Immunohistochemical Study of $3\beta$-Hydroxysteroid Dehydrogenase /$\Delta^5-\Delta^4$ Isomerase in the Rat Cardiovascular System*

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Summary. The enzyme complex $3\beta$-hydroxysteroid dehydrogenase (3$\beta$-HSD) is involved in the biosynthesis of all classes of active steroids. It is known that the enzymatic activity of 3$\beta$-HSD is present not only in classical steroidogenic tissues, but also in many peripheral tissues including cardiac tissue. To determine whether 3$\beta$-HSD is present in rat non-steroidogenic tissues, we examined cardiovascular tissues including the ventricle, atrium, aortic arch, abdominal aorta, and inferior vena cava by immunohistochemistry and Western blotting using polyclonal antibody raised against a synthetic peptide of human placental 3$\beta$-HSD.

By Western blotting, protein bands immunoreactive for anti-3$\beta$-HSD were detected at molecular weights of 42 and 37 kDa in both the ventricle and atrium, whereas only a 37 kDa band was recognized in both the aortic arch and abdominal aorta. By immunohistochemistry, immunoreactivity for 3$\beta$-HSD was detected in both the ventricular and atrial cardiocytes, while immunostaining was also found, though faintly, in the smooth muscles of the aortic arch, abdominal aorta, and inferior vena cava.

These results suggest that cardiocytes may synthesize the steroidogenic 3$\beta$-HSD enzyme.

The enzymatic complex $3\beta$-hydroxysteroid dehydrogenase /$\Delta^5-\Delta^4$ isomerase (3$\beta$-HSD) catalyzes the obligatory oxidation and isomerization of $\Delta^5$-3$\beta$-hydroxyprogren and $\Delta^5$-3$\beta$-hydroxyandrostene steroid precursors into $\Delta^4$-3-ketosteroids necessary for the formation of all classes of steroid hormones such as progesterone, mineralocorticoids, glucocorticoids, androgens, and estrogens (DUPONT et al., 1990, 1992; JUMEAU et al., 1993). The 3$\beta$-HSD enzymatic activity is present not only in classical steroidogenic tissues, but also in many peripheral tissues, including the mammary gland, kidney, liver, brain, sciatic nerve, skin, thymus, pancreas, lung, spleen, prostate and uterus (ZHAO et al., 1991; DUPONT et al., 1992; GUENNOUN et al., 1995a, b; KOENIG et al., 1995; MARTINI et al., 1996).

The widespread tissue distribution of 3$\beta$-HSD activity implies the physiological importance of steroids in peripheral tissues. Pregnenolone converts into progesterone in the brain and sciatic nerve (JUNG-TESTAS et al., 1989; MORFIN et al., 1992; AKWA et al., 1993; KOENIG et al., 1995). The progesterone synthesized within both central and peripheral nervous systems has been suggested to regulate neurotransmission and to be related to myelination (KOENIG et al., 1995; BAULIEU et al., 1996). In the skin, 3$\beta$-HSD localized in the sebaceous glands converts the adrenal precursor dehydroepiandrosterone (DHEA) into sex steroids (DUMONT et al., 1992; POCHI and STRAUSS, 1969). These demonstrate the importance of a further investigation of the role of 3$\beta$-HSD in steroid biosynthesis in many other peripheral tissues.

ZHAO et al. (1991) and MARTEL et al. (1994) have shown the presence of appreciable levels of 3$\beta$-HSD activity in the heart of the rat and rhesus monkey using [14C] pregnenolone and [14C] dehydroepiandrosterone. However, little is known about the expression of 3$\beta$-HSD, particularly its localization in the heart.

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In the present study, we therefore examined the presence of 3β-HSD in non-steroidogenic cardiovascular tissues of the rat by immunoblot and immunohistochemical studies.

**MATERIALS AND METHODS**

**Animals**

Sprague-Dawley male rats weighing 150–200 g were used for the present study.

**Antibody**

The affinity purified polyclonal anti-3β-HSD antibody was obtained after immunization of New Zealand white rabbits with human placental 3β-HSD oligopeptide (S256-D-D-T-P-H-Q-S-Y-D-D-L-N268).

**Immunoblot**

To examine the presence of 3β-HSD in cardiac (ventricular and atrial), aortic arch, and abdominal aortic tissues, extracts of these tissues were analyzed by Western blotting. For evaluation of the antibody, we also used tissue extracts from human placental tissue, and rat adrenal, skin, testicular and ovarian tissues in which 3β-HSD has been shown to be present (Leadhead et al., 1983; Dupont et al., 1990, 1992; Simard et al., 1993). From the placental extract, human type I 3β-HSD was isolated according to the method by Thomas et al. (1988). The tissues were homogenized in 0.1 M phosphate buffer, pH 7.2, and centrifuged for 15 min at 1000 g. The supernatants were subjected to 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and subsequently transferred to a nitrocellulose membrane. After treatment of the membrane with Tris-HCl buffer (10 mM Tris-HCl, 150 mM NaCl pH 7.4) containing 5% skim milk (DIFCO Lab, Detroit, USA), the blot was incubated for 1 h at room temperature with anti-3β-HSD antibody diluted to 1:1000 (20 μg/ml) in Tris-HCl buffer. After treatment with the secondary antibody, the blot was developed with 5-bromo-4-chloro-3-indolyl-phosphate (BCIP) and 4-nitro blue tetrazolium chloride (NBT).

**Immunohistochemistry**

After being anesthetized with pentobarbital (40 mg/kg, intraperitoneal), the rats were fixed by cardiac perfusion with 4% paraformaldehyde buffered with 0.1 M phosphate buffer, pH 7.2 for light microscopic immunohistochemistry. Various tissues including the...
Fig. 3. Immunohistochemical localization of 3β-HSD in rat steroidogenic tissues. Immunoreactivity is observed in ovarian theca cells (a), ovarian luteinizing granulosa cells (b), Leydig cells of testis (c), and adrenal cortex (d). Arrowheads indicate reactive sites of 3β-HSD. ×400
cardiac ventricle and atrium, aortic arch, abdominal aorta, inferior vena cava, ovary, testis, and adrenal gland were excised from the rats and further fixed with the same fixative for 24 h. After dehydration, they were embedded in paraffin and sections were cut at 4 μm with a microtome. The sections were deparaffinized, and treated with 3% H₂O₂ for 40 min to block endogenous peroxidase, and then pepsin solution (Research Genetics) for 5 min to expose the antigen. Tissue sections were incubated overnight at 4°C with anti-3β-HSD antibody diluted to 1:1000 (20 μg/ml) in Tris-HCl buffer and further incubated for 20 min at room temperature in biotinylated goat anti-rabbit IgG (DAKO Corporation Carpinteria, CA93013, USA). Following treatment with horse-radish peroxidase conjugated streptavidin for 10 min at room temperature, the sections were visualized with three-amino-a-ethylcarbazole (AEC).

RESULTS

Characterization of anti-3β-HSD antibody

The protein sequences of human placental 3β-HSD (S²⁵⁷-D-D-T-P-H-Q-S-Y-D-L-N²⁶⁹) used as antigen sources showed a high degree of identity (93%) with rat ovary 3β-HSD (S²⁵⁷-D-D-T-P-H-Q-S-Y-D-N-L-N²⁶⁹) as aspartic acid replaced asparagine in 267 of amino acid sequences.

The ability of the antiserum to recognize 3β-HSD was evaluated in human placental extracts and in various tissues of the rat by immunoblot and immunohistochemistry. Using affinity purified polyclonal anti-3β-HSD antibody for SDS-polyacrylamide gel electrophoresis (PAGE) and Western blot analysis, we detected a protein band at the molecular weight of 43 kDa in the human placental extract (Fig. 1). Moreover, the antibody also recognized a 42 kDa protein in rat tissue extracts from the adrenal gland, skin, testis, and ovary (Fig. 2). We further evaluated the anti-3β-HSD antibody by immunohistochemistry, noting intense staining of the interstitial and thecal cells of the ovary, the luteal cells of the corpus luteum, and the Leydig cells of the testis and adrenal cortex (Fig. 3 a–d) as previously reported for types I and II 3β-HSD (LEADHEAD et al., 1983; DUPONT et al., 1990, 1992; SIMARD et al., 1993). The results indicated that our present antibody raised against a synthetic peptide of human placental 3β-HSD can be applicable to both immunochemical and immunohistochemical approaches.

Immunoblot analysis of cardiovascular tissues

To examine the presence of the 3β-HSD proteins in cardiovascular tissues, we analyzed extracts from these tissues by Western blotting. The protein bands immunoreactive for anti-3β-HSD were identified at the molecular weights of 42 kDa and 37 kDa in both ventricular and atrial tissues, while only a 37 kDa protein band was detected in the tissues of the aortic arch and abdominal aorta (Fig. 4). Since there are four types of 3β-HSD whose molecular weights are commonly 42 kDa, the 42 kDa protein present in cardiac tissues is considered to be one of 3β-HSD isoforms, but the 37 kDa protein is non-specific.

Immunohistochemical localization of 3β-HSD

Immunostaining was positively detected in cardiocytes of both the ventricle and atrium. The immunoreactivity of the cells was relatively intense in the atrium, compared to that in the ventricle (Fig. 5 a, b). When sections of the aortic arch, abdominal aorta, and inferior vena cava were stained with anti-3β-HSD, weak or faint immunoreactivity was found in muscular cells in the tunica media (Fig. 5 c–e).

DISCUSSION

In the present study, we prepared a sequence specific antibody to human type 1 3β-HSD, using a synthetic peptide corresponding to the amino acid sequences of
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Fig. 5. Immunohistochemical localization of 3β-HSD in rat cardiovascular tissues. Immunoreactivity is observed in cardiocytes of the ventricle (a) and atrium (b). Weak or faint immunodeposits are also seen in smooth muscles of the aortic arch (c), abdominal aorta (d) and inferior vena cava (e). a: ×400, b-e: ×200

The antibody was confirmed to recognize a 42 kDa protein, 3β-HSD in the steroidogenic tissues, by both immunochemical and immunohistochemical methods. Using this antibody, we demonstrated the presence of 3β-HSD in cardiac tissues.

Threeβ-HSD activity has been found not only in steroidogenic but also in various other tissues including nervous and cardiovascular organs (LE GOASCOGNE et al., 1987; JUNG-TESTAS et al., 1989; ZHAO et al., 1991; MORFÉN et al., 1992; AKWA et al., 1993; MARTEL et al., 1994; KÖNIG et al., 1995; SANNE and KRUEGER, 1995). There have been many papers reporting that a major adrenal steroid hormone DHEA and sex steroid levels are strongly associated with cardiovascular diseases (ELDRUP et al., 1987; LACROIX et al., 1992; EICH et al., 1993; SLOWINSKA-SKZEDNICKA et al., 1995). Indeed, receptors of estrogen and progesterone have also been identified in respective rat cardiac myocytes and human left atrial appendages (INGEGNO et al., 1988; GROHE et al., 1997), indicating that the heart is one of the target organs for steroid hormones. The sources of the steroids, however, have not been elucidated. The present data showing the presence of 3β-HSD in cardiac tissues by Western blotting and immunohistochemistry suggest the possibility that cardiocytes themselves produce steroid hormones as ligands for the receptors of estrogen and
progesterone.

Besides cardiocytes, the immunoreactivity for 3β-HSD was also found in tissues such as the aortic arch, abdominal aorta, and inferior vena cava, although the reaction was weak or faint. By the present immunoblotting, we could detect only a 37 kDa protein band in these tissues. Because four types of rat 3β-HSD cDNAs all encode a 372-amino acid, they have a similar molecular weight of 42 kDa (Zhao et al., 1990, 1991; Simard et al., 1993). Therefore it seems reasonable to presume that the 37 kDa protein band found in two cardiac vascular tissues might be non-specific. At present, it remains unknown whether the faint or weak immunoreactivity detected in the vascular tissues by immunohistochemistry indicates the real presence of 3β-HSD. Further studies using, for example, in situ hybridization will be required to settle the question.

In conclusion, our present results demonstrated the presence of 3β-HSD in rat cardiac tissues, which offers a new possibility that certain steroids affecting physiological functions of the heart may be synthesized in situ.

REFERENCES


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