biochem. Biophys. Res. Commun. 248, 341–346.
[8] Kojima, S., Nomura, T., Icho, T., Kajiwara, Y., Kitabatake, K. and Kubota, K. (1993) FEBS Lett. 329, 125–128.
[9] Wede, I., Altindag, Z., Widner, B., Wachter, H. and Fuchs, D. (1998) Free Radic. Res. 29, 331–338.
[10] Oettl, K. and Reibnegger, G. (1999) Biochim. Biophys. Acta 1430, 387–395.
[11] Zakrevskaya, Y.V., Semenkova, G.N., Cherenkevich, S.N., Murzenok, P.P. and Gurin, V.N. (1993) Rep. Belarus Sci. Acad. 37 (6), 79–81.
[12] Böyum, A. (1976) Scand. J. Immunol. 5, 9–18.
[13] Vetohin, C.C., Semenkova, G.N. and Cherenkevich, S.N. (1986) in: Luminescent Analysis in Medicine and Biological Investigations, Medical Institute of Riga, pp. 81–5.
[14] Kovalenko, E.I., Semenkova, G.N., Cherenkevich, S.N. and Smirnova, E.N. (2001) in: Chemiluminescence at the Turn of the Millennium, Schweda-Werbedruck, Dresden, pp. 112–117.
[15] Semenkova, G.N., Smirnova, E.N., Kovalenko, E.I., Gerein, V., Savanovich, I.I. and Cherenkevich, S.N. (1997) Exp. Oncol. 19, 153–156.
[16] Babior, B.M. (1999) Blood 93, 1464–1476.
[17] Dröge, W. (2002) Physiol. Rev. 82, 47–95.
[18] Schobersberger, W., Hoffmann, G., Grote, J., Wachter, H. and Fuchs, D. (1995) FEBS Lett. 377, 461–464.
[19] Jourd'heuil, D., Jourd'heuil, F.L., Kutchukian, P.S., Musah, R.A., Wink, D.A. and Grisham, M.B. (2001) J. Biol. Chem. 276, 28799–28805.
[20] Steinbeck, M.J., Khan, A.U. and Karnovsky, M.J. (1992) J. Biol. Chem. 267, 13425–13433.
[21] Oettl, K., Wirleitner, B., Baier-Bitterlich, G., Grammer, T., Fuchs, D. and Reibnegger, G. (1999) Biochem. Biophys. Res. Commun. 264, 262–267.
[22] Wirleitner, B., Czaputa, R., Oettl, K., Böck, G., Widner, B., Reibnegger, G., Baier, G., Fuchs, D. and Baier-Bitterlich, G. (2001) Immunobiology 203, 629–641.
[23] Thomas, A.H., Lorente, C., Capparelli, A.L., Martinez, C.G., Braun, A.M. and Oliveros, E. (2003) Photochem. Photobiol. Sci. 2, 245–250.
[24] Roilides, E., Uhlig, K., Venzon, D., Pizzo, P.A. and Walsh, T.J. (1993) Infect. Immun. 61, 1185–1193.
[25] Murr, C., Fuith, L.C., Widner, B., Wirleitner, B., Baier-Bitterlich, G. and Fuchs, D. (1999) Anticancer Res. 19, 1721–1728.