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Inhibitory effect of neopterin on NADPH-dependent superoxide-generating oxidase of rat peritoneal macrophages

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The effect of the oxidized form of neopterin (NP) on the NADPH-dependent superoxide-generating oxidase (NADPH-oxidase) was investigated in both whole-cell and cell-free activation systems by using peritoneal macrophages of rats which had received an intraperitoneal injection of mineral oil. In the whole-cell system, NP remarkably inhibited the generation of superoxides in macrophages stimulated with phorbol myristate acetate (PMA). NP also showed an significant suppression of the activation of superoxide-generating NADPH-oxidase in the cell-free system using solubilized membranes and sodium dodecyl sulfate (SDS) as a stimulant. The 50%-inhibitory concentration (IC_{50}) of NP was about 1 μM in both assay systems. In a kinetic study, competitive inhibition of the NADPH-oxidase by NP was observed in the cell-free system with a calculated inhibition constant (K_i) of 6.50 μM. These findings suggest that NP may play an important role in the suppression of superoxide generation via the inhibition of the NADPH-oxidase in phagocytes.

Neopterin; Antioxidant; Superoxide; NADPH-oxidase

1. INTRODUCTION

Neopterin (NP) is an α-amino-hydroxypteridine derivative, and a precursor of biopterin (BP), which is derived from guanosine triphosphate (GTP) [1]. Namely, intracellular GTP is cleaved by GTP-cyclohydrolase to 7,8-dihydroneopterin triphosphate (NPH2TP). This compound is further converted into tetrahydrobiopterin (BPH4) by the actions of two enzymes, 6-pyruvoyltetrahydropterin synthase and sepiapterin reductase, in succession. This pathway has been established in liver, neuroendocrine tissues, and lymphocytes [2]. Human monocytes/macrophages, however, lack 6-pyruvoyltetrahydropterin synthase. As a consequence, these cells accumulate NPH2TP and excrete 7,8-dihydroneopterin (NPH2) and NP after cleavage of the phosphate moiety by phosphatase. It has been generally accepted that NP is released from monocytes/macrophages, that interferon-γ (IFN-γ), one of the immuno-modulators produced by activated T cells, is the only inducer for NP secretion from macrophages [3,4], and that IFN-γ increases the intracellular concentration of GTP as well as the conversion of it to NP [5]. Although the biological function of NP remains unknown, NP is used as a clinical marker for the diagnosis of patients with various malignant disorders, as well as viral infections including AIDS [6–9].

On the other hand, active oxygen species such as superoxide anion radicals have recently been suggested to be potent pathogenic factors in various viral diseases, so that the pathogenesis may involve no direct viral cytotoxicity, but reactivity of active oxygen species released by lymphocytes [10,11]. That is, an excess amount of active oxygen species following overreaction of the immunodefense response against the virus-infected organs, may participate in the mechanism of tissue injuries observed in various types of viral diseases. Based on these reports, we suspected that NP might play an important role as an endogenous scavenger of active oxygen species in these viral diseases. In previous reports, we have demonstrated potent antioxidant activities of pteridines, particularly the reduced form of NP, i.e. 5,6,7,8-tetrahydroneopterin (NPH4) [12–14]. Recently, the antioxidant potency of the reduced form of pteridines has also been confirmed by Weiss et al. [15]. Our investigations have made it clear that NPH4 directly reacts with the released superoxide anion radicals in both phagocyte/phorbol myristate acetate (PMA) and xanthine/xanthine oxidase (X/XOD) reaction systems. On the other hand, NP, an oxidized form of

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Abbreviations: NP, neopterin; NPH4, 5,6,7,8-tetrahydroneopterin; NPH2, 7,8-dihydroneopterin; NPH2TP, 7,8-dihydroneopterin triposphate; GTP, guanosine triphosphate; BP, biopterin; BPH4, 5,6,7,8-tetrahydrobiopterin; IFN-γ, interferon-γ; PMA, phorbol myristate acetate; X/XOD, xanthine/xanthine oxidase; PKC, protein kinase C; SDS, sodium dodecyl sulfate; SOD, superoxide dismutase; FAD, flavin adenine dinucleiotide.

neopterin, shows potent antioxidant activity in the phagocyte/PMA-superoxide reaction system, but not in the X/XOD superoxide reaction system. Those previous findings indicated that NP might inhibit the process of superoxide generation in phagocytes.

In order to elucidate the mechanism of the inhibitory effect of NP on superoxide generation in phagocytes, the effect of NP on the NADPH-dependent superoxide-generating oxidase was investigated in rat peritoneal macrophages, since superoxide radicals are produced primarily through the activation of plasma membrane-bound NADPH oxidase (EC1.6.99.6) in phagocytes, such as lymphocytes and macrophages, upon stimulation with various agents, such as concanavalin A, PMA, phospholipase C, endotoxin and detergents.

2. MATERIALS AND METHODS

2.1. *Reagents*

Cytochrome *c* from horse heart, β-NADPH (type I), phorbol myristate acetate (PMA), erythrocyte Cu, Zn-superoxide dismutase (SOD) (3,300 U/mg protein, grade IV), Hanks' balanced salt solution (HBSS), Percoll, and D-neopterin (NP) were purchased from Sigma Chemicals Co., Ltd (St. Louis, MO). 5,6,7,8-Tetrahydroneopterin (NPH4) was from Junsei Chemicals Co., Ltd. ATP, sodium deoxycholate, dimethyl sulfoxide (DMSO), EGTA, flavin adenine dinucleotide (FAD), glycerol, sucrose, sodium dodecyl sulfate (SDS), and PIPES were obtained from Wako Chemicals Co., Ltd. (Tokyo, Japan). Sodium deoxycholate was recrystallized from ethanol before use. All other chemicals used were of the highest purity grade available from commercial sources.

2.2. *Cell preparation and subfractionation*

Rats were injected intraperitoneally with 10 ml of mineral oil and exudates were harvested 5 days later. The cells were washed 3 times with HBSS by centrifugation at $350 \times g$ for 5 min. Erythrocytes were eliminated by hypotonic lysis. The cells suspension contained more than 90% macrophages.

2.3. *Solubilization of membranes and preparation of cytosol*

Solubilization of the membranes and preparation of the cytosol were basically done by the method of Umeki [16], with a slight modification. Briefly, cells were disrupted and homogenized by using a sonicator fitted with a titanium probe. The homogenate was freed of unbroken cells, nuclei, and large debris by centrifugation of $800 \times g$ for 10 min at 4°C, and the supernatant was subjected to subcellular fractionation. It was centrifuged at $48,000 \times g$ for 1 hr at 4°C in a fixed-angle rotor (SRP 70AT-230) of a Hitachi ultracentrifuge (SCP85H2). The pellet was resuspended in ice-cold phosphate-buffered saline (PBS)-sucrose buffer which was composed of 131 mM NaCl, 8 mM Na,K-phosphate buffer, pH 7, and 340 mM sucrose, to the original volume of the homogenate by sonication for 3 s and subjected to stepwise Percoll gradient fractionation [17]. The membranes were obtained by pooling the γ-fraction ($230,000 \times g$ for 2 h at 4°C). The pooled membranes were suspended at a concentration of 3×10^8 cells eq/ml in half-strength Borregaard's relaxation buffer, which contains 50 mM KCl 1.5 mM NaCl, 5 mM PIPES, pH 7.3, 1.75 mM $MgCl_2$, 0.5 mM ATP, 0.62 mM EGTA, and 0.34 M sucrose. The suspension was divided into aliquots and stored at −70°C. The membrane suspension was thawed at room temperature, mixed with an equal volume of extraction buffer containing 1 mM NaN_3, 0.0017 mM $CaCl_2$, 20 mM sodium glycinate (pH 8.0), 2.33% sodium deoxycholate (w/v), and 50% glycerol (v/v), vortexed briefly, incubated on ice for 30 min with occasional agitation, and finally centrifuged at $435,000 \times g$ for 1 h at 4°C. The supernatant from the $230,000 \times g$ spin was also divided into aliquots and stored at −70°C. This material served as the source of cytosolic activation factor. Protein content was assayed in macrophage homogenates and subcellular fractions by the methods of Lowry et al. [18].

2.4. *Assay of NADPH-dependent superoxide production*

Superoxide production by intact PMA-stimulated macrophages (whole-cell system) was assayed by a discontinuous method in which the SOD-inhibitable reduction of cytochrome *c* was monitored by absorbance measurement. That is, 2×10^5 cells were preincubated in an HBSS assay medium containing 0.12 mM cytochrome *c* and the desired concentration of NP for 2 min at 37°C before the reaction was started by adding PMA (0.3 μg/ml in DMSO). Assay mixtures were incubated for 4 min at 37°C, in a total volume of 1.0 ml. The reference cuvette contained 20 μg of SOD in addition to these components. Superoxide production in the cell-free system was assayed by the method of Curnutte et al. [19] with a slight modification. The assay mixture contained 0.1 mM cytochrome c, 3.6 mM $MgCl_2$, 89 mM KCl, 2.7 mM NaCl, 0.5 mM PIPES (pH 7.3), 0.9 mM ATP, 1.2 mM EGTA, 0.5 μM FAD, 6×10^6 cells eq of cytosol, 1.5×10^6 cells eq of membranes solubilized in deoxycholate, the desired concentration of NP, 0.04 mM SDS and 0.16 mM NADPH in a total volume of 0.75 ml. The reaction mixture except for NADPH was preincubated for 2 min at 37°C. Then the reaction was started by adding NADPH and the change in absorbance at 550 nm was followed for 3 min on a Hitachi 320 double-beam spectrophotometer. The reference cuvette contained 40 μg of SOD. In kinetic studies, concentrations of NADPH were varied in the cell-free system. The initial rate of superoxide production was calculated from the linear segment of the increase in absorbance at 550 nm and translated into nanomoles of super-oxide by using an extinction coefficient of $\varepsilon = 19.6\ mM^{-1} \cdot cm^{-1}$.

3. RESULTS AND DISCUSSION

Our previous study on the scavenging activity of pteridines against superoxide anion radicals had shown that the oxidized form of neopterin (NP) exhibits potent antioxidant activity in the splenic macrophage/PMA-superoxide generation system, though it has little effect in the X/XOD reaction system. Namely, the 50% inhibitory concentration (IC_{50}) of NP was about 1.0 μM in

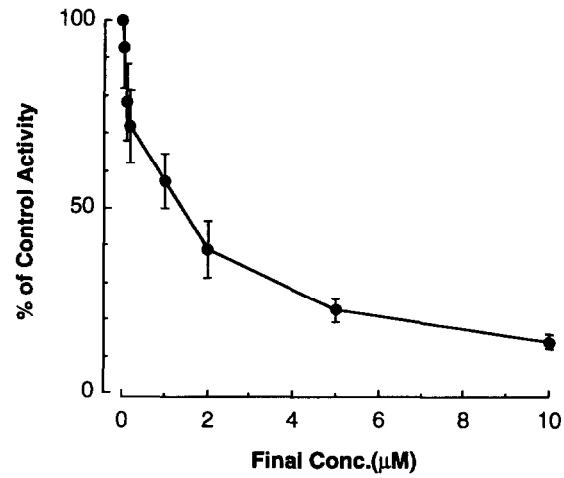

Fig. 1. Dose-dependent inhibition by NP of superoxide generation in intact rat macrophages stimulated with PMA. The assay method is described in section 2. Concentration of 1 to 10 μM of neopterins were used in the assays. Each value represents the mean ± S.D. of three independent assays.

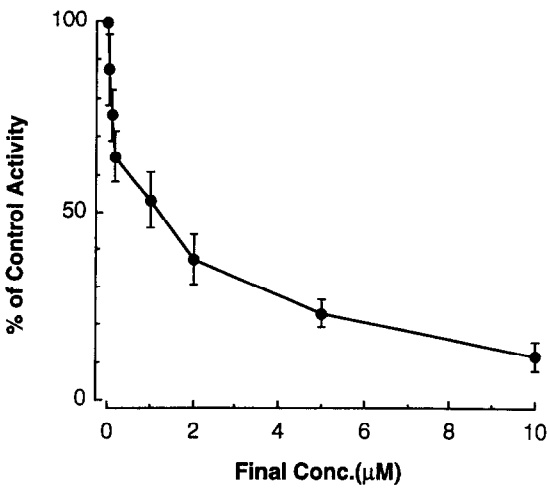

Fig. 2. Dose-dependent inhibition by NP of superoxide generation in the cell-free system. The assay method is described in section 2. Concentrations of 1 to 10 μM NP were used in the assays. Each value represents the mean ± S.D. of three independent assays.

the splenic macrophage/PMA reaction system, but more than 370 μM in the X/XOD reaction system [12]. These findings led us to speculate that NP interfered with the process of generation of the superoxide in phagocytes. In order to elucidate the antioxidant mechanism of NP in the macrophage/PMA reaction system, we investigated the effect of NP on rat peritoneal macrophage NADPH-oxidase activity.

As shown in Fig. 1, NP significantly inhibited the superoxide generation in the intact macrophage/PMA reaction system (whole-cell system) in a dose-dependent manner. The IC_{50} for NP was 1.39 μM and this value is in good agreement with those we previously obtained in the mouse splenic macrophage/PMA reaction system. In this whole-cell system, NP may enter into the cells through the membrane, and be intracellularly reduced to NPH2 or NPH4, which has potent antioxidant activity as previously reported [12,15], and these reduced forms of NP may directly scavenge the superoxides. However, if that is the case, the activity should be much stronger (close to that of NPH4) in this assay system.

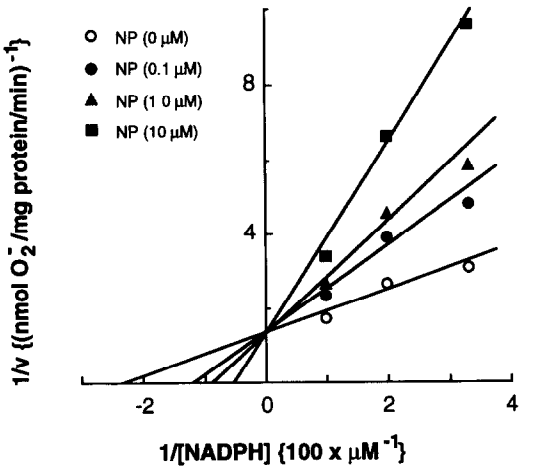

Fig. 3. Lineweaver–Burk plot of NADPH-oxidase-dependent superoxide generation by SDS-activated rat macrophage sonicates. The mean values of 3 independent assays are shown.

Elevation of the cytosol calcium concentration has also been considered to be an important factor for superoxide generation in phagocytes. Recently, it has been demonstrated that pteridine derivatives such as NP increase intracellular calcium concentration in human monocytes, and it was suggested that they are involved in the regulation of calcium-mediat-ed macrophage activation including superoxide generation [20]. Another important factor for the superoxide generation is protein kinase C (PKC). In the phagocytic cells/PMA superoxide generation system, PKC has been considered to be a receptor of and to be activated by PMA, which also activates the respiratory burst [21]. We also preliminarily examined the effect of NP on PKC activity in the rat peritoneal macrophage. NP did not show any effect on the PKC activity over the range of concentration which effectively inhibited superoxide generation. In order to examined the mechanism of the NP action, we examined the inhibitory effect of NP on the NADPH-oxidase using a cell-free system. As shown in Fig. 2, NP significantly inhibited the generation of the superoxides (IC_{50} of 1.23 μM). This value is almost the same as that in the intact whole-cell system. Since it is unlikely that NPH2 or NPH4 is formed from NP in this cell-free system, the inhibitory effect appears to be due to a direct effect of NP on NADPH-oxidase itself. To better understand the effect of NP on the activation of NADPH-oxidase in the cell-free system, we examined the inhibition at various concentrations of NP or NADPH. Fig. 3 shows a Lineweaver–Burk plot of the inhibition of the NADPH-oxidase by NP. The inhibition was competitive with respect to NADPH. The values of K_m and V_{max} were 42.64 μM and 0.74 nmol O_2^-/mg protein/min. The apparent K_i for NP was 6.50 μM. We also noted inhibitory effects of oxidized and reduced forms of other pteridine derivatives, including BP, pterin, etc. (unpublished data). These findings suggest that pterdine derivatives may be important suppressive factors of the production of superoxide in phagocytes. Detailed studies on the mechanisms involved are under way.

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