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Estimation of aldehyde oxidase activity in vivo from conversion ratio of N^1-methylnicotinamide to pyridones, and intraspecies variation of the enzyme activity in rats

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d) Abbreviation: NMN, N^1-methylnicotinamide; 2-PY, N^1-methyl-2-pyridone-5-carboxamide; 4-PY, N^1-methyl-4-pyridone-3-carboxamide; HPLC, high-performance liquid chromatography; RP value, the ratio of the amounts of pyridones to the total amounts of NMN and pyridones.

ABSTRACT:

The in vivo conversion ratio of N^1-methylnicotinamide (NMN) to N^1-methyl-2-pyridone-5-carboxamide (2-PY) and N^1-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.

Introduction

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 (Wright et al., 1999; Calzi et al., 1995; Terao et al., 2000). They have been suggested to be relevant to the pathophysiology of a number of clinical disorders (Berger et al., 1995; Moriwaki et al., 1997; Wright et al., 1995). 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, nitrosamines, hydroxamic acids, azo dyes, oximes, epoxides, aromatic nitro compounds and 1,2-benzisoxazole derivatives (Sugihara et al., 1996). Recently, aldehyde oxidase homologues, which may exhibit different metabolic role, were identified in mice (Vila et al., 2004; Garattini et al., 2003; Kurosaki et al., 2004). Marked inter-species 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 twelve 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 Caucasians 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.

N^1-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 N^1-methyl-2-pyridone-5-carboxamide (2-PY) and N^1-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).

(Fig. 1)

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 non-invasive prediction of aldehyde oxidase activity in rats. We also observed considerable inter-strain variations of RP value, which were correlated to those of liver aldehyde oxidase activity in rats.

Materials and Methods

Chemicals. NMN and *N*'-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; Jcl:SD from Clea Japan Inc., Tokyo; Crj:SD from Charles River Japan, Inc., Yokohama; 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.

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 hr for 30 hr.

Liver preparations. Rat livers were excised from several strains of rats. Liver samples were homogenized in four volumes of 1.15% KCl. The cytosolic fraction was obtained from the homogenate by successive centrifugation at 9,000 x *g* for 20 min and 105,000 x *g* for 60 min.

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 by HPLC. The incubation mixture consisted of 0.2 μmol of NMN and liver cytosol equivalent to 50 - 100 mg of liver wet weight in a final volume of 1 ml of 0.1 M K, Na-phosphate buffer (pH 7.4). The incubation was performed at 37º for 10 min. After incubation, the mixture, after addition of 10 μg of *N*'-methylnicotinamide (an internal standard) and 0.6 g of KCl was extracted 3 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.

Measurement of NMN, 2-PY, 4-PY and nicotinamide excreted in urine. NMN, 2-PY, 4-PY and nicotinamide in urine of humans and 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 μg of *N*'-methylnicotinamide as an internal standard and 0.6 g of KCl was added to the solution and the mixture was extracted 3 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 humans and 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 acetophenone on the ice. One ml of 6 N NaOH and 0.5 ml of formic acid were added to the mixture and stood on the ice for 10 min. After 10 min, the mixture was boiled in the water bath for 5 min. The amount of fluorometric compound, 1-methyl-7-phenyl-15-dehydro-5-oxo-1,6-naphthyridine formed from NMN and acetophenone was determined by using HPLC as below. Urine samples were measured twice and the average value was used. The difference between pairs of values was within 7 %. The detection limit was 0.05 nmol/ml.

HPLC. HPLC was performed using a Hitachi L-6000 chromatograph (Hitachi Co. Ltd., Tokyo, Japan) fitted with a Capcell pak C18 UG120 column (25 cm x 4.6 mm, Shiseido Co. Ltd., Tokyo, Japan) for the separation of 2-PY, 4-PY and nicotinamide. The mobile phase was acetonitrile-water (3:97, v/v). The chromatograph was operated at a flow rate of 0.5 ml/min and with a detection wavelength of 254 nm. The elution times of 4-PY, 2-PY, nicotinamide and *N* ‘-methylnicotinamide (an internal standard) were 13.5, 14.7, 16.6 and 30.0 min, respectively. For the detection of NMN, a LiChrospher Select B column (15 cm x 4.6 mm, Merck Co. Ltd., Tokyo, Japan) was used, and the mobile phase was acetonitrile-0.1 M KH_2PO_4 (1:4, v/v). The chromatograph was operated at a flow rate of 0.5 ml/min and with a fluorometric detection (extension of 382 nm and emmision of 440 nm). The elution times of 1-methyl-7-phenyl-15-dehydro-5-oxo-1,6-naphthyridine was 6.2 min. The amount of the product was determined from the peak area.

Statistical analysis. Correlation of in vitro aldehyde oxidase activity and RP value was tested using Spearman’s rank correlation test.

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, while the content of 2-PY was higher, and the one 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).

(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 (percent 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.

$$RP\ (\%) = [(2\text{-PY} + 4\text{-PY})/(\text{NMN} + 2\text{-PY} + 4\text{-PY})] \times 100$$

The RP values showed marked variations among the strains. The RP value was highest in Sea:SD, followed by Jcl:SD, Slc:Wistar/ST rats, while WKA/Sea rats showed the lowest value. The value of Sea:SD rats was about nine-fold higher than that of WKA/Sea rats (Fig. 2).

(Fig. 2)

Correlation of the ratio of pyridones in urine with the *in vitro* liver aldehyde oxidase activity. The oxidase activity of liver cytosols toward NMN from various strains of rats also exhibited significant strain differences and Sea:SD showed the highest activity and WKA/Sea was lowest (Fig. 3A). The amount of 4-PY was higher than 2-PY in vitro NMN oxidation, same as in urine. The major enzyme that catalyzes conversion of NMN to the pyridones is aldehyde oxidase, and similar strain differences were observed using benzaldehyde, 2-hydroxypyrimidine, phthalazine and methotrexate as substrates (Sugihara et al., 1995; Kitamura et al., 1999). The RP values in several strains of rats were found to be closely correlated with the in vitro liver aldehyde oxidase activities toward NMN (Fig. 3B). The RP values were also correlated with reported values of oxidase activities toward benzaldehyde and methotrexate (Sugihara et al., 1995; Kitamura et al., 1999) (Spearman's rank correlation coefficient rs=0.96, P=0.002 and rs=0.70, P=0.02, respectively).

(Fig. 3)

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 hr. As their amounts in non-treated urine were less than one micromol 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.

(Fig. 4)

Discussion

We attempted to find a non-invasive 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 with 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 were 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. 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, difference 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 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, while AOH2 and AOH3 are observed at low levels in skin and olfactory mucosa, respectively (Kurosaki et al., 2004). Further, 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, human 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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Footnotes

a) Unnumbered footnote

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Legends to Figures

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

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 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. RP (%) = [(2PY + 4PY)/(NMN + 2PY + 4PY)] x 100. Other details are described in Materials and Methods. Each value represents the mean ± SD of four rats.

Fig. 3. Aldehyde oxidase activity toward NMN in liver cytosol from several strains of rats (A), and correlation between in vitro aldehyde oxidase activity and in vivo RP values in six strains of rats (B).

The aldehyde oxidase activities toward NMN in rat livers were measured as described in Materials and Methods. Each value represents the mean ± SD of four rats. Aldehyde oxidase activity towrd NMN is positively correlated with *in vivo* RP values in six strains of rats (Spearman's rank correlation coefficient r_S=1, P=0.002). Aldehyde oxidase activities toward NMN are taken from (A), and RP values are taken from Fig. 2.

Fig. 4. Urinary excretion of NMN and its pyridones in Sea:SD and WKA/Sea rats treated with NMN (100 mg/kg).

Each value represents the mean ± SD of three rats. The urine was collected from rats dosed with NMN intraperitoneally at 100 mg/kg. NMN, 2-PY and 4-PY were extracted and determined by using HPLC. Other details are described in Materials and Methods.

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Table 1. Amounts of nicotinamide and its metabolites usually excreted in rat urine.

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

Each value represents the mean ± SD of four rats. The amounts of nicotinamide and its metabolites usually excreted in rat urine were measured by HPLC as described in Materials and Methods.

Fig. 1.

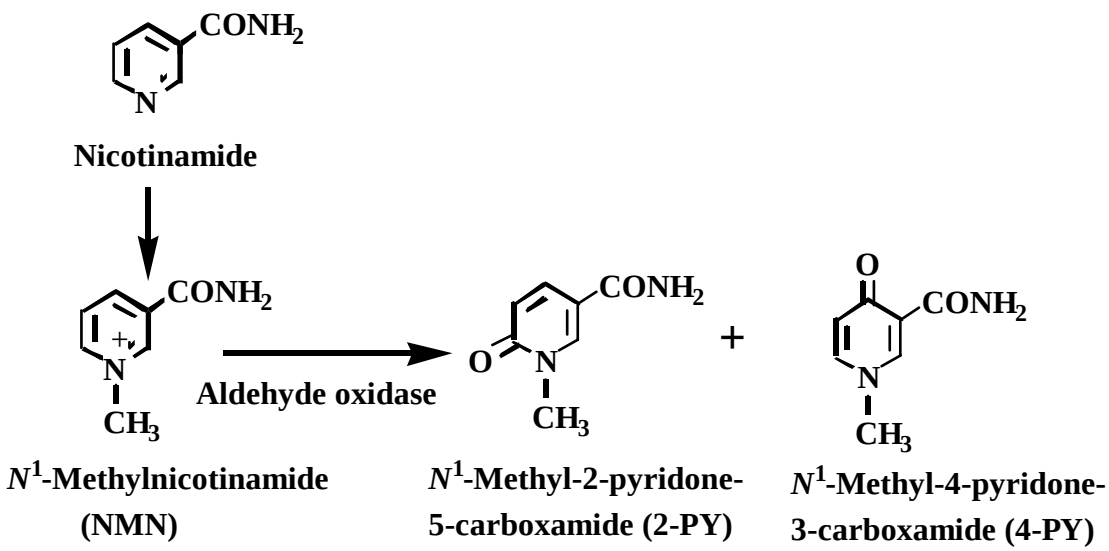


Fig. 2.

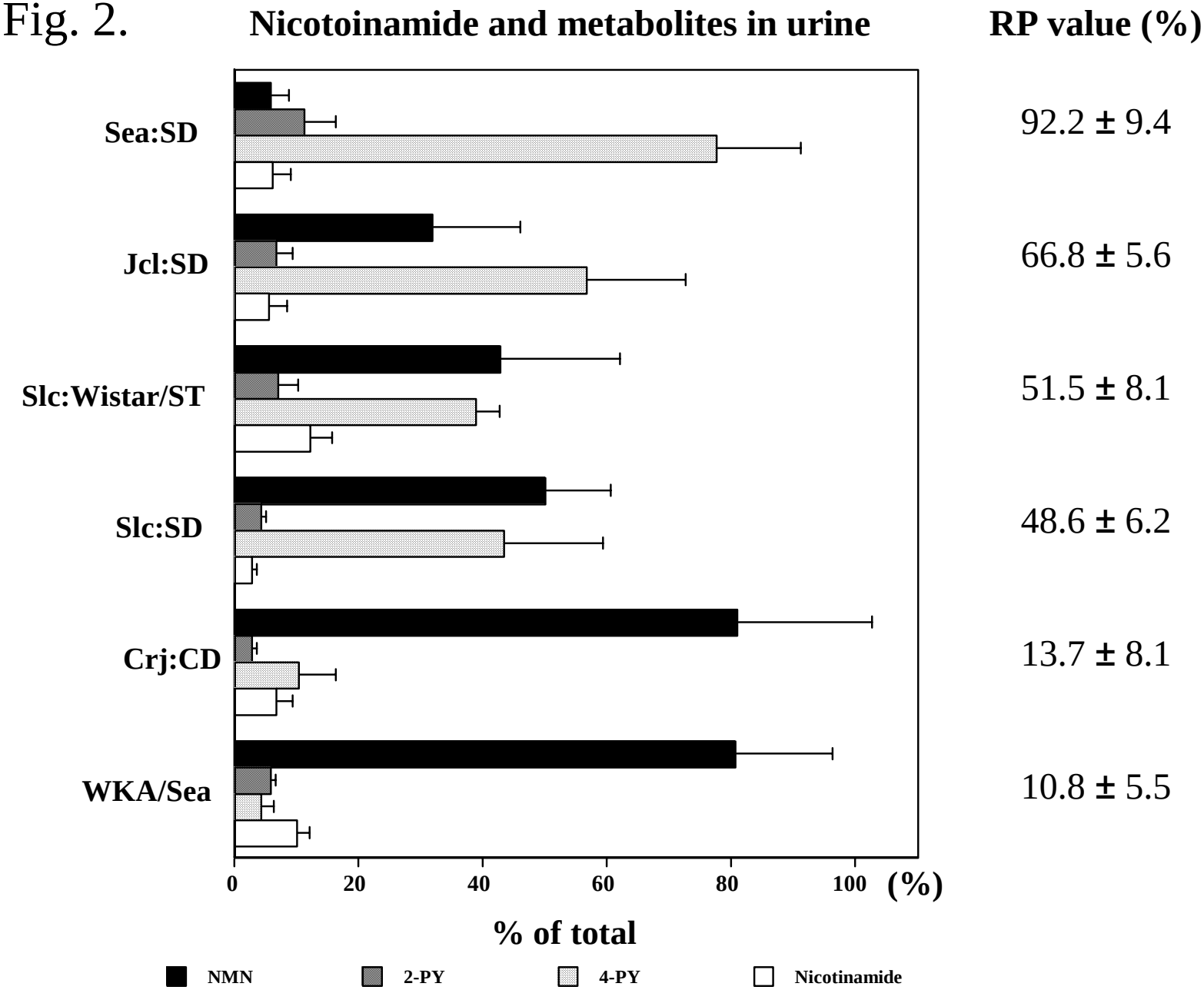
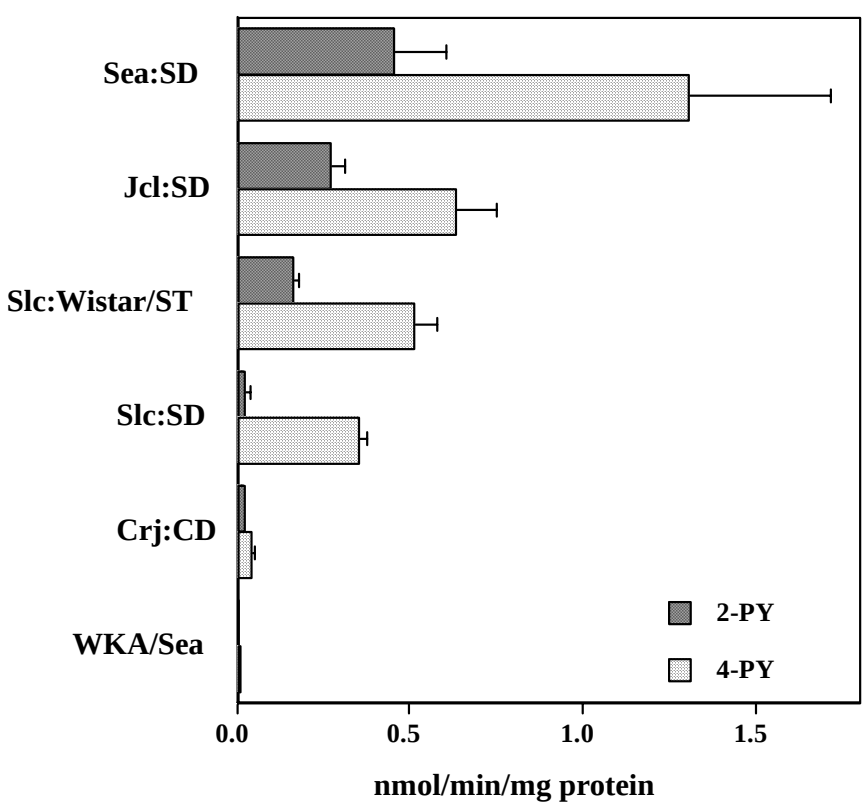


Fig. 3.

(A) Aldehyde oxidase activity in rat liver cytosol



(B) Correlation between *in vitro* aldehyde oxidase activity and *in vivo* RP value

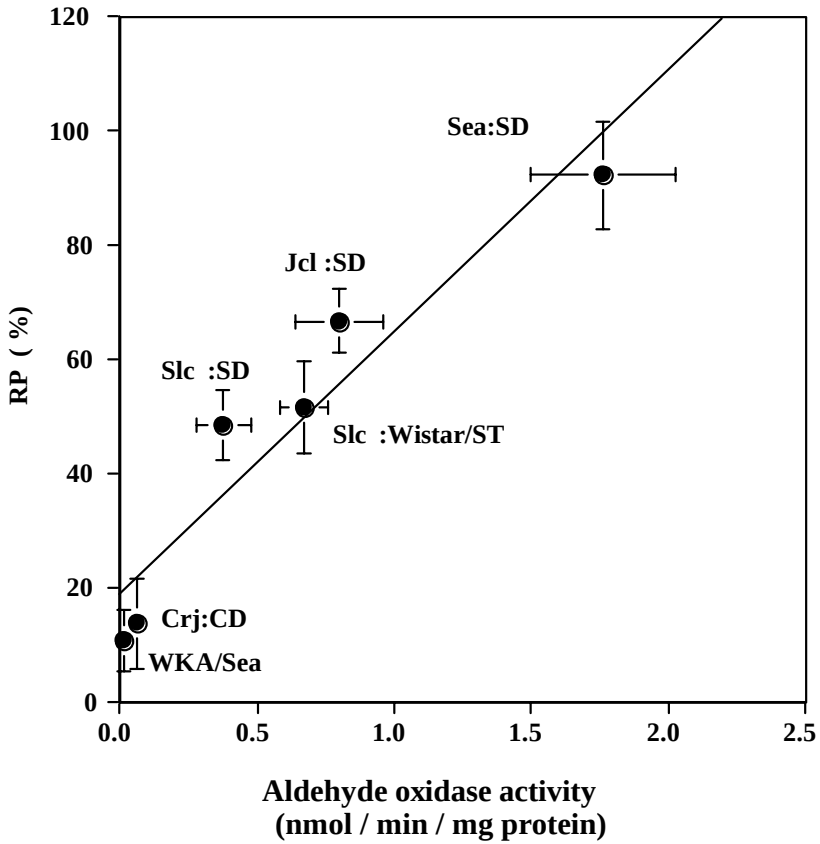


Fig. 4.