INVOLVEMENT OF MOLYBDENUM HYDROXYLASES IN REDUCTIVE METABOLISM OF NITRO POLYCYCLIC AROMATIC HYDROCARBONS IN MAMMALIAN SKIN

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Received April 24, 2005; accepted May 31, 2005

ABSTRACT:

Molybdenum hydroxylases, aldehyde oxidase and xanthine oxidoreductase, were shown to be involved in the nitroreduction of 2-nitrofluorene (NF), 1-nitropyrene, and 4-nitrobiphenyl, environmental pollutants, in the skin of various mammalian species. NF was reduced to 2-aminofluorene by hamster skin cytosol in the presence of 2-hydroxypyrimidine, 4-hydroxypyrimidine, N7-methyl-2-aminopyrimidinone, or benzaldehyde, but not hypoxanthine or xanthine. Inhibitors of aldehyde oxidase markedly inhibited these nitroreductase activities, but oxypurinol, an inhibitor of xanthine oxidoreductase, had little effect. In DEAE column chromatography of hamster skin cytosol, the major fraction exhibiting nitroreductase activity also showed aldehyde oxidase activity. 2-Hydroxypyrimidine-linked nitroreductase activities of skin cytosol from rabbits and guinea pigs were also inhibited by an inhibitor of aldehyde oxidase. In contrast, nitroreductase activities of skin cytosols of rats and mice were markedly inhibited by oxypurinol. When aldehyde oxidase activity was estimated in skin cytosol of various mammals using benzaldehyde oxidase activity as a marker, considerable variability of the activity was found. The highest activity was observed with hamsters, and the lowest activity with rats. On the other hand, the highest xanthine oxidoreductase activity was observed with rats, and the lowest activity with rabbits. These skin cytosols of various mammals also exhibited significant 2-hydroxypyrimidine-linked nitroreductase activities toward 1-nitropyrene and 4-nitrobiphenyl catalyzed by aldehyde oxidase and xanthine oxidoreductase. Thus, NF was mainly reduced by aldehyde oxidase and xanthine oxidoreductase in skins of animals. However, the contributions of these two molybdenum hydroxylases were considerably different among animal species.

Nitrated polycyclic aromatic hydrocarbons (nitro-PAHs) are widely distributed in the environment, chiefly as the result of incomplete combustion processes, and have various biological activities including mutagenicity and carcinogenicity through skin contact (El-Bayoumy et al., 1982; Beije and Möller, 1988; Purushit and Basu, 2000). 2-Nitrofluorene (NF) is classified as a nitro-PAH and requires metabolic activation, being converted into electrophilic metabolites, which ultimately bind to cellular macromolecules. Reduction of the nitro group is thought to be a key step in the metabolic activation (Fu, 1990). In addition, nitroreduction is considered the major reaction in the metabolism of NF in vivo (Möller et al., 1987; Ueda et al., 2001). Therefore, the nitroreduction of these compounds in animal livers has been investigated extensively, and it was reported that the reductive metabolism of nitro-PAHs is catalyzed by cytochrome P450, aldehyde oxidase, and/or xanthine oxidoreductase in mammalian liver (Saito et al., 1984; Tatsumi et al., 1986; Bauer and Howard, 1990).

Recently, we examined reductive metabolism of NF in rat skin preparations and showed that nitroreduction in skin was mainly catalyzed by cytosolic xanthine oxidoreductase, but not by microsomal cytochrome P450, cytosolic aldehyde oxidase, or DT-diaphorase (Ueda et al., 2003). However, an earlier study using rabbit liver had shown that aldehyde oxidase played a major role in the reduction of NF and other nitro-PAHs (Tatsumi et al., 1986). In sea bream liver, nitroreduction of NF was catalyzed by aldehyde oxidase (Ueda et al., 2002). Furthermore, our preliminary study using rat liver found that aldehyde oxidase was mainly involved in reduction of NF, with xanthine oxidoreductase playing a lesser role. These facts suggested that both of the molybdenum hydroxylases contribute to the nitroreduction of nitro-PAHs in mammalian tissues, especially in skin, which is one of target organs for environmental contaminants.

Moreover, there are marked species differences and strain differences (in rats and mice) of liver aldehyde oxidase (Krenitsky et al., 1974; Sugihara et al., 1995; Rashidi et al., 1997; Kitamura et al., 1999a). For example, in liver, high aldehyde oxidase activity is observed in rabbits and hamsters. Therefore, the enzyme primarily responsible for nitroreduction of nitro-PAHs in skin may well be species-dependent.

In the present study, we demonstrated the involvement of both aldehyde oxidase and xanthine oxidoreductase in nitroreduction of nitro-PAHs in skin cytosols of various mammals. Furthermore, the relative contributions of these molybdenum hydroxylases to the nitroreduction in skin cytosols of various mammals were determined.

ABBREVIATIONS: nitro-PAH, nitrated polycyclic aromatic hydrocarbon; NF, 2-nitrofluorene; NP, 1-nitropyrene; NB, 4-nitrobiphenyl; HPLC, high-performance liquid chromatography.
**Materials and Methods**

**Chemicals.** NF, 1-nitropyrene (NP), 4-nitrobi phenyl (NB), 2-aminofluorene, 1-aminopyrene, 4-aminobiphenyl, 2-hydroxy pyrimidine hydrochloride, 4-hydroxy pyrimidine, xanthine, 1-methylxanthine and benzaldehyde were purchased from Tokyo Chemical Industry Co., Ltd. (Tokyo, Japan). N'-Methyl nicotinamide, menadione, chlorpromazine, quercetin dihydrate, isovanillin, oxy purinol, hypoxanthine, and phenethylmethylsulfonyl fluoride were obtained from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). Other chemicals used were of the highest grade commercially available.

**Animals.** Male Syrian Golden hamsters (65–80 g), Japanese albino rabbits (2.0–2.5 kg), Hartley guinea pigs (150–190 g), and ddY mice (23–30 g) were purchased from Japan SLC Inc. (Shizuoka, Japan). Sea/Sprague-Dawley rats (200–240 g) were purchased from Seiwa Experimental Animals, Ltd. (Fukuoka, Japan).

**Preparation of Skin Cytosol.** Skin cytosol and microsomes were prepared by using a reported method (Ueda et al., 2003). Briefly, the skin was excised, and subcutaneous tissues were scraped off with scissors. Scraped sheets of skin were homogenized with 3 volumes of 0.1 M potassium/sodium phosphate buffer (pH 7.4) containing 0.1 mM dithiotheritol, 0.1 mM phenylmethylsulfonyl fluoride, 0.1 mM EDTA, and 0.1 mM ethylene glycol bis(β-aminoethyl)-N,N,N',N'-tetraacetic acid (buffer A). Microsomes and cytosol were obtained from the homogenate by successive centrifugation at 9000g for 20 min and at 105,000g for 90 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 200 volumes of 10-fold-diluted buffer A for 12 h. The dialyzed solution was centrifuged at 9000g for 20 min, and the supernatant 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 buffer A and eluted with a 100-ml linear gradient of 0 to 0.3 M sodium chloride in buffer A. The fractions were assayed for aldehyde oxidase activity, xanthine oxidase activity, and nitroreductase activity toward NF in the presence of 2-hydroxy pyrimidine, and the active fractions were pooled and stored at −80°C.

**Assays of Nitroreductase Activity.** The incubation mixture consisted of 0.1 μmol of NF, NP, or NB, 0.5 μmol of an electron donor, and a skin preparation in a final volume of 1 ml of 0.1 M potassium/sodium phosphate buffer (pH 7.4). The incubation was performed at 37°C for 30 min under an atmosphere of nitrogen using a Thunberg tube. The protein concentration used in assays was 2 mg/ml. In some cases, incubation was also performed in the presence of 10 μM menadione; 100 μM isovanillin, quercetin, or chlorpromazine, which are potent inhibitors of aldehyde oxidase; or 100 μM oxypurinol, an inhibitor of xanthine oxidoreductase. After incubation, 50 μg of phenacetin was added to the mixture as an internal standard, and then the mixture was extracted with 7 ml of ethyl acetate. The extract was evaporated to dryness in vacuo and the residue was subjected to high-performance liquid chromatography (HPLC). Nitroreductase activity was determined by measuring amino derivatives formed from NF, NP, and NB.

**Assay of Aldehyde Oxidase Activity.** Aldehyde oxidase activity was measured in terms of the oxidation of benzaldehyde to benzoic acid. Each incubation mixture consisted of 0.1 μmol of benzaldehyde and skin cytosol in a final volume of 0.5 ml of 0.1 M potassium/sodium phosphate buffer (pH 7.4). The incubation was performed at 37°C for 15 min. The protein concentration used in assays was 1 mg/ml. After incubation, the reaction was stopped by addition of 500 μl of methanol, and 50 μg of estriol was added to the mixture as an internal standard. After mixing, the solution was centrifuged at 10,000 rpm for 5 min. An aliquot of the supernatant was subjected to HPLC. For the determination of 2-aminofluorene, 1-aminopyrene, or 4-aminobiphenyl, the column was operated at a flow rate of 0.7 ml/min of acetonitrile/water (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), 4-aminobiphenyl, 2-aminofluorene, 1-aminopyrene, NF, and NP were 5.1, 8.5, 8.8, 13.6, 17.0, 18.9, and 30.7 min, respectively. For the determination of benzoic acid, the column was operated at a flow rate of 0.5 ml/min of 0.5% acetic acid/acetonitrile (1:1) at 40°C, using estriol as an internal standard, with the detector set at 272 nm. Retention times of authentic estriol (an internal standard), benzoic acid, and benzaldehyde were 6.5, 7.6, and 10.0 min, respectively. For the determination of 1-methyluric acid, the column was operated at a flow rate of 0.5 ml/min of 0.5% acetic acid/methanol (9:1) at 40°C, using acetaminophen as an internal standard, with the detector set at 280 nm. Retention times of authentic acetaminophen (an internal standard), 1-methyluric acid, and 1-methylxanthine were 11.3, 14.0, and 17.1 min, respectively.

**Results**

**Nitroreduction of NF in Hamster Skin Preparations.** Nitroreductase activity toward NF in hamster skin microsomes and cytosol in the presence of various electron donors was examined. The skin cytosol exhibited significant nitroreductase activity with 2-hydroxy pyrimidine or 4-hydroxy pyrimidine (electron donors of aldehyde oxidase and xanthine oxidoreductase), and N'-methyl nicotinamide or benzaldehyde (electron donors of aldehyde oxidase). However, NADPH and NADH (electron donors of DT-diaphorase) or hypoxanthine, xanthine, and 1-methylxanthine (electron donors of xanthine oxidoreductase) had no effect on the activity (Fig. 1A). The 2-hydroxy pyrimidine- or 4-hydroxy pyrimidine-linked nitroreductase activity was markedly inhibited by menadione, isovanillin, chlorpromazine, and quercetin (inhibitors of aldehyde oxidase), but oxypurinol, an inhibitor of xanthine oxidoreductase, had little effect. Moreover, N'-methyl nicotinamide- or benzaldehyde-linked activity was completely inhibited by menadione, but not by oxypurinol (Fig. 1B). In contrast, microsomal nitroreductase activity was enhanced with NADPH, but the full activity (3.5 pmol/min/mg protein) was much lower than that of skin cytosol. These facts suggest that the nitroreduction by hamster skin cytosol is mainly due to aldehyde oxidase.

**DEAE Column Chromatography of Hamster Skin Cytosol.** The hamster skin cytosol was subjected to DEAE-cellulose column chromatography, and the fractions were assayed for nitroreductase activity in the presence of 2-hydroxy pyrimidine, as well as for aldehyde oxidase and xanthine oxidase activities. The fractions that exhibited 2-hydroxy pyrimidine-linked nitroreductase activity were separated into two active fractions. The minor active fractions (fraction I; fraction 26–32) were coeluted with xanthine oxidoreductase, and the major active fractions (fraction II; fraction 37–46) were coeluted with aldehyde oxidase (Fig. 2).

Fraction I exhibited nitroreductase activity in the presence of 2-hydroxy pyrimidine or hypoxanthine. The 2-hydroxy pyrimidine-linked nitroreductase activity of fraction I was inhibited by oxypurinol, but not by menadione. On the other hand, the nitroreductase activity of fraction II was enhanced by addition of 2-hydroxy pyrimidine, N'-methyl nicotinamide, and benzaldehyde, but not by hypoxanthine. The 2-hydroxy pyrimidine-linked nitroreductase activity was inhibited by menadione, but not by oxypurinol (Fig. 3). These results confirmed that the nitroreductase activities in fractions I and II are due to
xanthine oxidoreductase and aldehyde oxidase, respectively. Fraction II accounted for the majority of the nitroreductase activity in hamster skin cytosol.

**Nitroreduction of NF in Skin Cytosols of Various Mammals.**  
2-Hydroxypyrimidine-linked nitroreductase activity toward NF in skin cytosol of various mammals was examined. The skin cytosols of rabbits, guinea pigs, mice, and rats all exhibited significant nitroreductase activity. The effects of various chemicals were then examined. In rabbits, similar to hamster, the nitroreductase activity in skin cytosol was markedly inhibited by menadione, but not by oxypurinol. In skin of guinea pigs, the nitroreductase activity was partly inhibited by both oxypurinol and menadione. Moreover, the activities in skin cytosols of mice and rats were significantly inhibited by oxypurinol but little affected by menadione (Fig. 4). These results suggest that aldehyde oxidase participates predominantly in nitroreduction of NF in skins of hamsters and rabbits, whereas xanthine oxidoreductase...
participates predominantly in mice and rats, and both flavoenzymes participate in guinea pigs.

**Aldehyde Oxidase and Xanthine Oxidoreductase Activities in Skin Cytosol.** When aldehyde oxidase activity in skin cytosol was measured in terms of the oxidation of benzaldehyde, the highest activity was observed with hamsters, followed by rabbits and guinea pigs. In mice and rats, the activities were slight. The difference in the activity between hamsters and rats was about 6-fold. When xanthine oxidase activity was assayed in the absence of NAD, marked differences were found in skin cytosols from various mammals (Table 1). The activities were completely inhibited by oxypurinol, but not menadione (data not shown). The highest activity was observed with rats, followed by mice, guinea pigs, and hamsters. However, very low activity was observed in rabbits. The difference in activity between...
1-Methyluric acid formed was determined using HPLC. in 0.1 M potassium/sodium phosphate buffer (pH 7.4) was incubated at 37°C for 20 min. Sodium phosphate buffer (pH 7.4) was incubated at 37°C for 15 min. Benzoic acid formed was nitroreductase activities were slightly inhibited by an inhibitor of xanthine oxidoreductase in the presence of 2-hydroxypyrimidine, and skin cytosol in 0.1 M potassium/sodium phosphate buffer (pH 7.4) was incubated at 37°C for 30 min under an anaerobic condition. The amounts of 1-aminopyrene and 4-aminobiphenyl formed were determined by HPLC. Other details are described under Materials and Methods.

## Discussion
Aldehyde oxidase, a cytosolic enzyme, contains FAD, molybdenum, and an iron-sulfur center, and is closely related to xanthine oxidoreductase (Turner et al., 1995; Hille, 2005). Both enzymes have similar primary and secondary structures, but their substrate specificities differ (Krenitsky et al., 1972; Beecham, 1985). Aldehyde oxidase can catalyze the oxidation of various aldehydes and nitrogenous heterocyclic compounds (Clarke et al., 1995; Jordan et al., 1999; Kitamura et al., 1999a), whereas xanthine oxidoreductase participates in purine metabolism such as caffeine, hypoxanthine, and 6-deoxy-adenosine (Krenitsky et al., 1984; Jones et al., 1987; Relling et al., 1992). Both enzymes in the presence of an electron donor can also mediate the reduction of a variety of compounds, such as sulfoxide, N-oxide, epoxide, and aromatic nitro compounds (Kitamura and Tatsumi, 1984; Tatsumi et al., 1986; Hirao et al., 1994; Sugihara et al., 1996). In a previous study, we demonstrated that NF was mainly reduced by xanthine oxidoreductase in the presence of 2-hydroxyxypyr imidine or 4-hydroxyxypyr imidine as an electron donor in skin of Sea/Sprague-Dawley strain rats, which exhibited high liver aldehyde oxidase activity (Ueda et al., 2003). However, in this study using hamster skin, 2-hydroxyxypyr imidine- and 4-hydroxyxypyr imidine-linked nitroreductase activities were slightly inhibited by an inhibitor of xanthine oxidoreductase and markedly inhibited by inhibitors of aldehyde oxidase. Moreover, electron donors to aldehyde oxidase enhanced the nitroreductase activity, showing that aldehyde oxidase plays an important role in the nitroreduction in hamster skin. In contrast, the activity in skin of guinea pigs was partially inhibited by oxypurinol and menadione. These results suggested that xanthine oxidoreductase mainly contributed to nitroreduction in skin of mice and rats, whereas both molybdenum hydroxylases contributed in skin of guinea pigs. Therefore, it appears that the patterns of participation of these molybdenum hydroxylases in nitroreduction in mammalian skin can be classified into three groups, i.e., 1) participation of mainly aldehyde oxidase (hamsters and rabbits), 2) participation of mainly xanthine oxidoreductase (mice and rats); and 3) participation of both molybdenum hydroxylases (guinea pigs) (Fig. 5).

A marked species difference exists in the liver aldehyde oxidase activity. We determined aldehyde oxidase activity using benzanldehyde as a substrate in skin cytosols from hamsters, rabbits, guinea pigs, mice, and rats, and clear species differences in the levels of the activity were observed. The aldehyde oxidase and xanthine oxidoreductase activities in skin of various species appeared to be complementary, i.e., when one was high, the other was low. In addition, the pattern of species difference observed in these experiments using skin cytosol was similar to that in liver, except that rats (Sea/Sprague-Dawley strain), which have high liver aldehyde oxidase activity, exhibited relatively high xanthine oxidoreductase and low aldehyde oxidase activity in skin cytosol. In humans, it was reported that methotrexate, benzaldehyde, N,N'-methylmhydantoinamide, and 6-methylpurine were metabolized by liver aldehyde oxidase (Rodrigues, 1994; Sugihara et al., 1997; Kitamura et al., 1999b), and farniclovir was metabolized by liver xanthine oxidoreductase (Fowles et al., 1994). Since human aldehyde oxidase and xanthine oxidoreductase both play important roles in the metabolism of exogenous compounds, they may both be involved in metabolism of nitro compounds, such as some hair dyes and various topically applied therapeutic antiviral agents and anticancer agents, in human skin (Van Duuren, 1980; Rashidi et al., 1997).

In this study, skin cytosols from various mammals also exhibited significant nitroreductase activity toward NP and NB. The highest activity toward NF was observed with hamsters, which also exhibited high aldehyde oxidase activity. On the other hand, the highest activities toward NP and NB were observed with rats, which exhibited high xanthine oxidoreductase activity. This substrate specificity of the nitroreduction observed in skin cytosol from various mammals presumably reflects the specificity of the two flavoenzymes i.e., aldehyde oxidase, in the reduction of NF and xanthine oxidoreductase in the reduction of NP and NB.

### Table 1: Aldehyde oxidase and xanthine oxidase/xanthine dehydrogenase activities in skin cytosols from various mammals

<table>
<thead>
<tr>
<th>Species</th>
<th>Aldehyde Oxidase</th>
<th>Xanthine Oxidase</th>
<th>Xanthine Dehydrogenase</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hamster</td>
<td>1.43 ± 0.26</td>
<td>0.15 ± 0.01</td>
<td>0.66 ± 0.14</td>
</tr>
<tr>
<td>Rabbit</td>
<td>0.91 ± 0.07</td>
<td>0.05 ± 0.01</td>
<td>0.24 ± 0.16</td>
</tr>
<tr>
<td>Guinea pig</td>
<td>0.47 ± 0.17</td>
<td>0.36 ± 0.12</td>
<td>1.81 ± 0.21</td>
</tr>
<tr>
<td>Mouse</td>
<td>0.26 ± 0.08</td>
<td>0.83 ± 0.14</td>
<td>1.95 ± 0.78</td>
</tr>
<tr>
<td>Rat</td>
<td>0.24 ± 0.11</td>
<td>1.23 ± 0.15</td>
<td>1.97 ± 0.39</td>
</tr>
</tbody>
</table>

### Table 2: Nitroreductase activity toward nitrogen PAHs in skin cytosols from various mammals

<table>
<thead>
<tr>
<th>Species</th>
<th>1-Nitropyrene</th>
<th>4-Nitrobiphenyl</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hamster</td>
<td>15.0 ± 6.2</td>
<td>132.7 ± 30.1</td>
</tr>
<tr>
<td>Rabbit</td>
<td>5.5 ± 3.3</td>
<td>11.7 ± 9.4</td>
</tr>
<tr>
<td>Guinea pig</td>
<td>16.4 ± 3.0</td>
<td>121.4 ± 19.3</td>
</tr>
<tr>
<td>Mouse</td>
<td>23.4 ± 3.8</td>
<td>175.7 ± 15.5</td>
</tr>
<tr>
<td>Rat</td>
<td>35.1 ± 6.7</td>
<td>173.7 ± 10.5</td>
</tr>
</tbody>
</table>

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*The mixture containing 0.1 μmol of benzaldehyde and skin cytosol in 0.1 M postassium/sodium phosphate buffer (pH 7.4) was incubated at 37°C for 15 min. Benzoic acid formed was determined using HPLC.

*The mixture containing 0.1 μmol of 1-methylxanthine and skin cytosol in 0.1 M potassium/sodium phosphate buffer (pH 7.4) was incubated at 37°C for 20 min. 1-Methyluric acid formed was determined using HPLC.

*The mixture containing 0.1 μmol of 1-methylxanthine, 0.27 μmol of NAD, and skin cytosol in 0.1 M potassium/sodium phosphate buffer (pH 7.4) was incubated at 37°C for 20 min. 1-Methyluric acid formed was determined using HPLC.
have been investigated extensively. Krenitsky et al. (1974) found no aldehyde oxidase activity in skin of Sprague-Dawley rats and rhesus monkeys. On the other hand, Kooij et al. (1992) reported that xanthine oxidoreductase was present in rat skin. Immunohistochemical analysis confirmed that xanthine oxidoreductase was distributed in epidermis, sebaceous glands, and hair follicle epithelium, whereas aldehyde oxidase was not detected in epidermis of Wistar rats (Morwak et al., 1996). Moreover, in mice, mRNA of aldehyde oxidase was detected in esophagus, lung, liver, and testis, but not skin (Kurosaki et al., 1999). In contrast, Terao et al. (2000) cloned cDNA for AOH1 and AOH2, novel molybdenum hydroxylases, and demonstrated that mRNA of AOH2 was present in the basal layer of the epidermis and hair follicle in mice. We detected aldehyde oxidase and xanthine oxidoreductase activities in skins of various mammals. However, the physiological roles of these enzymes are still unknown. Possible roles include homeostatic control of vitamins such as retinoids and other biologically active molecules, and elimination of biogenic wastes or breakdown products generated by ultraviolet radiation and other environmental stresses (Kishore and Boutwell, 1980; Siegenthaler et al., 1995; Beedham et al., 1995; Garattini et al., 2003).

The present study has demonstrated that nitrorductase activities among some nitro-PAHs in skin of various mammalian species are due to both aldehyde oxidase and xanthine oxidoreductase, and the species differences reflect differences of relative aldehyde oxidase and xanthine oxidoreductase activities. Although the physiological roles of aldehyde oxidase and xanthine oxidoreductase in skin are unknown, it is clear that both molybdenum hydroxylases play important roles in the metabolism of xenobiotics in skin.

References


Cloning of the cDNAs coding for two novel molybdo-flavoproteins showing high similarity with aldehyde oxidase and xanthine oxidoreductase. *J Biol Chem* **39:**30690–30700. 


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