Effects of Selenium Deficiency on Aldehyde Oxidase 1 in Rats

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Selenium deficiency has been reported to result in an extraordinary decrease of glutathione peroxidase (GSH-Px) and, reversely, an increase of detoxifying enzymes such as glutathione-S-transferase (GST), uridine-5'-diphosphate glucuronosyltransferase (UGT), nicotinamide-dependent quinone oxidoreductase (NQO1; DT-diaphorase), and epoxide hydrolase without significantly affecting cytochrome P450 activity. However, little is known about the effects on aldehyde oxidase 1 (AOX1) activity towards various kinds of aldehydes and N-heterocyclic aromatic compounds. The aim of this study is to clarify the effects of selenium deficiency on AOX1 in rats. As expected, selenium deficiency was confirmed by the extremely low activity of GSH-Px along with the increased activities of GST and DT-diaphorase. AOX1 activity towards vanillin and (5)-RS-8359 was increased by selenium deficiency, and that corresponded to an increase of AOX1 protein level but not to a decreased AOX1 mRNA level. It has been documented that the assembly of the catalytically active holoenzyme forms of the molybdo-flavoenzyme family is very complex and is controlled through transcriptional and translational events by many gene products. In addition, selenium deficiency has been known to cause oxidative stress that leads to an increase of AOX1 activity. Furthermore, aldehyde oxidase homolog 1 (AOH1) with properties similar to AOX1 is present in rodent liver. All the reports suggest that the mechanisms by which selenium deficiency increases AOX1 activity are highly complicated and investigated from different points of view.

Key words aldehyde oxidase 1; selenium deficiency; rat

Aldehyde oxidase 1 (AOX1, EC 1.2.3.1) and xanthine oxidase are major members of the molybdo-flavoenzyme family. Both enzymes consist of a homodimer with a subunit molecular mass of about 150 kDa. AOX1 catalyzes the oxidation of a wide range of endogenous and exogenous aldehydes and N-heterocyclic aromatic compounds.1–3) The representative N-heterocyclic-containing drugs that serve as substrates for AOX1 are famciclovir,4) methotrexate,5) 6-mercaptopurine,6) and cinchona alkaloids.7) In addition, the atypical antipsychotic drug, ziprasidone, is mainly metabolized by AOX1-catalyzed reductive ring cleavage in human.8) Retinal is an aldehyde derivative of vitamin A that is oxidized to transcriptional retinoic acid by retinal oxidase. Retinal oxidase was found to be identical to AOX1.9) Although retinal can be considered to be a physiological substrate of AOX1, the possibility was doubted by Garattini et al. in mouse liver cytosolic extracts due to higher activity of aldehyde dehydrogenase than that of the combined activities of AOX1 and aldehyde oxidase homologue 1 (AOH1).9) AOX1 might have an indirect role in biogenic neurotransmitter amine metabolism via biotransformation of their oxidatively deaminated intermediates to carboxylic acids.10) Thus, AOX1 is definitely a kind of drug-metabolizing enzyme, but its physiological role has not yet been revealed.

AOX1 has some interesting pharmacokinetic properties. It is well known that there are remarkable species differences in AOX1 activity depending on not only animal species but also on the chemical structure of the substrate. Roughly speaking, AOX1 activity is high in monkey and human, moderate to low in rat and mouse, and deficient in dog.1,2) Large strain differences in rat, and individual differences in some kinds of rat strains have also been reported.11–15) We demonstrated that an obvious individual difference in AOX1 activity in Donryu strain rats is derived from a single nucleotide substitution of the AOX1 gene consisting of approximately 4300 base pairs. In addition, the mechanism of the individual difference is true to strain differences in rat.16,17)

Many drug metabolizing enzymes are generally susceptible to change caused by a variety of internal and external factors. Genetic mutation is one of major internal factors as well as disease status and age. Nutritional condition is given as an example of an external factor along with enzyme induction and inhibition, which are often caused by concurrently administered medicines, herbs, and foods. As for AOX1, the induction by dioxine18) and the in vitro inhibition by a number of medicines such as raloxifen and ethinylestradiol19) have been reported. Furthermore, the amount of retinal oxidase, namely AOX1, has been demonstrated to be increased by zine deficiency.20,21) Selenium is an essential trace element similar to zinc. Selenium deficiency has been known to affect drug metabolizing enzymes differently: a severe decrease of Se-dependent glutathione peroxidase (GSH-Px)22–25) and sulfotransferase,22,23,26) flavin-containing monooxygenase,22,23) and, on the contrary, a profound increase of glutathione-S-transferase (GST),22–25) uridine-5'-diphosphate glucuronosyltransferase (UGT),22,23) epoxide hydrolase,25) aldo-keto reductase,27) and nicotinamide-dependent quinone oxidoreductase (NQO1; DT-diaphorase),25) without significantly affecting cytochrome P450 22–24) The drastic decrease of GSH-Px activity was mirrored by the decrease of GSH-Px mRNA that was attributed to the post-transcriptional regulation such as most possibly stabilization of mRNA.28,29) However, it is still unknown whether selenium deficiency will affect AOX1 activity. In this report, we studied selenium deficiency on AOX1 activity in rat, focusing on the molecular mechanisms involved.

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**MATERIALS AND METHODS**

**Chemicals and Reagents** The (S)-enantiomer of RS-8359 and its 2-keto metabolite were supplied by Ube Kosan Co., Ltd. (Yamaguchi, Japan). Hydrocortisone, an internal standard of HPLC analysis, and cytochrome c were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.). 1-Chloro-2,4-dinitrobenzene (CDNB), menadione, vanillin, vanillic acid, and imidazole were obtained from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). All other reagents were of reagent grade. Anti-His-tag antiserum was purchased from Medical & Biological Laboratories Co., Ltd. (Nagoya, Japan).

**Animals** Four-week old male Wistar strain rats were purchased from Clea Japan (Tokyo, Japan). The animals were divided into two groups and housed according to the Guidelines for Animal Experimentation (Tohoku Pharmaceutical University) in cages in unidirectional airflow rooms with controlled temperature (22±2°C), relative humidity (50±10%), and 12-h light/dark cycles (07:00—19:00 h). Tap water was available *ad libitum*. A Torula-yeast-based selenium-deficient and selenium-supplemented diet, shown in Table 1, was purchased from Oriental Yeast Co., Ltd. (Tokyo, Japan). The Se content of the basal diet was 0.02 mg/kg, while the Se-supplemented control diet contained 0.40 mg Se/kg. Se was added as sodium selenite mixed with finely powdered sucrose. The animals were fed with each diet for five weeks.

**Preparation of Liver Subcellular Fractions** Animals were sacrificed by bleeding from the carotid artery under anesthesia and the livers were immediately taken out. The livers were homogenized in three volumes of 10 mM phosphate buffer (pH 7.4) containing 1.15% KCl and 100 μM phenylmethanesulphonylfluoride by a Potter-Elvehjem Teflon homogenizer. Cytosolic and microsomal fractions were prepared by successive centrifugation at 9000 g for 20 min and then at 105000 g for 60 min. The microsomal fractions were washed twice with the same buffer and then suspended in 10 mM potassium phosphate buffer (pH 7.4) containing 30% glycerol and stored at −80°C until use. The liver cytosol was dialyzed for the assay of vanillin oxidation activity. Protein concentration was determined using the Protein Assay kit (Bio-Rad Laboratories). The proteins were electrophoretically transferred to a nitrocellulose membrane and blocked with 5% nonfat dry milk in phosphate-buffered saline (PBST). The target proteins were visualized by a Western Blotting Kit (Amersham Bioscience). The membrane was blocked with 5% nonfat dry milk in phosphate-buffered saline (PBS), then incubated successively with a primary rabbit anti-monkey aldehyde oxidase antibody at 0.5 μg/ml, a secondary antibody (anti-rabbit fluorescein-conjugated whole antibody) at a dilution of 1: 600, and a tertiary antibody (anti-fluorescein alkaline phosphatase conjugate) at a dilution of 1: 2500. The blocking and incubation at each immunoreaction step were performed at room temperature for 1 h, and the membrane was washed two or three times with PBS containing 0.1% Tween-20 (PBST). The target proteins on the membrane were detected by the enhanced chemiluminescence detection system (GE Healthcare). Relative densities were measured by a Fluorescent Image Analyzer FLA-3000G (Fuji Photo Film Co., Ltd., Kanagawa, Japan). An HMW Calibration Kit (GE Healthcare) was used for the molecular weight standards. Protein level was expressed as percentage of the Se-adequate control (defined as 100%).

**Enzyme Activity Assay** AOX1 activity was determined using vanillin[20] and (S)-RS-8359[21] known to be a substrate of AOX1 as follows. Vanillin (2.5—400 μM) was incubated at 37°C for 20 min in a reaction mixture (0.25 ml) consisting of 100 mM phosphate buffer (pH 7.4), 1.0 mM K$_2$Fe(CN)$_6$, 0.13 mM ethylenediaminetetraacetic acid (EDTA), and liver cytosol. The reaction was stopped by the addition of acetonitrile (0.50 ml) containing 0.2 mg/ml of p-nitrobenzylalcohol as an internal standard. The mixture was then centrifuged at 10000 g for 5 min. Aliquots (25 μl) of the supernatant were analyzed for quantification of the oxidation product by reverse-phase HPLC on a YMC-Pack ODS AQ-302 column (4.6 mm i.d.×150 mm, YMC Co., Ltd., Kyoto, Japan). The mobile phase was 15% acetonitrile adjusted to pH 2.9 with phosphoric acid; the flow rate was 0.8 ml/min. The HPLC instrument was a Shimadzu model 6A High Performance Liquid Chromatograph System (Shimadzu Seisakusho Co., Ltd., Kyoto, Japan). The peaks were monitored for absorbance at 260 nm; the peak area was calculated on a Chromatopac C-R4A (Shimadzu). The 2-oxidation activity of (S)-RS-8359 (100 μm) was measured according to a previous report by using HPLC.[31] GSH-Px activity was determined by the method of Paglina and Valentine.[32] modified by Lawrence and Burk with hydrogen peroxide as the substrate. GST activity was assayed by the method of Habig et al.[33] with CDNB as the substrate. DT-diaphorase was measured by following the dicumarol-sensitive reduction of cytochrome c by menadione at 550 nm according to the method reported by Yoshimura et al.[34] The change of absorbance at each wavelength was monitored with a Beckman DU-650 spectrophotometer.

**Western Blot Analysis** Cytosolic proteins were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), which was performed on the PhastSystem using PhastGel gradient 8—25 in PhastGel SDS buffer strips (GE Healthcare Ltd., Buckinghamshire, U.K.). The proteins were electrophoretically transferred to a polyvinylidene difluoride (PVDF) membrane (Daiichi Pure Chemicals Co., Ltd., Ibaraki, Japan) in transfer buffer (15% methanol containing Tris 25 mM and glycine 192 mM, pH 8.3). Detection of aldehyde oxidase was performed using an ECF Western Blotting Kit (Amersham Bioscience). The membrane was blocked with 5% nonfat dry milk in phosphate-buffered saline (PBS), then incubated successively with a primary rabbit anti-monkey aldehyde oxidase antibody at 0.5 μg/ml, a secondary antibody (anti-rabbit fluorescein-linked whole antibody) at a dilution of 1: 600, and a tertiary antibody (anti-fluorescein alkaline phosphatase conjugate) at a dilution of 1: 2500. The blocking and incubation at each immunoreaction step were performed at room temperature for 1 h, and the membrane was washed two or three times with PBS containing 0.1% Tween-20 (PBST). The target proteins on the membrane were detected by the enhanced chemiluminescence detection system (GE Healthcare). Relative densities were measured by a Fluorescent Image Analyzer FLA-3000G (Fuji Photo Film Co., Ltd., Kanagawa, Japan). An HMW Calibration Kit (GE Healthcare) was used for the molecular weight standards. Protein level was expressed as percentage of the Se-adequate control (defined as 100%).

**cDNA Synthesis and Real-Time Reverse Transcription Polymerase Chain Reaction (RT-PCR)** The PCR and subsequent cloning were performed to generate a standard for the quantitative PCR of AOX1 and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as follows. Total RNA was isolated from rat liver with an SV Total RNA Isolation System (Promega Co., Madison, WI, U.S.A.) according to

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Percent of diet</th>
</tr>
</thead>
<tbody>
<tr>
<td>Torula yeast</td>
<td>30</td>
</tr>
<tr>
<td>Sucrose</td>
<td>55.7</td>
</tr>
<tr>
<td>Lard</td>
<td>5.0</td>
</tr>
<tr>
<td>Cod-liver oil</td>
<td>3.0</td>
</tr>
<tr>
<td>Mineral mix</td>
<td>5.0</td>
</tr>
<tr>
<td>Vitamin mix</td>
<td>0.85</td>
</tr>
<tr>
<td>DL-Methionine</td>
<td>0.30</td>
</tr>
<tr>
<td>Choline chloride</td>
<td>0.15</td>
</tr>
</tbody>
</table>
the manufacturer’s instructions. An aliquot of 1 μg of total RNA was used to synthesize the first-strand cDNA with SuperScript II and Oligo (dT)$_{12-18}$ (Invitrogen, Carlsbad, CA, U.S.A.). PCR amplification was conducted with cDNA (1 μg), AmpliTaq Gold (AmpliTaq DNA Polymerase), and respective oligonucleotide primers for AOX1 and GAPDH as follows: AOX1-forward gtccagaagcttccaga, AOX1-reverse gagttcaactgagaccaaga, GAPDH-forward cgaccccttcattgacctca, GAPDH-reverse tgtactgcttgctgaagcct, which were designed using Primer Express Software (Applied Biosystems, Warrington, U.K.). The primers for aldehyde oxidase were designed with reference to the nucleotide sequences of male Sprague-Dawley rat AO reported by Wright et al. 35) The PCR conditions were as follows: denaturation at 50 °C for 2 min and 95 °C for 10 min, 35 amplification cycles of 95 °C for 30 s, 60 °C for 30 s, and 72 °C for 30 s. The amplified DNA fragment (1 μg) was subcloned in the pCRII-TOPO vector using a TOPO TA Cloning Kit (Invitrogen). The resulting plasmids were purified using a Wizard Plus Miniprep DNA Purification System (Promega). The DNA sequences of the products were determined by a CEQ 8000 Analysis System (Beckman-Coulter Inc., Fullerton, CA, U.S.A.) with a Dye Terminating Cycle Sequencing Quick Start Kit (Beckman-Coulter) according to the recommended protocol. The respective standard curves for aldehyde oxidase and GAPDH were constructed using serial dilutions of plasmid DNA to determine the amount of template in each reaction. Plasmid DNA were linearized and quantified by spectrophotometry for amplification.

Quantitative real-time PCR analyses were performed using the PE ABI 7700 PRISM Sequence Detection System (Perkin-Elmer Life Science, Boston, MA, U.S.A.) with SYBR Green PCR Master Mix (Applied Biosystems), reverse transcribed cDNA (1 μg), and the same gene-specific primers as described above. The PCR reactions were performed at 95 °C for 10 min, followed by 35 cycles of 95 °C for 30 s, 60 °C for 30 s, and 72 °C for 30 s. The amplified DNA were linearized and quantified by spectrophotometry to determine the amount of template in each reaction. Plasmid DNA were linearized and quantified by spectrophotometry for amplification.

Statistical Analysis The results are expressed as the mean±S.E. of three to four experiments. Statistical significance was compared by Student’s t-tests. Values with $p<0.05$ were considered statistically significant.

RESULTS

Activities of GSH-Px, GST, and DT-Diaphorase The body weight of the Se-deficient group (134±6.2 g) was reduced to 72% of the Se-adequate control (185±4.8 g) at age 9 weeks, while the liver weight and liver to body weight ratio were not affected by selenium deficiency. Decreased body weight is an effect of prolonged selenium deficiency in rat. 22, 23 The activities of the three enzymes of GSH-Px, GST, and DT-diaphorase present in cytosol and reported to be sensitive to dietary selenium were determined (Table 2). GSH-Px activity in the Se-deficient group reduced to 4.7% of the Se-adequate control, while GST activity increased about 3.2 times when CDNB was used as the substrate and DT-diaphorase activities also increased approximately 6.6 times compared to the control. Selenium deficiency has been demonstrated to affect various kinds of drug metabolizing enzymes. 22—29 In particular, GSH-Px activity has been used as a marker of physiological selenium status because the activity decreases to an almost undetectable level depending on the degree of selenium deficiency. 22—28 In contrast, GST activity for CDNB has been shown to increase a few fold by dietary selenium deficiency. 22—27 Low activity of Se-dependent GSH-Px in combination with an increase of cytosolic GST activity has been shown to be indicative of severe selenium deficiency. 22—28 DT-diaphorase activity has also been known to be enhanced by about a 7-fold increase in selenium deficiency. 25 The results obtained in this study indicate that the rats fed with Se-deficient food were unquestionably in a selenium-deficient state.

### Table 2. Effect of Selenium Deficiency on Enzymes of Liver Cytosolic Fraction

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Group</th>
<th>$K_m$ (μM)</th>
<th>$V_{max}$ (pmol/min/mg protein)</th>
<th>$V_{max}/K_m$ (ml/min/mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vanillin</td>
<td>Control</td>
<td>14.0±1.39</td>
<td>62.8±14.1</td>
<td>4.35±0.67</td>
</tr>
<tr>
<td></td>
<td>Se deficient</td>
<td>15.8±1.68</td>
<td>215±18.6**</td>
<td>13.7±0.69*</td>
</tr>
<tr>
<td></td>
<td>Control</td>
<td>34.1±7.57</td>
<td>7.38±1.77</td>
<td>1.91±0.69</td>
</tr>
<tr>
<td></td>
<td>Se deficient</td>
<td>40.7±8.80</td>
<td>13.8±2.05*</td>
<td>3.39±1.24</td>
</tr>
</tbody>
</table>

The values are the mean±S.E. of five or six animals. $a$) GSH-Px activity was expressed as μmol NADPH oxidized/min/mg protein. $b$) GST activity was expressed as mmol CDNB conjugated/min/mg protein. $c$) DT-diaphorase activity was expressed as μmol cytochrome $c$ reduced/min/mg protein. $∗p<0.05$, $∗∗p<0.01$, and $∗∗∗p<0.001$; significantly different from the control group value.
were analyzed to consider the mechanisms involved. An SDS-PAGE/Western blotting method was employed to measure AOX1 protein levels (Fig. 1). The AOX1 level in the Se-deficient group increased by about 2.4 times compared to that of the Se-adequate control. The level of AOX1 mRNA was evaluated by Real-time RT-PCR. AOX1 mRNA of the Se-deficient group decreased to about 60% of that of the Se-adequate control (Fig. 2). The present study showed that the Se deficiency resulted in an increase in AOX1 activity that corresponded to the increased protein level but not to the decreased mRNA level.

**DISCUSSION**

Zinc deficiency has been reported to result in an increase of retinal oxidase, namely AOX1, activity in rat. A similar increase of AOX1 activity was shown by selenium deficiency in this study accompanied by strikingly diminished GSH-Px activities. Christensen et al. reported that despite the steady-state levels of GSH-Px activity and its mRNA were markedly reduced by selenium deficiency, there was no significant decline in the transcription rate of the GSH-Px gene. They speculated that dietary selenium exerts its effect on pretranslational GSH-Px gene expression, for example at the level of mRNA stabilization. As to the effects of selenium deficiency on drug-metabolizing enzymes, Davies et al. considered that dietary selenium appears to enhance potential conjugative detoxication mechanisms such as GST and UGT rather than to decrease the possible activation of chemicals via the hepatic cytochrome P450 system. Olsson et al. discussed that the changes observed in enzyme activities in connection with selenium deficiency are responses to changed levels of endogenously generated metabolites, which in turn might relate to effects on endocrine tissues. The increase in retinal oxidase (AOX1) activity by zinc deficiency was postulated to be a compensatory mechanism to maintain a normal flow of retinol to retinoic acid in the presence of reduced growth and/or food intake.

Although those several papers have documented the influences of selenium and zinc deficiency on typical drug-metabolizing enzymes, the molecular mechanisms are still poorly understood. In this study, we aimed to clarify the molecular mechanisms of AOX1 activity enhancement by selenium deficiency. A gap was observed between the increase of activity and protein level of AOX1 and the decrease of its mRNA level. Kurosaki et al. reported that in the liver of male mice, despite similar amounts of AOX1 mRNA in female mice, the levels of the corresponding protein and enzyme activity are significantly higher than are those in females. It is likely that androgens have an important role in controlling the amount of the catalytically active form of AOX1 by a translational or post-translational mechanism. According to Rivera et al., there is a remarkable time lag between the accumulation of the AOX1 mRNA and its translational products during the induction of AOX1 by dioxin, suggesting that factors other than increased gene transcription contribute to the regulation of AOX1 by dioxin in vivo. A correlation between AOX1 mRNA and protein levels in various tissues has been shown to not always be evident, suggesting that both transcriptional and translational events control the expression of AOX1 in a tissue-specific fashion. Garattini et al. documented that the assembly of the catalytically active holoenzyme forms of the molybdo-flavoenzyme family is very complex and controlled by many gene products. Thus, expression of AOX1 is potentially regulated at several levels. Cisplatin-induced nephrotoxicity in rats resulted in decreased GSH-Px and increased AOX1 activities, suggesting that AOX1 might be activated under the oxidative stress status caused by cisplatin. Selenium deficiency causes a significant reduction in the activity of GSH-Px in several tissues, particularly in liver, inducing increased oxidative stress that might be caused via transforming growth factor-β1. In addition to AOX1 and xanthine oxidase, the presence of aldehyde oxidase homolog 1 (AOH1) has been reported as a new member of the molybdo-flavoenzyme family in rodent liver.

![Fig. 1. AOX1 Protein Expression in Liver of Selenium-Deficient Rats](image1)

**Hepatic cytoxin from control and selenium-deficient rats was resolved by SDS-PAGE and transferred to PVDF membranes. The blots were probed with antibodies raised against monkey AOX1 expressed in Escherichia coli. Immunoactive protein was detected using the ECF detection system (A) and relative densities were measured using a Fluorescent Image Analyzer FLA-3000 (B). +p<0.05; significantly different from the control group value.**

![Fig. 2. AOX1 mRNA Expression in Liver of Selenium-Deficient Rats](image2)

**The expression level was determined by real-time RT-PCR. Relative quantification of the target gene expression was normalized with the mRNA expression of an endogenous reference gene, GAPDH. The values are the mean±S.E. of five or six animals. *p<0.05; significantly different from the control group value.**
isoenzyme shows a similar substrate specificity as does that of AOX1. Although the properties of rat AOH1 have been less characterized compared to those of mouse, there is a possibility that AOH1 might be induced by selenium deficiency. All the reports suggest that the mechanisms by which selenium deficiency increases AOX1 activity should be investigated from a variety of viewpoints, which will be the subjects of future studies.

Recently, AOX1 was identified as an ATP-binding cassette transporter A1 (ABC A1) interacting protein to affect ABC A1-dependent phospholipid and cholesterol efflux.\(^{65}\) AOX1 has also been reported to be essential for adipogenesis from the fact that a knockdown of AOX1 in adipocytes led to impaired lipid storage and adiponectin release in the differentiated cells.\(^{46}\) Thus, elucidation of the physiological functions of AOX1 has just begun. Revealing the molecular mechanism of AOX1 increase due to oxidative stress caused by selenium deficiency might be helpful in understanding the physiological role of AOX1.

In conclusion, selenium deficiency resulted in an increase of AOX1 activity that corresponded to an increase of AOX1 protein but not to a decreased AOX1 mRNA level in rat. A dichotomy between mRNA and protein levels is not unusual for molybdo-flavoenzymes, suggesting that the gene expression is under regulation of many translational and post-translational events. Furthermore, selenium deficiency causes oxidative stress responsible for AOX1 variation, and the presence of AOH1 with properties similar to AOX1 in rodent liver has been known. The reports suggest that the mechanisms of the increase of AOX1 activity by selenium deficiency should be investigated in detail from different points of view.

REFERENCES