ABSTRACT:

The reductive metabolism of 2-nitrofluorene, a carcinogenic air pollutant, was investigated in rat skin microsomes and cytosol. 2-Nitrofluorene was reduced to the corresponding amine by the microsomes with NADPH and by the cytosol with 2-hydroxy pyrimidine or 4-hydroxy pyrimidine under anaerobic conditions. The cytosolic activity was much higher than that of skin microsomes. The 2- or 4-hydroxy pyrimidine-linked nitroreductase activity was inhibited by oxyxpurinol and (+/-)-8-(3-methoxy-4-phenylsulfanylphenyl) pyrazolo[1,5-a]-1,3,5-triazine-4(1H)-one (BOF-4272), inhibitors of xanthine oxidase, but not by menadione, chlorpromazine and isovanillin, inhibitors of aldehyde oxidase. When skin cytosol was applied to a DEAE-cellulose column, the fractions containing xanthine oxidase exhibited a marked 2-hydroxy pyrimidine-linked nitroreductase activity. In contrast, the aldehyde oxidase fraction showed little activity. Nitroreductase fractions obtained by ion exchange chromatography showed a band in Western blotting analysis using anti-rat xanthine oxidase. Moreover, the xanthine oxidase fraction exhibited a significant nitroreductase activity in the presence of 2-hydroxy pyrimidine, 4-hydroxy pyrimidine or 4-hydroxy pyrimidine or hydroxanthine, and these activities were inhibited by inhibitors of xanthine oxidase. These results indicated that reduction of 2-nitrofluorene in the skin was mainly catalyzed by xanthine oxidase.

Nitrat ed polycyclic aromatic hydrocarbons (nitro-PAHs\(^1\)), which are found in particulate emissions from diesel engines, in exhaust from kerosene heaters, in urban air, and in river sediments, are generated by the incomplete combustion of fossil fuels and photochemical reactions (El-Bayoumy et al., 1982; Rosenkranz and Merkelstein, 1983). They are potentially mutagenic and carcinogenic to humans through inhalation, ingestion, and skin contact (IARC, 1989; Fu, 1990; Purohit and Basu, 2000). 2-Nitrofluorene is one of the nitro-PAHs and is found in ambient air together with other nitro-PAHs (Beije and Möller, 1988). It has been investigated in a large number of studies as a model substance for nitro-PAHs. The International Agency for Research on Cancer has classified 2-nitrofluorene as carcinogenic in experimental animals and possibly carcinogenic in humans (IARC, 1989).

The mechanism of genotoxicity is thought to involve metabolic reduction of these nitro-PAHs, so that reduction of the nitro group is considered a key metabolic reaction in the activation of 2-nitrofluorene to mutagens (McCoy et al., 1981; Vance et al., 1987) and ultimate carcinogen (Beije and Möller, 1988). In our previous study, it was demonstrated that 2-nitrofluorene was mainly metabolized to 2-aminofluorene and its acylated metabolites in rat and dog (Ueda et al., 2001). Möller et al. (1987) also examined the in vivo metabolism of 2-nitrofluorene in rats and identified two metabolites, 7-hydroxy-2-acetylanilino fluorene and 5-hydroxy-2-acetylanilino fluorene; they speculated that 2-nitrofluorene was reduced to 2-aminofluorene, which was acetylated to 2-acetylanilino fluorene and further metabolized via the known 2-acetylanilino fluorene metabolic pathway. Therefore, it is considered that nitroreduction plays the key role in the metabolism of 2-nitrofluorene in vivo. Reduction of nitro-PAHs and aromatic nitro compounds proceeds with microsomal and cytosolic fractions of mammalian liver. Previous studies showed that cytochrome P450 systems, xanthine oxidase and/or aldehyde oxidase are involved in the reduction of 1-nitropyrene, 4-nitrobiphenyl, 1-nitronaphtalene, 2-nitrofluorene, and 9-hydroxy-2-nitrofluorene to the corresponding amines (Poirier and Weisburger, 1974; El-Bayoumy et al., 1982; El-Bayoumy and Hecht, 1983; Kitamura et al., 1983; Saito et al., 1984). However, little is known about nitroreduction of nitro-PAHs in extrahepatic tissues, for example skin, that are potential targets for environmental contaminants.

Skin is constantly exposed to a variety of environmental chemicals, cosmetics, and drugs. It is not merely a passive structural barrier between the body and environmental chemicals but also may be an important site of metabolism. In recent years, although cutaneous metabolic reactions with skin preparations, tissue-cultured skin, and slices have been well studied, most of the work has dealt with oxidative reactions catalyzed by cytochrome P450 (Kao and Carver, 1990; Jugert et al., 1994; Ahmad et al., 1996; Cottovio et al., 1996) and alcohol dehydrogenase (Boehlein et al., 1994); conjugative reactions catalyzed by glutathione S-transferase (Mukhtar and Bickers, 1981; Agarwal et al., 1992); UDP-glucuronosyltransferase (Moloney et al., 1982); and sulfor transferase (Wong et al., 1993); and hydrolytic reac-
tions catalyzed by esterases (McCracken et al., 1993). Little is known about reductive reactions in skin.

In the present study, in vitro metabolism in rat skin was examined, focusing on nitroreduction of 2-nitrofluorene, and it was demonstrated that xanthine oxidase plays the major role in the nitroreduction of 2-nitrofluorene in the skin.

Materials and Methods

Materials. 2-Nitrofluorene, 2-aminofluorene, 2-hydroxypyrimidine, benzaldehyde, 1-methylxanthine, and xanthine were purchased from Tokyo Chemical Industry Co., Ltd. (Tokyo, Japan). Menadione, oxyuroxinol, chlorpromazine, 4-hydroxypyrimidine, phenylmethylsulfonyl fluoride, N,N,N,N-tetraacetic acid (buffer A), and then homogenized with a Polytron tissue homogenizer (Kinematica GmbH, Zurich, Switzerland). Microsomes and cytosol were obtained from the homogenate by successive centrifugation at 9000g for 20 min and at 105,000g for 60 min. The microsomal fraction was washed by resuspension in the 0.1 M K,Na-phosphate buffer (pH 7.4) containing 0.1 mM dithiothreitol, 0.1 mM phenylmethylsulfonyl fluoride, and stored at −80°C. Protein was determined by the method of Lowry et al. (1951) with bovine serum albumin as a standard.

Assays of Nitroreductase Activity. The incubation mixture consisted of 0.1 μmol of 2-nitrofluorene (the concentration was chosen on the basis of the Kₐ value of 10.1 μM and the accuracy of the assay), 0.5 μmol of an electron donor, and a skin preparation or 0.07 U of milk xanthine oxidase in a final volume of 7 ml of ethyl acetate. The extract was evaporated to dryness in vacuo and the residue was subjected to high-performance liquid chromatography (HPLC).

HPLC. HPLC was performed in an LC-10ADVP (Shimadzu Co., Ltd., Kyoto, Japan) chromatograph fitted with a 250 × 4.6 mm column of CAPCELL PAK UG120 (Shiseido Co., Ltd., Tokyo, Japan). For the determination of 2-aminofluorene, the column was operated at a flow rate of 0.7 ml/min of CH₃CN/H₂O (6:4) at 40°C, using phenacetin as an internal standard, with the detector set at 280 nm. Retention times of authentic phenacetin (an internal standard), 2-aminofluorene, and 2-nitrofluorene were 5.1, 8.7, and 18.5 min, respectively.

Assays of Xanthine Oxidase Activity. The assay was performed by measuring the increase in absorbance at 292 nm, which accompanies the oxidation of xanthine to uric acid.

Western Blot Analysis. Rat skin cytosol and DEAE-fractions containing 5 μg of protein were separated by 7.5% SDS-polyacrylamide gel electrophoresis, and proteins were transferred onto a polyvinylidene fluoride membrane (0.2 mm; Bio-Rad, Hercules, CA) using semidy electrotransfer in glycerine, SDS (0.1%), and methanol (5%). The membrane was blocked for 60 min with blot buffer [20 mM Tris-HCl buffer (pH 8.0) containing 5% (w/v) nonfat milk] at room temperature, then briefly washed with the blot buffer and incubated overnight after addition of anti-rat aldehyde oxidase rabbit serum (1 in 100 dilution) or anti-rat xanthine oxidase rabbit serum (1 in 200 dilution). The membrane was washed four times with the blot buffer, incubated with goat anti-rabbit IgG horseradish peroxidase (Daiichi Pure Chemicals Co., Ltd., Tokyo, Japan) (1 in 2000 dilution) for 2 h at room temperature, and then washed four times with Tris-buffered saline (25 mM Tris-HCl, 0.5 M NaCl, pH 7.5). Development was performed in Tris-buffered saline containing 0.06% diaminobenzidine hydrochloride and 0.036% H₂O₂ for about 5 min.

DEAE-Cellulose Column Chromatography. The skin cytosolic fraction was subjected to ammonium sulfate fractionation, and proteins that precipitated between 30 and 60% ammonium sulfate saturation were collected. The precipitate was dissolved in buffer A and dialyzed against 100 volumes of 10-fold diluted buffer A for 12 h. The dialyzed solution was adsorbed on a column (1.5 × 12 cm) of DE-52, which was equilibrated with buffer A. The column was washed with 50 ml of buffer A and eluted with a 100-mM linear gradient of 0 to 0.3 M sodium chloride in buffer A. The fractions collected were assayed for nitroreductase activity toward 2-nitrofluorene in the presence of 2-hydroxypyrimidine or hypoxanthine, and the active fractions were pooled and stored at −80°C.

Results

Metabolism of 2-Nitrofluorene by Rat Skin Preparations. The in vitro metabolism of 2-nitrofluorene by rat skin preparations was examined. When 2-nitrofluorene was anaerobically incubated with skin microsomes plus NADPH or cytosol plus 2-hydroxypyrimidine, a major metabolite was detected in HPLC chromatograms of the extracts of these incubation mixtures (Fig. 1). In the case of boiled skin preparations, the metabolite was not detected. The metabolite was identified as 2-aminofluorene by comparison of its HPLC, and UV and mass spectral comparison with authentic 2-aminofluorene (data not shown).

The time course of the reduction of 2-nitrofluorene to 2-aminofluorene by rat skin cytosol in the presence of 2-hydroxypyrimidine under an anaerobic condition was essentially linear for 30 min, but little activity was detected under aerobic conditions (Fig. 2A). When the nitroreductase activities of rat skin microsomes plus NADPH or cytosol plus 2-hydroxypyrimidine were assayed with various protein concentrations in incubation mixtures under anaerobic conditions, the amount of 2-aminofluorene formed increased linearly with microsomal and cytosolic protein concentrations up to 3.0 mg/ml. However, the microsomal nitroreductase activity was about one-third of cytosolic activity (Fig. 2B). In the other experiments of this study, incubations were carried out for 30 min at a protein concentration of 2.0 mg/ml under anaerobic conditions.

Skin microsomes catalyzed the reduction of 2-nitrofluorene to 2-aminofluorene in the presence of NADPH under anaerobic conditions. The NADPH-linked activity was completely inhibited by carbon monoxide (data not shown). In contrast, skin cytosol exhibited nitroreductase activity in the presence of 2-hydroxypyrimidine and 4-hydroxypyrimidine and showed weak activity in the presence of benzaldehyde and hypoxanthine. However, N,N,N,N-tetramethylnicotinamide, xanthine, and 1-methylxanthine were not effective for the cytosolic nitroreductase activity. Moreover, NADPH and NADH, electron donors to NAD(P)H:quinone oxidoreductase (DT-diaphorase), had no effect on the cytosolic nitroreductase activity. The full activity of
cytosol supplemented with electron donor was much higher than that of microsomes supplemented with NADPH. These facts suggest that 2-nitrofluorene was mainly reduced to 2-aminofluorene in rat skin by molybdenum hydroxylases (aldehyde oxidase or xanthine oxidase). Furthermore, the effect of some aldehyde oxidase or xanthine oxidase inhibitors on nitroreductase activity in skin cytosol was examined. The 2-hydroxypyrimidine-linked nitroreductase activity was remarkably inhibited by oxypurinol and BOF-4272, which are inhibitors of xanthine oxidase (Yamamoto et al., 1993). However, the nitroreductase activity was not inhibited by inhibitors of aldehyde oxidase, such as menadione, chlorpromazine, and isovanillin (Clarke et al., 1995; Jordan et al., 1999). Hypoxanthine-linked nitroreductase activity was also inhibited by oxypurinol and BOF-4272 (Fig. 3). Furthermore, commercial bovine milk xanthine oxidase exhibited nitroreductase activity toward 2-nitrofluorene in the presence of 2-hydroxypyrimidine but not xanthine. The 2-hydroxypyrimidine-linked nitroreductase activity (1.82 nmol/min/mg of protein) was markedly inhibited by addition of oxypurinol but not menadione. These facts suggest that xanthine oxidase mainly catalyzed the nitroreduction of 2-nitrofluorene in rat skin.

**DEAE Column Chromatography of Skin Cytosol.** When the skin cytosol was fractionated with ammonium sulfate as described under Materials and Methods, the nitroreductase activity was exclusively recovered between 30 and 60% ammonium sulfate saturation (data not shown). Furthermore, the ammonium sulfate precipitate was chromatographed on a DEAE-cellulose column. As shown in Fig. 4, 2-hydroxypyrimidine- and hypoxanthine-linked nitroreductases were coeluted with xanthine oxidase (fraction I; fraction 33–38). However, 2-hydroxypyrimidine-linked nitroreductase was also eluted in another fraction (fraction II; fraction 42–49).

**Western Blot Analysis of Rat Skin Aldehyde Oxidase and Xanthine Oxidase.** Fractions I and II were subjected to Western blot analysis. The primary antibodies used to probe the blots were rabbit anti-rat aldehyde oxidase and rabbit anti-rat xanthine oxidase antisera. As shown in Fig. 5, rabbit anti-rat xanthine oxidase antiserum reacted with skin cytosol and strongly reacted with fraction I, indicating that

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**Fig. 1.** HPLC of the metabolites of 2-nitrofluorene generated by rat skin microsomes with NADPH (A) or by cytosol with 2-hydroxypyrimidine (B). The absorbance was measured at 280 nm. AF, 2-aminofluorene; NF, 2-nitrofluorene.

**Fig. 2.** Reduction of 2-nitrofluorene to 2-aminofluorene with rat skin microsomes and cytosol; A, time course of the cytosolic activity; B, skin microsomes and cytosol dependence. Each value represents the mean ± S.D. of three rats. In A, the mixture contained 0.1 μmol of 2-nitrofluorene, 0.5 μmol of 2-hydroxypyrimidine and 2.0 mg of skin cytosol in a final volume of 1 ml of 0.1 M K,Na-phosphate buffer (pH 7.4). The incubation was performed at 37°C for 0, 5, 10, 15, 20, and 30 min under anaerobic (●) or aerobic (○) conditions. In B, the same procedure as in (A) was used except that protein concentrations (0, 0.25, 0.5, 1.0, 2.0 and 3.0 mg/ml) of skin cytosol (●) and microsomes (○) were varied, and the incubation time was 30 min.
these bands correspond to xanthine oxidase. However, no band was detected from fraction II. On the other hand, rabbit anti-rat aldehyde oxidase antiserum reacted with skin cytosol, and a faint band was detectable in fraction II, but not in fraction I. These facts suggest that nitroreductase activity toward 2-nitrofluorene is exhibited mainly by xanthine oxidase and slightly by aldehyde oxidase.

**Metabolism of 2-Nitrofluorene by DEAE Column Chromatography Fractions.** Fraction I exhibited significant nitroreductase activity in the presence of 2-hydroxypyrimidine, 4-hydroxypyrimidine, or hypoxanthine. However, \(N^1\)-methyl nicotinamide and benzaldehyde supported little activity. The full activities with 2-hydroxypyrimidine and 4-hydroxypyrimidine were approximately 9-fold and 45-fold higher than those of skin cytosol, respectively. The 2-hydroxypyrimidine-linked nitroreductase activity of fraction I was inhibited by oxypurinol and BOF-4272 but not by menadione, chlorpromazine, or isovanillin (Fig. 6). Hypoxanthine-linked activity was also inhibited by oxypurinol and BOF-4272. On the other hand, the nitroreductase activity of fraction II was also enhanced by addition of 2-hydroxypyrimidine and 4-hydroxypyrimidine but to a much lesser extent than in the case of fraction I. The 2-hydroxypyrimidine- and 4-hydroxypyrimidine-linked nitroreductase activities were inhibited by menadione (data not shown). These results confirmed that the nitroreductase activities in fraction I and II were due to xanthine oxidase and aldehyde oxidase, respectively. Fraction I accounted for the majority of the activity.
2-aminofluorene generated was determined by HPLC. Other details are described under Materials and Methods.

Menadione was added at 1° the presence of an inhibitor. The incubation was performed at 37°C for 30 min with 2.0 mg/ml of fraction I and various electron donors under anaerobic conditions.

Discussion

There is no conclusive evidence that 2-nitrofluorene in ambient air is carcinogenic to human skin. However, El-Bayoumy et al. (1982) reported that 3-nitropyrene and 6-nitrochrysenes induced skin tumors in laboratory animals. In rat, 2-nitrofluorene induced skin tumors after dermal application (Morrison et al., 1950). 2-Nitrofluorene lacked initiating capacity in a mouse skin two-stage carcinogenesis system, and nitroreductase activity toward nitro-PAH in mouse skin was very low (Möller et al., 1993). This suggests that 2-nitrofluorene may be carcinogenic to skin, but this nitro-PAH is not activated by nitroreduction in mouse skin. The reductive metabolites may be more reactive electrophiles, which react with nucleic acid to produce adducts. Our findings suggest that 2-nitrofluorene is carcinogenic after nitroreduction to 2-aminofluorene in rats. On the other hand, 2-nitrofluorene may be activated by ultraviolet radiation to various reactive intermediates that can bind to RNA and protein (Wierckx et al., 1992). Furthermore, Asokan et al. (1986) reported that the skin is a major target tissue of airborne nitroarenes. Bus drivers and tramway employees were at increased risk of developing skin cancer (Soll-Johanning et al., 1998). These results indicate that numerous environmental chemicals, including nitro-PAHs, may induce skin damage.

In the present study, it has been demonstrated that 2-nitrofluorene is metabolized to 2-aminofluorene in rat skin preparations. In a previous study, nitro-PAHs were metabolized to the corresponding amines by reconstituted cytochrome P450 (Saito et al., 1984). Our previous studies showed that rabbit liver microsomes and cytosol generated 2-nitrofluorene reductive metabolites, hydroxylamine and amine, and provided evidence that aldehyde oxidase functions as a major liver enzyme responsible for 2-nitrofluorene reduction (Tatsumi et al., 1986). 1-Nitropyrene and 3-nitrofluoranthenes were transformed to reductive metabolites by xanthine oxidase in animal livers (Bauer and Howard, 1990). Therefore, it was supposed that several enzymes catalyze the nitroreduction of nitro-PAHs. In the present study, nitroreductase activity in skin cytosol was greater than that of microsomes and was catalyzed mainly by xanthine oxidase. In mammary gland, cytosolic nitroreductase activity toward 9-oxo-2-nitrofluorene was supported by addition of hypoxanthine and N1-methylnicotinamide, and the activity was not inhibited by menadione (Ritter et al., 2000). However, our findings suggest that the nitroreduction of nitro-PAHs in mammary gland also involves xanthine oxidase. 2-Nitro-4-aminoaniline and 3-nitro-4-hydroxyaniline, hair dye constituents, were mutagenic in mammalian cells (Van Duuren, 1980). In the skin, these chemicals may be nitroreduced by xanthine oxidase, and such reduction would play an important role in their toxicity. Furthermore, xanthine oxidase is known to produce toxic superoxide anions, which are implicated as a possible mediator of tissue damage. Reactive oxygen species generated by xanthine oxidase are thought to cause lipid peroxidation, inflammation, and symptoms of aging in the skin.

In the current study, the cytosolic nitroreductase activity was enhanced by addition of 2-hydroxypyrimidine and 4-hydroxypyrimidine, which are electron donors to aldehyde oxidase. The electron donor requirements of xanthine oxidase in rat skin seem contradictory. However, the 2-hydroxypyrimidine- and 4-hydroxypyrimidine-linked nitroreductase activities were not inhibited by inhibitors of aldehyde oxidase but were inhibited by inhibitors of xanthine oxidase. In addition, the requirements of 2-hydroxypyrimidine and 4-hydroxypyrimidine, and the susceptibility to xanthine oxidase inhibitors of commercial bovine milk xanthine oxidase, are consistent with the involvement of skin xanthine oxidase. The results of Western blotting analysis using anti-rat xanthine oxidase also supported the involvement of xanthine oxidase, but not aldehyde oxidase, in the nitroreduction. These experiments are consistent with the idea that 2-hy-
Fig. 7. Xanthine oxidase-catalyzed reduction of 2-nitrofluorene in rat skin.

droxyuridine and 4-hydroxyuridine also function as electron donors of xanthine oxidase and that the nitroreduction by rat skin cytosol mainly involves xanthine oxidase but not aldehyde oxidase (Fig. 7).

The findings of this and other studies suggest that many xenobiotics and endogenous compounds as well as aromatic nitro compounds, might be metabolized in skin. When various drugs, cosmetics, toiletries, industrial chemicals, agricultural chemicals, and detergents come into contact with the skin, metabolites produced in the skin may cause irritation, sensitization, cytotoxicity, mutagenicity, or carcinogenicity.

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