ELSEVIER

FEBS Letters 338 (1994) 223–226

FEBS 13607

FEBS LETTERS

Enhancement of hydrogen peroxide-induced luminol-dependent chemiluminescence by neopterin depends on the presence of iron chelator complexes

Christian Murr[a], Dietmar Fuchs[a], Walter Gössler[b], Arno Hausen[a], Gilbert Reibnegger[a], Ernst R. Werner[a], Gabriele Werner-Felmayer[a], Hermann Esterbauer[c], Helmut Wachter[a,*]

[a]*Institute for Medical Chemistry and Biochemistry, University of Innsbruck, Fritz Pregl Str. 3, A-6020 Innsbruck, Austria*
[b]*Institute for Analytical Chemistry, University of Graz, A-8010 Graz, Austria*
[c]*Institute for Biochemistry, University of Graz, A-8010 Graz, Austria*

Received 14 December 1993

Abstract

We have previously shown that neopterin, 6-D-erythro-trihydroxypropyl-pteridine, synthesized by human monocytes/macrophages upon stimulation by interferon-γ, enhances toxicity of reactive oxygen at neutral or slightly alkaline pH (7.5), but not at acidic pH (below 6.5). In the present study, we explored in more detail the necessary requirements for neopterin to modulate the effects of hydrogen peroxide in a luminol-dependent chemiluminescence assay. We demonstrate that neopterin enhances hydrogen peroxide effects only in the presence of iron chelator complexes like iron-(III)- or iron-(II)-EDTA or iron-(III)-DTPA. Thus, iron chelator complexes together with neopterin may play an important role in macrophage-mediated effector mechanisms.

Key words: Neopterin; Macrophage; Hydrogen peroxide; Iron; Chelator

1. Introduction

Activated macrophages produce, besides a lot of other secretory products, reactive metabolites of oxygen such as hydrogen peroxide, superoxide, hydroxyl radicals and possibly singlet oxygen [1]. Hydrogen peroxide is a central molecule in mediating macrophage-mediated extracellular cytolysis [2]. Upon stimulation by interferon-γ, macrophages produce and release large amounts of neopterin [3], which is synthesized from guanosine triphosphate (GTP); GTP is converted by GTP-cyclohydrolase I (EC 3.5.4.16) to 7,8-dihydroneopterin triphosphate. Because human macrophages in contrast to a variety of other cells have only a very low activity of 6-pyruvoyl tetrahydropterin synthase, the second enzyme in tetrahydrobiopterin biosynthesis, 7,8-dihydroneopterin triphosphate accumulates and is cleaved by phosphatases to 7,8-dihydroneopterin [4], which is then partly oxidized to neopterin. Elevations of neopterin have been found in viral infections, various malignant disorders, autoimmune diseases and during allograft rejection episodes [5–11]. From these studies neopterin has turned out to be a useful marker for monitoring of activation of cellular immunity in patients, but so far no biological role of neopterin has been clarified.

However, Heales et al. [12] showed that 7,8-dihydroneopterin scavenges luminol-dependent chemiluminescence of zymosan-activated human macrophages, and Kojima et al. [13] found a scavenging activity by neopterin on superoxide radical anion. On the other hand, we demonstrated [14] that neopterin enhances hydrogen peroxide and chloramine T activity in a luminol-dependent chemiluminescence assay and also strenghtens toxicity of these agents against bacteria at slightly alkaline pH (pH 7.5), while 7,8-dihydroneopterin was shown to be a scavenger independently from pH value. Finally Kojima et al. [15] reported a suppression of superoxide-generating NADPH-oxidase by neopterin using macrophages stimulated with phorbol myristate acetate. In order to clarify these seemingly contradictory findings about the interaction between neopterin and activated oxygen species, we investigated in more detail which requirements govern the influence of neopterin on hydrogen peroxide-induced luminol-dependent chemiluminescence.

*Corresponding author. Fax: (43) (512) 507 2279.

SSDI 0014-5793(93)E1524-P

2. Materials and methods

2.1. Reagents

Dulbecco's phosphate-buffered saline (PBS) was purchased from Serva Co., Ltd. (Heidelberg, Germany) and Sigma Chemicals Co., Ltd. (St. Louis, MO), phosphate-buffered saline from BioMérieux (Marcy l'Etoile, France). Disodium hydrogen phosphate, potassium dihydrogen phosphate, sodium chloride, potassium chloride, dimethyl sulfoxide (DMSO), iron-(II)-sulfate, iron-(III)-chloride, ethylenediamine tetraacetic acid disodium salt (EDTA), diethylenetriamine pentaacetic acid (DTPA), potassium hexacyanoferrate-(III), tin-(II)-chloride, barium chloride and manganese chloride were from Merck (Darmstadt, Germany) at the highest purity grade available. Luminol (5-amino-2,3-dihydro-1,4-phthalazinedione) was also from Sigma. D-Erythro-neopterin was obtained from Dr. B. Schircks laboratories (Jona, Switzerland).

2.2. Preparation of buffer solutions

Effects of neopterin were investigated in four solutions from distinct manufacturers. Buffer salts from Serva (= solution A), that we have been using in previous studies [14], buffer salts from Sigma (= solution C) and BioMérieux (= solution D) were dissolved in distilled water according to preparation instructions by the manufacturers, yielding a final concentration of 4.2 mM potassium, 153 mM sodium, 140 mM chloride, 1.47 mM dihydrogen phosphate and 8.1 mM hydrogen phosphate in solutions A and C, respectively. Additionally a buffer solution was prepared with high grade purity reagents from Merck (= solution B) in identical final concentrations of ions as mentioned above. Solution D was registrated by the manufacturer to contain 150 mM phosphate-buffered saline. If necessary, pH was adjusted to pH 7.5 by addition of hydrochlorous acid or sodium hydroxide.

For further investigations solution B was supplemented with aqueous solutions of iron-(II)-sulfate (= solution a), iron-(III)-chloride (= solution b), iron-(II)-sulfate and EDTA (= solution c), iron-(III)-chloride and EDTA (= solution d), iron-(III)-chloride and DTPA (= solution e), and potassium hexacyanoferrate-(III) (= solution f) maintaining an iron concentration of 2.3 μM and an EDTA or DTPA concentration of 4.6 μM.

2.3. Luminol-dependent measurement of chemiluminescence

Neopterin was dissolved in solutions A, B, C or D at pH of 7.5 yielding a final concentration of 30 μM. Luminol was dissolved in DMSO at a concentration of 10 mM and a 40 μM working solution was freshly prepared by dilution with solution A–D or a–f. 120 μl of luminol solution and 600 μl of solutions A–D and a–f without (= control) and with neopterin were incubated for 60 s in microcuvettes at 37°C in a luminometer (LKB 1251, Pharmacia, Piscataway, NJ). 140 μl of 1% hydrogen peroxide (0.29 M) solution were injected and light output was measured for 30 s at 37°C. Maximum light output of samples was compared to that of controls.

2.4. Analysis of elements

Analysis of elements was done by inductively coupled plasma mass spectrometry (ICP-MS) on a VG Plasma Quad 2 Turbo Plus (VG Elemental Ltd., Winsford, UK), equipped with a Gilson Miniplus-2 peristaltic pump, a Meinhard nebulizer (type TR-30-A3) and a water-cooled (5°C) Scott-type double pass spray chamber. 1 ml of each sample was diluted with 8.4 ml of distilled water after addition of 500 μl of double distilled nitric acid and 100 μl of a 87 μM indium solution (supplied by Merck, Darmstadt, Germany). A blank solution was prepared by dissolving 1.7 mmol sodium chloride in 10 ml of distilled water after addition of 500 μl of a double distilled nitric acid and 100 μl of a 87 μM indium solution. Data were quantified with respect to a single internal standard (indium) and corrected for mass bias by means of a measured mass response curve. For the mass response curve a multi-element standard solution containing magnesium, cobalt, indium, lanthanum, lead, bismuth and uranium with a concentration of 100 μg/l was measured. To compensate matrix effects this standard solution was made with a concentration of 17 mM sodium chloride. According to the indium counts found in the sample solutions all concentrations of the other elements were calculated with the response curve. From each sample the counts of the blank solution mentioned above were subtracted.

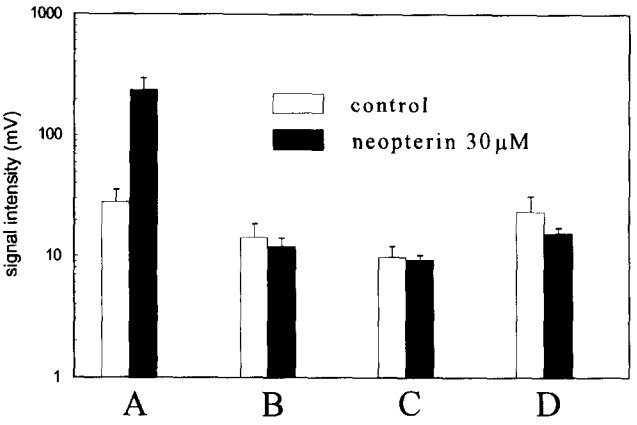

Fig. 1. Effect of neopterin on hydrogen peroxide-induced luminol-dependent chemiluminescence in different phosphate buffers (A, Serva; B, Merck; C, Sigma; D, BioMérieux) at pH 7.5. Light output after addition of hydrogen peroxide (final concentration 53 mM) to luminol and the buffer solution was used as control. Peak values of the investigated samples without (control) and with neopterin are shown. Plots show mean values (mean + one standard deviation) in a logarithmic scale for two independent experiments each of which was performed in duplicate.

3. Results

As pointed out in Fig. 1, neopterin enhances hydrogen peroxide-induced chemiluminescence about ninefold using solution A. In contrast there was no effect of neopterin in solution C, solution B and D showing even a slight scavenging effect of neopterin.

An ICP-MS analysis of the four solutions confirmed that these solutions contained certain elements in varying concentrations. As seen in Table 1, solution A has a higher content of iron, manganese, tin and barium than all other solutions. Other differences of elements of solutions A–D did not appear to be associated with distinct results obtained in luminol reaction.

When we supplemented solution B with iron-(II)-sulfate, iron-(III)-chloride, manganese chloride, tin-(II)-chloride or barium chloride alone and in various combinations, we could not find any striking influence on the effect induced by neopterin in hydrogen peroxide-induced luminol-dependent chemiluminescence (results of iron supplementation are shown in Fig. 2).

In contrast, when we supplemented solution B with iron-(II)-sulfate or iron-(III)-chloride in combination with EDTA, neopterin enhanced hydrogen peroxide-induced chemiluminescence eight- and eleven-fold, respectively (Fig. 2). In case of supplementation with iron-(III)-chloride in combination with DTPA, there was a twelvefold enhancement of the signal. Iron supplementation of solution B with potassium hexacyanoferrate-(III) caused an immense increase of the signal intensity compared to controls without iron, however, addition of neopterin resulted in scavenging but not enhancing chemiluminescence.

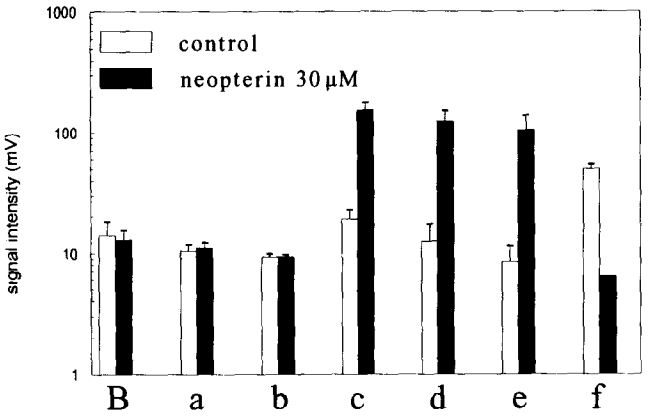

Fig. 2. Effect of neopterin on hydrogen peroxide-induced luminol-dependent chemiluminescence of solution B as control and after supplementation of solution B with iron-(II)-sulfate (a), iron-(III)-chloride (b), iron-(II)-sulfate and EDTA (c), iron-(III)-chloride and EDTA (d), iron-(III)-chloride and DTPA (e), and potassium hexacyanoferrate-(III) (f).

4. Discussion

Our data clearly indicate that enhancement of hydrogen peroxide-induced luminol-dependent chemiluminescence by neopterin requires an iron chelator complex. In preparations of buffer solutions using high purity grade reagents (solution B) neopterin did not exhibit any enhancing effect. Similarly, upon supplementation of these preparations by iron salts, no effect was observed. Only solution A (Serva) was capable of enhancing chemiluminescence. Therefore we suspected that solution A contained not only iron but also a certain so far not identified chelator. Indeed, supplementing solution B with iron ions and a chelating agent resulted in similar effects as those observed in solution A. A possible explanation for the necessity of an iron chelator could be that especially iron-(III) is poorly soluble in aqueous solution at pH 7.5, since hydrated polynuclear iron complexes are formed which readily precipitate, and a chelator seems to be necessary to keep iron ions in dissolved form. The presence of one aquo coordination site, remaining after complex formation, is necessary for the ability of chelated iron to catalyze the formation of hydroxyl radicals by the Haber–Weiss reaction [16,17]. However, it is obviously not essential for the enhancement of hydrogen peroxide-induced luminol-dependent chemiluminescence by neopterin, since EDTA (pentadentate ligand) and DTPA (hexadentate ligand) show essentially the same effects.

Our data may provide an additional explanation of the contradictory observations in literature about the interaction of neopterin and reactive oxygen species: On the one hand, neopterin enhances toxicity by reactive oxygen species only at neutral or slightly alkaline pH (7.5) but not at acidic pH (below 6.5) as described earlier [14]. On the other hand, the presence of certain iron chelator complexes seems to be necessary for the enhancement of hydrogen peroxide toxicity by neopterin and may therefore play an important role in macrophage-mediated effector mechanisms. In contrast to this observations, in chloramine T-induced luminol-dependent chemiluminescence the enhancement by neopterin was independent from the presence of iron chelator complexes. Using chloramine T we observed that neopterin enhanced signal strength not only in buffer solution A, but also in solution B, where neopterin was unable to enhance hydrogen peroxide-mediated reaction. The scavenging effect of 7,8-dihydroneopterin in hydrogen peroxide-induced luminol-dependent chemiluminescence did not depend on pH value and the presence of iron chelator complexes (unpublished observations).

Further investigations of the mechanism of enhancement of hydrogen peroxide-induced chemiluminescence by neopterin and the role of biologically important chelators of iron are currently under way.

Table 1
Concentrations in μM of elements in solutions A–D. Semiquantitative values obtained from two determinations are shown as means

Element	Solution A	Solution B	Solution C	Solution D
Mg	1.0	0.9	2.0	1.6
Al	1.1	3.6	12.8	12.7
P	5653	6285	4808	3257
Ca	1.8	9.4	8.2	*
Fe	2.1	*	*	*
Mn	0.8	*	0.02	0.05
Ni	0.2	0.1	0.4	0.7
Cu	0.2	0.3	0.7	0.6
Zn	0.5	0.1	0.2	0.5
Br	108	70	345	300
Ag	0.006	0.002	0.005	0.009
Sn	0.05	*	*	*
Ba	0.88	0.02	0.01	0.07
Pb	0.05	0.04	0.03	0.06

*denotes lower concentrations than the blank value.

Acknowledgements: This work was supported by the Austrian Funds 'Zur Förderung der Wissenschaftlichen Forschung', Project FWF 9257.

References

[1] Nathan, C.F., Murray, H.W. and Cohn, Z.A. (1980) N. Engl. J. Med. 303, 622–626.
[2] Nathan, C.F., Brukner, L.H., Silverstein, S.C. and Cohn, Z.A. (1979) J. Exp. Med. 149, 84–99.
[3] Huber, C., Batchelor, J.R., Fuchs, D., Hausen, A., Lang, A., Niederwieser, D., Reibnegger, G., Swetly, P., Troppmair, J. and Wachter, H. (1984) J. Exp. Med. 160, 310–316.
[4] Werner, E.R., Werner-Felmayer, G., Fuchs, D., Hausen, A., Reibnegger, G., Yim, J.J., Pfeiderer, W. and Wachter, H. (1990) J. Biol. Chem. 265, 3189–3192.
[5] Wachter, H., Fuchs, D., Hausen, A., Reibnegger, G. and Werner, E.R. (1989) Adv. Clin. Chem. 27, 81–141.

[6] Reibnegger, G., Egg, D., Fuchs, D., Günther, R., Hausen, A., Werner, E.R. and Wachter, H. (1986) Arthrit. Rheum. 29, 1063–1070.
[7] Weiss, G., Kronberger, P., Conrad, F., Bodner, E., Wachter, H. and Reibnegger, G. (1993) Cancer Res. 53, 260–265.
[8] Fuchs, D., Hausen, A., Reibnegger, G., Werner, E.R., Dierich, M.P. and Wachter, H. (1988) Immunol. Today 9, 150–155.
[9] Fahey, J.L., Taylor, J.M.G., Detels, R., Hofmann, B., Melmed, R., Nishanian, P. and Giorgi, J.V. (1990) N. Engl. J. Med. 322, 166–172.
[10] Reibnegger, G., Aichberger, C., Fuchs, D., Hausen, A., Spielberger, M., Werner, E.R., Margreiter, R. and Wachter, H. (1991) Transplantation 52, 58–63.
[11] Wachter, H., Fuchs, D., Hausen, A., Reibnegger, G., Weiss, G., Werner, E.R. and Werner-Felmayer, G. (1992) Neopterin: Biochemistry – Methods – Clinical Application, De Gruyter, Berlin/New York.
[12] Heales, S.J.R., Blair, J.A., Meinschad, C. and Ziegler, I. (1988) Cell. Biochem. Funct. 6, 191–195.
[13] Kojima, S., Icho, T., Kajiwara, Y. and Kubota, K. (1992) FEBS Lett. 304, 163–166.
[14] 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.
[15] Kojima, S., Nomura, T., Icho, T., Kajiwara, Y., Kitabatake, K. and Kubota, K. (1993) FEBS Lett. 329, 125–128.
[16] Klebanoff, S.J. (1992) in: Inflammation: Basic Principles and Clinical Correlates (Gallin, J.I., Goldstein, I.M. and Snyderman, R., Eds) Oxygen Metabolites from Phagocytes, pp. 541–588, Raven Press, New York.
[17] Graf, E., Mahoney, J.R., Bryant, R.G. and Eaton, J.W. (1984) J. Biol. Chem. 259, 3620–3624.