XANTHINE OXIDASE-CATALYZED METABOLISM OF 2-NITROFLUORENE, A CARCINOGENIC AIR POLLUTANT, IN RAT SKIN

OSAMU UEDA, SHIGEYUKI KITAMURA, KOJI OHASHI, KAZUMI SUGIHARA, AND SHIGERU OHTA

Graduate School of Biomedical Sciences, Hiroshima University, Hiroshima, Japan
(Received September 23, 2002; accepted December 18, 2002)

This article is available online at http://dmd.aspetjournals.org

ABSTRACT:
The reductive metabolism of 2-nitrofluorene, a carcinogenic air pollutant, in rat skin microsomes and cytosol was investigated. 2-Nitrofluorene was reduced to the corresponding amine by the microsomes with NAPDH and by the cytosol with 2-hydroxyiminidine or 4-hydroxyiminidine under anaerobic conditions. The cytosolic activity was much higher than that of skin microsomes. The 2- or 4-hydroxyiminidine-linked nitroreductase activity was inhibited by oxyxpurinol and (±/-)8-(3-methoxy-4-phenylsulfinylphenyl) 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-hydroxyiminidine-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-hydroxyiminidine, 4-hydroxyiminidine or hypoxanthine, 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.

Nitro-PAHs are found in particulate emissions from diesel engines, 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-acetylaminofluorene and 5-hydroxy-2-acetylaminofluorene; they speculated that 2-nitrofluorene was reduced to 2-aminofluorene, which was acetylated to 2-acetylaminofluorene and further metabolized via the known 2-acetylaminofluorene 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-nitrophenyl, 1-nitronaphthalene, 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 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 (Boehnlein 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 sulfotransferase (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-hydroxyxypuridine, benzaldehyde, 1-methylxanthine, and xanthine were purchased from Tokyo Chemical Industry Co., Ltd. (Tokyo, Japan). Menadione, oxypurinol, chlorpromazine, 4-hydroxyxypuridine, phenylmethylsulfonyl fluoride, N\textsuperscript{3}-methylnicotinamide and isovanillin were from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). BOF-4272 was obtained from Otsuka Pharmaceutical Factory, Inc. (Tokushima, Japan). Hypoxanthine and bovine milk xanthine oxidase (14.43 U/ml, 10.5 mg/ml) were purchased from Calbiochem (San Diego, CA). Anti-rat aldehyde oxidase rabbit serum was prepared by the method of Sugihara et al. (1995). Anti-rat xanthine oxidase rabbit antibody was kindly provided by Dr. T. Nishino (Nippon Medical School, Tokyo, Japan). Other chemicals used were of the highest grade commercially available.

Preparations of Tissue Microsomes and Cytosol. Male Sea/Sprague Dawley rats (5 weeks old) from Seiwa Experimental Animals, Ltd. (Fukuoka, Japan) were used. The animals were exsanguinated, and the back of each animal was immediately shaved with an electric clipper over an area of approximately 4 × 5 cm. The skin was excised, and the sheets were placed with the epidermal side down on an ice–cooled glass plate, and subcutaneous tissues were scraped off with scissors. Scraped sheets of skin free from fat were cut into pieces with scissors and mixed with three volumes of 0.1 M K\textsubscript{2}Na\textsubscript{2} phosphate buffer (pH 7.4) containing 0.1 mM dithiothreitol, 0.1 mM phenylmethanesulfon fluoride, and 0.1 mM EDTA, and recentrifugation at 105,000 g for 60 min. The 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\textsubscript{2}Na\textsubscript{2} phosphate buffer (pH 7.4) containing 0.1 mM EDTA, and recentrifugation at 105,000g for 60 min. The 105,000g pellet (microsomal fraction) was resuspended in the same buffer. The cytosol fraction was dialyzed against 400 volumes of buffer A for 18 h. The microsomes and cytosol were 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 Km 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 1 ml of 0.1 M K\textsubscript{2}Na\textsubscript{2} phosphate buffer (pH 7.4). The incubation was performed by mea-
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

---

**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.

---

**METABOLISM OF 2-NITROFLUORENE IN RAT SKIN**
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, $N^\text{6}$-methylaminopyrimidine 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 oxyxpurinol and BOF-4272 but not by menadione, chlorpromazine, or isovanillin (Fig. 6). Hypoxanthine-linked activity was also inhibited by oxyxpurinol 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.
(A) anti-rat xanthine oxidase  (B) anti-rat aldehyde oxidase

cytosol  Fraction II  Fraction I  cytosol  Fraction II  Fraction I

Fig. 5. Western blots probed with anti-rat xanthine oxidase antibody (A) and anti-rat aldehyde oxidase antibody (B) for rat skin cytosol, fraction I and fraction II.

Blots were loaded with 5 μg of protein of rat skin cytosol or DEAE-fraction (fraction I and fraction II). Other details are described under Materials and Methods.

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-nitrochrysene induced skin tumors in laboratory animals. In rat, 2-amino-1-nitrofluorene induced skin tumors after dermal application (Morris 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-amino-1-nitrofluorene 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, Aoskan 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-Johannening et al., 1998), and the incidence of skin cancer was significantly elevated among bus drivers in Denmark (Netterstrøm, 1988). 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-amino-1-nitrofluorene 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-nitrofluoranthene 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 mammery 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 mammery 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-hydroxyxypyrrimidine and 4-hydroxyxypyrrimidine, which are electron donors to aldehyde oxidase. The electron donor requirements of xanthine oxidase in rat skin seem contradictory. However, the 2-hydroxyxypyrrimidine- and 4-hydroxyxypyrrimidine-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-hydroxyxypyrrimidine and 4-hydroxyxypyrrimidine, 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. 6](image-url)  

Fig. 6. Reduction of 2-nitrofluorene to 2-amino-1-nitrofluorene by fraction I separated by DEAE-cellulose column chromatography.

Each bar represents the mean ± S.D. of three experiments. A, requirement of electron donor. B, the effect of inhibitors on 2-hydroxyxypyrrimidine- or 4-hydroxyxypyrrimidine-linked nitroreductase activity. C, the effect of inhibitors on hypoxanthine-linked nitroreductase activity. Open column, in the absence of an inhibitor; hatched column, in 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. Menadione was added at 1 × 10-3 M; chlorpromazine, isovanillin, and oxypurinol were added at 1 × 10-4 M; and BOF-4272 was added at 20 μg/ml. The amount of 2-amino-1-nitrofluorene formed was determined by HPLC. Other details are described under Materials and Methods.
2-hydroxypyrimidine
4-hydroxypyrimidine

\[
\begin{align*}
\text{Hypoxanthine} & \quad \text{Xanthine oxidase} \\
& \quad \text{xanthine} \quad \text{uracil}
\end{align*}
\]

Fig. 7. Xanthine oxidase-catalyzed reduction of 2-nitrofluorene in rat skin.

droxypyrimidine and 4-hydroxypyrimidine 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 carcinogeticity.

Acknowledgments. We thank Dr. Takeshi Nishino, Nippon Medical School, who provided anti-rat xanthine oxidase rabbit antibody. We also thank Dr. Shinshu Naito, Otsuka Pharmaceutical Factory, Inc., who provided BOF-4272.

References


Rosenkranz HS and Mermelstein R (1983) Mutagenicity and genotoxicity of nitroarenes. All nitro-containing chemicals were not created equal. Mutat Res 114:217–267.


