Important role of the C-terminal region of pig aldo-keto reductase family 1 member C1 in the NADPH-dependent reduction of steroid hormones

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The NADPH-dependent reduction activities of two paralogous pig AKR1C1s with and without 19 additional amino acid residues in C-terminus were evaluated against steroid hormones including 5α-dihydrotestosterone, testosterone, progesterone, androstenedione and 5α-androstane-3,17-dione, which act as substrates of the AKR1C1s. Among the hormones, the AKR1C1s exhibited the highest activity against 5α-dihydrotestosterone and the lowest activity against testosterone and progesterone. Furthermore, the AKR1C1s showed the largest differential activities against 5α-dihydrotestosterone, but no such change of activities was found against progesterone and testosterone. These results suggest that the C-terminal region of AKR1C1 plays an important effect in the reduction activities of pig AKR1C1. Thus, the differential activities of two AKR1C1 paralogs observed in the present study provide important insights in understanding the molecular evolution.

Keywords: AKR1C1, NADPH-dependent reduction, Steroid hormone.

Aldo-keto reductases (AKRs) which exist in all living organisms involve in biosynthesis, metabolism and detoxification as NAPDH-dependent oxido-reductase to mediate reduction of various aldehydes and ketones to corresponding alcohols in vivo. AKRs have been divided into 14 super-families from AKR1A to AKR1G. AKR1 family has been classified into 6 sub-families AKR1A to AKR1G. AKR1C plays a role as steroid 5β-reductases and members of AKR1C sub-family mediate 25 enzymatic functions. Endogenous substrates for AKR1C sub-family include aldehyde group of sugar or lipid, prostaglandins, retinoids and steroids. The sub-family plays important roles in detoxification of xenobiotics, such as various drugs or carcinogens.

AKR1C1 (20α-hydroxysteroid dehydrogenase (HSD)/dihydrodiol dehydrogenase (DD1), AKR1C2 (type 3 3α-HSD/bile acid binding protein), AKR1C3 (type 2 3α-HSD, type 5 17β-HSD/prostaglandin F synthase) and AKR1C4 (type 1 3α-HSD/choledochol reductase) isoforms have shown homology up to 86%. Especially, AKR1C1 (20α-HSD) converts progesterone, a steroid hormone to inactive 20α-dihydropregosterone (20α-OHP); progesterone is involved in the menstrual cycle, pregnancy and embryogenesis of human and other species and plays an important role for pregnancy and pregnancy maintenance and exhibits the highest levels at before and after delivery. AKR1C1 also inhibits synthesis of compounds, including estrogen, benzodiazepines and non-steroid anti-inflammatory drugs.

Recently, AKR1C1 in pig has shown differential expression associated with estrous cycle, thus it is deduced that AKR1C1 plays a critical role for hormone regulation during the estrous cycle. However, it remains to be discovered whether additional amino acids of AKR1C1-like C-terminus identified in pig play important physiological or enzymatic roles.

In this study, we report two paralogous AKR1C1s exhibiting single nucleotide variations and have also investigated, whether the variant AKR1C1 T-type when compared with AKR1C1 C-type shows differential enzymatic activities to steroid hormones, including 5α-dihydrotestosterone, testosterone, progesterone, androstenedione and 5α-androstane-3,17-dione. We have assessed the NADPH-dependent reduction activity of pig AKR1C1 and found that the reduction activity is dependent significantly on the C-terminal region, truncated by the non-synonymous variation.
Materials and Methods

Ethics statement
Pigs used in this study were slaughtered in accordance with the guidelines on animal care and use established by the Animal Care and Use Committee of GNTECH and with the Korea Animal Protection Act and related law. In detail, pigs weighing approximately 110 kg were transported to an abattoir near the experimental station. They were slaughtered by stunning with electrical tongs (300 volts for 3 s) after 12 h of feed restriction. The shocked pigs were exsanguinated while being hung.

RNA extraction
The livers were collected from 5 individual pigs of Landrace, Yorkshire, Duroc and stored in liquid nitrogen until use. Total RNA was isolated from the collected livers using Trizol reagent, according to the manufacturer’s instruction (Life Technologies, Invitrogen). Briefly, 2 ml of Trizol reagent was added to 0.1–0.2 g of grounded tissue, the mixture was well-suspended with homogenizer and 1 ml aliquot of the mixture was transferred into a 1.5 ml e-tube. Then, the mixture was left alone at room temperature (RT) for 10 min, centrifuged at 12,000 rpm for 10 min and the solution excluding the cell debris was saved into a new tube. Two hundred µl of chloroform was added to the supernatant and then 500 µl isopropanol was added to precipitate the total RNA, which was then washed with 70% ethanol and dried at RT. The extracted total RNA was dissolved in water and quantified spectrophotometrically at 260 nm. For each sample, a total RNA aliquot was subjected to electrophoresis in 1.5% agarose gel to verify the extracted total RNA.

cDNA synthesis
For RT-PCR, the first strand cDNA was synthesized by using Superscript II Reverse Transcriptase, according to the manufacturer’s protocol (Invitrogen, USA). Briefly, 5µl of extracted RNA was added to a reaction mixture consisting of 4 µl of 5X First strand buffer (Invitrogen, Carlsbad, CA), 1 µl of 10 mM deoxynucleotide triphosphates (dNTPs) (Promega, USA) dissolved in DEPC-water, 2 µl of 0.1 M dithiothreitol (DTT) (Invitrogen, USA), 1 µl (200 U/µl) of SuperScript Reverse Transcriptase II (Invitrogen, USA), 1 µl (0.5 µg/µl) of oligo-d(T) 12 to 18 primer (Invitrogen, USA), 1 µl of RNase inhibitor (Invitrogen, USA) and RNase-free water. Then, the reverse transcription step was carried out at 42°C for 1 h, followed by heating at 70°C for 15 min and adding 1 µl RNase H at 37°C for 20 min before storage at 4°C. RT-PCR products of two genes were separated on a 2% Tris-acetate-ethylene diaminetetraacetic acid (TAE) agarose gel and visualized by ultraviolet (UV) after ethidium bromide staining.

Cloning, overexpression and purification of recombinant AKR1C1
cDNA construct encoding the C-terminal-deleted AKR1C1 paralog (AKR1C1 T type) was prepared by reverse transcription and PCR using the following oligonucleotides with restriction sites: 5'-GGATCCATGGATCCAAATGGATCCCAAAAGCCAGCG-3' and 5'-AAGCTTCAATGGATCCAAATGGATCCCAAAAGCCAGCG-3' as forward and reverse primers, respectively. Next, the construct encoding the AKR1C1 paralog with the C-terminal region from R320 to L337 (AKR1C1 C-type) was prepared by a site-directed mutagenesis using AKR1C1 T-type cDNA as a template. The employed primers were designed to contain the desired nucleotide change (T→C) as shown in Table 1. Finally, two types of AKR1C1 constructs were subcloned into the pPROEXHTb vector (Invitrogen) for the production of His-tagged fusion protein. After isopropylthio-β-galactoside (IPTG) induction, the two recombinant proteins were overexpressed and purified from E. coli BL21 cells.

Measurement of AKR1C1 reductase activity
The reductase activity was measured under conditions described previously14. Reaction mixtures included 60 mM sodium phosphate (pH 6.5), purified recombinant proteins such as AKR1C1 C- and T-types, 0.1 mM NADPH and 0.1 mM substrates (the reproductive steroid hormones indicated above) and were incubated in a total volume of 0.5 ml at 37°C. The assay of reductase activity was spectrophotometrically carried out by monitoring the decrease in absorbance at 340 nm with time.

| Table 1—Oligonucleotides used in this study |
|-----------------|-----------------|
| No. | Oligonucleotides | Nucleotide sequences (5'-3') |
| 1 | SDM-F1 | 5'-GTAAAGGAGCGAGGTGACCTGAAC-3' |
| 2 | SDM-R1 | 5'-TTTGCAGCTGCTGCTTACCTTAC-3' |
| 3 | SDM-F2 | 5'-GAATTCATGGATCCCAAAAGCCAGCG-3' |
| 4 | SDM-R2 | 5'-GTCGACCTCACAGCTCGGAGCATG-3' |
Data were analyzed by the GLM procedure of SAS (SAS Inst. Inc., Cary, NC) and presented as means ± SE. Results were considered significant, when the $P$ value was less than 0.05.

**Results and Discussion**

To investigate whether the truncated C-terminus has an important effect on the reductase activity of pig AKR1C1, the reductase activities of two AKR1C1 paralogs with different C-terminal lengths were compared using spectrophotometric analyses. The results of SDS-PAGE revealed that the AKR1C1 T-type having the non-synonymous single base variation (C→T) was expressed as a recombinant protein with a size (about 37 kDa) smaller than that (about 39 kDa) of AKR1C1 C-type (Fig. 1), indicating that the variation led to deletion of the C-terminal region from R320 to L337.

For measurement of reductase activity, reproductive steroid hormones, such as testosterone, 5α-dihydrotestosterone, progesterone, androstenedione and 5α-androstane-3,17-dione were used as substrates of the NADPH-dependent reductase activity, because the pig AKR1C1 has been suggested to be associated with reproductive traits. The AKR1C1 C- and T-types exhibited difference in their reductase activities (Fig. 2); AKR1C1 T-type converted the active form to the inactive form of most steroid hormones, except testosterone, (AKR1C1 mediates active hormone to inactive one, active → inactive) at a rate higher than that of AKR1C1 C-type. Especially, 5α-dihydrotestosterone was reduced more readily by AKR1C1 T-type than by AKR1C1 C-type ($P < 0.05$). Thus, these results strongly indicated that the C-terminal region from R320 to L337 truncated by a non-synonymous variation had an important effect on the NADPH-dependent reductase activity of pig AKR1C1.

When the amino acid sequence of pig AKR1C1 C-terminal region was compared with other animals, pig AKR1C1 was found to have 14 amino acids more
than those of other animals (Fig. 3). It was assumed that the truncated AKR1C1 T-type was similar in amino acid lengths and enzymatic activities of AKR1C1s from other animals, as carbonyl reductase revealed via non-sense single base variation. Therefore, it was deduced that the additional 14 amino acids in AKR1C1 C-type play an important role for hormonal regulation via C-loop, such as AKR4C9.

Previously, AKR1C homologs have been shown to comprise a gene cluster in the homologous region on a chromosome. For example, a cluster of AKR1C homologs has been identified in the homologous region on mouse chromosome 13. In human, a region near the telomere chromosome 10p15 also contains at least six AKR1C homologs. Similarly, the pig chromosome-10 contains the AKR1C gene cluster, which includes QTL (quantitative traits) having possible associations with reproductive traits. These AKR1C clusters have been suggested to be created by gene duplications during evolution. Although the AKR1C paralogs at the gene cluster maintain a high degree of sequence similarity, they have been reported to differ significantly in their substrate specificity and biochemical activity; in human and mouse, AKR1Cs have shown different substrate preferences, in spite of their high sequence homology.

The pig AKR1C1, an AKR1C cluster gene on chromosome-10 has been recently identified to be expressed as two different sizes of proteins with or without the C-terminal region from R320 to L337 caused by the non-synonymous single nucleotide change (C/T) (Fig. 1A), but genotyping via a pig population in this study revealed no minor allele frequencies (data not shown). Accordingly, the non-sense single nucleotide change was likely to be generated by gene duplication during evolution and thus it was considered as the paralogous sequence variation, especially a duplicated gene nucleotide variation. Moreover, our enzymatic comparison between AKR1C1 C- and T-types revealed that they had different NADPH-dependent reductase activities, dependent on the C-terminal region. Altogether, these suggested the divergent evolutionary history of two paralogous AKR1C1s, induced by the duplicated gene nucleotide variation.

In conclusion, the truncated C-terminal region of pig AKR1C1 plays an important role of in the NADPH-dependent reduction of steroid hormones. Although further kinetic studies are warranted to examine the effects of the truncated C-terminal region on substrate affinity and enzymatic efficiency, the present study provides an important insight in understanding the molecular evolution of two paralogous AKR1C1s, induced by the duplicated gene nucleotide variation.

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