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ANTIOXIDATIVE ACTIVITY OF 5,6,7,8-TETRAHYDROBIOPTERIN AND ITS INHIBITORY EFFECT ON PARAQUAT-INDUCED CELL TOXICITY IN CULTURED RAT HEPATOCYTES

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The *in vitro* antioxidative activity of 5,6,7,8-tetrahydrobiopterin (BPH4) was measured and the ability of BPH4 to prevent paraquat-induced cell damage was examined in cultured hepatocytes. The scavenging activity of BPH4 against superoxide anion radicals was assayed in two systems, i.e., xanthine/xanthine oxidase (X/XOD) and rat macrophage/phorbol myristate acetate (Mφ/PMA) radical-generating systems. BPH4 showed an extremely strong superoxide anion radical-scavenging activity in both assay systems. Biopterin (BP) itself did not show any activity in the X/XOD system, but was effective in the Mφ/PMA system. The antioxidative activities of BPH4 against both superoxide anion and hydroxyl radicals were confirmed by spin trapping-ESR spectrometry. BPH4 also protected rat brain homogenate against auto-oxidation. We further examined the effect of BPH4 on paraquat-induced cell toxicity in cultured rat hepatocytes. The paraquat-induced elevation of the release of lactate dehydrogenase (LDH), a marker enzyme for cytotoxicity from cultured hepatocytes was suppressed by BPH4 in a dose-dependent manner. The elevation of lipid peroxides simultaneously induced by paraquat was also inhibited by BPH4 in the same manner. These results suggest that BPH4 might be useful in the treatment of various diseases whose pathogenesis is active oxygen-related.

KEY WORDS: 5,6,7,8-tetrahydrobiopterin, antioxidant, paraquat, cell damage.

INTRODUCTION

5,6,7,8-Tetrahydrobiopterin (BPH4) is biosynthesized from guanosine triphosphate (GTP).[1,2] GTP is converted into 7,8-dihydroneopterin triphosphate (NH2TP) by the enzyme GTP cyclohydrolase. NH2TP is dephosphorylated and reduced by 6-pyruvoyltetrahydropterin synthase and sepiapterin reductase (EC 1.1.1.153) to yield BPH4 (Figure 1). The biochemical function of BPH4 has been well established; it acts as a cofactor for phenylalanine, tyrosine and tryptophan hydroxylase.[3–5] BPH4 plays an important role in regulating the *in vivo* activities of tyrosine and tryptophan hydrolases, which are the rate-limiting enzymes in the biosynthesis of the catecholamines and serotonin, respectively. BPH4 also has a regulatory role in cytokine-induced NO biosynthesis in various cells.[6–9]

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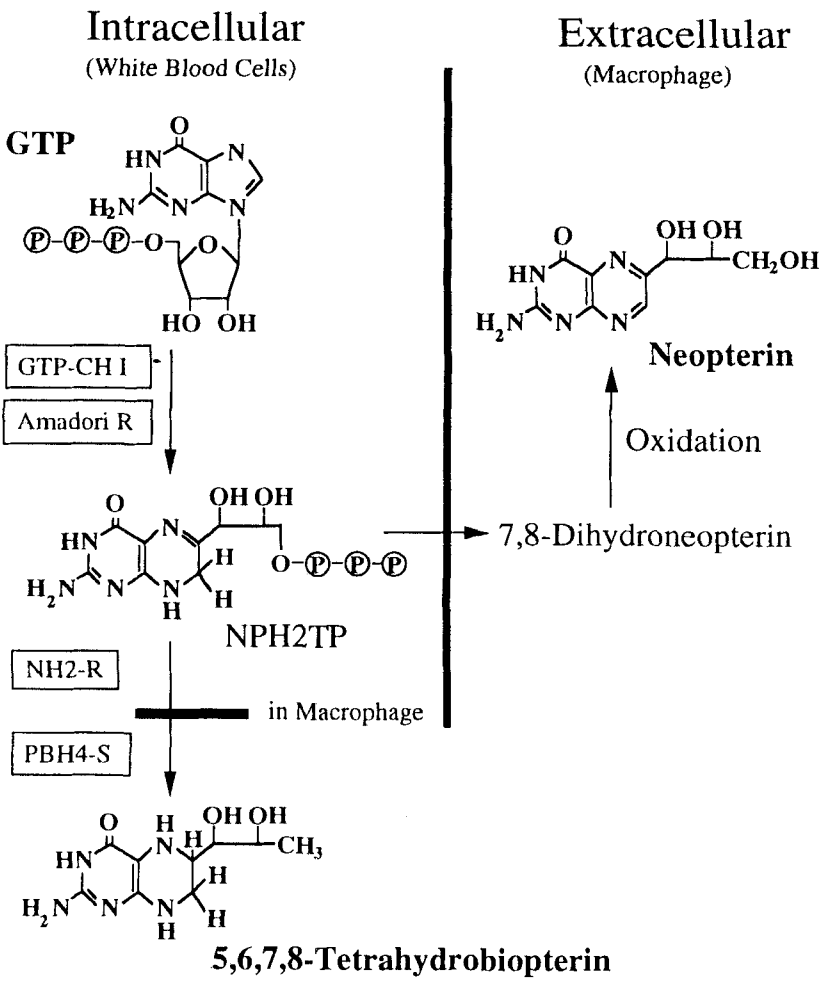

FIGURE 1 Biosynthesis of 5,6,7,8-tetrahydrobiopterin in white blood cells. Abbreviations: GTP, guanosinetriphosphate; GTP-CH I, guanosine-triphosphate cyclohydrolase; Amadori R, amadori reaction; 7,8 NH2TP, 7,8-dihydroneopterin triphosphate; NH2-R, 7,8-dihydroneopterin reductase; PBH4-S, 6-pyruvoyltetrahydropterin synthetase.

We have recently found a strong antioxidative activity of 5,6,7,8-tetrahydroneopterin (NPH4), a precursor of BPH4, which suggests a possible function for NPH4 as an endogenous antioxidant.[10-12] BPH4 might have a similar role. In this study, the antioxidative activity of BPH4 was determined in various ways, and we examined the efficacy of this compound against the superoxide-induced cell toxicity caused by paraquat.

MATERIALS AND METHODS

Materials

Dulbecco's modified Eagle's medium (DMEM), L-15 medium, and fetal bovine serum (FBS) were purchased from Nissui Co., Ltd., Tokyo, Japan. Collagen type I, aprotinin (6000 KIU/mg) dexamethasone, N-2-hydroxyethylpiperazine-N′-2-ethanesulfonic acid (HEPS), glycoletherdiamine-N,N,N′,N′-tetraacetic acid (GEDTA), ethylenediaminetetraacetic acid (EDTA), diethylenetriamine-N,N,N′,N″,N″-pentaacetic acid (DTPA), 1,1-diphenyl-2-picrylhydrazyl (DPPH), paraquat, ascorbic acid (Asc), and cysteamine (CysNH2) were obtained from Wako Chemicals Co., Ltd., Tokyo, Japan. Penicillin G potassium, and streptomycin sulfate were from Meiji Seika Co., Ltd., Tokyo. 2-Methyl-6-phenyl-3,7-dihydroimidazo-(1,2-α)-pyrazin-3-one (CLA) was from Tokyo Kasei Kogyo Co., Ltd., Tokyo, Japan. Reduced and oxidized forms of neopterin, biopterin and pterin were gifts from Asahi Breweries Ltd., Tokyo, Japan. 5,5-Dimethyl-1-pyrroline-N-oxide (DMPO) was purchased from Labotec Co., Ltd., Tokyo, Japan.

Chemiluminescence assay of superoxide anion radical-scavenging activity

The scavenging activities of BPH4 and other antioxidants, i.e., Asc and CysNH2, were assayed in terms of reduction of chemiluminescence induced by superoxide anion radicals according to the method of Nakano.[13] The activity was measured in xanthine/xanthine oxidase (X/XOD) and rat macrophage/phorbol myristate acetate (Mφ/PMA) radical-generating systems. The reaction mixture of the X/XOD system contains 50 μM hypoxanthine, 1.5 μM CLA, 25 mU of XOD, and an appropriate concentration of BPH4 or other antioxidant in 1 ml of 50 mM Tris-HCl buffer (pH 7.6). The reaction was started by adding XOD. In the Mφ/PMA system, the reaction mixture contains 5×10^5 rat Mφ, 1.0 μM DTPA, 1.0 μM CLA and 0.4 μg of PMA in 1 ml of Hanks' balanced salts solution (HBSS, pH 7.6). The reaction was started by adding PMA. Luminescence was continuously recorded for 5 min with a luminescence reader (Aloka BLR 102). The 50% inhibitory concentration (IC_{50}) was calculated from the inhibition curve. Rat Mφ were prepared as described elsewhere.[12]

Briefly, male Wistar strain rats, weighing about 200 grams, were intraperitoneally injected 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.

Analysis of antioxidant activity of BPH4 by spin trapping-ESR spectrometry

Superoxide anion radicals were generated by the X/XOD system. The reaction was started by adding 0.25 U/ml of XOD to a mixture of 0.5 mM xanthine, 1 mM DTPA, 460 mM DMPO, and an appropriate concentration of BPH4 in 50 mM phosphate buffer (pH 7.4). The reaction mixture was quickly placed into an ESR quartz flat cell ($60 \times 10 \times 0.3$ mm inner size, with an effective sample volume of 160 μl). The cell was placed in the cavity of ESR spectrometer, and the ESR spectrum was recorded exactly 1 min after addition of XOD on a JEOL X-band spectrometer, Model JES-FR80, at 100 kHz magnetic field modulation (magnetic field 335 ± 5 mT, micro-wave power

10 mW, amplitude 5×10^2, modulation amplitude 0.02 mT, response time 0.1 sec, sweep time 2 min).

Hydroxyl radicals were generated by the Fenton reaction of Cohen and Sinet.[14] The reaction was started by adding 1 mM hydroperoxide to 50 mM phosphate buffer (pH 7.4) containing 50 μM $FeSO_4$, 100 μM DTPA, and 10 mM DMPO. The spectra were recorded in the same way as in the case of superoxide anion radicals.

Reactivity of BPH4 with chemically stable radical, 1,1-diphenyl-2-picrylhydrazyl (DPPH)

The reactivity of BPH4 with DPPH was examined by the method of Blois.[15] An appropriate concentration of BPH4 dissolved in ethanol was added to an ethanol solution containing 0.1 mM DPPH. The reaction mixture was kept for 20 min at room temperature and the absorbance was followed at 517 nm. The reduction of absorbance was regarded as indicating the quenching activity of BPH4. The effects of other antioxidants including Asc and CysNH2 were examined similarly.

Male Wistar strain rats were killed and the brains were removed. The whole brain tissues were homogenized in 50 mM phosphate buffer (pH 7.4) with a Potter-type glass homogenizer to give a 5% (W/V) homogenate. One ml of the homogenate was incubated with or without BPH4 or another antioxidant at 37°C for 1 hr and lipid peroxides produced by autooxidation were measured according to the method of Ohkawa *et al.*[16] Each antioxidant was added to the homogenate to a final concentration of 125 μM. The lipid peroxides content was represented as the content of malondialdehyde (MDA)/mg protein. The protein content was measured by the method of Lowry *et al.*[17]

Tissue culture

Rat hepatocytes were prepared according to the method of Ichihara *et al.*[18] with a slight modification. A male Wistar rat, weighing about 200 g, was anesthetized with pentobarbital and the abdomen was opened. The liver was first perfused through the portal vein with 200 ml of Ca^{2+}-free Hanks'-HEPS buffer (pH 7.2) containing 0.5 mM GEDTA and then with Hanks'-HEPS buffer (pH 7.2) containing 0.04% collagenase (type I) for 7 min. The liver was removed, put into DMEM, gently cut into small pieces, and pressed through a sterilized gauze. The filtrate containing the isolated hepatocytes was washed 3 times with DMEM by centrifugation at $60 \times g$ for 1 min. The final cell pellet was suspended in DMEM containing 10% FBS. The cell number was adjusted to 5×10^5 cells/ml, and the suspension was seeded into dishes (60 mm × 10 mm) at a concentration of 2×10^6 cells per dish. After 5 hr the medium was changed to 4 ml of L-15 medium containing aprotinin (5U/ml) and the hepatocytes were cultured for 24 hr. Appropriate concentrations of BPH4 and other antioxidants were prepared with HBSS. Paraquat, BPH4 and other antioxidants including Asc and CysNH2 were then added to the medium and incubation was continued for another 24 hr in a CO_2 incubator.

Assay of lactate dehydrogenase (LDH)

LDH released into the medium from the hepatocytes was assayed as a marker of the cell damage. An aliquot of the supernatant of the cultured medium was collected at each time interval and subjected to LDH assay with LDH CII-Test Wako (Wako Chemicals Co., Ltd., Tokyo, Japan). The activity was expressed in international units (IU/L).

Assay of lipid peroxides

The hepatocytes were incubated for 24 hr, then the cells were killed and disrupted by adding 2 ml of 20% TCA to the medium. The mixture was transferred into a glass test tube and subjected to the assay of lipid peroxides by the method of Gavino *et al.*[19] The concentration of lipid peroxides were expressed as nmol of malondialdehyde (MDA) per dish.

Statistical analysis

Student's *t*-test was used to evaluate the significance of differences between groups. The criterion of significance was taken as $P < 0.05$.

RESULTS

Superoxide anion radical-scavenging activity of BPH4

The scavenging activities of BPH4 and other antioxidants including Asc and CysNH2 were determined by using two superoxide anion radical-generating systems, i.e., the X/XOD and the Mφ/PMA systems. As shown in Table 1, the reduced form of biopterin, BPH4, showed a strong antioxidative activity with the IC_{50} of 0.40–0.45 μM in both assay systems. Asc also had a strong antioxidative activity, but its activity was considerably less than that of BPH4. CysNH2 showed only weak activity in these assays. On the other hand, the oxidized form, BP, showed strong activity in the Mφ/PMA system, but not in the X/XOD system. Other pteridine derivatives, i.e., neopterin and pterin, showed similar characteristics.

Analysis of antioxidative activity of BPH4 by ESR

The scavenging activities of BPH4 against superoxide anion and hydroxy radicals were further examined by using spin trapping-ESR spectrometry. The ESR spectra obtained from the X/XOD superoxide anion radical-generating system containing DMPO with and without BPH4 are shown in Figure 2. The DMPO-OOH signal was selectively decreased in a dose-dependent manner by BPH4. The value of IC_{50} was obtained as

TABLE 1
Antioxidant activity of 5,6,7,8-tetrahydrobiopterin and other antioxidants against superoxide anion radical[a]

Antioxidants	50% inhibitory concentration (IC_{50}, μM)	
	X/XOD	Mφ/PMA
5,6,7,8-Tetrahydroneoperin	0.45	0.45
5,6,7,8-Tetrahydrobiopterin	0.45	0.40
5,6,7,8-Tetrahydropetrin	0.55	0.30
Neopterin	400	0.90
Biopterin	>1000	3.50
Pterin	65	1.50
Ascorbic acid	1.50	1.50
Cysteamine	64	60

[a]The activity was assayed in xanthine/xanthine oxidase (X/XOD) and rat macrophages/phorbol myristate acetate (Mφ/PMA) systems. Luminescence from CLA with and without antioxidant was continuously recorded and the IC_{50} to the control was calculated from the inhibition curves. Each value is the mean of three determinations.

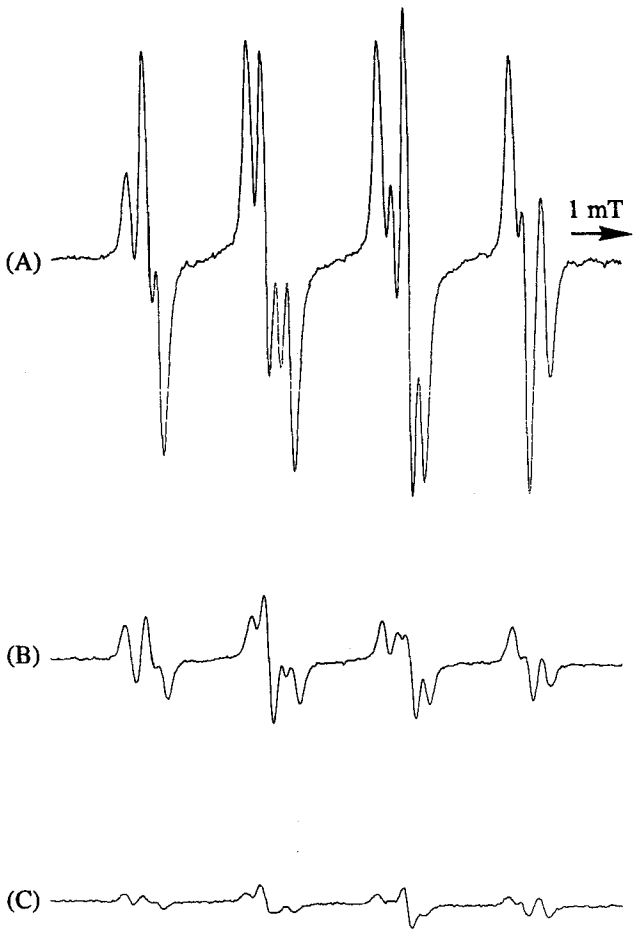

FIGURE 2 ESR spectra of DMPO spin adducts formed in a xanthine/xanthine oxidase (X/XOD) system in the absence of BPH4 (A) and in the presence of 0.5 mM (B) and 1.0 mM (C) BPH4. Each spectrum was obtained at 60 sec after adding XOD. The species present include DMPO-OOH and DMPO-OH.

200 μM. Moreover, the ESR spectra of hydroxyl radicals generated by means of the Fenton reaction in the presence of DMPO with and without BPH4 are shown in Figure 3. The DMPO-OH signal was decreased in the same fashion by BPH4. The IC_{50} value was also about 200 μM.

Reactivity of BPH4 with DPPH

In order to estimate the scavenging capacity of BPH4 for active oxygen radicals, we examined how many molecules of DPPH react with BPH4. As shown in Figure 4, the reaction of BPH4 with DPPH was saturated at a concentration of about 2.5×10^{-5} μM.

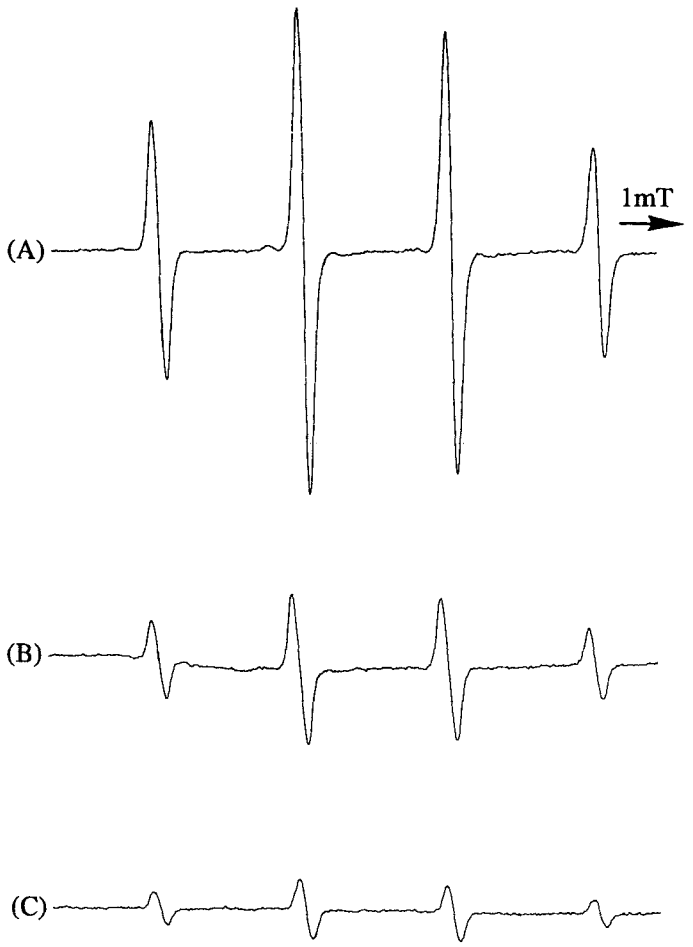

FIGURE 3 ESR spectra of DMPO spin adducts formed in the Fenton reaction in the absence of BPH4 (A) and in the presence of 0.5 mM (B) and 1.0 mM (C) BPH4. Each spectrum was obtained at 60 sec after adding hydroperoxide. The main species present is DMPO-OH.

Since the concentration of DPPH was 1×10^{-4} μM, four mol of DPPH react with one mol of BPH4. In the case of Asc, two mol of DPPH react with one mol of Asc.[15]

Effect of BPH4 on the autooxidation of rat brain homogenate

The effect of BPH4 on the autooxidation of brain homogenate, which is considered to be induced by hydroxyl radical and lipid peroxides, was examined. As shown in Figure 5, BPH4 significantly lowered the MDA level to about half that of the control at a concentration of 125 μM. Asc showed almost the same efficacy as BPH4. The oxidized form, BP itself, was ineffective (data not shown).

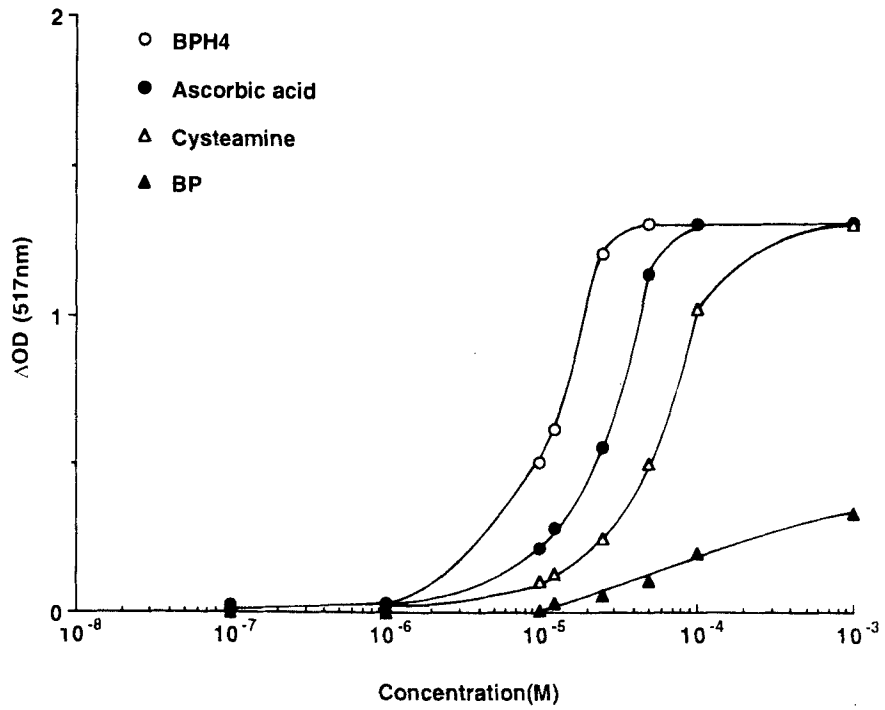

FIGURE 4 Reactivities of BPH4 and other antioxidants with DPPH. An appropriate concentration of BPH4 dissolved in ethanol was added into 0.1 mM DPPH ethanol solution. The reaction mixture was kept for 20 min at room temperature and the absorbance was followed at 517 mm. The difference of absorbance from the control was regarded as reflecting the quenching activity, ○, BPH4; ●, Ascorbic acid; △, Cysteamine; ▲, BP. Each point represents the mean value of 3 determinations.

Effects of BPH4 on the LDH release and on lipid peroxides content

We preliminarily investigated the dose-dependent effect of paraquat on LDH release from the hepatocytes (data not shown). The isolated hepatocytes were incubated with 1, 5 and 10 mM paraquat and the release of LDH from the cells was assayed as a function of time for 24 hr. Significant increases of LDH in the cultures were not observed at 1 mM and 5 mM paraquat. However, a dramatic increase began at 12 hr after adding 10 mM paraquat and continued until 24 hr without loss of cell viability. Thus, we examined the effect of BPH4 on the release of LDH and the elevation of lipid peroxides in hepatocytes incubated with 10 mM paraquat for 24 hr. LDH was slightly increased in control cultures incubated for 24 hr. As shown in Figure 6, the paraquat-induced elevation of LDH was inhibited by BPH4 in a dose-dependent manner at concentrations ranging from 5 μM to 125 μM. BPH4 alone did not increase the release of LDH in the cultures. CysNH2 and Asc showed no significant efficacy at 125 μM. The level of MDA in the hepatocytes was drastically elevated by the incubation with 10 mM paraquat. The elevation was also suppressed by BPH4 in a dose-dependent manner at the same concentration range as in the case of LDH. CysNH2 had no effect

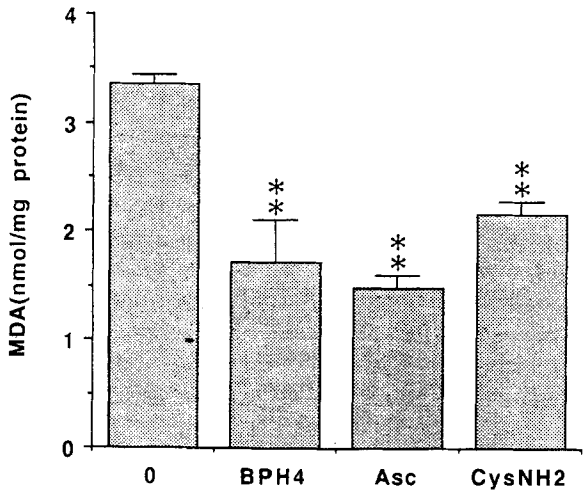

FIGURE 5 Effects of BPH4 and other antioxidants on the autooxidation of brain homogenate. BPH4 or another antioxidant was added to the 5% brain homogenate to a final concentration of 250 μM. Lipid peroxides are represented as the content of malondialdehyde (MDA). Asc, ascorbic acid; CysNH2, cysteamine. Each value is the mean ± S.E.M. of 3 independent assays. $^{**}P < 0.01$; significantly different from control (0).

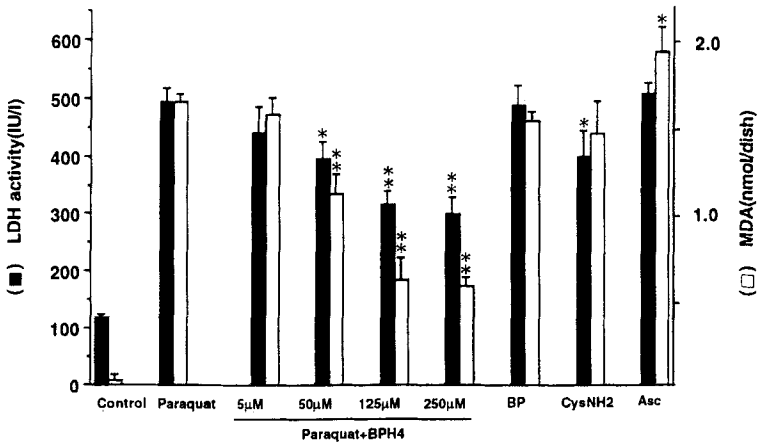

FIGURE 6 Effect of BPH4 and other antioxidants on the release of lactate dehydrogenase (LDH) and on the lipid peroxides level in cultured hepatocytes. An appropriate concentration of BPH4 or another antioxidant was added to the medium simultaneously with 10 mM paraquat, and the mixture was incubated for 24 hr. LDH activity in the medium and lipid peroxides in medium containing the hepatocytes were assayed. LDH activity and lipid peroxides content are expressed in international units (IU/L) and as malondialdehyde contents per mg protein (MDA/mg protein), respectively. Asc, ascorbic acid; CysNH2, cysteamine. Each value indicates the mean ± S.E.M. of 3 determinations. $^{*}P < 0.05$; $^{**}P < 0.01$; significantly different from paraquat-treated group.

on the elevation of MDA, while Asc seemed to increase it somewhat. The oxidized form, BP, had no effect on either parameter.

DISCUSSION

Shen and Zhang[20] have suggested the presence of a BPH4-mediated antioxidation system in pheochromocytoma (PC 12) cells. This system required the simultaneous presence of BPH4, NADH, and dihydropteridine reductase (DHPR), and it may have two components: a non-enzymatic action, and the peroxidase-catalyzed reduction of H_2O_2 to H_2O by BPH4 with the regeneration of BPH4 by DHPR using NADH as a cofactor. However, the reaction mechanism remains to be established. We have suggested an important role of the reduced form of neopterin (NPH4), which is one of the precursors of BPH4, as an endogenous antioxidant.[10–12] In the present study, we further examined the antioxidative activity of BPH4 *in vitro* by using various assay methods. We also investigated the efficacy of BPH4 to counteract the cytotoxicity induced by paraquat, which is well established to produce active oxygen radicals, such as superoxide anion radicals.

The *in vitro* antioxidative activity of BPH4 was evaluated in terms of the reduction of chemiluminescence induced by superoxide anion radicals in two generation systems, i.e., X/XOD and Mφ/PMA systems. BPH4 showed strong scavenging activity against superoxide anion radicals generated in both systems. In contrast, the oxidized form, BP, had little antioxidative activity in the former system, but showed strong activity in the latter system. Other pteridine derivatives including neopterin and pterin, exhibit a similar pattern to this (Table 1). We concluded previously that the reduced form of neopterin (NPH4) directly scavenges superoxide anion radicals, while the oxidized form (NP) suppresses the superoxide generation in macrophages through the inhibition of the membrane NADPH-oxidase.[10,12] A similar mechanism may operate with biopterin as well, i.e., BP may inhibit NADPH oxidase, resulting in the inhibition of superoxide anion generation, while some antioxidative activity is also generated by the formation of BPH4 from BP in macrophages. As shown in Figures 2 and 3, we confirmed by spin trapping-ESR spectrometry that BPH4 scavenged both superoxide anion radical and hydroxyl radical. We further examined the effect of BPH4 on the autooxidation of brain homogenate, which is considered to be induced by hydroxyl radicals and/or lipid peroxides. BPH4 protected the brain homogenate from the oxidation as potently as Asc and more potently than CysNH2, used as positive controls.

Paraquat is an effective herbicide which is also quite toxic to man and animals. Cyclic oxidation-reduction reactions involving oxygen and cellular components are thought to be involved in the toxicity:[21] paraquat ion is converted into a paraquat cation radical by accepting an electron from NADPH in the cells, and this reacts with oxygen molecules and hydroperoxides to produce hydroxyl and superoxide anion radicals, respectively. These active oxygen radicals oxidize cell-membrane lipids to form lipid peroxides, leading to cell damage. This mechanism is supported by the protective effect of antioxidants. For example, dimethylthiourea (DMTU), a hydroxy radical scavenger, and vitamin E, a chain reaction inhibitor for lipid peroxidation, significantly suppressed the paraquat-induced tissue damage such as pulmonary edema[22] and reduced the mortality of the animals treated with paraquat.[23]

Parkinsonism is pathologically manifested as a premature degeneration of dopamine neurons, leading to depletion of dopamine, and it has been suggested that the possible elevation of tyrosine hydrolase activity in surviving dopamine neurons by

BPH4 administration may provide a therapeutic means of increasing striatal synaptic dopamine.[24–26] It has been speculated that the degeneration of dopamine neurons in Parkinson's disease is induced, at least in part, by active oxygen radicals.[27] 1-Methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) treatment has been used to generate an animal model of this disease.[28] Mitsumoto *et al.*[29] have indicated that MPTP and its derivatives produce superoxide anion radicals in some living cells and that the cells' growth was inhibited by these compounds in proportion to the potential of superoxide generation.

Paraquat is structurally analogous to MPTP, a potent inducer of a model of Parkinson's disease. In the present study, we examined the effect of BPH4 on the release of LDH and the elevation of lipid peroxides in cultured hepatocytes treated with paraquat. As shown in Figure 6, paraquat greatly elevated the release of LDH from the hepatocytes, and the elevation was suppressed by BPH4 in a dose-dependent manner at concentrations ranging from 5 μM to 125 μM. CysNH2 and Asc were ineffective at 125 μM. The elevation of MDA induced by paraquat was similarly suppressed by BPH4.

BPH4 has been suggested as a potential therapeutic agent for Parkinson's disease based on its ability to increase the supply of dopamine through activating synthesis in surviving dopamine neurons. Our data obtained here suggest that another important factor may be the antioxidant efficacy of this compound. BPH4 may also have therapeutic potential against various other diseases where active oxygen radicals are involved in the pathogenesis.

We further preliminarily examined the effect of BPH4 on MPTP-induced nerve cell damage. In this study, we have already obtained the fact that MPP^+, which is considered as an ultimate active metabolite for generating Parkinson's disease in an animal model, easily induces PC 12 cell damage and BPH4 significantly prevent the cells from the damage. The detailed preventive mechanism is now under investigation.

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