Lack of Formation of Aldehyde Oxidase Dimer Possibly Due to 377G>A Nucleotide Substitution

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ABSTRACT:

In addition to the many articles reporting on the marked differences in species and large differences in rat strains in response to aldehyde oxidase (AO), individual differences in some rat strains have also been reported. However, little has been clarified about any related molecular biological mechanisms. We previously revealed that nucleotide substitutions of 377G>A and 2604C>T in the AO gene might be responsible for individual differences in AO activity in Donryu strain rats. By using native polyacrylamide gel electrophoresis/Western blotting in this study, the lack of formation of the AO dimer protein, which is essential for catalytic activity, was shown in poor metabolizer Donryu rats, and this could be a major reason for the individual differences. Rat strain differences were also verified from the same perspectives of nucleotide substitutions and expression levels of a dimer protein. Rat strains with high AO activity showed nucleotide sequences of (377G, 2604C) and a dimer protein. In the case of those with low AO activity, the nucleotide at position 2604 was fixed at T, but varied at position 377, such as G, G/A, and A. An AO dimer was detected in the liver cytosols of rat strains with (377G, 2604T), whereas a monomer was observed in those with (377A, 2604T). These results suggest that the lack of formation of a dimer protein leading to loss of catalytic activity might be due to 377G>A nucleotide substitution. Individual and strain differences in AO activity in rats could be explained by this 377G>A substitution, at least in the rat strains used in this study.

Both aldehyde oxidase (AO) and xanthine oxidase are major members of the molybdenum hydroxylase family. They consist of a homodimer with a subunit molecular mass of about 150 kDa. Each subunit of AO contains a molybdopterin cofactor, a FAD, and two modifiers with a subunit molecular mass of about 150 kDa. Each

activity might be due to 377G>A nucleotide substitution. Individual and strain differences in AO activity in rats could be explained by this 377G>A substitution, at least in the rat strains used in this study.

A large variation in rat strains has also been demonstrated in the oxidation activity of benzaldehyde (Sugihara et al., 1995) and methotrexate (Kitamura et al., 1999a). An interesting individual difference has been reported in Wistar rats (Gluecksohn-Waelsch et al., 1967) and in Sprague-Dawley (SD) rats (Beedham et al., 1998). Similar to those reports, we observed remarkable species differences (Takasaki et al., 2005; Itoh et al., 2006), large differences in rat strains (Sasaki et al., 2006), and striking individual differences in Donryu rats (Itoh et al., 2007b) in the AO-catalyzed oxidation of the (S)-enantiomer of RS-8359 [(±)-4-(4-cyanoanilino)-5,6-dihydro-7-hydroxy-7H-cyclopenta[d]-pyrimidinone (Itoh et al., 2007b)]. The compound is a reversible and selective MAO-A inhibitor (Yokoyama et al., 1989; Miura et al., 1993) and has been developed as an antidepressant (Plenker et al., 1997; Puchler et al., 1997). One of the major metabolic pathways of RS-8359 is the AO-catalyzed 2-oxidation on the pyrimidine ring to give the 2-keto metabolite (Itoh et al., 2005). The AO-catalyzed 2-oxidation activity of (S)-RS-8359 in Donryu strain rats was shown to be divided into three groups: ultrarapid metabolizer (UM), extensive metabolizer (EM), and poor metabolizer (PM). Nucleotide substitutions at positions 377 and 2604 were suggested to be primarily responsible for differences in aldehyde oxidase activity observed in that strain. In particular, 377G>A substitution was supposed to be more central for the deficiency of the catalytic

ABBREVIATIONS: AO, aldehyde oxidase; SD, Sprague-Dawley; RS-8359, (±)-4-(4-cyanoanilino)-5,6-dihydro-7-hydroxy-7H-cyclopenta[d]-pyrimidinone; MAO-A, monoamine oxidase A; PM, poor metabolizer; EM, extensive metabolizer; UM, ultrarapid metabolizer; PCR, polymerase chain reaction; native PAGE, native polyacrylamide gel electrophoresis; HPLC, high-performance liquid chromatography; PVDF, polyvinylidene difluoride.
activity of AO than 2604C>T substitution, because the former substitution accompanies an amino acid change from a symmetric Gly to an asymmetric Ser very close to the second Fe-S redox center (Itoh et al., 2007b). In another report, we demonstrated that rat strains with high AO activity exhibited a dimeric protein on native PAGE/Western blot in contrast to rat strains with low AO activity, which produced only a monomer protein (Itoh et al., 2007a). The results clearly showed that discussions on rat strain differences in AO activity should be based on the expression levels of the dimeric protein itself. From a combined consideration of the results obtained from the two separately conducted studies on individual differences in Donryu rats and differences in rat strains, we assumed that individual differences in Donryu rats might be due to a deficiency in formation of the AO dimeric protein. In this study, we investigated the possibility that 377G>A nucleotide substitution in the AO gene might result in the differences in rat strains.

Materials and Methods

Chemicals. The (S)-enantiomer and 2-keto metabolite of RS-8359 were supplied by Ube Kosan Co. Ltd. (Yamaguchi, Japan). Hydrocortisone, an internal standard of HPLC analysis, was purchased from Sigma Chemical Co. (St. Louis, MO). All other reagents were of reagent grade.

Preparation of Liver Cytosolic Fractions. Eight-week-old male rats of 10 strains were used. Crj/Donryu strain rats were purchased from Charles River Japan (Yokohama, Japan). Wistar strain rats were obtained from Charles River Japan (Crl:Wistar and LEW/Crlj), Japan Slc (Shizuoka, Japan); Slc:Wistar, WKY/Izm, and WKAH/Hkm), Clea Japan (Tokyo, Japan; Jcl:Wistar), and Imamichi Institute for Animal Reproductions (Saitama, Japan; Iar:Wistar or Wistar-Imamichi). Sprague-Dawley strain rats were obtained from Charles River Japan (Crl:SLD) and Japan Slc (Slc:SDL). Fisher strain rats (F344/Ducrl-Crlj) were purchased from Charles River Japan. F344/Ducrl-Crlj, LEW/Cel-Crlj, WKY/Izm, and WKAH/Hkm are inbred; the others are a closed colony. The animals were housed according to the Guidelines for Animal Experimentation (Tohoku Pharmaceutical University) in cages in rooms with a unidirectional airflow at a controlled temperature (22 ± 2°C), relative humidity (50 ± 10%), and 12-h light/dark cycles (7:00 AM—7:00 PM). Tap water was supplied by Ube Kosan Co. Ltd. (Yamaguchi, Japan). Hydrocortisone, an internal standard of HPLC analysis, was purchased from Sigma Chemical Co. (St. Louis, MO). All other reagents were of reagent grade.

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Direct Sequence Analysis of Genomic DNA. Genomic DNA was isolated with a DNeasy Blood and Tissue Kit (QIAGEN, Hilden, Germany) according to the manufacturer’s instructions. Primer sets were designed in reference to the nucleotide sequences of rat (GenBank accession number NW047816) on introns as follows: 377 forward: 5‘-gaggtcttctcaataacctgtg-3‘, 377 reverse: 5‘-gatgttgtgctgataaagtcta-3‘, 2604 forward: 5‘-ctccataactacaagcata-3‘, 2604 reverse: 5‘-ggtgagctctatgcttatta-3‘. The PCR was conducted in a solution of 50 μl containing 1× Ex Taq buffer, 200 μM dNTP mix solution, 2.5 U Ex TaqDNA polymerase (TaKaRa Bio Inc., Shiga, Japan), 2 mM MgCl2, 0.2 μM concentration of forward and reverse primer, and 2 μl of the template. PCR amplification was conducted using a PCR thermal cycler MP (TaKaRa) as follows: initial denaturation at 94°C for 5 min, and 30 cycles of 94°C for 30 s, 60°C for 30 s, and 72°C for 30 s, and a final extension at 72°C for 10 min. The PCR products were purified using the Wizard SV Gel and PCR Clean-Up System (Promega, Madison, WI). The DNA sequences were determined using the CEQ 8000 Genetic Analysis System (Beckman Coulter Inc., Fullerton, CA) and a DTCs Quick Start Kit, for which the above-mentioned forward or reverse primer was used as the direct sequence primer.

Statistical Analysis. The results are expressed as the mean ± S.E. for the number of experiments. Statistical significance was compared between low and high activity groups by Student’s t test. Values with p < 0.05 were considered statistically significant.
different between UM and PM in Donryu strain rats, suggesting that catalytic activity. Only two nucleotides among 4032 base pairs were polymorphism was caused by a lack of dimer formation necessary for onset of 2604T), respectively. The current study revealed that this polymorphism was identical nucleotide sequences. However, Crlj:Wistar rats showed three different types of nucleotide sequences, (377G, 2604C), (377G/A, 2604C/T), and (377A, 2604T), which were the same as those of the Donryu strain (Itoh et al., 2007b). In addition, the two SD strains of Crlj:SD and Slc:SD had other sequence patterns, (377G, 2604T), (377A, 2604T), and (377G/A, 2604T), thus being heterozygous at position 377, but homozygous at position 2604.

Characteristics of AO in SD Strain Rats. Because new combinations of nucleotide substitutions were found in the AO of SD strain rats, detailed analyses of nucleotide sequences, enzyme activities, and immunoreactive 300-kDa expression levels were performed using 14 Crlj:SD strain rats. The nucleotide sequences (Table 2) were divided into three groups: group 1 (n = 3; 377G, 2604T), group 2 (n = 5; 377G/A, 2604T), and group 3 (n = 6; 377A, 2604T). Group 1 exhibited nearly the same activity as that of group 2. In contrast, group 3 showed significantly lower activity (Fig. 3). The presence of AO dimer protein was confirmed in group 1 but not in group 3, in which only a monomer protein was detected; both the dimer and monomer proteins were present in group 2 according to the respective native PAGE/Western blot analyses (Fig. 4). Almost the same results were obtained in the experiment using 13 Slc:SD strain rats (Table 2).

Discussion

In addition to marked species differences and large variations in rat strains (Beedham et al., 2002; Kitamura et al., 2006), individual differences have been reported in AO activity in Wistar rats (Gluecksohn-Waelsch et al., 1967), SD rats (Beedham et al., 1998), and Donryu rats (Itoh et al., 2007b). However, little has been known about the mechanisms contributing to the differences. In the previous report (Itoh et al., 2007b), we demonstrated polymorphism in the AO of the Donryu rat strain such that UM, EM, and PM possess nucleotide substitutions of (377G, 2604C), (377G/A, 2604C/T), and (377A, 2604T), respectively. The current study revealed that this polymorphism was caused by a lack of dimer formation necessary for onset of catalytic activity. Only two nucleotides among 4032 base pairs were different between UM and PM in Donryu strain rats, suggesting that the two nucleotide substitutions are very critical for dimer formation. The nucleotide substitutions of 377G>A and 2604C>T result in the amino acid changes of Gly110Ser and Ala852Val, respectively (Itoh et al., 2007b). The results suggest that changing from a symmetric Gly to an asymmetric Ser in a position very near the second Fe-S redox center (Wright et al., 1999) might cause a more serious conformational change in AO protein structure and loss of enzyme activity than would the amino acid change of Ala852Val.

The above-mentioned mechanism of individual differences in AO activity in Donryu strain rats, i.e., a genetic polymorphism, was also postulated to be the case for the difference in AO activity among rat strains. The postulate was verified with direct sequence analysis of genomic DNA and native PAGE/Western blot analysis of AO dimer protein in 10 rat strains. However, the situations were considerably more complex for the differences in rat strains than for the individual differences in Donryu rats. Rat strains with high AO activity showed simple nucleotide sequences of (377G, 2604C) and a dimer protein, whereas among rat strains with low activity, Slc:Wistar and F344/DuCrjCrlj rats had different types of nucleotide substitutions of (377A, 2604T) and a monomer protein. In contrast, Crlj:SD and Slc:SD rats possessed more complex sequences of (377G, 2604T), (377G/A, 2604T), and (377A, 2604T) and a mixture of a dimer and a monomer protein. Furthermore, high activity Crlj:Wistar rats exhibit-
The same three nucleotide substitutions and protein profiles as those of Donryu rats, suggesting that Crlj:Wistar rats will exhibit polymorphism in AO activity. As shown previously (Itoh et al., 2007a), the larger variation seen in the activities and expression levels of AO for Crlj:Wistar than those for other high activity rat strains might be ascribed to polymorphic metabolism. Thus, the nucleotide substitutions at positions 377 and 2604 were obviously responsible not only for the individual differences in the Donryu strain rats, but also for the rat strain differences in AO activity.

The complex nucleotide substitutions at positions 377 and 2604 were observed in both Crl:SD and Slc:SD rats. In those cases, the nucleotide at 2604 was fixed to T, but was varied at 377; for example, as G, G/A, and A. A 300-kDa dimer protein showing high AO activity was found in rats with the nucleotide substitutions of (377G, 2604T). In contrast, only a 150-kDa monomer protein showing low AO activity was found in rats with substitutions of (377A, 2604T). The mechanism of individual differences in the AO activity of Donryu strain rats was presumed to be the 377G/H11022A substitution among 4032 base pairs, as mentioned above. Judging from the results obtained in the SD strain rats and the possible mechanism of individual differences in the Donryu strain rats, it is suggested that the 377G/H11022A substitution might be the major cause of the lack of dimer formation of AO. It is possible that the nucleotide substitution of 2604C/H11022T is associated with the dimer formation of AO because of the relatively low expression level of the 300-kDa protein in rats with the nucleotide substitutions of (377A, 2604T). This will be an interesting subject for future clarification.

In conclusion, the 377G/H11022A nucleotide substitution in the AO gene accompanying amino acid change from a symmetric Gly to an asymmetric Ser at the 110 position was suggested as the reason for the lack of formation of AO dimer protein and the loss of its catalytic activity. The substitution might be primarily responsible not only for individual differences in AO of the Donryu and Crlj:Wistar strains of rats, but also for differences in AO activity in rat strains, at least in the rat strains used in this study.

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References


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<th>Nucleotide Position</th>
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<th>High AO Activity</th>
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<td></td>
<td></td>
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<td>F344/DuCrl/Crlj</td>
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<tr>
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<td>G</td>
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<tr>
<td>2604</td>
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TABLE 2
Nucleotide substitutions of AO genomic DNA in Crl:SD and Slc:SD rat strains

Both Crl:SD and Slc:SD strains belong to the low AO activity group. The number in each group is indicated in parentheses.

<table>
<thead>
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<th>Nucleotide Position</th>
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<th>Slc:SD</th>
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<tr>
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<td>377</td>
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<td>T</td>
<td>A</td>
</tr>
<tr>
<td>2604</td>
<td>T</td>
<td>G/A</td>
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</table>

![Graph](image1)

Fig. 3. AO-catalyzed 2-oxidation activity of (S)-RS-8359 in liver cytosols of Crl:SD strain rats. The (S)-enantiomer of RS-8359 (200 μM) was incubated at 37°C for 10 min with cytosol (20 mg/ml, 0.10 ml) and the oxidation product was quantified by reversed-phase HPLC.

![Graph](image2)

Fig. 4. Native PAGE/Western blot analysis of AO protein in liver cytosols of Crl:SD strain rats. Cytosolic proteins were separated by native PAGE, transferred electrophoretically to a PVDF membrane, and immunoreacted against rabbit anti-monkey AO antibody. The target proteins on the membrane were detected by the enhanced chemiluminescence detection system.

![Graph](image3)

Fig. 5. Activity (pmol/min/mg protein) of AO in liver cytosols of Crl:SD strain rats. The activity was measured as described in the methods section.

![Graph](image4)

Fig. 6. Western blot analysis of AO protein in liver cytosols of Crl:SD strain rats. Cytosolic proteins were separated by native PAGE, transferred electrophoretically to a PVDF membrane, and immunoreacted against rabbit anti-monkey AO antibody. The target proteins on the membrane were detected by the enhanced chemiluminescence detection system.


