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Neopterin as an endogenous antioxidant

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The in vitro potency of neopterin (NP) as an antioxidant and its in vivo activity to suppress alloxan-induced diabetes were investigated. The reduced form of neopterin, 5,6,7,8-tetrahydroneopterin (NPH-4), showed an extremely high superoxide anion radical scavenging activity in two assay systems, i.e. xanthine/xanthine oxidase- and macrophage/phorbol myristate acetate (PMA)-reaction systems. NPH-4 also inhibited the oxidation of linoleic acid about as effectively as uric acid. Furthermore, NPH-4 and NP effectively suppressed alloxan-induced mouse diabetes. These results suggest that pteridines play an important role as endogenous antioxidants.

Neopterin; Antioxidant; Alloxan diabetes

1. INTRODUCTION

Neopterin (NP) is an α-amino-hydroxypteridine derivative, and a precursor of biopterin (BP), which is derived from guanosine triphosphate [1]. It is now generally accepted that NP is released from monocytes/macrophages, that interferon-γ (INF-γ), one of the immunomodulators produced by activated T cells, is the only inducer for NP secretion from macrophages [2,3], and that INF-γ augments the intracellular concentration of guanosine triphosphate as well as the conversion of it to NP [4]. Several biological roles of pteridines have been reported so far. Kaufman [5] showed that 5,6,7,8-tetrahydro-biopterin serves as a cofactor for mammalian aromatic amino acid mono-oxygenases, which hydroxylate phenylalanine, tyrosine, and tryptophan, and thus regulate the biosynthesis of neurotransmitters including dopamine, norepinephrine and serotonin. A tetrahydropteridine derivative is also reported to be a cofactor for the enzymic oxidation of glycerly ethers to alchohols [6]. Even though the exact physiological functions of pteridines are still obscure, it has been suggested that NP might act as an endogenous inhibitor of folate synthesis in view of the major role of INF-γ in the induction of antibacterial, antiprotozoal, and antifungal states in parasitized cells [7].

More recently, there have been many reports of significant elevation of urinary or serum pteridine levels in patients with various malignant disorders, as well as in patients with viral diseases such as acquired immunodeficiency syndrome [8–11].

Active oxygen species such as superoxide anion radicals are thought to be potent pathogenic factors in various viral diseases [12,13], so that the pathogenesis may involve not direct viral cytotoxicity and/or reactivity of active oxygen species. That is, an excess amount of active oxygen species following overreaction of immunodefense response against the virus-infected organs, may participate in the mechanism of tissue injuries observed in various types of viral diseases. We suspected that pteridines might play an important role as endogenous scavengers for these active oxygen species. Therefore we have investigated the potency of pteridines as antioxidants in vitro and in vivo.

2. MATERIALS AND METHODS

2.1. *Reagents*

2-Methyl-6-phenyl-3,7-dihydroimidazo(1,2-α)-pyrazin-3-one (CLA) was purchased from Tokyo Kasei Kogyo Co., Ltd. Phorbol myristate acetate (PMA), bovine erythrocyte Cu, Zn-superoxide dismutase (SOD) (grade IV), xanthine and xanthine oxidase (XOD) were from Sigma Chemicals Co., Ltd (St. Louis, MO). D-Neopterin (NP) and biopterin (BP) were also from Sigma Chemicals Co., Ltd. 5,6,7,8-Tetrahydroneopterin (NPH-4) was from Junsei Chemicals Co., Ltd.

2.2. *Isolation of splenic cells*

Splenic cells were prepared from spleen of male ddY mouse. Blood was withdrawn from the abdominal aorta, and the spleen was removed. Total splenic cells including T-cells, B-cells, and macrophages, etc., were prepared by teasing the spleen with a syringe in cold Hanks' balanced salt solution (HBSS). After filtration through sterilized nylon wool mesh and centrifugation at 400×*g* for 10 min, the cells were suspended in HBSS and adjusted to a cell concentration of 1×10^7 cells per ml.

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Abbreviations: NP, neopterin; NPH-4, 5,6,7,8-tetrahydroneopterin; BP, biopterin; CLA, 2-methyl-6-phenyl-3,7-dihydroimidazo-(1,2-α)-pyrazin-3-one; SOD, superoxide dismutase; PMA, phorbol myristate acetate; XOD, xanthine oxidase; INF-γ, interferon-γ.

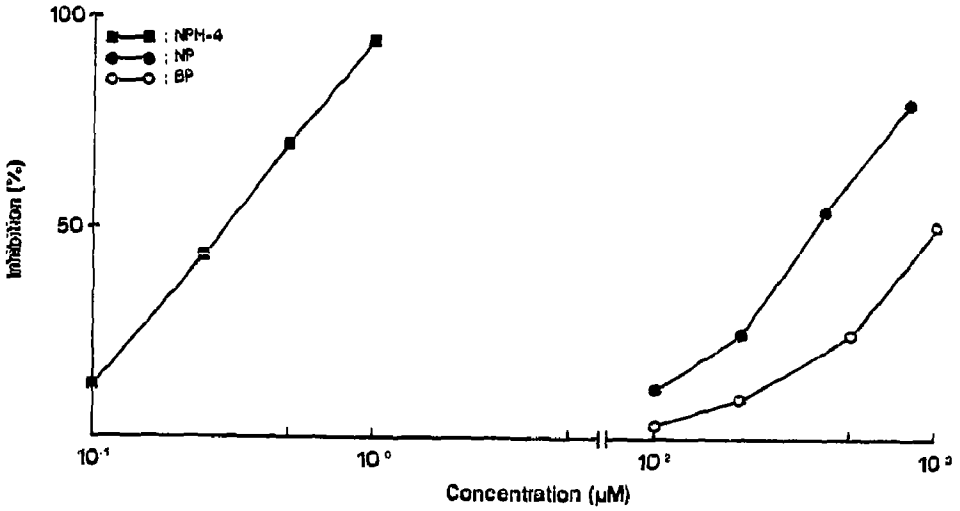

Fig. 1. Inhibitory effect of pteridines on superoxide anion radicals released in xanthine/XOD-reaction system.

2.3. *Assay of superoxide radical-scavenging activity by chemiluminescence*

The scavenging activity of pteridines for superoxide anion radicals was assayed in terms of reduction of chemiluminescence induced by O_2^- according to the method of Nakano et al [14]. As O_2^--generating systems, xanthine/XOD- and macrophage/PMA-reaction systems were used in this experiment. The reaction mixture of the xanthine/XOD system contains 50 μM xanthine, 1.5 μM CLA, 15 mUnits of XOD, and an appropriate concentration of pteridine in 1 ml of 50 mM Tris-HCl buffer (pH 7.6). The reaction was started by addition of XOD. In the case of the macrophage/PMA system, the reaction mixture contains 1×10^6 splenic cells, 1.0 μM diehtylene triamine-*N,N,N,N',N'*-pentaacetic acid, 1.5 μM CLA and 0.4 μg of PMA in 1 ml of HBSS. The reaction was started by the addition of PMA. Luminescence was continuously monitored for 10 min with a Luminescence reader (Aloka BLR 102).

2.4. *Inhibitory effect of pteridines on oxidation of linoleic acid*

The oxidation of linoleic acid was conducted by the method of Smith and Lawing (15), with a slight modification. Briefly, linoleic acid (0.65 mmol) was dissolved in 1 ml of 0.65 M NaOH and diluted to 100 ml with 0.2 M borate buffer (pH 9.0). Four ml of linoleic acid solution was added to a 50 ml glass vessel containing 11 ml of 0.2 M borate buffer, 1 ml of 0.1 mM copper sulfate, and 1 ml of 4 mM pteridine to give a total volume of 20 ml. Uric acid was used as a positive control antioxidant in this assay. The reaction vessels were shielded from light with tin foil and incubated at 37°C for 30 h. One ml of the reaction mixture was taken out at each time interval, 0.5 ml of 35% trichloroacetic acid was added to it, and the level of thiobarituric acid-positive materials was assayed as described as Uchiyama and Mihara [16].

2.5. *Effect of pteridines on alloxan-induced diabetes*

Male ddY mice were purchased from Tokyo Experimental Animals Co., Ltd., Tokyo, Japan. Alloxan was freshly dissolved in physiological saline and intravenously injected at a dose of 75 mg/kg body weight via tail vein. The animals were killed 24 h after the injection. NPH-4, NP, and BP were intra-peritoneally injected into mice 10 min before alloxan treatment at a dose of 3 mg/kg body weight. SOD was also administered at a dose of 5 mg/kg body weight in the same manner. Blood specimens were withdrawn from the abdominal aorta under sodium pentobarbital anesthesia. Sera were obtained by centrifugation of blood at 2500 rpm for 10 min and subjected to glucose assay, by using glucose C[II]-TEST WAKO (Wako Pure Chemical, Industries, Ltd., Osaka).

3. RESULTS AND DISCUSSION

The scavenging activity of NPH-4, NP, and BP for superoxide anion radical was determined by using two anion radical-generating systems, i.e. xanthine/XOD- and the splenic macrophage/PMA-reaction systems. As shown in Fig. 1, NPH-4 showed a significant scavenging activity for the superoxide anion radical in the xanthine/XOD-reaction system. The 50% inhibitory concentration (IC_{50}) was about 0.3 μM. In contrast, only weak activity were seen in NP and BP which gave IC_{50} values of 370 μM and over 1 mM, respectively. The activity was also assayed in another O_2^--generating system, i.e. the splenic macrophage/PMA-reaction system (Fig. 2). In this assay system, high activities were observed with all pteridines: the values of IC_{50} were 0.33, 1.1, and 3.0 μM, respectively. The differences of IC_{50} of pteridines obtained from these assays suggest that NPH-4 directly

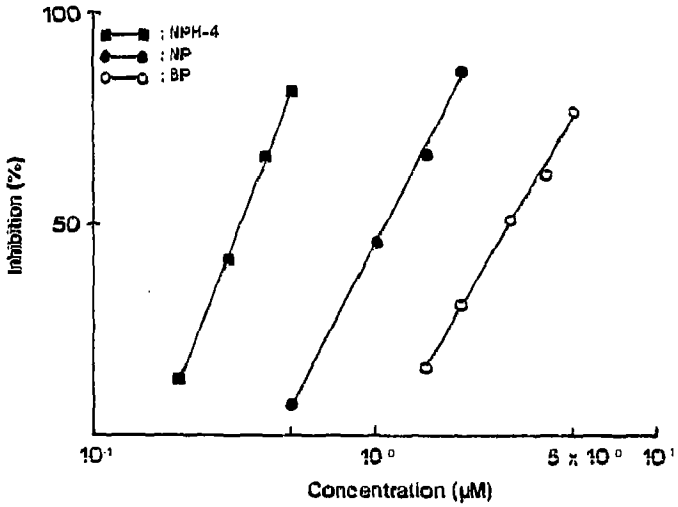

Fig. 2. Inhibitory effect of pteridines on superoxide anion radicals released in splenic macrophage/PMA-reaction system.

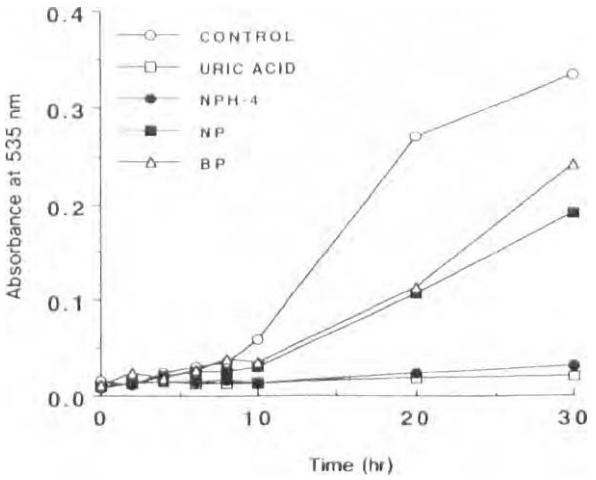

Fig. 3. Inhibitory effect of pteridines on oxidation of linoleic acid.

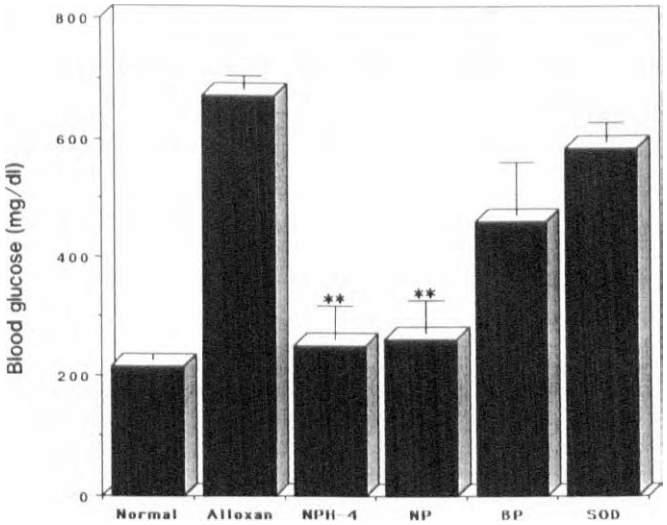

Fig. 4. Effect of pteridines on the elevated blood glucose level induced by alloxan. **Significantly differ from alloxan-treated group ($P<0.01$).

reacts with superoxide anion radical released in the xanthine/XOD and macrophage/PMA-reaction systems, whereas NP and BP act on macrophages to decrease the generation of O_2^-. These results are consistent with the speculation that pteridines such as NPH-4, NP and BP play important roles as endogenous SOD like substance, scavenging for the superoxide anion radical.

Stimulation, such as viral infection, activates macrophages to produce active oxygen species for defense, but large amounts of active oxygen species can sometimes cause serious cell/tissue injury. Thus, it would not be surprising if macrophage produced SOD-like substances to protect themselves.

From a different point of view, the potency of pteridines as free radical scavengers was also investigated in the air auto-oxidation of linoleic acid.

As shown in Fig. 3, linoleic acid was conspicuously oxidized from 10 h and the oxidation reached saturation at 30 h after the start of incubation. The presence of pteridines protected linoleic acid from the oxidation for 30 h. In particular, NPH-4 protected linoleic acid as effectively as uric acid, a typical free radical scavenger. The other pteridines, i.e. NP and BP, also inhibited the oxidation to about half that of the control at 20 h after the start of incubation.

Active oxygen species have been suggested to be involved in various processes such as inflammation, carcinogenesis, and ageing, as well as experimental alloxan-induced diabetes. A variety of agents with direct or indirect reducing activity toward active oxygen species have been shown to protect against alloxan-induced diabetes [17–19]. As shown above, NPH-4 has a novel scavenging potency against not only superoxide anion radicals but also active oxygen radicals. Thus, we examined the protective efficacy of NPH-4 against alloxan-induced diabetes. As shown in Fig. 4, NPH-4 completely blocked the elevation of blood glucose level, as we had expected. NP was also active, but BP was not very effective under these experimental conditions. These data indicate that the antioxidant activity of pteridines is manifested in vivo. However, the protective potency of pteridines was not always in accordance with in vitro antioxidant or radical scavenging activities.

This observation indicates that lipophilic NP may easily enter the cells through the cell membrane, following to reduce to NPH-4 in living cells. On the basis of these results, we suggest that pteridines may play important role as endogenous antioxidants and also be useful as therapeutic agents against diseases in which active oxygen species play as the potent pathogenic factors.

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