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Nonenzymatic Reduction of *N*-Hydroxy-2-acetylaminofluorene to 2-Acetylaminofluorene by Heme in the Presence of Hydroquinones

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This study demonstrates the reduction of *N*-hydroxy-2-acetylaminofluorene (*N*-OH-AAF) to 2-acetylaminofluorene by heme in the presence of hydroquinones. *N*-OH-AAF was reduced by hemoglobin and hematin in the presence of menadiol or 1,4-dihydroxynaphthalene under anaerobic conditions. However, protoporphyrin, ferric chloride and ferrous chloride did not catalyze the reduction of *N*-OH-AAF, even in the presence of hydroquinones. *N*-OH-AAF was also reduced by liver microsomes and cytosol, and by rat blood in the presence of 1,4-dihydroxynaphthalene. When liver preparations and blood were boiled, these activities were not diminished. The reduction is considered to occur nonenzymatically at the heme group of catalytic hemoproteins in the presence of hydroquinones as an electron donor.

Key words —— *N*-hydroxy-2-acetylaminofluorene, reduction, 2-acetylaminofluorene, hemoprotein, hydroquinone, rat blood

INTRODUCTION

2-Acetylaminofluorene (AAF) is activated to *N*-hydroxy-2-acetylaminofluorene (*N*-OH-AAF) by *N*-hydroxylation in the animal body.[1–4] The active metabolite is also reduced to the parent AAF in animal tissues *in vitro*,[5–9] and this reduction is a main detoxification step of *N*-OH-AAF. Therefore, the concentration of *N*-OH-AAF in tissues, resulting from the balance of *N*-hydroxylation of AAF and reduction of *N*-OH-AAF, is of importance in determining carcinogenicity.[10] *N*-Hydroxylation of AAF requires NADPH and oxygen, and has been shown to occur in liver microsomes from several mammalian species.[5,11] It was also reported that *N*-OH-AAF is reduced to AAF by the cytochrome P450 system in liver microsomes.[9] Recently, we showed the nonenzymatic reduction of *N*-OH-AAF by cytochrome P450.[12] In this system, *N*-OH-AAF was reduced to AAF by cytochrome P450 2B1 as if the reduction was an enzymatic reaction. On the other hand, the liver cytosolic enzymes responsible for the reduction of *N*-OH-AAF have also been investigated in our laboratory. An enzyme capable of reducing *N*-OH-AAF was purified from rabbit liver cytosol and was tentatively designated as "*N*-hydroxy-2-acetylaminofluorene reductase".[13] Furthermore, liver cytosolic aldehyde oxidase (EC 1.2.3.1) was found to be responsible for the reduction of *N*-OH-AAF to the amide.[14]

In previous work, the reduction of tertiary amine *N*-oxides to the corresponding amines by mammalian liver cytosols was investigated with imipramine *N*-oxide. The cytosols exhibited significant *N*-oxide reductase activity when menadione (vitamin K_3) was added together with NADH or NADPH.[15,16] In the menadione-dependent *N*-oxide reduction in liver cytosol, it was demonstrated that menadiol (reduced form of menadione) formed from menadione by liver quinone reductase, reduced *N*-oxide compounds to the corresponding amines through catalysis by heme.

The present study provides evidence that *N*-OH-AAF is reduced to AAF by the heme of various hemoproteins present in rat liver and blood in the presence of hydroquinones.

MATERIALS AND METHODS

Chemicals —— AAF, 1,4-dihydroxynaphthalene and protoporphyrin IX disodium salt were purchased from Nacalai Tesque Inc. (Kyoto, Japan). Beef blood hemoglobin, bovine blood hematin, NADH and NADPH were obtained from Sigma Chemical Co. (St.

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Louis, MO, U.S.A.). *N*-OH-AAF was prepared by the reported method.[1] Reduced menadione, menadiol, was prepared by the method of Fieser.[17]

Preparations of Livers and Blood from Rats —— Male rats (180—220 g, 6—7 weeks old, Slc: Wistar/ST strain, Japan Slc, Shizuoka, Japan) were exsanguinated. The liver was immediately perfused with 1.15% KCl and homogenized in 4 volumes of KCl solution, using a Potter-Elvehjem homogenizer. Microsomes and cytosol were obtained from the homogenate by successive centrifugation at 9000 *g* for 20 min and 105000 *g* for 60 min. Blood samples were collected from the jugular vein into polystyrene tubes containing heparin as an anticoagulant. Protein contents of the specimens were determined by the method of Lowry *et al.*[18] using bovine serum albumin as a standard protein.

Assay of Reductase Activity —— The incubation mixture consisted of 0.2 μmol of *N*-OH-AAF, 1 μmol of hydroquinones, 0.1 mmol of sodium fluoride, and liver preparations, blood or a related compound in a final volume of 1 ml of 0.1 M K, Na-phosphate buffer (pH 7.4). Microsomes or cytosol equivalent to 100 mg wet weight of liver, 20 μl of blood, 0.5 mg of hemoglobin, 5 μg of hematin, 1 mg of $FeCl_2$ or $FeCl_3$, or 1 mg of protoporphyrin was used. In the case of blood, 0.01 M phosphate buffer was used. The incubation was performed using a Thunberg tube under anaerobic conditions. The side arm contained *N*-OH-AAF and the body contained all other components. The tube was gassed for 3 min with nitrogen, evacuated with an aspirator for 5 min, and again gassed with nitrogen. The reaction was started by mixing the components of the side arm and the body, and continued for 10 min at 37℃. The mixture, after the addition of 20 μg of phenacetin as an internal standard, was extracted once with 5 ml of ether and the extract was evaporated to dryness *in vacuo*. The residue was dissolved in 0.1 ml of methanol and then subjected to HPLC on an L-6000 chromatograph (Hitachi, Tokyo, Japan) fitted with a 130 mm × 4 mm Lichrosphere 100RP-18 column (Merck, Darmstadt, Germany). The mobile phase was acetonitrile−water (1 : 1) and the chromatograph was operated at a flow rate of 0.4 ml/min at a wavelength of 254 nm. The elution times of AAF and phenacetin were 4.2 and 8.0 min, respectively. AAF formed was determined from its peak area.

Table 1. Reduction of *N*-Hydroxy-2-acetylaminofluorene by Hemoglobin and Hematin in the Presence of Hydroquinones

Addition	2-Acetylaminofluorene formed (nmol/10 min/mg)		
	Hemoglobin		Hematin
	Aerobic	Anaerobic	Anaerobic
None	ND	ND	ND
Menadiol	13±5	110±12	2559±242
1,4-Dihydroxynaphthalene	11±4	87±4	2457±160
Menadione	ND	6±3	−

Each value represents the mean ± S.D. of three experiments.
ND: not detected.

RESULTS

Reduction of *N*-OH-AAF by Heme

N-OH-AAF was reduced by hemoglobin in the presence of a hydroquinone such as menadiol or 1,4-dihydroxynaphthalene under anaerobic conditions. When menadiol was replaced with menadione, the activity was not observed. The reducing activity was inhibited when the incubation was performed under aerobic conditions, indicating that the activity is susceptible to inhibition by oxygen (Table 1). When boiled hemoglobin was used, the activity was not diminished (data not shown). Hematin was also effective when these hydroquinones were added (Table 1). However, protoporphyrin, ferric chloride and ferrous chloride were ineffective in the reduction even in the presence of hydroquinones. The results lead to the speculative conclusion that the heme group of hemoglobin catalyzes the reduction of *N*-OH-AAF using hydroquinones as an electron donor.

Reduction of *N*-OH-AAF by Rat Blood and Liver Preparations

The reduction of *N*-OH-AAF by rat blood was enhanced significantly by the addition of 1,4-dihydroxynaphthalene under anaerobic conditions. Under aerobic conditions the activity was decreased. When the blood was boiled, the hydroquinone-dependent reduction occurred similarly (Table 2). The rat liver microsomes also exhibited the reducing activity toward *N*-OH-AAF in the presence of 1,4-dihydroxynaphthalene under anaerobic conditions (Fig. 1). The liver cytosol showed some reducing activity, and the activity was enhanced about four-fold by the

Table 2. Reduction of *N*-Hydroxy-2-acetylaminofluorene by Rat Blood

Addition	2-Acetylaminofluorene formed (nmol/10 min/mg protein)		
	Blood		Boiled blood[a]
	Aerobic	Anaerobic	Anaerobic
None	1.0±0.1	1.2±0.1	4.8±0.6
1,4-Dihydroxynaphthalene	1.7±0.3**	35.5±1.5*	37.0±1.7*

Each value represents the mean ± S.D. of three experiments. *a*) Blood was boiled for 5 min. *$p<0.001$ compared with control. **$p<0.05$ compared with control.

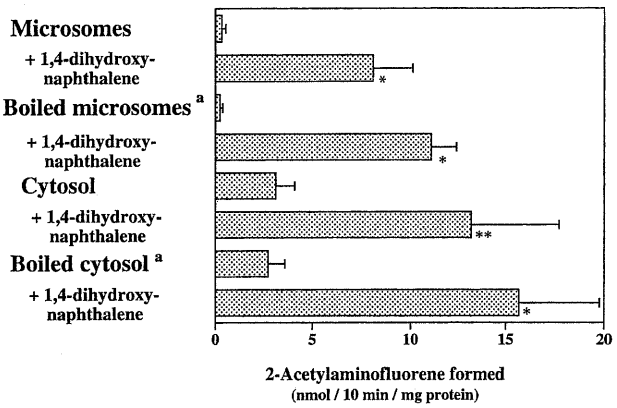

Fig. 1. Reduction of *N*-Hydroxy-2-acetylaminofluorene by Rat Liver Microsomes and Cytosol under Anaerobic Conditions

Each bar represents the mean ± S.D. of three experiments. *a*) The microsomes and cytosol were boiled for 5 min. *$p<0.005$, **$p<0.05$ compared with control.

addition of 1,4-dihydroxynaphthalene. The boiled microsomes and cytosol also exhibited the reducing activity (Fig. 1). These activities of liver preparations were decreased under aerobic conditions, as in the case of blood (data not shown). These facts suggested that the heme moieties of hemoproteins in liver preparations and blood show nonenzymatic reducing activity towards *N*-OH-AAF in the presence of hydroquinones.

DISCUSSION

Hydroquinones, two-electron-reduced quinones, are formed from quinones by quinone reductases such as DT-diaphorase, NADPH-cytochrome P450 reductase, NADH-cytochrome b_5 reductase and ubiquinone oxidoreductase, and have been detected in various tissues including blood.[19-23] Some of the toxicity of quinones has been reported to be attributed to the corresponding hydroquinones.[24-30] Hydroquinone (1,4-dihydroxybenzene) and 2-bromohydroquinone are nephrotoxic and nephrocarcinogenic in rats and formation of the glutathione conjugate is thought to be an activation pathway.[28,29] Tetrachloro-1,4-hydroquinone, a metabolite of hexachlorobenzene, has been implicated in the toxic effects, including the carcinogenicity, of hexachlorobenzene.[26] Hydroquinones formed from the quinones of polycyclic aromatic hydrocarbons by DT-diaphorase also exert toxicity through the generation of reactive oxygen species.[24,27] Some hydroquinones are also known to possess an antioxidant activity, and to inhibit lipid peroxidation by scavenging lipid peroxyl radicals.[23,31] The cytotoxicity of menadione was blocked by the antioxidant effect of menadiol formed by DT-diaphorase.[32,33] However, the function of hydroquinones in detoxification as a reducing agent for a hydroxamic acid, *N*-OH-AAF, is described for the first time in this report.

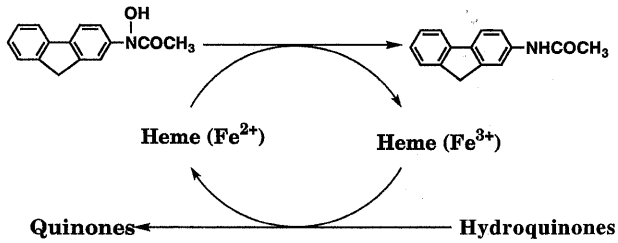

Fig. 2. Possible Mechanism for Hydroquinone-Dependent Reduction of *N*-Hydroxy-2-acetylaminofluorene by Heme

Based on the present results, the following mechanism for the reduction of *N*-OH-AAF by hemoproteins in liver preparations and in the blood of rats is proposed (Fig. 2). *N*-OH-AAF is catalytically reduced by the heme group of hemoproteins in the presence of hydroquinones as an electron donor. In preliminary experiments, when hemoglobin was incubated with 1,4-dihydroxynaphthalene, the UV maximum at 406 nm due to the oxidized form of hemoglobin was changed to a maximum at 432 nm due to the reduced form, while addition of *N*-OH-AAF restored the peak at 406 nm concomitantly with the formation of AAF. This fact suggested that the interconversion between oxidized and reduced forms of heme was involved in the catalytic action.

Yamazoe *et al.*[9] reported that *N*-OH-AAF was enzymatically reduced by the cytochrome P450 system. In contrast, we demonstrated the nonenzymatic reduction of *N*-OH-AAF in rat

liver microsomes. Furthermore, we previously provided evidence that *N*-OH-AAF was nonenzymatically reduced by cytochrome P450.[12] *N*-OH-AAF was reduced by cytochrome P450 2B1 and NADPH-cytochrome P450 reductase in the presence of NADPH and FAD. The reduction also proceeded with native or boiled cytochrome P450 2B1 only in the presence of reduced FAD. In that paper, we proposed a mechanism for the reduction of *N*-OH-AAF via a nonenzymatically catalyzed process via the heme moiety of cytochrome P450 in the presence of reduced FAD as an electron donor.

It has been reported that aldehyde oxidase reduces a variety of hydroxamic acids, including *N*-OH-AAF, in the presence of its electron donor.[13,34] Moreover, a new enzyme responsible for the reduction of *N*-OH-AAF to AAF, which was named "*N*-hydroxy-2-acetylaminofluorene reductase", was purified from rabbit liver cytosol.[12] The enzyme required cysteine, glutathione, dithiothreitol, 2-mercaptoethanol, and either NADPH or NADH as an electron donor. Considerable variability of the two cytosolic enzyme activities exists among species. In contrast, the hydroquinone-dependent heme-catalyzed system responsible for the reduction of *N*-OH-AAF is distributed widely among tissues of various animal species.

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