

Effect of 5,6,7,8-Tetrahydroneopterin on the Bovine Endothelial Cell Injury Induced by Cumene Hydroperoxide

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ABSTRACT—Neopterin is an 2-amino-4-hydroxypteridine derivative and a precursor of biopterin, which is derived from guanosine triphosphate. Previously, we have reported that 5,6,7,8-tetrahydroneopterin (NPH₄), a reduced form of neopterin, possesses an antioxidant activity in various systems. In this study, we investigated the activity in more detailed manner and discussed the possible applications of this antioxidant. Analysis by electron spin resonance spectrometry indicated that NPH₄ scavenged superoxide anion radicals and hydroxyl radicals as well. Moreover, NPH₄ protected the rat brain homogenate from autoxidation. Next, we examined the effect of NPH₄ on the cell injury induced by cumene hydroperoxide (CHP) in cultured bovine artery endothelial cells. The activity of lactate dehydrogenase, a marker enzyme of cell injury, was elevated by CHP in a dose-dependent manner, and this elevation was dose-dependently suppressed by NPH₄. The elevation of lipid peroxide content was also inhibited by NPH₄ in the same fashion. These data suggest that NPH₄ would be effective against various diseases whose pathogenesis is active oxygen-related.

Keywords: 5,6,7,8-Tetrahydroneopterin, Cumene hydroperoxide, Active oxygen, Antioxidant, Endothelial cell

Neopterin is an 2-amino-4-hydroxypteridine derivative and a precursor of biopterin, which is derived from guanosine triphosphate (GTP) (1). Namely, intracellular GTP is cleaved by GTP-cyclohydrolase to 7,8-dihydroneopterin triphosphate. The latter compound is further converted to tetrahydrobiopterin by the actions of two enzymes, 6-pyruvoyltetrahydropterin synthase and sepiapterin reductase, in succession. This pathway has been established in the liver, neuroendocrine tissues and lymphocytes (2). Neopterin has been used as a clinical marker for the diagnosis of various malignant diseases including AIDS (3–6). Previously, we have investigated the *in vitro* activity of neopterin as an endogenous antioxidant (7–9). The reduced form of neopterin (NPH₄: 5,6,7,8-tetrahydroneopterin) showed an extremely high scavenging activity against superoxide anion radicals in two assay systems: hypoxanthine/xanthine oxidase (HPX/XOD) and macrophage/phorbol myristate acetate (Mφ/PMA) superoxide reaction systems. The IC₅₀ of NPH₄ was about 0.3 μM. The radical scavenging capacity of NPH₄ was also esti-

mated by using a stable radical, 1,1-diphenyl-2-picrylhydrazyl (DPPH). NPH₄ strongly reacted with the radical, showing that two moles of DPPH reacted with one mole of NPH₄. The capacity was almost the same as that of ascorbic acid (Asc).

In this paper, we examined in detail the antioxidant activity of NPH₄. For this purpose, we used electron spin resonance (ESR) spectrometry to assess the scavenging activities of NPH₄ against the superoxide anion radicals and the hydroxyl radicals. The superoxide anion- and the hydroxyl-radicals were generated by the HPX/XOD-system and the Fenton-reaction-system, respectively. If antioxidants and other reducing agents inhibit the formation of peroxy radicals and lipid peroxides, these should interfere with the subsequent formation and breakdown of cyclic endoperoxides to malondialdehyde (MDA). Thus, we investigated the antioxidant activity of NPH₄ on the autoxidation of the rat brain homogenates that contain lipids as a main component. Since it has been well known that cumene hydroperoxide (CHP) injures cells through the formation of lipid peroxidation, we further investigated the protective effect of NPH₄ on the en-

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dothelial cell-injury induced by CHP.

MATERIALS AND METHODS

Chemicals

NPH₄ was prepared by Asahi Breweries, Ltd. (Tokyo). The following reagents were obtained from commercial sources: 5,5-dimethyl pyrroline-1-oxide (DMPO) and diethylenetriamine *N,N,N',N'',N'''*-pentaacetic acid (DETAPAC) (Labotec Co., Ltd., Tokyo); CHP (Aldrich Chemical Co., Ltd., Milwaukee, WI, USA); xanthine oxidase (XOD) (Boehringer Mannheim Yamanouchi, Tokyo); Dulbecco's modified Eagle's medium (DMEM) and Dulbecco's phosphate-buffered saline (PBS) (Nissui Co., Ltd., Tokyo); hypoxanthine (HPX), hydrogen peroxide, Asc and cysteamine (CysNH₂) (Wako Pure Chemicals Co., Ltd., Osaka).

Analysis of antioxidant activity of NPH₄ by ESR spectrometry

Superoxide anion-radicals were generated by the HPX/XOD reaction system. The reaction was started by adding 0.25 U/ml of XOD to 75 mM PBS (pH 7.8) containing 0.5 mM HPX, 0.96 mM DETAPAC and 0.69 mM DMPO with or without an appropriate concentration of NPH₄, and then the reaction mixture was quickly put into an ESR quartz flat cell. The cell was placed in the cavity of the ESR spectrometer, and the ESR spectrum was recorded exactly 1 min after adding XOD. ESR spectra were recorded on a JEOL X-band spectrometer, Model JES-FR80 (Jeol Co., Tokyo), at 100 kHz magnetic field modulation by using an aqueous quartz flat cell (inner size 60 mm × 10 mm × 0.31 mm) with an effective sample volume of 160 μ l. The magnetic field was 335 ± 5 mT; microwave power, 8 mW; amplitude, 5×10^2 ; modulation amplitude, 0.02 mT; response time, 0.1 sec; sweep time, 2 min; and the hydroxyl radicals were generated by the Fenton-reaction. The reaction was started by adding 1 mM hydrogen peroxide to 75 mM PBS (pH 7.8) containing 50 μ M FeSO₄, 100 μ M DETAPAC and 10 mM DMPO in the presence or absence of NPH₄. The spectra were obtained in the same way as those for the superoxide anion-radicals.

Inhibitory effect of NPH₄ on lipid peroxidation in rat brain homogenates

The inhibitory effects of NPH₄ and other antioxidants on the autoxidation in rat brain homogenates (10) were examined by measuring the amount of MDA formed according to the method of Ohkawa et al. (11). The rat brain was quickly removed after decapitation and washed three times with ice-cold 0.9% NaCl. The whole brain tissues were homogenized with a Potter-type glass homogenizer

in 50 mM phosphate buffer (pH 7.5) to give 5.0% (W/V) homogenates. An aliquot of the homogenates was incubated with or without NPH₄ and other antioxidants, i.e., Asc and CysNH₂, at 37°C for 1 hr. NPH₄ was added to the brain homogenates to a final concentration of 2.5, 25 or 250 μ M. The dose of the other antioxidants was fixed at 250 μ M. The content of lipid peroxide was represented as the content of MDA/mg protein.

Culture of endothelial cells

Bovine artery endothelial cells (BAEC) prepared from the carotid arteries were kindly given to us by Prof. Dr. K. Kataoka, Department of Basic Engineering, Science University of Tokyo. BAEC were routinely cultured in DMEM (pH 7.5) supplemented with 10% fetal bovine serum (FBS) at 37°C in an atmosphere of 95% O₂ and 5% CO₂. Cells in monolayer were treated with PBS containing 0.1% (W/V) trypsin and 0.01% (W/V) EDTA and then subcultured in a growth medium, i.e., DMEM with 10% (w/v) FBS. Experiments were performed with the cells in the 10th to 20th passages.

Protection of CHP-induced endothelial cell injury by NPH₄

The endothelial cells (1.6×10^5) were suspended in DMEM supplemented with 10% (W/V) FBS and cultured in a 24-well plate for 4 hr at 37°C in a CO₂-incubator, and then the medium was changed to the fresh DMEM without FBS. The cells were incubated with various concentrations of CHP at 37°C for another 2 hr. The dose-dependent effect of NPH₄ on the cell injury induced by CHP was examined at the fixed concentration of 1.0 mM CHP; That is, the cells were exposed to the medium containing CHP and 0.1, 0.2, 0.5 or 1.0 mM of NPH₄ for 2 hr. The inhibitory effect of NPH₄ on the elevation of lactate dehydrogenase (LDH) activity induced by 1 mM CHP was compared with those of other antioxidants including Asc and CysNH₂. The concentration of NPH₄ and other antioxidants was 0.5 mM in all cases.

Estimation of endothelial cell injury

The degree of endothelial cell injury was estimated by assessing the activity of LDH released from the cells. An aliquot of the supernatant of the cultured medium was collected at each time interval and subjected to the LDH assay. The LDH activity was measured by the Wroblewski-La Due method (12), with a slight modification. Briefly, the reaction was started by adding 50 μ l of the supernatant to the mixture containing 1.05 mM sodium pyruvate, 0.15 mM NADH and 45 mM phosphate buffer (pH 7.5). The change of absorbance at 340 nm was recorded for 3 min with a Hitachi 320 double-beam spectrophotometer.

Measurement of lipid peroxide

Lipid peroxide was assayed by measuring MDA, basically according to the method of Ochi et al. (13), as follows: The confluent monolayer of the cells in the 24-well plate was cultured in the medium containing CHP or CHP plus NPH₄ for the indicated periods, and then the medium and the cells were collected and sonicated. Two milliliters of 20% trichloroacetic acid and 4 ml of 0.67% 2-thiobarbituric acid were added to the homogenates. The mixture was heated at 97°C for 20 min. After cooling, *n*-butanol was added to the mixture and then it was shaken vigorously. After centrifugation at 3,000 rpm for 20 min, the absorbance of the butanol layer was measured at the wavelength of 532 nm. The amount of MDA was estimated on the basis of the standard curve obtained with 1,1,3,3-tetramethoxypropane. The content of lipid peroxides was expressed as nmol MDA per well.

Statistical analyses

Statistical significance was determined by Student's *t*-test for the comparison between two groups or by two-way repeated measures analysis of variance (ANOVA) and Dunnett's tests for multiple comparison where appropriate. The criterion of significance was taken as $P < 0.05$.

RESULTS

Determination of the scavenging activity of NPH₄ against superoxide anion- and hydroxyl-radicals by ESR spectrometry

The scavenging activity of NPH₄ against superoxide anion- and hydroxyl-radicals was assessed by ESR. The superoxide anion-radicals generated in the HPX/XOD reaction system containing DMPO were scavenged by NPH₄ in a dose-dependent manner. As shown in Fig. 1, NPH₄ showed strong activity to scavenge superoxide anion-radicals. The concentration of 50% inhibition (IC₅₀) of NPH₄ for the radicals was about 0.1 mM, and 1 mM NPH₄ almost completely scavenged the superoxide anion-radicals.

The DMPO-OH signal, which was produced by the reaction of this trapping agent with hydroxyl radicals generated in the Fenton-reaction, was also decreased by NPH₄ in the same fashion as the superoxide anion-radicals (Fig. 2). The IC₅₀ was about 0.4 mM, and 1 mM NPH₄ almost completely scavenged the hydroxyl radicals.

Effect of NPH₄, Asc and CysNH₂ on lipid peroxidation in rat brain homogenates

The level of autoxidation was expressed as nmol of MDA that was formed through the autoxidation of lipids. As shown in Fig. 3, NPH₄ remarkably inhibited the MDA

formation in a dose-dependent manner, and the efficacy was much higher than that of CysNH₂ and close to that of Asc.

The effect of NPH₄ on the cytotoxicity induced by CHP

As noted above, NPH₄ was found to possess a strong antioxidant activity. Thus, we examined the effect of NPH₄ on the BAEC injury induced by CHP. The degree of the cell injury was estimated by assessing the activity of LDH released in the medium as the result of cell damage. As shown in Fig. 4, the release of LDH from the cells into the medium was increased by CHP dose-dependently, reaching a plateau at a concentration of around 1.0 mM CHP. NPH₄ inhibited the elevation of LDH activity induced by 1.0 mM CHP in a dose-dependent manner (Fig.

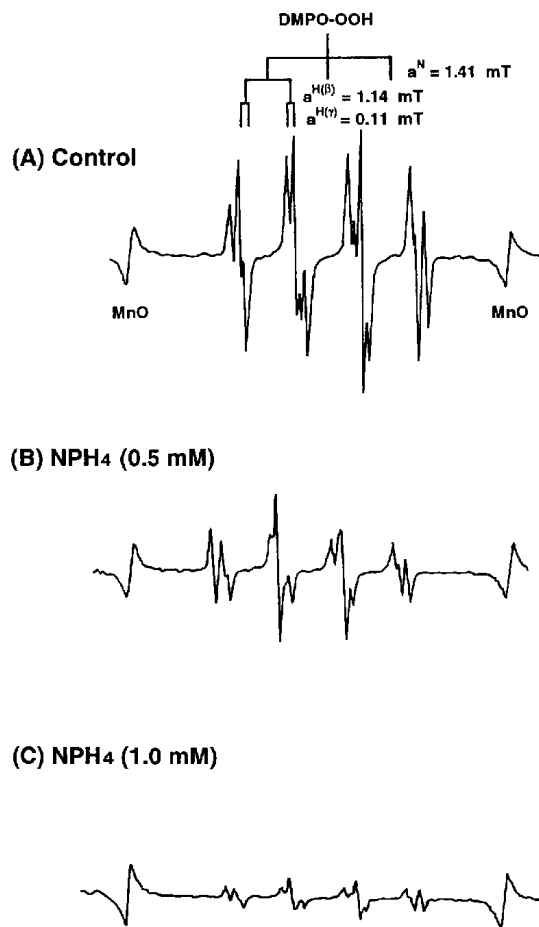


Fig. 1. ESR spectra of DMPO spin adducts formed in the HPX/XOD reaction system in the absence of NPH₄ (A) and presence of 0.5 mM (B) and 1.0 mM (C) of NPH₄. Signals appearing both at high and low field correspond to Mn²⁺ installed in the ESR cavity as a reference. The DMPO-OOH is indicated by the stick diagrams. Hyperfine splitting constants (hfsc's) were calculated based on the Mn²⁺ (MnO) marker. The hfsc's are a^N (1.41 mT), $a^{H(\beta)}$ (1.14 mT) and $a^{H(\gamma)}$ (0.11 mT), which are consistent with those of DMPO-OOH.

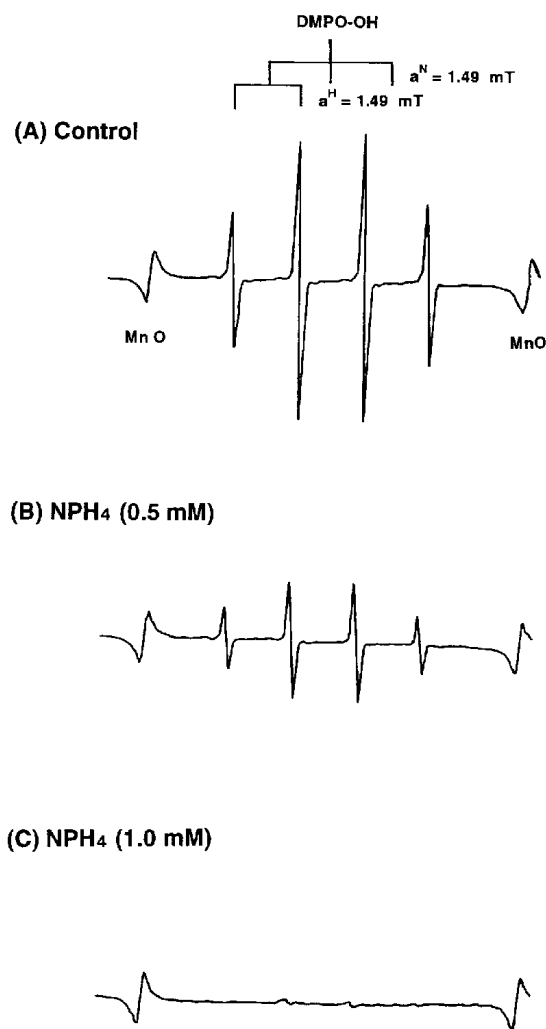


Fig. 2. ESR spectra of DMPO spin adducts formed in the Fenton-reaction in the absence of NPH₄ (A) and presence of 0.5 mM (B) and 1.0 mM (C) of NPH₄. Signals appearing both at high and low field correspond to Mn²⁺ installed in the ESR cavity as a reference. The DMPO-OH is indicated by the stick diagrams. Hyperfine splitting constants (hfsc's) were calculated based on the Mn²⁺ (MnO) marker. The hfsc's are a^N (1.49 mT) and a^H (1.49 mT), which are consistent with those of DMPO-OH.

5).

The suppressive efficacy of NPH₄ on the elevation of LDH activity induced by 1.0 mM CHP was compared with other antioxidants, i.e., Asc and CysNH₂. As can be seen in Fig. 6, the elevation of LDH activity was significantly inhibited by 0.5 mM NPH₄, whereas CysNH₂ showed a weak efficacy, and Asc seemed to increase the level of LDH activity somewhat.

Finally, the effects of various NPH₄ doses and other antioxidants on MDA content induced by CHP treatment was further examined. The content of MDA in the cultured cells was increased by 1 mM CHP (Fig. 7). NPH₄ significantly suppressed the elevation of MDA content

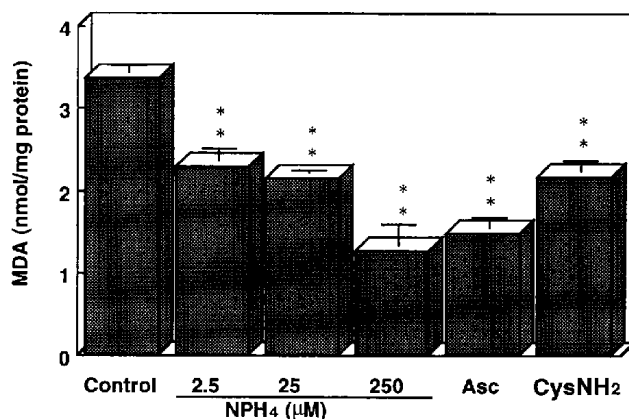


Fig. 3. Effect of NPH₄ and other antioxidants on the autooxidation of rat brain homogenates. NPH₄ was added to the 5% brain homogenates at concentrations of 2.5, 25 and 250 μ M. The activity of NPH₄ was compared with those of Asc and CysNH₂ at 250 μ M. Each value indicates the mean \pm S.E.M. of 4 independent assays. **Statistical significance of the difference from the control group at $P < 0.01$. The control group represents the MDA content of the brain homogenates without antioxidant.

($P < 0.05$) at concentrations ranging from 0.1 to 0.5 mM, whereas Asc and CysNH₂ showed no effect on the elevation of MDA content.

DISCUSSION

Accumulated evidence indicates that active oxygen species such as superoxide anion radical, hydroxyl radical, hydroperoxide and lipid peroxide are involved in many diseases including virus infection, cancer, autoimmune disease and diabetes (14–17). In fact, the potential useful-

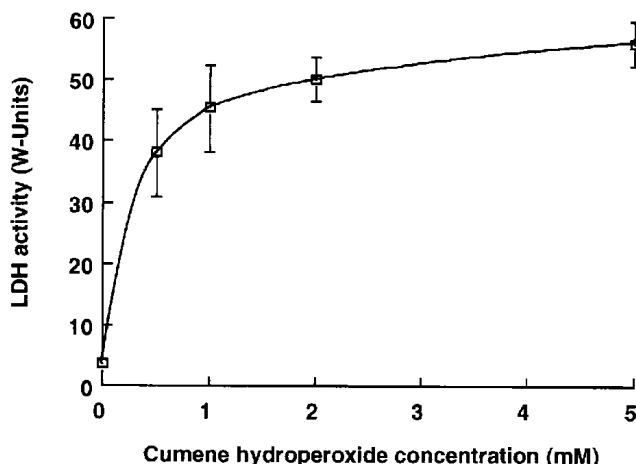


Fig. 4. Effect of CHP on the release of LDH in cultured bovine artery endothelial cells. LDH activity is represented as Wroblewski (W) units. Each value indicates the mean \pm S.E.M. of 3 determinations.

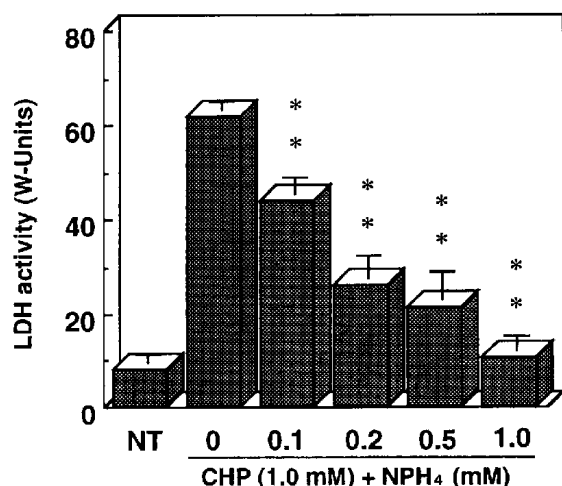


Fig. 5. Effect of NPH₄ on the release of LDH in cultured bovine artery endothelial cells treated with CHP. An appropriate concentration of NPH₄ was added to the medium simultaneously with 1.0 mM CHP, and the mixture was incubated for 2 hr. LDH activity is expressed as Wroblewski (W) units. Each value indicates the mean \pm S.E.M. of 4 determinations. **Statistical significance of the difference from the control group at $P < 0.01$. NT, group not treated with CHP.

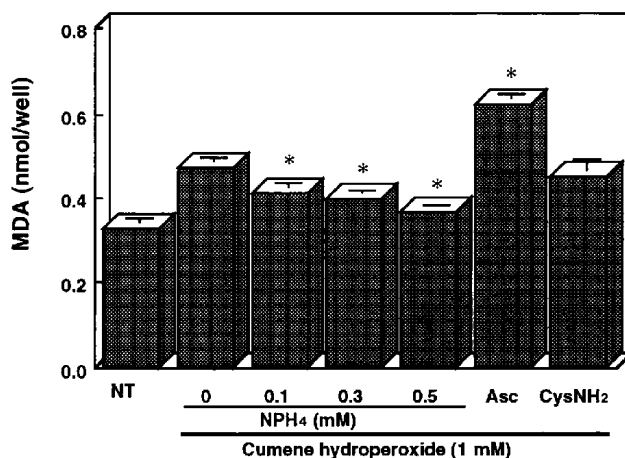


Fig. 7. Effect of NPH₄ and other antioxidants on the lipid peroxide level in cultured bovine artery endothelial cells. NPH₄ and other antioxidants were added to the medium simultaneously with 1 mM CHP, and the mixture was incubated for 2 hr. Doses of NPH₄ and other antioxidants were fixed at 0.5 mM. Lipid peroxide content is expressed as malondialdehyde contents per well (MDA, nmol/well). Each value indicates the mean \pm S.E.M. of 4 determinations. *Statistical significance of the difference from the group not treated with NPH₄ (0) at $P < 0.05$. NT, group not treated with CHP.

ness of various kinds of antioxidants and active oxygen scavenging enzymes such as superoxide dismutase and catalase against these active oxygen-related diseases has already been proposed.

We have reported that NPH₄, a reduced form of neo-

pterin, showed potent antioxidant activity in various systems (7). Namely, NPH₄ was confirmed to possess an extremely high superoxide anion-radical scavenging activity in both HPX/XOD and M ϕ /PMA-superoxide anion radical reaction systems by using the chemiluminescence method, and we concluded that NPH₄ directly scavenges superoxide anion radicals. We further examined how many molecules of radicals react with NPH₄ by using DPPH in order to estimate the scavenging capacity of this compound for active oxygen radicals (8). The reaction of NPH₄ with $1 \times 10^{-4} \mu\text{M}$ DPPH was saturated at a concentration of about $1 \times 10^{-4} \mu\text{M}$, suggesting that one mole of NPH₄ reacts with two moles of DPPH as well as one mole of Asc. The postulated reaction of NPH₄ with free radicals is shown in Fig. 8.

In this study, we conducted ESR spectrometric analysis to evaluate the antioxidant activity of NPH₄ in more detail and to examine its protective efficacy on the BAEC injury induced by CHP.

ESR spectrometric analysis indicated that NPH₄ scavenged both superoxide anion radicals and hydroxyl radicals. It is well known that superoxide anion radicals generate hydroxyl radicals via hydroperoxide in cells, enabling them to be an initiator for lipid peroxidation.

To confirm the scavenging activity of NPH₄ for lipid peroxide, the effect of NPH₄ on the autoxidation of brain homogenate, which is considered to be induced by hydroxyl radicals and lipid peroxide (10), was further examined. As shown in Fig. 3, NPH₄ and Asc completely inhibited

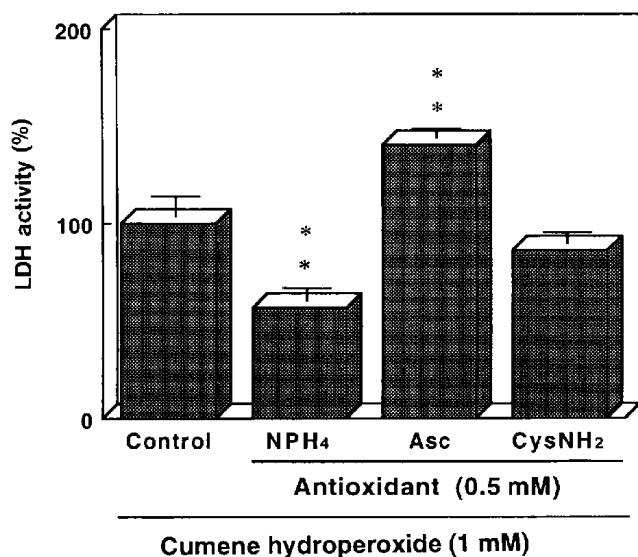


Fig. 6. Effect of NPH₄ and other antioxidants on the release of LDH in cultured bovine artery endothelial cells treated with CHP. Doses of NPH₄ and antioxidants were fixed at 0.5 mM. LDH activity is expressed as % of the control with 1 mM CHP. Each value indicates the mean \pm S.E.M. of 4 determinations. **Statistical significance of the difference from the control group at $P < 0.01$.

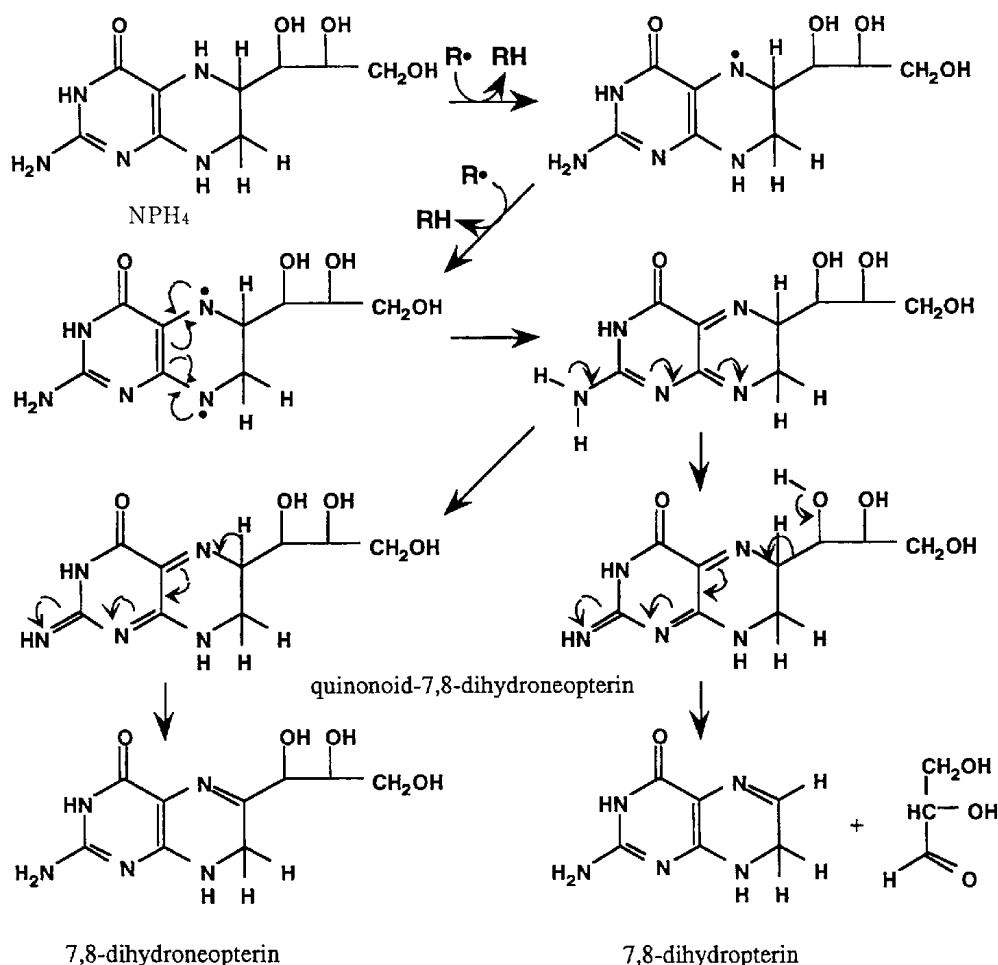


Fig. 8. Speculative reaction mechanism of NPH₄ with radicals (R[•]).

the autoxidation of the rat brain homogenates, while CysNH₂ did only slightly. These results may show that NPH₄ scavenges not only active oxygen radicals such as superoxide anion radicals and hydroxyl radicals, but also lipid peroxide.

It has generally been considered that CHP easily changes to peroxy radicals and hydroxyl radicals, resulting in induction of cytotoxicity against various kinds of cells. To observe the possible *in vivo* potency of NPH₄, we preliminarily examined the effect of NPH₄ on cell injury induced by CHP in cultured BAEC. As shown in Fig. 5, NPH₄ effectively inhibited the CHP-induced BAEC injury. The inhibition may be attributed to the potency of NPH₄ to scavenge hydroxyl radicals and peroxy radicals, which initiates lipid peroxidation, because NPH₄ is a water-soluble antioxidant and seems not to act as a chain breaker of lipid peroxidation in the cell membrane. However, no significant efficacy was found for CysNH₂. Conversely, Asc increased the LDH activity somewhat. Asc might act as a pro-oxidant under our experimental conditions in which free metal ions such as

ferric ion were present.

Taking the endothelial cell injury into consideration, from other stand points such as the generation of atherosclerosis, the cell injury may be an initiating event for the formation of these lesions (18). Briefly, the endothelial cell injury may induce growth factor release and auto-crine or paracrine stimulation of cells in the artery, resulting in migration and proliferation of smooth muscle which are emphasized as the key event in the development of the advanced lesions of atherogenesis. Protection of the cells from injury or stimulation may therefore lead to prevention of atherosclerosis.

Low density lipoprotein (LDL) can be oxidatively modified via endothelial cells, or smooth muscle cells within the artery wall, resulting in its uptake by macrophages through scavenger receptors. This process is also considered to be a key step in the formation of cholesterol-laden macrophage foam cells in atherosclerosis. Antioxidants and antioxidative enzymes would therefore play significant roles in this process, i.e., atherogenesis. It had been already established that addition of α -

tocopherol, butylated hydroxytoluene or probucol, effective free radical scavengers, inhibited the oxidative modification of LDL and the other accompanied-changes such as a marked increased electrophoretic mobility, increased density, hydrolysis of lecithin to lysolecithin, degradation of apoprotein B, and peroxidation as well (19, 20). We also preliminarily examined the effect of NPH₄ on the Cu²⁺-mediated oxidation of LDL and already obtained its inhibitory efficacy (data not shown). However, further studies are needed to reveal the efficacy of NPH₄ on atherogenesis.

Based on these data, it can be suggested that NPH₄ possesses scavenging activity toward not only active oxygen radicals including superoxide anion- and hydroxyl-radical, but also lipid peroxide; and it is also expected to have potential usefulness for the treatment of various diseases such as atherosclerosis, whose pathogenesis is active oxygen radicals-related.

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