Biochemical Pharmacology, Vol. 45, No. 10, pp. 1953–1958, 1993.
Printed in Great Britain.

0006-2952/93 $6.00 + 0.00

PROTECTIVE EFFECTS OF TETRAHYDRONEOPTERIN AGAINST FREE RADICAL-INDUCED INJURY

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(*Received* 21 *December* 1992; *accepted* 1 *March* 1993)

Abstract—The therapeutic potency of 5,6,7,8-tetrahydroneopterin (NPH4) was investigated in ischemic paw edema, Adriamycin® (ADR)-induced cardiotoxicity, and endotoxin [lipopolysaccharide (LPS)]- and carbon tetrachloride (CCl_4)-induced hepatotoxicity in mice. Ischemic paw edema was completely suppressed by pre-administration of NPH4. ADR-induced cardiotoxicity and LPS-induced hepatotoxicity were significantly decreased by post-administration of NPH4. Furthermore, NPH4 ameliorated CCl_4-induced hepatotoxicity. These results suggest that NPH4 may be useful for the treatment of free radical, especially superoxide radical, -related tissue injury.

It is well known that free radical damage to the cell membrane occurs upon ischemia and subsequent reperfusion [1] and during organ dysfunction caused by toxic drugs [2]. A relatively subtle indicator of ischemic injury to a tissue is enhanced capillary permeability, which results in edema formation. More pronounced injury may be manifested as leakage of cytosolic enzymes into the systemic circulation or as microscopic or gross morphologic changes and tissue destruction. Recent evidence suggests that oxygen-derived free radicals may be produced abundantly in ischemic tissues, accounting for at least part of the injurious effects.

Adriamycin® [doxorubicin (ADR§)], an anthracycline antibiotic, is one of the most commonly used anticancer agents, having a therapeutic effect against a broad spectrum of human acute leukemias and neoplasms. However, it also exhibits cardiotoxicity [3], which restricts its clinical usefulness. One of the possible mechanisms for the cytotoxicity is the production of superoxide radical during intracellular metabolism of ADR [4].

Gram-negative bacterial endotoxin [lipopolysaccharide (LPS)] has been shown to induce many injurious reactions [5], among which endotoxin shock is the most important from the clinical viewpoint. Despite the rapid advances in the treatment of shock, mortality caused by endotoxin shock remains high, and is mainly due to multiple organ failure. The liver is the most important organ in the control of energy metabolism. When endotoxin shock is severe and prolonged, it is difficult to maintain or restore these liver functions. Metabolic events in animals suffering from shock are regarded as secondary effects of decreased tissue perfusion, which leads to generalized cellular hypoxia, ischemia and finally organ damage.

It has been established that lipid peroxide formation in liver subjected to ischemia and subsequent reperfusion causes cellular damage [6]. Administration of coenzyme Q_{10} as well as an antioxidant suppressed lipid peroxide formation and enhanced survival of rats subjected to hepatic ischemia followed by reperfusion [6]. An antioxidant, α-tocopherol, showed a similar protective effect on ischemic liver cell damage [7]. These results are of interest because ischemia and shock result in a similar physiologic condition, that is, cellular hypoxia due to decreased tissue blood flow.

Carbon tetrachloride (CCl_4) is most frequently used as a chemical inducer of experimental liver cirrhosis. It is metabolized to the CCl_3 radical in the microsomes of hepatocytes, and the CCl_3 radical forms lipid peroxide by reacting with unsaturated fatty acids in the plasma membranes of hepatocytes. Microsomes, mitochondria and the nuclei of the hepatocytes are impaired by the lipid peroxide, and the hepatocytes are destroyed.

Recently, we reported that the reduced form of neopterin, 5,6,7,8-tetrahydroneopterin (NPH4), showed extremely efficient superoxide radical-scavenging activity [8]. We suspected that NPH4 might be effective against diseases in which active oxygen species play a role as potent pathogenic factors. Therefore, we investigated the effect of NPH4 treatment on superoxide radical-related tissue injury, i.e. ischemic paw edema, ADR-induced cardiotoxicity and endotoxin-induced hepatotoxicity. In addition, we examined the inhibition by NPH4 of CCl_4-induced liver injury as a model in which the pathogenesis does not involve superoxide radical.

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§ Abbreviations: NPH4, 5,6,7,8-tetrahydroneopterin; ADR, Adriamycin®; LPS, lipopolysaccharide; CCl_4, carbon tetrachloride; SOD, superoxide dismutase; TBARS, 2-thiobarbituric acid reactive substances; GPT, alanine aminotransferase; GPx, glutathione peroxidase; CLA, 2-methyl-6-phenyl-3,7-dihydroimidazo(1,2-α)-pyrazin-3-one; PMA, phorbol myristate acetate; XOD, xanthine oxidase.

MATERIALS AND METHODS

Materials. NPH4 was obtained from Junsei Chemicals Co. Ltd (Tokyo, Japan). 2-Methyl-6-phenyl-3,7-dihydroimidazo(1,2-α)-pyrazin-3-one (CLA) was from Tokyo Kasei Kogyo Co. Ltd. ADR was from Kyowa Hakko Kogyo Co. Ltd (Tokyo, Japan). Cuprozinc superoxide dismutase [EC 1.15.1.1 (SOD), 3200 U/mg protein] from bovine liver, phorbol myristate acetate (PMA), xanthine and xanthine oxidase [EC 1.1.3.22 (XOD), 1 U/mg protein] from buttermilk were from the Sigma Chemical Co. (St Louis, MO, U.S.A.). All other chemicals used were of the highest purity grade available from Wako Pure Chemicals Co. Ltd (Osaka, Japan). ICR (male, 6 weeks old) and ddY (male, 6 weeks old) mice were from Tokyo Experimental Animals Co. Ltd (Tokyo, Japan).

Assay of superoxide radical-scavenging activity by chemiluminescence. The scavenging activity of NPH4, ascorbic acid and cysteamine was assayed according to the previous report [8].

Ischemic paw edema. Male ddY mice, 6 weeks old, were used in this experiment. Effects of NPH4 and SOD on ischemic paw edema were examined by the basis of the method of Ōyanagui [9] with a slight modification. Briefly, each mouse was placed in a plastic holder, and a rubber band (38 mm diameter) was bound eight times around the right paw. After 20 min, the rubber band was removed, and the paw thickness was measured with a gauge every 10 min for 40 min, starting just after release. NPH4 (3.0 mg/kg of body weight) or SOD from bovine liver (5.0 mg/kg of body weight) was dissolved in saline, and was administered i.v. just before application of the rubber band. In the placebo group, saline was administered. Ischemia paw edema was determined as the increase of paw thickness after removing the rubber band.

LPS-induced hepatotoxicity. Male ICR mice, 6 weeks old, were used. LPS was dissolved in saline solution, and a dose of 15 or 30 mg/kg of body weight was administered s.c. NPH4 in saline was administered i.p. at a dose of 3.0 mg/kg of body weight once an hour for 8 hr after LPS administration. In the placebo group, saline was administered. After LPS administration, the mice were allowed food and water *ad lib.* and they were monitored for 24 hr to determine survival. The mice, given LPS at a dose of 15 mg/kg of body weight, were killed after 24 hr, and the livers were excised. Lipid peroxidation in the liver was measured by the method of Uchiyama and Mihara [10] as 2-thiobarbituric acid reactive substances (TBARS) concentration, and protein was assayed by the method of Lowry *et al.* [11].

ADR-induced cardiotoxicity. Male ICR mice, 6 weeks old, were used. The mice were administered s.c. a solution of ADR in physiological saline at a dose of 20 mg/kg of body weight. The dosing regimen of NPH4 was as used by Olson *et al.* [12]. After ADR administration, NPH4 in saline was administered i.p. at a dose of 3.0 mg/kg of body weight twice a day for 6 consecutive days. At the 11th day after ADR administration, when cardiotoxicity was expected to be manifest, the mice were killed and the hearts were excised. TBARS and protein concentrations in the heart were estimated as described above.

Table 1. Inhibitory effect of NPH4, ascorbic acid and cysteamine on superoxide released in xanthine/XOD- and splenic macrophage/PMA-reaction systems

Compound	IC_{50} (μM) X/XO*	IC_{50} (μM) MØ/PMA†
NPH4	0.3	0.3
Ascorbic acid	1.5	1.5
Cysteamine	84	80

* Xanthine/XOD-reaction system.
The reaction mixture contains 50 μM xanthine, 1.5 μM CLA, 15 mU of XOD and appropriate concentration of the compound in 1 mL of 50 mM Tris–HCl buffer (pH 7.6). The reaction was started by addition of XOD.

† Splenic macrophage/PMA acetate-reaction system. The reaction mixture contains 1×10^6 splenic cells, 1.0 μM diethylene triamine-*N*,*N*,*N*′,*N*″,*N*″-pentaacetic acid, 1.5 μM CLA and 0.4 μg of PMA in 1 mL of Hank's balanced salt solution. The reaction was started by addition of PMA.

CCl_4-induced acute hepatotoxicity. Male ddY mice, 6 weeks old, were used. CCl_4 (5% w/v) was dissolved in olive oil, and a dose of 5.0 mL/kg of body weight was orally administered to the mice. NPH4 in saline was administered i.p. at a dose of 3.0 mg/kg of body weight once an hour for 8 hr after CCl_4 administration. At 24 hr after CCl_4 administration, blood was withdrawn from the abdominal aorta, and the liver was excised. The plasma was obtained by centrifugation at 3000 rpm for 10 min. The activity of alanine aminotransferase (GPT) in the plasma was measured by using GPT-test kit WAKO (Wako Pure Chemicals Co. Ltd, Osaka, Japan). TBARS and protein concentrations in the liver were estimated as described above.

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 radical-scavenging activity of NPH4

The scavenging activity of NPH4, ascorbic acid and cysteamine was determined by using two anion radical-generating systems, i.e. the xanthine/XOD- and splenic macrophage/PMA-reaction systems. As shown in Table 1, NPH4 showed a significant scavenging activity for the superoxide rather than ascorbic acid and cysteamine in both systems.

Effect of NPH4 on ischemic paw edema of mice

The time course of ischemia paw edema formation is shown in Fig. 1. Paw thickness of the placebo group increased with time after release from ischemia, reaching a plateau after 20 min. NPH4 administration just before ischemia completely suppressed the formation of paw edema. Pre-administration of SOD also suppressed the paw

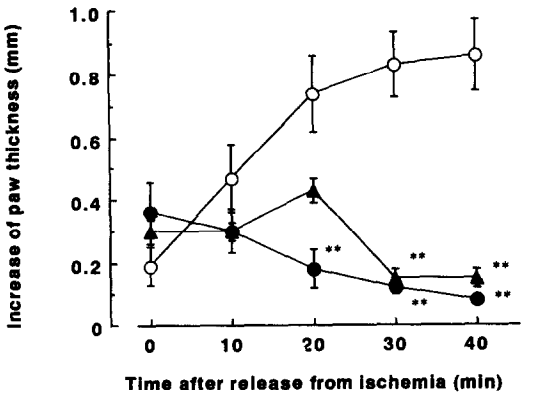

Fig. 1. Effect of NPH4 and SOD on the development of ischemic paw edema of mice. Open circles (○) show the placebo group, closed circles (●) show the NPH4 group and closed triangles (▲) show the SOD group. Each point represents the mean ± SE of five mice. NPH4 or SOD was administered i.v. just before ischemia at a dose of 3.0 or 5.0 mg/kg of body weight, respectively. Significantly different from the control, $^{**}P < 0.01$.

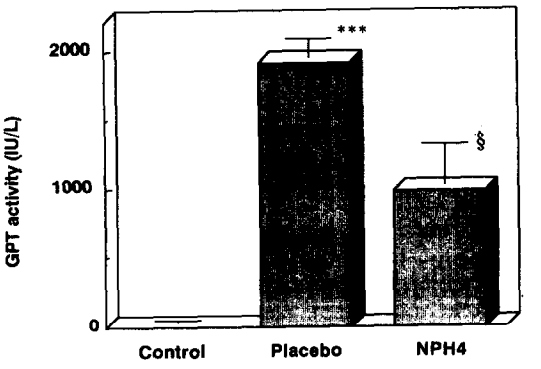

Fig. 2. Effect of NPH4 on GPT activity in the plasma 24 hr after CCl_4 (250 mg/kg of body weight, p.o.) administration. Results are expressed as the mean ± SE of five mice. NPH4 was administered i.p. at a dose of 3.0 mg/kg of body weight once an hour for 8 hr after CCl_4 administration. Significantly different from the control, $^{***}P < 0.001$. Significantly different from the placebo, §$P < 0.05$.

Table 2. Effect of NPH4 on lipid peroxidation*

Group‡	Lipid peroxidation† (nmol/mg protein) Liver LPS	Liver CCl_4	Heart ADR
Control	0.853 ± 0.222	1.019 ± 0.148	0.488 ± 0.029
Placebo	0.797 ± 0.055	2.997 ± 0.382§	1.530 ± 0.171§
NPH4	0.990 ± 0.114	2.274 ± 0.228	0.736 ± 0.089‖

* The lipid peroxidation value is expressed as TBARS (nmol/mg protein). Each value is the mean ± SE of five animals.

† Animals were killed 24 hr after LPS or CCl_4 treatment, or 11 days after ADR treatment.

‡ LPS or ADR was given s.c. dissolved in saline at a dose of 15 or 20 mg/kg of body weight, respectively. CCl_4 was given orally as a 5% (w/v) solution in olive oil at a dose of 5 mL/kg of body weight. NPH4 was given i.p. dissolved in saline at a dose of 3 mg/kg of body weight once an hour for 8 hr after LPS or CCl_4 treatment, or twice a day for 6 days after ADR treatment.

§ Significantly different from its respective control, $P < 0.01$.

‖ Significantly different from the placebo, $P < 0.01$.

edema, but appeared to be effective only at more than 20 min after the release.

Effect of NPH4 on LPS-induced hepatotoxicity

The survival rates for the placebo- and NPH4-treated groups were 45.5% and 81.8%, respectively. The effect of NPH4 on the liver TBARS concentration was also examined in mice given LPS at a dose of 15 mg/kg of body weight. However, as shown in Table 2, no significant difference was observed among the control, placebo and NPH4 groups.

Effect of NPH4 on ADR-induced cardiotoxicity

The effect of NPH4 on the elevated heart lipid peroxidation induced by high doses of ADR (20 mg/kg of body weight) was examined. At the 11th day after ADR administration, TBARS concentration in the heart was increased, as shown in Table 2. The NPH4 treatment markedly reduced the elevation of TBARS concentration in the heart.

Effect of NPH4 on CCl_4-induced hepatotoxicity

The effect of NPH4 on the CCl_4-induced liver injury was evaluated by estimation of GPT activity in the plasma and TBARS concentration in the liver. As shown in Fig. 2, GPT activity in the plasma was drastically elevated at 24 hr after CCl_4 administration. The elevation was significantly inhibited by NPH4 treatment ($P < 0.05$). Table 2 shows TBARS concentration in the liver at 24 hr after CCl_4 administration. In the liver of the CCl_4-treated group, TBARS concentration was significantly higher than that of the control. Post-CCl_4 administration of NPH4 tended to decrease the TBARS concentration in the liver, but without statistical significance.

DISCUSSION

Neopterin is an α-amino-hydroxypteridine derivative, and a precursor of biopterin, which is derived from guanosine triphosphate [13]. It is now generally accepted that neopterin is released from monocytes/macrophages, that interferon-γ, one of the immunomodulators produced by activated T cells, is the only inducer for neopterin secretion from macrophages [14], and that interferon-γ augments the intracellular concentration of guanosine triphosphate as well as the conversion of it to neopterin [15]. Several biological roles of pteridines have been reported so far. Kaufman [16] showed that 5,6,7,8-tetrahydrobiopterin serves as a cofactor for mammalian aromatic amino acid monooxygenases, which

hydroxylate phenylalanine, tyrosine and tryptophan, and thus regulate the biosynthesis of neurotransmitters including dopamine, norepinephrine and serotonin. A tetrahydropteridine derivative was also reported to be a cofactor for the enzymatic oxidation of glyceryl ethers to alcohols [17], but the exact physiological functions of pteridines are still obscure.

Recently, we reported that NPH4 showed an extremely potent superoxide radical-scavenging activity, and that pteridines pretreatment provided significant protection against alloxan-induced diabetes [8], the pathogenesis of which is thought to be related to active oxygen species such as superoxide. These effects were especially shown in NPH4 treatment, and as shown in Table 1, the superoxide radical-scavenging activity of NPH4 was much stronger than those of ascorbic acid and cysteamine, which have been well known to be anti-oxidants. On the basis of these results, we suggested that pteridines, particularly NPH4, might be useful as therapeutic agents against diseases in which active oxygen species play a role as potent pathogenic factors.

Ischemic paw edema is considered to be a model of organic ischemia. Superoxide has been implicated as playing a major role in postischemic or reperfusion injury in a wide variety of tissues. One major source of superoxide are polymorphonuclear leukocytes (neutrophils) [18], which have been implicated as mediators of free radical damage in several tissues. It has been suggested that ischemia and reperfusion result in XOD-generated, superoxide-dependent accumulation of neutrophils in the intestinal mucosa, where neutrophil-derived oxidants mediate or exacerbate injury, or both [19]. Another source of superoxide is the enzyme XOD. Granger *et al.* [20] proposed that XOD-mediated reperfusion injury involves two important events during the ischemic period to poise the tissue for injury once oxygen becomes available again. One of these events, the breakdown of ATP to AMP to hypoxanthine, would provide the substrate for XOD. The second event, the intracellular conversion of xanthine dehydrogenase to XOD, would allow the enzyme to utilize oxygen, rather than NAD, as an oxidant. As a result of the conversion, the active enzyme would produce superoxide, rather than NADH, as hypoxanthine was oxidized. Thus, when oxygen was reintroduced into the tissue the action of the oxidase would provide a burst of superoxide and subsequently derived active oxygen species that would inflict damage on the organ. Allopurinol, a competitive inhibitor of XOD, is highly protective against certain types of reperfusion injury [21], as is SOD. As shown in Fig. 1, administration of NPH4 completely suppressed ischemic paw edema, as did that of SOD from bovine liver. This suggests that NPH4 may be useful as a therapeutic agent to protect various organs against ischemia and reperfusion injury.

Gram-negative bacterial endotoxin, LPS, induces many biological reactions [22], and two major mechanisms have been postulated to be involved in these reactions. One is the effect of anaphylatoxin produced via an alternative pathway, and the other is the effect of chemical mediators such as histamine, serotonin, kinin and platelet-activating factor, which are released from the reticuloendothelial system [23]. Other biological effects of LPS include the inhibition of both glucose metabolism and lipid metabolism, the activation of protein kinase C, lipid peroxidation and direct cellular damage [24].

Lipid peroxide formation or free radical formation is known to cause toxic effects in cellular membranes. Free radicals are scavenged by antioxidants, such as α-tocopherol, the reduced glutathione/glutathione peroxidase (GPx) system, SOD, or catalase [2, 25]. Since NPH4 is also an antioxidant, we suspected that administration of NPH4 might at least partly suppress free radical-associated toxicity. As was expected, the survival rate of NPH4-treated mice given LPS was enhanced in comparison with that of placebo-treated mice (81.8% and 45.5%, respectively). While as shown in Table 2, TBARS concentration in the liver was not different between control, placebo-treated and NPH4-treated mice. Sugino *et al.* [26] reported that TBARS concentration in the liver of placebo group was increased 5-fold 16 hr after LPS administration and returned to the normal level at 24 hr. To elucidate the effect of NPH4 on LPS-induced lipid peroxidation, experiments need to be done at an earlier stage. But the survival data suggest that treatment with NPH4 is effective against LPS toxicity.

ADR, an anthracycline antibiotic, is one of the most important anticancer agents, showing a broad therapeutic activity spectrum against human neoplasms [27]. However, the clinical use of ADR is limited, mainly by its cardiotoxicity [3]. The mechanism of this cardiotoxicity has not yet been established, although numerous hypotheses have been presented [28]. It is well known that ADR has a variety of deleterious effects: induction of free radical-dependent lipid peroxidation, enzyme inhibition, injury to mitochondrial function, modification of calcium transport and release of vasoactive substances [29]. The current investigations suggest that ADR-induced free radicals formed through a redox cycling interaction may have an important role in ADR cardiotoxicity. Bacher *et al.* [4] demonstrated that ADR can be enzymatically activated to the semiquinone free radical by microsomal NADPH-cytochrome P450 reductase, and this enhances microsomal NADPH oxidation, oxygen consumption and subsequently the production of superoxide radical. They also suggested that a free radical scavenger, α-tocopherol, can prevent the one-electron reduction of oxygen by ADR in the microsomal system. The superoxide radical can be dismutated by SOD to hydrogen peroxide, which is catalytically reduced in the cell to H_2O by catalase or GPx, though the latter predominates in cardiac muscles. These systems constitute the major intracellular hydrogen peroxide decomposition processes. In the presence of ADR, Adachi *et al.* [30] demonstrated that the formation of superoxide radical by heart mitochondria was several times higher than that by liver mitochondria, and that administration of ADR induced increases in SOD and catalase contents. Several investigations have been reported on the effect of intracellular free

radical scavengers. Jackson *et al.* [31] showed that the cardiac reduced glutathione/GPx system was activated at the onset of cellular damage by ADR treatment. Alessandro *et al.* [32] reported an increase of mouse heart catalase activity following multiple ADR doses. Droshow *et al.* [33] reported that a single dose of ADR (15 mg/kg) caused an acute depression of cardiac GPx activity 24 hr after administration, and the activity returned to the normal level by 96 hr after administration. On the basis of these reports, we suspect that induction of endogenous active oxygen scavengers or administration of antioxidants such as α-tocopherol and coenzyme Q may decrease the cardiotoxicity of ADR. As shown in Table 2, TBARS concentration in the heart of the NPH4-treated group was lower than that of the placebo-treated group. The changes of SOD, catalase and GPx activities have not been tested, and yet this result suggests that the administration of NPH4 is effective in decreasing cardiac lipid peroxidation induced by ADR.

CCl_4-induced liver injury is a widely used experimental model of hepatotoxicity. Experiments from several laboratories suggest that CCl_4 hepatotoxicity is not simply due to a solvent action on the liver structural components but is more likely caused by the free radicals presumably arising during a CCl_4 activation step mediated by the drug-metabolizing enzymes located in the endoplasmic reticulum [34]. According to several authors [34], these free radicals would initiate a process of lipid peroxidation. The $CCl_3^{\cdot}$ and $CCl^{\cdot}$ free radicals generated during the initial activation step, as well as the peroxyl free radicals produced later during lipid peroxidation, were considered to be responsible for the initial alterations finally leading to necrosis. Castro *et al.* [35] reported that administration of cysteamine significantly suppressed CCl_4-induced hepatotoxicity. Both NPH4 and cysteamine have strong antioxidant activity [16], and so we investigated the effect of NPH4 on CCl_4-induced hepatotoxicity. As shown in Fig. 2 and Table 2, NPH4 treatment tended to suppress the hepatotoxicity induced by CCl_4, but the effect of NPH4 on lipid peroxidation was not statistically significant. There are several reports indicating that a strict correlation may not always exist between the degree of lipid peroxidation and the toxicity of CCl_4. Doses of the lipid antioxidant α-tocopherol which protect against CCl_4 toxicity *in vivo* [36] do not affect the production of conjugated dienes in microsomal lipids or the changes in microsomal properties associated with lipid peroxidation [37]. NPH4 seems to have an analogous action.

In conclusion, we consider that treatment with NPH4 may be effective for protection against radical-induced damage, especially superoxide-induced damage. Further studies on the mechanism of action of NPH4 for amelioration of ADR-induced cardiotoxicity are in progress.

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