Effect of 5,6,7,8-Tetrahydroneopterin on Oxidative Modification of Low-Density Lipoprotein, and Its Uptake in the Macrophage-Like Cell Line J774

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ABSTRACT—Since oxidative modification of low-density lipoprotein (LDL) and uptake of the oxidized LDL by macrophages are closely related to the pathogenesis of atherosclerosis, we examined the inhibitory effect of 5,6,7,8-tetrahydroneopterin (NPH_4) on Cu(II)-induced lipid peroxidation of LDL. NPH_4 significantly inhibited both lipid peroxidation and the associated increase of electrophoretic mobility of ApoB protein. Furthermore, NPH_4 suppressed the uptake of oxidized [^{14}C]oleyl-esterified LDL by the macrophage-like cell line J774. NPH_4 may be a candidate for the treatment of atherosclerosis and other active oxygen-related diseases.

Keywords: 5,6,7,8-Tetrahydroneopterin, Active oxygen, Antioxidant, Low-density lipoprotein, Macrophage

Neopterin (NP) is a 2-amino-4-hydroxypteridine derivative and a precursor of biopterin, which is synthesized from guanosine triphosphate (1). Intracellular GTP is cleaved by GTP-cyclohydrolase to 7,8-dihydroneopterin triphosphate. The latter substance is converted to 5,6,7,8-tetrahydrobiopterin by 7,8-dihydroneopterin reductase in white blood cells and to NP in macrophages. NP has been used as a clinical marker for the diagnosis of various malignant diseases, especially active oxygen related-diseases, including atherosclerosis (2) and AIDS (3). We have shown that the reduced form of neopterin, 5,6,7,8-tetrahydroneopterin (NPH_4), has extremely high antioxidant activity, e.g., scavenging activity on superoxide anion radical (4), and an inhibitory effect on lipid peroxidation. In addition, NPH_4 has a potent inhibitory effect on cumene hydroperoxide-induced bovine endothelial cell injury (5) and active oxygen related-disease models (6, 7).

Recently, it has been suggested that active oxygen is important in the pathogenesis of atherosclerosis (8, 9); that is, superoxide anion radical is supposed to be an inducer of oxidative modification of low-density lipoprotein (LDL), and oxidatively modified LDL causes endothelial cell injury, which may be the initial step in the formation of atheroma (10, 11). Furthermore, in the progression of atherosclerosis, oxidized LDL is closely connected with foam cell formation (9). Oxidized LDL characteristically shows elevation of malondialdehyde (MDA) content, a parameter of lipid peroxidation, and increased electrophoretic mobility of ApoB protein. It has been suggested that naturally occurring antioxidants, polyphenol (12), α-tocopherol and probucol (13), inhibit oxidative modification of LDL and uptake of oxidized LDL by macrophages. These observations have focused attention on the potential usefulness of antioxidants to treat atherosclerosis. In this study, we investigated the inhibitory effects of NPH_4, an extremely potent antioxidant, on the lipid peroxidation of LDL, the increase of electrophoretic mobility of ApoB, and the uptake of oxidized LDL by a macrophage-like cell line J774.

MATERIALS AND METHODS

Chemicals

NPH_4 was kindly provided by Asahi Breweries, Ltd. (Tokyo). The following reagents were purchased from commercial sources: egg yolk LDL (Funakoshi Co., Ltd., Tokyo); Dulbecco's modified Eagle's medium (DMEM) (Nissui Co., Ltd., Tokyo); 2,2′-azobis (2-amidinopropane) dihydrochloride (AAPH), $CuSO_4$, L-ascorbic acid (Asc) and cysteamine (Cys) (Wako Pure Chemicals Co.,

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Ltd., Osaka); [^{14}C]sodium oleate (NEN Research Laboratories, Boston, MA, USA); sodium oleate and bovine serum albumin (BSA) (Sigma Co., Ltd., St. Louis, MO, USA).

Inhibitory effect of NPH_4 on AAPH-induced lipid peroxidation of PC liposomes

Phosphatidyl choline (PC) from turkey egg yolk (100 mg) was dissolved in chloroform : methanol (2 : 1), and then 10 ml of PBS was added and mixed vigorously for 10 min to obtain a liposome suspension. This was incubated at 37℃ for 2 hr in the presence of 20 μM AAPH with or without an antioxidant, NPH_4, Asc or Cys. After the incubation, the content of MDA, a marker of lipid peroxidation was measured as follows: A 0.3-ml aliquot of the incubation mixture was removed into a glass tube and 2 ml of 20% trichloroacetic acid and 4 ml of 0.67% 2-thiobarbituric acid were added to it. The mixture was heated at 97℃ for 30 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 (14).

Inhibitory effect of NPH_4 on Cu(II)-induced lipid peroxidation of LDL

Egg yolk LDL (1 mg protein) was incubated at 37℃ for 0−24 hr in the presence of 10 μM $CuSO_4$ with or without an antioxidant, NPH_4, Asc or Cys. After incubation, the content of MDA was measured (14).

Inhibitory effect of NPH_4 on increase of electrophoretic mobility of ApoB

A solution of oxidized LDL, prepared as above, was diluted with phosphate-buffered saline (PBS), and oxidized LDL (50 μg protein) was subjected to electrophoresis on a 1.0% agarose gel in TBE (Tris/Boric acid/EPTA) buffer (pH 8.6) for 1 hr at 90 V. After the electrophoresis, lipoprotein was stained with Coomassie Brilliant Blue R250.

Culture of the macrophage-like cell line J774

The macrophage-like cell line J774 was obtained from Riken Cell Bank. Cells were cultured in DMEM (pH 7.4) supplemented with 10% (V/V) fetal bovine serum (FBS) at 37℃ in 95% O_2 and 5% CO_2. The medium was changed at 2- to 3-day intervals until assay.

Inhibitory effect of NPH_4 on oxidized LDL uptake in the macrophage-like cell line J774

Oxidized LDL uptake was estimated by measuring the radioactivity of [^{14}C]oleyl esterified LDL accumulated in J774 cells (15). To prepare the [^{14}C]oleate-BSA complex, 50 μCi of [^{14}C]-oleate was dried under an N_2 gas stream in a glass tube. A portion of the residue was suspended in 12.7 mM oleate solution containing 12% (W/V) BSA. The suspension was stirred overnight and stored at −20℃ until assay.

Cells were seeded at a density of 2.0×10^6 cells/well in 24-well plates. After the cells had grown to confluence, the medium was changed to serum-free DMEM containing 10 μM $CuSO_4$, LDL (1 mg protein/well), various concentrations of antioxidant and suspended [^{14}C]oleate-BSA complex (100,000 dpm). Incubation was carried out at 37℃ for 6 hr in 95% O_2 and 5% CO_2. After incubation, the medium was aspirated, and the cells were washed twice with 50 mM Tris-HCl buffer (pH 7.4) containing 150 mM NaCl and 12% (W/V) BSA and then washed once with 50 mM Tris-HCl buffer (pH 7.4) containing 150 mM NaCl. [^{14}C]Oleyl esterified oxidized LDL accumulated in the cells was extracted twice with hexane : isopropanol (3 : 2). The organic layers were combined, placed in a glass tube and evaporated under an N_2 gas stream. An aliquot of the residue was dissolved in 100 μl of hexane, and subjected to TLC using hexane : ethyl ether : acetic acid (90 : 30 : 1) as the solvent. The spot of cholesteryl ester was identified with iodine vapor and then scraped off, placed in a scintillation vial and counted for radioactivity in a toluene scintillation cocktail.

Statistical analyses

Statistical significance was determined by means of the unpaired 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

Inhibitory effect of NPH_4 on AAPH-induced lipid peroxidation of PC liposomes

AAPH at 20 μM caused a marked increase in lipid peroxidation of PC liposomes. NPH_4 at 50 μM significantly inhibited this AAPH-induced lipid peroxidation. Asc was also inhibitory, but Cys showed no effect. NPH_4 was the most potent among the antioxidants we studied (Fig. 1).

Inhibitory effect of NPH_4 on Cu(II)-induced lipid peroxidation of LDL

In the presence of 10 μM Cu(II), LDL was oxidatively modified; the MDA content in the reaction mixture was time-dependently elevated, reaching a plateau from 6 to 24 hr (Fig. 2). In the following experiment, accordingly, the incubation time was fixed at 6 hr. NPH_4 significantly inhibited the elevation of MDA in a dose-dependent man-

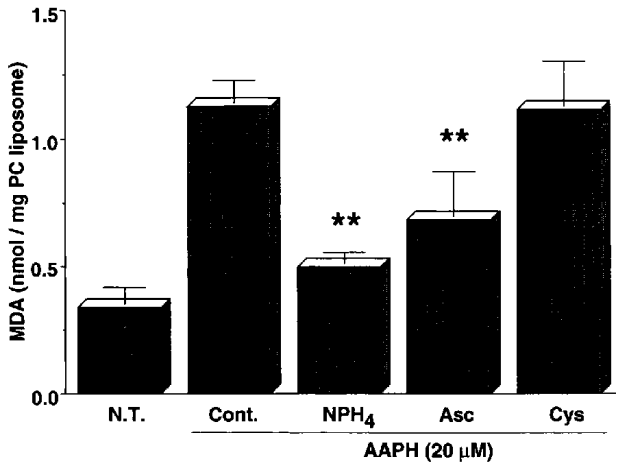

Fig. 1. Inhibitory effect of NPH_4, Asc and Cys on 20 μM AAPH-induced oxidation of PC liposomes. The PC liposome suspension was incubated at 37℃ for 2 hr with or without antioxidant simultaneously with 20 μM AAPH. Each antioxidant was added at the concentration of 50 μM. Each point represents the mean±S.E.M. of triplicate determinations. **: Significantly difference from the control group at $P<0.01$. N.T., group not treated with AAPH.

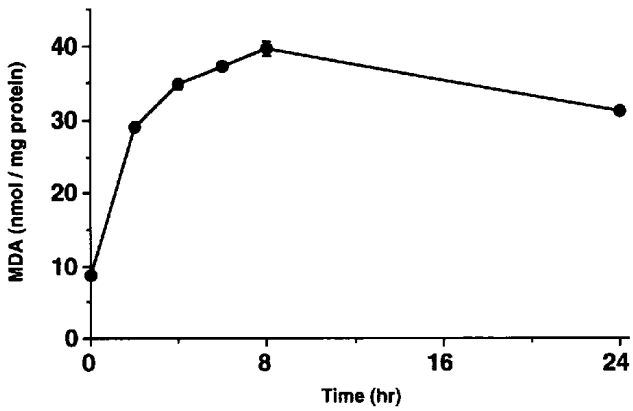

Fig. 2. Time course of lipid peroxidation of LDL induced by 10 μM $CuSO_4$. Lipid peroxidation of LDL was expressed as malondialdehyde (MDA) content. Each point represents the mean±S.E.M. of triplicate determinations.

ner over the concentration range of 5−500 μM. Asc showed a strong inhibitory effect at 500 μM, while Cys showed weak inhibition at the same concentration (Fig. 3).

Inhibitory effect of NPH_4 on the increase of electrophoretic mobility of ApoB

Agarose gel electrophoretic analysis of ApoB in oxidized LDL was performed. The electrophoretic mobility of ApoB was clearly elevated by the Cu(II)-induced oxidation of LDL, and the elevation of mobility was effectively suppressed by NPH_4 at concentrations of more than 50 μM. Asc and Cys also significantly inhibited the elevation of electrophoretic mobility at 500 μM (Fig. 4).

Inhibitory effect of NPH_4 on oxidized LDL uptake in the macrophage-like cell line J774

NPH_4 dose-dependently inhibited [^{14}C]oleyl cholesteryl

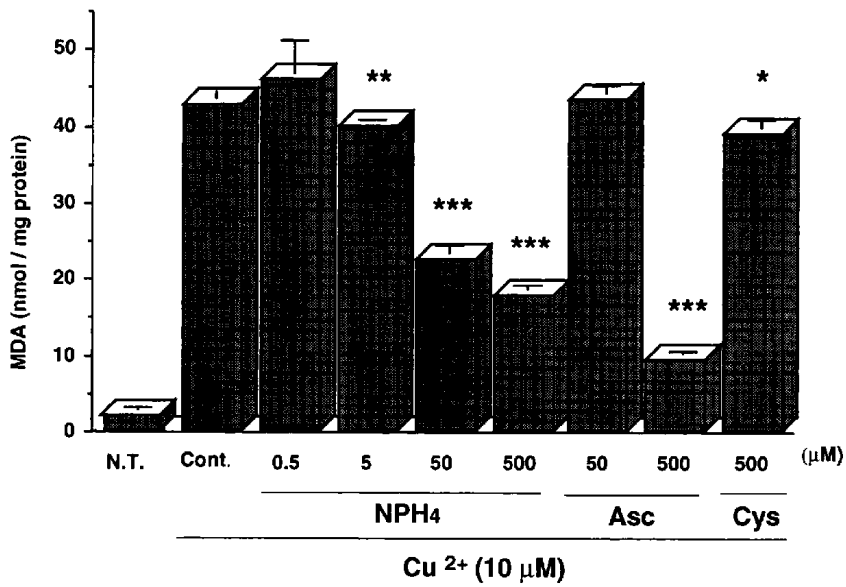

Fig. 3. Inhibitory effects of NPH_4, Asc and Cys on lipid peroxidation of LDL induced by 10 μM of $CuSO_4$. An appropriate concentration of NPH_4 or another antioxidant was added to the reaction mixture simultaneously with 10 μM $CuSO_4$, and the mixture was incubated at 37℃ for 6 hr. Each column represents the mean±S.E.M. of triplicate determinations. *, **, ***: Significantly difference from the control group at $P<0.05$, $P<0.01$ or $P<0.001$, respectively. N.T., group not treated with $CuSO_4$.

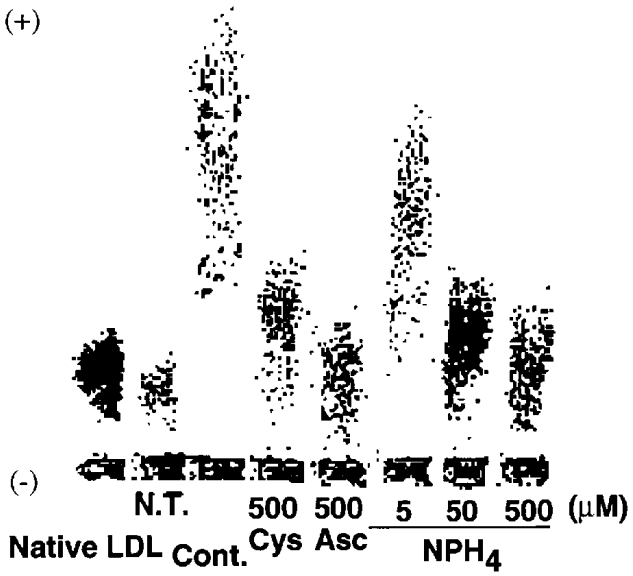

Fig. 4. Inhibitory effects of NPH_4, Asc and Cys on the elevation of the electrophoretic mobility of ApoB. Oxidatively modified LDL obtained in the presence or absence of each antioxidant was subjected to 1% agarose electrophoretic analysis in TBE buffer (pH 8.6). Lipoprotein was detected by Coomassie Brilliant Blue R250 staining. N.T., group not treated with $CuSO_4$.

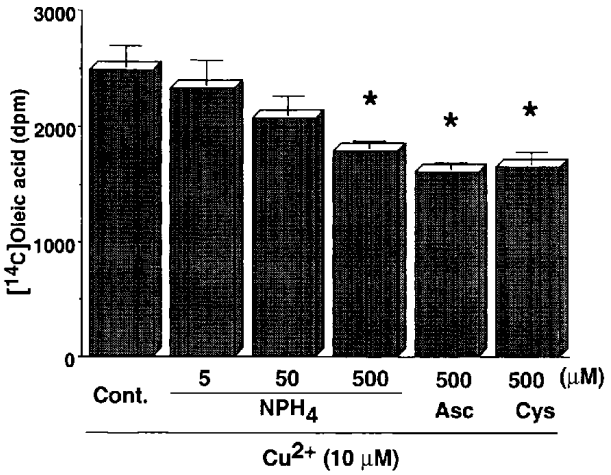

Fig. 5. Inhibitory effect of NPH_4, Asc and Cys on oxidized LDL uptake by macrophage-like J774 cells induced with 10 μM $CuSO_4$. [^{14}C]Oleate-BSA complex was added to the culture medium simultaneously with 10 μM $CuSO_4$, with or without an antioxidant, and the mixture was incubated at 37℃ for 6 hr in 95% O_2 / 5% CO_2. Oxidized LDL uptake was expressed as radioactivity of [^{14}C]oleyl esterified LDL taken up in J774 cells. Each column represents the mean±S.E.M. of triplicate determinations. *: Significant difference from the control group at $P<0.05$.

ester accumulation in the macrophage-like cell line J774. Accordingly, it appears that NPH_4 inhibits modified LDL accumulation in macrophages. Asc and Cys also showed an inhibitory effect at 500 μM (Fig. 5).

DISCUSSION

The effect of 5,6,7,8-tetrahydroneopterin (NPH_4) on oxidative modification of LDL and on the uptake of oxidized LDL by the macrophage-like cell line J774 were compared with those of two other antioxidants, L-ascorbic acid (Asc) and cysteamine (Cys). NPH_4 seemed to be the most potent inhibitor, significantly inhibiting both the AAPH-induced lipid peroxidation of PC liposomes and the Cu(II)-induced oxidative modification of LDL. It was no wonder that NPH_4 formed a complex with Cu(II) ion; however, our preliminary experiment showed that there was little interaction between NPH_4 and Cu(II) ion (data not shown). It also suppressed the uptake of oxidized LDL by J774 and suppressed the elevation of the electrophoretic mobility of ApoB. Asc at high concentration had rather similar activities to NPH_4. Interestingly, although Cys showed a weak inhibitory effect on lipid peroxidation (see Figs. 1 and 3), it strongly suppressed the increase of electrophoretic mobility of ApoB and the uptake of oxidized LDL. It was reported that the inhibition by α-tocopherol of oxidized ^{125}I-labeled LDL uptake by murine peripheral macrophages was due not to an inhibitory effect on lipid peroxidation, but rather to a protective effect against protein degradation in LDL (13). In our experiments, Cys appeared to act in a similar way. These results suggest that degradation of ApoB is a key step in the uptake of oxidized LDL.

NPH_4 has scavenging activity towards hydrogen peroxide, which is thought to be a main factor for modification of ApoB (16). It is thought that this effect inhibited the modification of ApoB, leading to the suppression of oxidized LDL uptake.

The LDL particle contains α-tocopherol and other endogenous antioxidants (12, 13), and it has been reported that supplementation of α-tocopherol inhibits cholesteryl ester accumulation in macrophages (13). It is still unclear whether NPH_4 shows a protective effect on the level of α-tocopherol in LDL. However, superoxide anion radical is known to decrease α-tocopherol in LDL and to oxidize LDL, and since we have shown that NPH_4 has an extremely high superoxide-scavenging activity (4), this action of NPH_4 may reduce the loss of α-tocopherol in LDL.

It has been reported that interferon-γ inhibits rat peritoneal macrophage-induced oxidation of LDL via a direct action on the macrophages (17). The precise mechanism remains unclear, but interferon-γ stimulates the release of NP, which is an inhibitor of the superoxide-generating enzyme NADPH oxidase located on the membrane surface (18), and NPH_2, which we showed to be a superoxide scavenger (4). Thus, NP and NPH_2 may

act as endogenous defensive factors against active oxygen species. Serum NP level was found to be significantly correlated with the extent of carotid atherosclerosis (2), and it was speculated that the release of NP reduces the cytotoxic potency of macrophages. Pteridine derivatives may play defensive roles for the pathogenesis of atherosclerosis. Since we adapted egg yolk LDL to investigate the basal inhibitory effect of NPH_4 on oxidative modification of LDL, further precise examination was needed. However, the above results, especially the inhibition by NPH_4 of the uptake of oxidized LDL by macrophages, taken together with our previous findings that NPH_4 inhibits cumene hydroperoxide-induced endothelial cell injury (5), imply that NPH_4 would be expected to be effective in the treatment of active oxygen-related diseases such as atherosclerosis.

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