Demonstration of an Androgen Receptor in Rat Pancreas

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[1,2,6,7-³H]Testosterone (250 μ Ci) was administered to castrated male rats; after 30 min a labelled testosterone–receptor protein complex with a pI of 5.1 was recovered from the pancreatic cytosol. A labelled testosterone–receptor complex with an identical pI was also extracted from the nuclear fraction of rat pancreas after incubation of minced pancreatic tissue with 0.1 μ M-[1,2,6,7-³H]testosterone for 30 min at 37°C. Studies *in vitro* showed that [1,2,6,7-³H]testosterone was bound to a receptor protein focusing at a pI of 5.1 and with a K_d of 2 nM and a number of binding sites of 4.7 fmol/mg of protein in castrated male rats. The testosterone–receptor complex sedimented at 3.5S in high-salt sucrose-density gradients, was excluded from Sephadex G-200 and Ultragel ACA-34, was stable towards treatment with dextran-coated charcoal, was relatively sensitive to heat, and was stable to treatment with deoxyribonuclease and ribonuclease, but was sensitive to treatment with proteinase. It is suggested that the pancreatic androgen receptor, which was also present in castrated female rats, may play a role in sex-steroid regulation of pancreatic function.

In a previous study (Gustafsson & Pousette, 1975) we investigated androgen uptake in several peripheral organs after intraperitoneal administration of [1,2,6,7-³H]testosterone to castrated male rats. The pancreas was found to have the highest amount of radioactivity per g of tissue of all the organs investigated 30 min after injection of the labelled steroid. The surprisingly high retention of androgen in the pancreas (more than three times that in the classical androgen-target organs: prostate, coagulation glands and seminal vesicles) prompted us to further investigate the mechanism by which androgen is accumulated in pancreatic tissue. The present study describes the binding of testosterone to a receptor protein in cytosol and nuclei from rat pancreas.

Materials and Methods

Steroids

[1,2,6,7-³H]Testosterone (specific radioactivity 84 Ci/mmol) and 5 α -[1,2,4,5,6,7-³H]dihydrotestosterone (specific radioactivity 100 Ci/mmol) were from The Radiochemical Centre, Amersham, Bucks., U.K. Unlabelled testosterone, 5 α -dihydrotestosterone, androstenedione, oestradiol and corticosterone were generously supplied by Dr. J. Babcock (Upjohn Co., Kalamazoo, MI, U.S.A.).

Animal experiments

Sprague–Dawley rats (8 weeks old) were used in all experiments. In cases where castrated rats were

used, the operations were performed under ether anaesthesia 14–16 h before the animals were killed.

In one series of experiments, castrated male rats were given an intraperitoneal injection of 250 μ Ci of [1,2,6,7-³H]testosterone in 120 μ l of acetone; 30 min later the animals were killed by a blow on the head. The pancreas was removed and the cytosol was prepared in Eagle's (1959) medium with a final protein concentration of about 13 mg/ml. The cytosol preparations were then chromatographed on Sephadex G-25 columns (10 cm $\times$ 10 cm) equilibrated in Eagle's medium. The void volumes were taken for isoelectric focusing or for identification of radioactive metabolites by t.l.c. and radio-g.l.c. (Gustafsson & Pousette, 1975). A steroid was considered identified if it had the same t.l.c. mobility and the same retention time on 1% SE-30 and on 1% OV-17 as the reference steroid.

In another series of experiments, pancreatic tissue from two castrated male rats was cut into pieces and incubated for 30 min at 37°C in 10 ml of Eagle's medium containing 80 μ Ci of [1,2,6,7-³H]testosterone. The tissue was washed twice, and then cytosol and nuclei were prepared according to previously described methods (Gustafsson & Pousette, 1974). In order to extract protein from the nuclei, the nuclear pellet was suspended in 2 ml of 0.4 M-KCl/1 mM-EDTA/0.01 M-Tris/HCl, pH 7.4, and kept at 0–2°C for 4 h. The nuclear extract obtained after centrifugation at 30000g for 30 min was chromatographed on Sephadex G-25 columns equilibrated in TKE buffer (0.01 M-KCl/1 mM-

EDTA/0.01 M-Tris/HCl, pH 7.4 (Puca & Brescianai, 1968), and the void volume was taken for analysis by isoelectric focusing. In another similar experiment 10 vol. of acetone/ethanol (1:1, v/v), was added to the nuclear pellet for extraction of radio-active metabolites retained in the nuclei. After filtration, the extract was evaporated to dryness and the residue was dissolved in double-distilled water and passed through an Amberlite XAD-2 column. The steroids were eluted with methanol and analysed by t.l.c. and radio-g.l.c.

Protein binding of androgen in vitro

Cytosol from pancreatic tissue of two male rats was prepared in Eagle's medium. Portions (0.5 ml) of the cytosol preparation were added to test tubes containing ^3H -labelled steroids in varying amounts. The steroids were added to the test tubes in organic solvents, and the solutions were taken to dryness under N_2 . The cytosol was incubated in the test tubes at 37°C for 30 min; it was ascertained that the steroid was rapidly dissolved in the cytosol. Time-studies showed that no further specific binding occurred after 30 min. When incubations were performed at $0-2^\circ\text{C}$ instead of 37°C , no high-affinity binding could be detected. The incubation mixture was then cooled and passed through a Sephadex G-25 column (1 cm $\times$ 10 cm) equilibrated in Eagle's medium. A sample of the void volume was taken for measurement of radioactivity with a Packard liquid-scintillation spectrometer model 2425. Calculation of radioactivity in d.p.m. was performed with a correction factor automatically determined by using an external standard. Protein determinations were performed by the method of Lowry *et al.* (1951) with bovine serum albumin as standard. Dissociation constants and number of binding sites for androgen-receptor protein were determined as described by Scatchard (1949). Other samples of the void volume from the Sephadex G-25 chromatography of radioactively labelled cytosol were taken for analysis by isoelectric focusing and sucrose-density-gradient centrifugation, and for treatment with RNAase,* DNAase and proteinase.

Isoelectric focusing

This was carried out essentially as described by Katsumata & Goldman (1974). Samples (3 ml) were distributed in the gradients, 10 ml columns were used and focusing was performed at 800 V for 15–20 h. The columns were fractionated into counting vials and 0.5 ml of double-distilled water was added before measurement of pH. Ferritin ($\text{pI} = 5.0$)

* Abbreviations: RNAase, ribonuclease; DNAase, deoxyribonuclease.

and haemoglobin ($\text{pI} = 7.2$ and 7.6) (Sigma Chemical Co., St. Louis, MO, U.S.A.) were used as standards.

Density-gradient centrifugation

The steroid-protein mixture was layered on top of a 5 ml linear 10–30% (w/v) sucrose density gradient in Eagle's medium. The tubes were centrifuged at 50000 rev./min for 20 h at 2°C in an SW 50.1 Beckman Spinco rotor. At the end of the centrifugation the bottom of the tube was punctured and 0.2 ml fractions were collected into counting vials and measured for radioactivity in a liquid-scintillation counter. Bovine serum albumin (4.6S) and rabbit anti-androstenedione (7S) were used as markers. Treatment with dextrane-coated charcoal was carried out as described by Beato & Feigelson (1972). Chromatography on Sephadex G-100 and Ultrogel ACA-34 (LKB Produkter AB, Stockholm, Sweden) was performed in 300 ml columns equilibrated in TKE buffer (0.01 or 0.4 M with respect to KCl).

Enzyme digestions

Treatment with proteinase, RNAase and DNAase (Sigma) was performed in Eagle's medium at 37°C . The residual amount of the steroid macromolecular complex was determined by chromatography on Sephadex G-25.

In separate experiments, the ligand specificity of the androgen receptor was investigated *in vitro* by incubating cytosol with 2.0 nM -[1,2,6,7- ^3H]testosterone plus $0.2\text{ }\mu\text{M}$ competing unlabelled steroid. Protein binding was assayed on Sephadex G-25 as described above.

Results

[1,2,6,7- ^3H]Testosterone was injected into castrated male rats; 30 min later more than 90% of the radioactivity recovered in the macromolecular fraction of the pancreatic cytosol was identified as testosterone. Isoelectric focusing of this fraction showed a single peak of radioactivity focusing at pH 5.1 (Fig. 1). This testosterone-protein complex was stable towards treatment with dextran-coated charcoal. A testosterone-protein complex with a pI of 5.1 was also recovered in the nuclear protein extract after incubation of minced pancreatic tissue with $0.1\text{ }\mu\text{M}$ -[1,2,6,7- ^3H]testosterone.

After incubation of cytosol from pancreatic tissue with 1 nM -[1,2,6,7- ^3H]testosterone for 30 min at 37°C , more than 90% of the incubated steroid was unmetabolized. Fig. 2 shows a typical Scatchard (1949) plot of the results obtained from incubations *in vitro*. The K_d for testosterone was calculated as 2.0 nM and for 5α -dihydrotestosterone as 1.5 nM . The number of binding sites were in both cases 4.7

Fig. 1. Isoelectric focusing of cytosol from pancreas of a castrated male rat given 250 μ Ci of [1,2,6,7- 3 H]testosterone 30 min before death

The cytosol was filtered on a Sephadex G-25 column and the void volume was taken for electrofocusing. Each fraction contained 0.3 ml. ●, Radioactivity; ○, pH.

Fig. 2. Scatchard plot of [1,2,6,7- 3 H]testosterone binding in cytosol from pancreas

Cytosol from pancreas of castrated male rats was incubated with [1,2,6,7- 3 H]testosterone at 37°C for 30 min. Bound radioactivity was determined by using gel filtration on Sephadex G-25. The dissociation constant was 2 nM and the number of binding sites 4.7 fmol/mg of protein.

fmol/mg of protein. Identical results were obtained when binding was analysed by chromatography on Sephadex G-25 and with dextran-coated-charcoal treatment. The [3 H]testosterone-protein complexes formed *in vitro* were also analysed by isoelectric focusing. When samples from the 'steep' part of the Scatchard plot (i.e. samples containing high-affinity binding protein) were electrofocused, the complex with pI = 5.1 appeared. Analysis of samples from the 'shallow' part of the plot (i.e. samples containing low-affinity binding proteins) showed the presence of several peaks, none of which focused at pH 5.1.

Table 1. Effects of some unlabelled steroids on high-affinity binding of [1,2,6,7- 3 H]testosterone to cytosol protein of pancreas from castrated male rats

Samples of cytosol preparations were incubated with 1 nM-[1,2,6,7- 3 H]testosterone alone or with 0.1 μ M of non-radioactive steroids.

Non-radioactive steroid	Bound radioactivity (% of control)
None	100
Testosterone	0
5 α -Dihydrotestosterone	0
Androstenedione	40
Oestradiol	60
Corticosterone	100

Table 1 summarizes the effects of various unlabelled steroids on high-affinity binding of [1,2,6,7- 3 H]testosterone to pancreatic cytosol. Corticosterone did not compete for the binding sites, whereas androstenedione and oestradiol competed more efficiently. The experiment with 5 α -dihydrotestosterone as competing ligand is a further indication that the androgen receptor in rat pancreas binds 5 α -dihydrotestosterone with about the same affinity as it binds testosterone.

When samples containing high-affinity binding protein labelled *in vitro* with [1,2,6,7- 3 H]testosterone were analysed by sucrose-gradient centrifugation, a radioactive fraction sedimented at 3.5S. This peak was displaced when 0.1 μ M of unlabelled testosterone was added to the incubation mixture. Further studies showed that the testosterone-receptor complex was excluded from Sephadex G-100 as well as from Ultragel ACA-34. The testosterone-receptor complex was stable at 50°C, but was quickly destroyed at higher temperatures. Further, the complex was stable towards treatment with RNAase and DNAase, but was destroyed when treated with proteinase.

Experiments were also performed to compare the low-capacity high-affinity binding of testosterone in pancreas from castrated male and female rats. Scatchard (1949) analysis of the results indicated that the K_d values were identical in the two sexes, but that the number of binding sites was slightly higher in male (4.7 fmol/mg of protein) than in female (3.0 fmol/mg of protein) rats.

Discussion

The present paper describes an androgen-binding protein in rat pancreas that binds testosterone and dihydrotestosterone with similar affinities *in vitro*. The partially characterized protein has many of the properties regarded as typical for androgen receptors: high-affinity low-capacity ligand binding, a sedimentation coefficient of 3.5S in high-salt sucrose-density

gradients and a pI of approx. 5 (Mainwaring & Mangan, 1973; Bullock *et al.*, 1975). Further, the testosterone-receptor complex was excluded from Sephadex G-200 and Ultrogel ACA-34 and was relatively sensitive to heat.

Experiments *in vivo* indicated that the physiological ligand for the pancreatic androgen receptor is testosterone. Even if the receptor has the capacity to bind 5 α -dihydrotestosterone at least equally well, this ligand normally appears to be absent from the pancreas. After administration of [1,2,6,7-³H]-testosterone to castrated male rats, the radioactivity recovered in the cytosol consisted of testosterone (75–90%), androstenedione (5–15%) and polar steroids (5–20%), and that in the nuclear fraction of testosterone (70–90%) and androstenedione (10–30%) (Gustafsson & Pousette, 1975).

The amount of androgen receptor in pancreas (4.7 fmol/mg of protein) is relatively high compared to other 'non-classical' androgen-target organs and tissues, e.g. kidney (1.5 fmol/mg of protein) and thigh muscle (1.0 fmol/mg of protein) (Gustafsson & Pousette, 1975). It does not seem likely, however, that the presence of an androgen receptor protein in pancreas can be the sole explanation for the high uptake of androgen in this tissue after intraperitoneal administration of [1,2,6,7-³H]testosterone. The large amounts of fat tissue closely associated with the pancreas probably lead to substantial non-specific retention of radioactivity. The presence of a receptor protein in pancreas specific for androgens is in line with our recent suggestions that a common class of testosterone receptors is present in most organs and that these receptors can be detected both *in vitro* and *in vivo* provided methods sensitive enough are used.

At the present time it is difficult to evaluate the biological significance of the described receptor protein. The consensus of current research opinion supports the view that steroid hormones are bound to high-affinity low-capacity proteins before evoking biological effects (King & Mainwaring, 1974). The presence of high-affinity binding of steroids in a tissue is not always linked to a known biological response of the tissue to the steroid in question (Wilson & Goldstein, 1972). However, androgens are known to affect most tissues (Kochakian, 1946) and after castration the weight of the pancreas is decreased (Dorfman & Shipley, 1956) indicating

an effect of androgen on pancreatic tissue. The regulation of endocrine and exocrine secretion from pancreas is complex and still not completely understood.

Sandberg & Kirdani (1973) have described the presence of an oestrogen receptor in pancreas from dog, baboon and man, and these authors speculated that oestrogens may play a role in the regulation of the endocrine and/or exocrine functions of the pancreas. In view of the results presented in this paper, the role of androgens may now be added to this discussion. It is evident that at least certain biochemical conditions precedent for a role of sex steroids in regulation of pancreatic function are fulfilled.

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