Short Communication

ESTIMATION OF ALDEHYDE OXIDASE ACTIVITY IN VIVO FROM CONVERSION RATIO OF N¹-METHYLNICOTINAMIDE TO PYRIDONES, AND INTRASPECIES VARIATION OF THE ENZYME ACTIVITY IN RATS

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ABSTRACT:
The in vivo conversion ratio of N¹-methylnicotinamide (NMN) to N¹-methyl-2-pyridone-5-carboxamide (2-PY) and N¹-methyl-4-pyridone-3-carboxamide (4-PY) as a parameter for the estimation of aldehyde oxidase level in rats was examined. NMN and its pyridones (2-PY and 4-PY) are usually detected in the urine of rats. When we measured the ratio of the amount of pyridones to the total amount of NMN and pyridones (RP value) in the urine of rats, marked intraspecies variations were observed. The variation in RP value among strains was closely related to the differences of liver aldehyde oxidase activity measured with NMN as a substrate. RP values after administration of NMN to different strains of rats confirmed the existence of strain differences of aldehyde oxidase activity in vivo. We demonstrated that measurements of NMN and its pyridones usually excreted in the urine can be used to predict the in vivo level of aldehyde oxidase.

Aldehyde oxidase (EC 1.2.3.1), a cytosolic enzyme, contains FAD, molybdenum, and iron-sulfur centers, and is closely related to xanthine oxidase (Beedham, 1987). The two enzymes have a very close evolutionary relationship, based on the recent cloning of the genes, and they show a high degree of amino acid sequence homology (Calzi et al., 1995; Wright et al., 1999; Terao et al., 2000). They have been suggested to be relevant to the pathophysiology of a number of clinical disorders (Berger et al., 1995; Wright et al., 1995; Moriwaki et al., 1997). Aldehyde oxidase commonly exists in vertebrates. The enzyme in liver of various species catalyzes the oxidation of a number of aldehydes and nitrogenous heterocyclic xenobiotics, such as methotrexate and cyclophosphamide (Beedham, 1987), and also catalyzes the metabolism of physiological compounds such as retinaldehyde and monoamine neurotransmitters (Huang and Ichikawa, 1994). Moreover, the enzyme in the presence of its electron donor can mediate the reduction of a variety of compounds, such as sulfoxides, N-oxides, nitrosoamines, hydrogenic acids, azo dyes, oximes, epoxides, aromatic nitro compounds, and 1,2-benzisoxazole derivatives (Sugihara et al., 1996). Recently, aldehyde oxidase homologs, which may exhibit different metabolic roles, were identified in mice (Garattini et al., 2003; Kurosaki et al., 2004; Vila et al., 2004). Marked interspecies variation of the enzyme activity in oxidative and reductive reactions was reported (Sugihara et al., 1996; Schofield et al., 2000). We found a significant variation of liver aldehyde oxidase activity in 12 strains of rats in an assay using benzaldehyde or methotrexate as a substrate (Sugihara et al., 1995; Kitamura et al., 1999). Variations of benzaldehyde oxidase in white people and Japanese have also been reported (Rodrigues, 1994; Sugihara et al., 1997). However, no report is available on the prediction of aldehyde oxidase levels in vivo.

N¹-Methylnicotinamide (NMN), which is formed from nicotinamide by nicotinamide methyltransferase, is widely distributed in animals, like nicotinamide (Yan et al., 1997). NMN is neurotoxic, and is detoxified by oxidation to N¹-methyl-2-pyridone-5-carboxamide (2-PY) and N¹-methyl-4-pyridone-3-carboxamide (4-PY) (Fig. 1). The conversion of NMN to 2-PY and 4-PY has been reported to be catalyzed by aldehyde oxidase, but the activity shows marked strain differences in mice (Stanulovic and Chaykin, 1971a).

In this study, we attempted to estimate the in vivo level of aldehyde oxidase in rats by using NMN as a substrate. The amounts of NMN and its pyridones excreted in the urine were measured by using high-performance liquid chromatography (HPLC). The conversion ratio of NMN to pyridones (RP value) in urine may be a useful parameter for noninvasive prediction of aldehyde oxidase activity in rats. We also observed considerable interstrain variations of RP value, which were correlated to those of liver aldehyde oxidase activity in rats.

Materials and Methods

Chemicals. NMN and N¹-methylnicotinamide were obtained from Tokyo Chemical Industry Co., Ltd. (Tokyo, Japan). 2-PY and 4-PY were prepared by the method of Shibata et al. (1988).

Animals. Six different strains of male rats (6–7 weeks of age) were used. Slc:Wistar/ST and Slc:SD rats were obtained from Japan Slc, Inc. (Shizuoka, Japan); Jcl:SD from Clea Japan Inc. (Tokyo, Japan); Crj:SD from Charles River Japan, Inc. (Yokohama, Japan); and WKA/Sea and Sea:SD from Seiwa Experimental Animals, Ltd. (Fukuoka, Japan). Among these strains, Slc: Wistar/ST and WKA/Sea are Wistar strains, and Jcl:SD, Sea:SD, Crj:SD, and Slc:SD are Sprague-Dawley strains. WKA/Sea is inbred, and the others are from closed colonies.
were measured by HPLC. The incubation mixture consisted of 0.2 nmol of NMN and liver cytosol equivalent to 50 to 100 mg of liver wet weight 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 10 min. After incubation, and after addition of 10 μg of N'-methylnicotinamide (an internal standard) and 0.6 g of K₂CO₃, the mixture was extracted three times with 5 ml of ether and the extract was evaporated to dryness. The residue was redissolved in 0.1 ml of methanol, and an aliquot was subjected to analysis by HPLC.

Assay for Aldehyde Oxidase Activity. Aldehyde oxidase activity was measured with NMN as a substrate. The amounts of 4-PY and 2-PY formed were measured with NMN as a substrate. The amounts of 4-PY and 2-PY formed were measured with NMN as a substrate. The amounts of 4-PY and 2-PY formed were measured with NMN as a substrate. The amounts of 4-PY and 2-PY formed were measured with NMN as a substrate. The amounts of 4-PY and 2-PY formed were measured with NMN as a substrate. The amounts of 4-PY and 2-PY formed were measured with NMN as a substrate. The amounts of 4-PY and 2-PY formed were measured with NMN as a substrate.

Fig. 1. Metabolic pathways of N'-methylnicotinamide.

Administration of NMN to Rats. NMN dissolved in saline (100 mg/ml) was administered to male rats intraperitoneally at a single dose of 100 mg/kg. After treatment, urine was collected at intervals of 6 h for 30 h.

Liver Preparations. Rat livers were excised from several strains of rats. Liver samples were homogenized in 4 volumes of 1.15% KCl. The cytosolic fraction was obtained from the homogenate by successive centrifugation at 105,000 g for 20 min and 105,000 g for 60 min.

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Measurement of NMN, 2-PY, 4-PY, and Nicotinamide Excreted in Urine. NMN, 2-PY, 4-PY, and nicotinamide in urine of rats were determined according to the method of Shibata et al. (1988). To measure 2-PY, 4-PY, and nicotinamide, the urine (0.1 ml) was diluted with 0.4 ml of water. Ten micrograms of N'-methylnicotinamide as an internal standard and 0.6 g of K₂CO₃ were added to the solution and the mixture was extracted three times with 5 ml of ether. The amounts of 2-PY, 4-PY, and nicotinamide in the extract were determined by using HPLC. To measure NMN in the urine of rats, the urine (0.01 ml) was added to the mixture of 1 ml of 0.2 M isonicotinamide and 0.5 ml of 0.1 M acetonitrile on ice. Then, 1 ml of 6 N NaOH and 0.5 ml of formic acid were added to the mixture and stood on ice for 10 min. After 10 min, the mixture was boiled in a water bath for 5 min. The amount of the fluorometric compound, 1-methyl-7-phenyl-15-dehydro-5-oxo-1,6-naphthyridine, formed from NMN and acetophenone was determined by using HPLC as described under Materials and Methods.

TABLE 1

<table>
<thead>
<tr>
<th>Strain</th>
<th>Nicotinamide</th>
<th>NMN</th>
<th>2-PY</th>
<th>4-PY</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>nmol/ml</td>
<td></td>
<td></td>
<td></td>
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</tr>
<tr>
<td>Sea:SD</td>
<td>34.0 ± 1.7</td>
<td>43.0 ± 1.6</td>
<td>64.2 ± 2.9</td>
<td>446.1 ± 20.0</td>
<td>587.3 ± 26.2</td>
</tr>
<tr>
<td>WKA/Sea</td>
<td>69.9 ± 1.6</td>
<td>580.3 ± 11.2</td>
<td>39.9 ± 0.6</td>
<td>30.4 ± 1.4</td>
<td>720.5 ± 14.8</td>
</tr>
</tbody>
</table>

Results

Variation of Urinary Excretion of Nicotinamide, NMN, and the Oxidative Metabolites, 2-PY and 4-PY, in Rats. NMN and its oxidative metabolites, 2-PY and 4-PY, are usually excreted in the urine from Sea:SD and WKA/Sea strains of rats. The contents of nicotinamide and NMN were lower in Sea:SD rats than in WKA/Sea rats, whereas the content of 2-PY was higher, and that of 4-PY was much higher. The NMN content in urine of WKA/Sea rats was 13 times that in Sea:SD rats. In contrast, the 4-PY content in urine of Sea:SD rats was 15 times that of WKA/Sea rats. The total amounts of nicotinamide and its metabolites in urine were similar in the two strains (Table 1).

When nicotinamide, NMN, 2-PY, and 4-PY were assayed in the urine of various strains of rats, the amounts of the pyridones and NMN showed clear variation among strains. The levels of pyridones (percentage of the sum of nicotinamide, NMN, 2-PY, and 4-PY) in the urine of Sea:SD rats were higher than those in WKA/Sea rats. In contrast, the level of NMN in the urine of WKA/Sea rats was higher than that in Sea:SD rats. Other strains showed a range of patterns, but the amount of 4-PY excreted was higher than that of 2-PY in all the strains except WKA/Sea (Fig. 2). The ratio of the amounts of the pyridones to the total amounts of NMN and the pyridones excreted in the urine (RP) was examined to estimate the aldehyde oxidase level in vivo in rats: RP (%) = [(2-PY + 4-PY)/(NMN + 2-PY + 4-PY)] × 100.

The RP values showed marked variations among the strains. The RP value was highest in Sea:SD, followed by Jcl:SD and Slc: Wistar/ST rats, whereas WKA/Sea rats showed the lowest value. The value of Sea:SD rats was about 9-fold higher than that of WKA/Sea rats (Fig. 2).
aldehyde and methotrexate (Sugihara et al., 1995; Kitamura et al., 1999) (Spearman’s rank correlation coefficient, $r_s = 0.96, P = 0.002$ and $r_s = 0.70, P = 0.02$, respectively).

Strain Difference of the in Vivo Metabolism of NMN to 2-PY and 4-PY in Rats after Administration of NMN. When NMN was administered to Sea:SD and WKA/Sea rats, NMN and its oxidative metabolites, 4-PY and 2-PY, were rapidly excreted in the urine. The amounts of NMN excreted in the urine of WKA/Sea rats were much higher than those of Sea:SD rats. In contrast, the levels of 2-PY and 4-PY in the urine of Sea:SD rats were higher than those of WKA/Sea rats (Fig. 4). The accumulated amounts of these compounds in urine did not further increase after 30 h. Since their amounts in urine of untreated rats were less than 2 to 3 $\mu$mol a day, the excreted NMN and pyridones were metabolites of treated NMN. This confirms that the RP values reflect the in vivo metabolism of NMN in rats.

### Discussion

We attempted to find a noninvasive method of determining aldehyde oxidase level in vivo in rats. In this study, we show that the ratio of the amount of pyridones to the total amount of NMN and pyridones (RP value) excreted in the urine is useful as a predictor of the aldehyde oxidase level in vivo. We found a substantial intraspecies variation of aldehyde oxidase activity in rats. The variation of RP values in rats in vivo was essentially identical with that of liver aldehyde oxidase activity among various strains. This result suggests that RP values do reflect the liver aldehyde oxidase activity. Therefore, measured RP values could be a good predictor of aldehyde oxidase activity in vivo.

Individual variation of the levels of 2-PY and 4-PY detected in the urine of rats paralleled those observed in the livers. The amount of 4-PY excreted was higher than that of 2-PY in rats. The product ratio of 2-PY and 4-PY reflected the liver oxidase activity toward NMN in rats. In this study, we assumed that the conversion of NMN to both 2-PY and 4-PY was due to the same enzyme activity. However, the enzyme responsible for the 2-hydroxylation of NMN has not been absolutely identified. Stanulovic and Chaykin (1971b) proposed that 2-hydroxylation of NMN was catalyzed by xanthine oxidase in rat in vivo. In contrast, we found that the ratio of 4-PY to 2-PY formed in rat liver was constant among rat strains. Furthermore, the formation of 4-PY and 2-PY from NMN by rat livers was completely inhibited by menadione, an inhibitor of aldehyde oxidase, but not by oxypurinol, an inhibitor of xanthine oxidase (data not shown). These facts suggest that aldehyde oxidase catalyzes 2- and 4-hydroxylation of NMN in vivo.

We have already reported marked strain differences of liver aldehyde oxidase activity in rats in an assay using benzaldehyde as a substrate.

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**Fig. 2.** Comparison of the relative amounts of nicotinamide and its metabolites in urine and the RP values for several strains of rats. Each value was calculated as a percentage of the total amount of nicotinamide, NMN, 2-PY, and 4-PY usually were excreted in the urine. NMN, 2-PY, 4-PY, and nicotinamide were determined by HPLC. The RP values were calculated from the amounts of NMN, 2-PY, and 4-PY excreted in the urine of rats according to the following formula: $\text{RP} (%) = [(2\text{-PY} + 4\text{-PY})/(\text{NMN} + 2\text{-PY} + 4\text{-PY})] \times 100$. Other details are described under Materials and Methods. Each value represents the mean ± S.D. of four rats.
Among the strains tested, the highest activity was observed with Sea:SD rats and the lowest with WKA/Sea rats. The differences between the activities toward benzaldehyde and methotrexate in the two strains of rats were 63.5- and 104-fold, respectively (Sugihara et al., 1995; Kitamura et al., 1999). We suggested that quantitative, but not qualitative, differences of aldehyde oxidase exist among different strains of rats, since there was little variation of $K_m$ values of the enzyme among the strains of rats examined, and Western blot analysis showed that the strains possessed
different quantities of the enzyme. In the present study, we confirmed the strain differences of aldehyde oxidase in rat liver with NMN as a substrate and thus showed that the oxidase activity correlated with the activity in vivo. Thus, the quantitative variation of the enzyme in livers appears to be the cause of strain differences of NMN metabolism in rats in vivo, as observed by the measurement of NMN and its pyridones in urine in the current study.

In contrast, aldehyde oxidase activity is regulated by androgen in mice, which contain four isozymes of the oxidase (AOX1, AOH1, AOH2, and AOH3) (Kurosaki et al., 2004). The aldehyde oxidase gene cluster in rat chromosome 9 is similar to that in mice and contains Aox1, Aoh1, Aoh2, and Aoh3. AOX1 and AOH1 are active aldehyde oxidase species in mouse liver, and they have similar substrate specificities, whereas AOH2 and AOH3 are observed at low levels in skin and olfactory mucosa, respectively (Kurosaki et al., 2004). Furthermore, a similar gene cluster exists in humans (Garattini et al., 2003). We have found different substrate specificities of aldehyde oxidase activity in several strains of mice and in individual humans in vitro (Sugihara et al., unpublished data). However, rats do not show such strain differences of substrate specificity, only strain differences of the activity. Nevertheless, the different substrate specificities of liver aldehyde oxidase/NMN oxidase activities in vitro in mice, humans, and other species are also correlated with in vivo RP values in those species (data not shown). Therefore, we consider that measurement of RP values in urine is a useful method to determine the aldehyde oxidase level in animals and humans in vivo.

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