An NADPH-Dependent Reductase in Neonatal Pig Testes That Metabolizes Androgens and Xenobiotics

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We have isolated an NADPH-dependent reductase from neonatal pig testes that metabolizes androgens and a variety of xenobiotics. This enzyme is distinct from $3\alpha/\beta,20\beta$ -hydroxysteroid dehydrogenase or its homologue, carbonyl reductase, as judged by its immunological and molecular properties and its much narrower specificity for steroids. This reductase and $3\alpha/\beta,20\beta$ -hydroxysteroid dehydrogenase may be part of a mechanism for regulating androgen levels in the neonatal pig testes. Interestingly, we could not find multiple isoforms of $3\alpha/\beta,20\beta$ -hydroxysteroid dehydrogenase/carbonyl reductase in pig testes unlike human and rat testes and other organs in which multiple isoforms are expressed.

Key words NADPH-dependent reductase; $3\alpha/\beta,20\beta$ -hydroxysteroid dehydrogenase; carbonyl reductase; androgen; xenobiotic; purification

An important mechanism for regulating the concentrations and actions of steroids and prostaglandins involves oxidoreductases that can catalyze the interconversion of the biologically active hormone and either less active or inactive compounds.^{1,2)} Among the enzymes that may perform this function is mammalian $3\alpha/\beta,20\beta$ -hydroxysteroid dehydrogenase ($3\alpha/\beta,20\beta$ -HSD), which metabolizes androgens and progestins.^{3,4)} This enzyme was first isolated from pig neonatal testes, suggesting that $3\alpha/\beta,20\beta$ -HSD may regulate steroid hormone concentrations in the testes during development.^{3,5)} However, later studies using antibodies to $3\alpha/\beta,20\beta$ -HSD revealed that it is expressed in many other tissues including kidney, liver, brain, lung, thymus, heart, spleen and adrenal gland, suggesting a broader role in steroid hormone action.⁶⁾

The cloning and sequencing of $3\alpha/\beta,20\beta$ -HSD⁷⁾ showed that it belongs to the short-chain alcohol dehydrogenase superfamily,⁸⁻¹¹⁾ which includes steroid and prostaglandin dehydrogenases.¹²⁾ $3\alpha/\beta,20\beta$ -HSD is 85% identical to carbonyl reductase, an enzyme first isolated from human brain by Wermuth,¹³⁾ who showed that carbonyl reductase metabolizes steroids, prostaglandins and a variety of xenobiotic carbonyl-containing compounds. Indeed, $3\alpha/\beta,20\beta$ -HSD also metabolizes prostaglandins and xenobiotics, leaving open the possibility of multiple functions of $3\alpha/\beta,20\beta$ -HSD and carbonyl reductase, depending on the tissue in which they are expressed.

However, despite the 85% sequence identity between pig $3\alpha/\beta,20\beta$ -HSD and human carbonyl reductase,¹⁴⁾ which allows them to be considered isozymes, they exhibit some differences in substrate specificity.⁷⁾ Moreover, compared with $3\alpha/\beta,20\beta$ -HSD, carbonyl reductase has 12 fewer amino acids at its carboxyl terminus. This suggests some complementary functions, which may be one reason for the presence of isozymes of carbonyl reductase in many tissues. Iwata *et al.*¹⁵⁾ and Wermuth and his group^{16,17)} found three isozymes of carbonyl reductase in human and rat testes, and Iwata *et al.*¹⁸⁾ and Inazu *et al.*¹⁹⁾ found multiple forms in rat ovaries and adrenals. These reports prompted us to attempt to isolate comparable isozymes from neonatal pig testes. As described in this paper, we found only one species of $3\alpha/\beta,20\beta$ -HSD/carbonyl reductase in neonatal pig testes. However, we

also isolated an NADPH-dependent reductase that metabolizes androgens and some carbonyl compounds. This enzyme is distinct from either $3\alpha/\beta,20\beta$ -HSD or carbonyl reductase. Interestingly, compared with $3\alpha/\beta,20\beta$ -HSD, this reductase has much narrower specificity for androgens, suggesting that it may be important in regulating hormone concentrations during development.

MATERIALS AND METHODS

Materials and Chemicals Fresh testes from neonatal pigs (strain, LWD; 2 weeks of age) were obtained from local farms after castration and were immediately transported to the laboratory in ice-cold 10 mM Tris-HCl buffer (pH 7.4) including KCl (0.15 M) and EDTA (1 mM). Fresh rat testes were obtained from male rats (5 weeks of age, Wister). Polybuffer exchanger PBE 94, polybuffer 74, 2',5'-ADP-Sepharose, Sephadex G-75 and molecular weight markers for electrophoresis were obtained from Pharmacia Fine Chemicals (Uppsala, Sweden). Epalrestat was generously provided by Ono Yakuhin Kogyo Co., Ltd. (Osaka, Japan). The reduced form of β -nicotinamide adenine dinucleotide phosphate (β -NADPH) was purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Prostaglandins were obtained from Funakoshi Chemicals (Tokyo, Japan). Various materials were obtained from sources already reported^{4,6)} except for the above materials. Other reagents were of the highest grade available and were obtained from Iwai Chemicals Co. (Tokyo, Japan).

Preparation of Cytosol from Testes The testes were decapsulated, weighed and dissected into smaller pieces with scissors. The weighed samples were homogenized with 4.5 vol. 10 mM Tris-HCl buffer (pH 7.4) including KCl (0.15 M), EDTA (0.5 mM), dithiothreitol (DTT, 0.5 mM), phenylmethylsulfonyl fluoride (PMSF, 0.025%) and pepstatin A (1.0 μ M) and the homogenate was centrifuged at 10000 $\times g$ for 20 min. The resulting supernatant was further centrifuged at 105000 $\times g$ for 60 min and this final supernatant was used as the cytosol fraction. All of the above procedures were carried out at 4 °C or in an ice bath.

Purification of NADPH-Dependent Reductase from

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Neonatal Pig All purification procedures were carried out in a cold room at 4 °C or in an ice bath.

Step 1: Ammonium Sulfate Fractionation The cytosol was fractionated by adding solid ammonium sulfate as described previously.³⁾ The precipitate obtained from 30 to 75% saturation was dissolved in buffer A: 10 mM Tris-HCl buffer (pH 7.4) including EDTA (0.5 mM), dithiothreitol (DTT) (0.5 mM) and pepstatin A (1.0 μ M), and the solution was subjected to molecular sieve chromatography.

Step: 2 Gel Filtration by Sephadex G-75 The sample was applied to a Sephadex G-75 column (5 $\times$ 67 cm) and eluted with buffer A. The enzyme fraction was pooled and concentrated by an ultrafiltration apparatus with a PM 10 Diaflo membrane.

Step 3: Chromatofocusing To exchange the buffer, the Sephadex G-75 fraction was applied to a Sephadex G-25 column (2.5 $\times$ 52 cm), which had been equilibrated with buffer B: 25 mM imidazole-HCl (pH 7.4) including DTT (1 mM). The eluate was then applied to a chromatofocusing column (1.5 $\times$ 54 cm) packed with Polybuffer exchanger PBE 94 and equilibrated with buffer B. A linear pH gradient from 7.4 to 5.0 was generated through the column by application of 15 bed volumes of Polybuffer 74/acetic acid (pH 5.0) containing DTT (0.5 mM). Under these conditions, enzyme activity was clearly separated into two fractions. The fractions eluted at pH 6.6 and pH 6.2 were designated as fraction-I and -II, respectively.

Further Purification of Fraction-I Fraction-I obtained from chromatofocusing was applied to an affinity column using 2',5'-ADP-Sepharose 4B (1.5 $\times$ 3.5 cm), which had been equilibrated with buffer C: 10 mM potassium phosphate buffer (KPB), pH 6.5, including DTT (0.5 mM). Then the column was washed with the same buffer and elution was carried out with a linear concentration gradient from 0 to 0.3 mM 2'-AMP in buffer C. Enzyme activity was eluted at around 0.15 mM 2'-AMP concentration. Enzyme fractions were pooled and dialyzed with buffer D: 3 mM KPB (pH 7.4) including DTT (0.1 mM) overnight. After dialysis, the enzyme fraction was applied to a DE-52 column (1.5 $\times$ 14 cm) which was equilibrated with buffer D. Elution was carried out with a linear gradient of increasing KPB concentration from 3 to 100 mM KPB (pH 7.4) including DTT (0.1 mM). Enzyme activity was eluted as a sharp peak at 10 mM KPB.

Further Purification of Fraction-II Fraction-II obtained from chromatofocusing was applied to a DE-52 column (1.5 $\times$ 14 cm), which was equilibrated with buffer D, and washed with the same buffer. Elution was carried out as described above. The enzyme activity was eluted at around 20 mM KPB. The enzyme fraction was further purified by rechromatography using a DE-52 column.

These purified enzymes were concentrated by ultrafiltration and then stored at -30 °C prior to use.

3 α / β ,20 β -HSD 3 α / β ,20 β -HSD/carbonyl reductase was purified from the testes obtained from neonatal pigs (strain, LWD; 2 weeks of age) after castration as reported previously.³⁾

Polyacrylamide Gel Electrophoresis Polyacrylamide gel electrophoresis with sodium dodecyl sulfate (SDS-PAGE) was carried out according to the method of Laemmli.²⁰⁾ The molecular weight markers (molecular weight in parentheses) used for SDS-PAGE were phosphorylase b (94 kDa), bovine

Fig. 1. Elution Profile of NADPH-Dependent Reductases of Neonatal Pig Testicular Cytosol by Chromatofocusing with PBE 94

The enzyme fraction after Sephadex G-75 gel filtration was applied to a PBE 94 column for chromatofocusing. Chromatofocusing was carried out as described in the Materials and Methods. For each fraction the pH and absorbance at 280 nm (---) were determined and the enzyme activity (—○—) was assayed using 4-nitrobenzaldehyde (1 mM) and NADPH (80 μ M). The enzyme activity was expressed as μ mol NADPH consumption per min per ml of eluate.

serum albumin (67 kDa), ovalbumin (43 kDa), carbonic anhydrase (30 kDa), soybean trypsin inhibitor (20.1 kDa) and α -lactalbumin (14.4 kDa).

Western Blot Analysis Western blot analysis of cytosolic proteins was carried out as previously described.^{5,6)}

Protein Determination Protein concentrations were estimated by the method of Lowry *et al.*²¹⁾

Enzyme Assay NADPH-dependent reductase activity was determined spectrophotometrically by measuring the oxidation rate of NADPH at 37 °C. The reaction mixture consisted of 0.1 M KPB (pH 6.5), 80 μ M NADPH, 1 mM 4-nitrobenzaldehyde and 20–200 μ l enzyme solution at the desired dilution in a total volume of 1 ml. Substrates that were poorly soluble in water were dissolved in ethanol (100 $\times$ stock solutions). Reactions were initiated by the addition of enzyme, and controls without substrate or enzyme were routinely included. The enzyme activity was expressed as the mean of three determinations.

RESULTS

Purification of NADPH-Dependent Reductase from Neonatal Pig Testes An enzyme fraction, which was purified from cytosol by ammonium sulfate precipitation (30–75% saturation) followed by gel filtration (Sephadex G-75), was applied to a PBE 94 column for chromatofocusing as shown in Fig. 1. The enzyme activity was separated into two peaks which were focused and eluted at pH 6.6 and 6.2 and were designated as fraction-I and II, respectively. The two active fractions, I and II, were further purified to homogeneity as judged by SDS-PAGE (Fig. 2). The yield was 2.8 mg for fraction-I and 19 mg for II from 1.18 g total cytosolic protein, when 44 testes were used as starting material. The molecular weights of each enzyme estimated by SDS-PAGE were 35 kDa for fraction-I and 31 kDa for II. The pI values of fraction-I and II were respectively estimated to be 6.6 and 6.2 by chromatofocusing.

Fraction-II is 3 α / β ,20 β -HSD We used antibody against 3 α / β ,20 β -HSD/carbonyl reductase from neonatal pig testes to examine the identity of the two enzyme fractions. As

Fig. 2. SDS-PAGE of Purified Fraction-I and -II from Neonatal Pig Testes

Electrophoresis was carried out using 10% acrylamide gel, pH 8.8, as the separation gel. Gel was stained with Coomassie brilliant blue R-250. Lane A, purified fraction-I (0.22 µg); Lane B, purified fraction-II (0.54 µg); Lane C, molecular weight marker.

Fig. 3. Blotting Analysis of Purified Fraction-I and -II with Anti- $3\alpha/\beta,20\beta$ -HSD Antibody

Purified samples incubated with 0.5% SDS in 0.25 M Tris-HCl (pH 6.8) were blotted onto a nitrocellulose membrane. Immunostaining was carried out as described in the Materials and Methods. Lane A, purified fraction-I; Lane B, purified fraction-II; Lane C, pig testicular $3\alpha/\beta,20\beta$ -HSD.

shown in Fig. 3, the antibody reacts strongly with purified fraction-II, while there is no reaction with fraction-I. Furthermore, fraction-II shows the same wide substrate specificity to $3\alpha/\beta,20\beta$ -HSD for various carbonyl-containing compounds (Table 1). These suggest that fraction-II is $3\alpha/\beta,20\beta$ -HSD.

Western Blot Analysis of the Protein Recognized by Anti- $3\alpha/\beta,20\beta$ -HSD Antibody We also used Western blot analysis to characterize purified fraction-II. As a positive control, we used rat testicular cytosol, which is known to have multiple forms of carbonyl reductase.^{15,16} As shown in Fig. 4, antibody to $3\alpha/\beta,20\beta$ -HSD/carbonyl reductase could recognize only one band in the purified fraction-II lane. The molecular weight of 31 kDa corresponds to that of pig $3\alpha/\beta,20\beta$ -HSD. At least three bands corresponding to a molecular weight of 31–33 kDa were recognized in the rat testicular cytosol lane. This indicates that purified fraction-II is $3\alpha/\beta,20\beta$ -HSD and fraction-I is a second reductase in pig testicular cytosol. Moreover, in contrast to rat testes, only one

Fig. 4. Western Blot Analysis of the Proteins Recognized by Anti- $3\alpha/\beta,20\beta$ -HSD Antibody in the Testicular Cytosol from Rat and Neonatal Pig

The cytosol was subjected to SDS-PAGE, transferred to nitrocellulose, and immunostained with anti- $3\alpha/\beta,20\beta$ -HSD antiserum. Lane A and B, rat (18 and 9 µg cytosolic protein); Lane C and D, neonatal pig (1.8 and 0.9 µg cytosolic protein); Lane E, pig testicular $3\alpha/\beta,20\beta$ -HSD (25 ng).

$3\alpha/\beta,20\beta$ -HSD/carbonyl reductase is present in neonatal pig testes.

Substrate Specificity of Purified Reductase-I The results of testing various substrates for metabolism by purified fraction-I are summarized in Table 1. We find that fraction-I reduces testosterone and androstane-3,17-dione (5α and 5β). Fraction-II, that is, $3\alpha/\beta,20\beta$ -HSD, has a broader substrate specificity, reducing not only dihydrotestosterone (5α and 5β) but also androstane-3,17-dione (5α and 5β), pregnane-3,20-dione (5α and 5β), and dihydrocortisol (5α and 5β). Interestingly, although fraction-I did not reduce prostaglandins, such as prostaglandin D_2 , prostaglandin E_2 and 13,14-dihydro-15-ketoprostaglandin $F_{2\alpha}$, $3\alpha/\beta,20\beta$ -HSD could. We also examined various ketones and aldehydes for metabolism by reductase-I. In contrast to $3\alpha/\beta,20\beta$ -HSD, which is capable of reducing a wide variety of ketones and aldehydes, fraction-I only reduced pyridine-3-aldehyde and pyridine-4-aldehyde among the pyridine derivatives, 4-nitrobenzaldehyde among the aromatic aldehydes, and methylglyoxal among the aliphatic ketones. 9,10-Phenanthrenequinone which a good substrate for carbonyl reductase was reduced by fraction-II, but only weakly by reductase-I. Glyceraldehyde and glucuronide were reduced by fraction-I better than by fraction-II.

Inhibition of Enzyme Activity by Various Agents The effects of various agents on the enzyme activity of purified fraction-I are shown in Table 2. For comparison, we show the effects of these compounds on fraction-II, $3\alpha/\beta,20\beta$ -HSD. Barbitol, valproate and diphenic acid, which are known to be inhibitors of aldehyde reductase, inhibited fraction-I, but not $3\alpha/\beta,20\beta$ -HSD. Fraction-I was inhibited by *p*-chloromercuribenzoic acid indicating that one or more sulfhydryl groups are important for activity. Epalrestat, which is a strong inhibitor of aldose reductase, failed to inhibit either fraction-I or -II.

DISCUSSION

We have isolated an NADPH-dependent reductase, fraction-I, from neonatal pig cytosol. Fraction-I reduces testosterone and androstane-3,17-dione (5α and 5β) as well as some general substrates with either aldehyde or ketone substituents (Table 1). However, reductase-I does not reduce prostaglandins, in contrast to $3\alpha/\beta,20\beta$ -HSD. The failure of

Table 1. Substrate Specificity of Purified Fraction-I and -II from Neonatal Pig Testes

Substrate	Final conc. (mM)	Specific activity	
		Purified fraction-I ($\mu\text{mol/min/ mg}$)	Purified fraction-II
Steroids			
5 α -Dihydrotestosterone	0.08	nd ^{a)}	0.130
5 β -Dihydrotestosterone	0.08	nd	0.077
Testosterone	0.08	0.044	nd
5 α -Androstane-3,17-dione	0.08	0.025	0.113
5 β -Androstane-3,17-dione	0.08	0.025	0.095
Androstenedione	0.08	nd	0.009
5 α -Pregnane-3,20-dione	0.08	nd	0.034
5 β -Pregnane-3,20-dione	0.08	nd	0.027
Progesterone	0.08	nd	0.006
17 α -Hydroxyprogesterone	0.08	nd	0.004
5 α -Dihydrocortisol	0.08	nd	0.250
5 β -Dihydrocortisol	0.08	nd	0.130
Prostaglandins			
Prostaglandin D ₂	0.08	nd	0.006
Prostaglandin E ₂	0.08	nd	0.014
13,14-dihydro-15-ketoprostaglandin F _{2α}	0.08	nd	0.047
Quinones			
Menadione	0.08	nd	1.20
9,10-Phenanthrenequinone	0.08	0.20	38.8
Aliphatic ketones			
Methylglyoxal	0.8	1.17	0.11
Diacetyl	0.8	0.37	0.57
Pyridine derivatives			
Pyridine-3-aldehyde	0.8	21.1	0.22
Pyridine-4-aldehyde	0.8	2.0	1.27
2-Benzoyl pyridine	0.8	nd	2.20
3-Benzoyl pyridine	0.8	nd	0.30
4-Benzoyl pyridine	0.8	nd	2.13
Aromatic ketone and aldehydes			
4-Nitroacetophenone	0.8	nd	0.40
2-Nitrobenzaldehyde	0.8	nd	1.50
3-Nitrobenzaldehyde	0.8	0.44	0.67
4-Nitrobenzaldehyde	0.8	2.12	1.12
Sugars			
D-Glyceraldehyde	0.8	0.38	0.014
D-Glucuronate	0.8	0.58	0.008
D-Glucose	0.8	nd	nd
D-Xylose	0.8	nd	nd
D-Galactose	0.8	nd	0.007

a) nd: not detected.

Table 2. Effect of Various Agents on Enzyme Activity of Purified Fraction-I and -II

Addition	Final conc. (mM)	Enzyme activity	
		Purified fraction-I (% of control)	Purified fraction-II (% of control)
None		100	100
Barbital	1.0	0	97.4
Valproate	0.1	20.0	97.8
Diphenic acid	0.05	28.0	97.1
Epalrestat	0.001	91.5	93.1
p-Chloromercuri -benzoic acid	0.001	11.6	0.7

fraction-I to react with antibody to 3 α / β ,20 β -HSD indicates that fraction-I is not closely related to pig 3 α / β ,20 β -HSD. This is supported by their different molecular weights: fraction-I is 35 kDa, while 3 α / β ,20 β -HSD is 31 kDa. Moreover,

fraction-I and 3 α / β ,20 β -HSD have different substrate specificities (Table 1).

Although the exact biological function of fraction-I is not known, its narrow steroid specificity is noteworthy. This, combined with its expression in pig testes, suggests a role for fraction-I in regulating androgen levels during development of the neonatal pig.

To our surprise, we find only one molecular form of 3 α / β ,20 β -HSD/carbonyl reductase in neonatal pig testicular cytosol, as judged by Western blot analysis. This result was obtained with an antibody that could identify the three isozymes of 3 α / β ,20 β -HSD/carbonyl reductase in rat testes cytosol, in agreement with reports from two other laboratories.¹⁵⁻¹⁷⁾ The biological function of the three isozymes in human and rat testes is not yet understood. Comparative studies with carbonyl reductases from pig and rat testes may resolve this question.

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