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SEXUAL DIFFERENTIATION OF HEPATIC STEROID METABOLISM IN THE RAT

JAN-ÅKE GUSTAFSSON, SVEN A. GUSTAFSSON, MAGNUS INGELMAN-SUNDBERG, ÅKE POUSETTE, ÅKE STENBERG and ÖRJAN WRANGE

Department of Chemistry and Department of Germfree Research, Karolinska Institutet, S-10401 Stockholm 60, Sweden

SUMMARY

Steroid metabolizing enzyme activities in rat liver may be classified according to their regulation. One group consists of enzymes, the activities of which are irreversibly determined at birth by androgens, a second group encompasses enzymes that are reversibly affected by androgens and a third group is made up of enzymes essentially independent of androgens. The mechanisms by which androgens influence hepatic enzyme activities are still not clear but the hypophysis seems to be essential for the sexual differentiation of liver metabolism in the rat. Possibly this control is exerted by a hypophyseal feminizing factor that feminizes a basal masculine enzyme pattern in rat liver. Some kind of direct androgen action on the liver cell may also be postulated since a radioactively labelled complex between 4-androstene-3,17-dione and a high affinity protein can be extracted from liver cytosol and nuclei after intrapertitoneal administration of [1,2,6,7-^{3}H]-testosterone to castrated male and female rats. This androgen "receptor" moves as a 10 S protein when analysed by sucrose density gradient centrifugation, has a pI of about H5 when analysed by isoelectric focusing and is selectively concentrated in unlabelled liver nuclei when these are incubated with liver cytosol containing labelled androgen-protein complex.

INTRODUCTION

Sexual differences in the hepatic metabolism of steroid hormones and drugs in the rat have been recognized for a long time[1–3]. Comparatively limited knowledge, however, is available about factors regulating hepatic steroid and drug metabolism. We felt that the rat liver should constitute a suitable model system for studies on hormone action on target cells and during recent years we have therefore undertaken a series of experiments to investigate regulation of liver steroid metabolism with special reference to the mechanisms behind the differentiating role of androgens.

EXPERIMENTAL

Experiments on gonadal and hypophyseal regulation of liver metabolism

Male and female rats of the Sprague–Dawley strain were castrated, hypophysectomized or sham-operated at various ages from birth until adulthood. Some castrated newborn male and female rats were given 500 μg of testosterone propionate subcutaneously at birth. Some castrated adult rats were treated for two weeks by intramuscular injections of 100 μg of testosterone propionate or 100 μg of estradiol benzoate in 0·5 ml of propylene glycol when adult. Control rats received vehicle only.

The rats were killed by cervical dislocation and the liver was excised quickly and cooled in a modified Bucher medium, pH 7·4[4]. Liver homogenates, 20% (w/v), were prepared with a Potter–Elvehjem homogenizer equipped with a loosely fitting teflon pestle. The microsomal and cytosol (105,000 *g* supernatant) fractions were prepared using differential centrifugation[5] and the microsomal fraction was resuspended. Microsomal suspension from 0·10 g of liver or cytosol from 0·60 g of liver was incubated in 3 ml of Bucher medium with 500 μg of [4-^{14}C]-4-androstene-3,17-dione at 37°C for 8 min in the presence of an NADPH-regenerating system. Incubations were also carried out with [4-^{14}C]-5α-androstane-3α,17β-diol and [4-^{14}C]-5α-androstane-3α,17β-diol 3,17-disulphate using 200 μg of each substrate and microsomal suspension from 0·20–0·50 g liver in 4·0 ml of Bucher medium containing the same amount of NADPH-regenerating system as in the incubations with [4-^{14}C]-4-androstene-3,17-dione.

The incubation mixtures were extracted and analysed by thin-layer chromatography–radioautography (the sulphurylated steroids were solvolysed prior to applica-

tion on thin-layer plates). The radioactive zones were localized, scraped off separately and measured for radioactivity in a Packard Liquid Scintillation Spectrometer, Model 4322. The steroid metabolites in the various zones were identified by gas chromatography–mass spectrometry (LKB 9000 instrument) and by radio–gas chromatography (Hewlett–Packard Gas Chromatograph, Model 402, equipped with a Barber–Colman Radioactivity Monitoring System Model 5190).

Experiments on intracellular binding and transport of androgens in rat liver

Male 56-day-old rats that had been castrated on the previous day were injected intraperitoneally with 250 μCi of [1,2,6,7-^{3}H]-testosterone (sp. act. 84 Ci/mmol; The Radiochemical Centre, Amersham, England) in 120 μl of acetone. The rats were killed 30 min after injection. The liver was perfused with about 50 ml of ice-cold saline via the portal vein, excised and homogenized in a small volume of 0·01 M Tris–HCl buffer pH 7·4—0·01 M KCl–0·001 M EDTA solution. The nuclear and cytosol (105,000 *g* supernatant) fractions were prepared[6]. The cytosol fraction was passed through a 10 ml Sephadex G-25 column and part of the void volume was chromatographed on a 250 ml Sephadex G-100 column. 0·2 ml of the void volume from the Sephadex G-25 column was applied on a 5·0 ml linear 5–20% (w/v) sucrose gradient, 0·40 M with respect to KCl, and run for 20 h at +2°C in an SW 50·1 rotor at 50,000 rev./min (Beckman Model L3-50 Ultracentrifuge). Ovine and bovine serum albumin having sedimentation coefficients of 3·7 and 4·6 S, respectively, were used as markers. The androgen-receptor complex eluted from the Sephadex G-100 column (see below) was also analysed by isoelectric focusing essentially as described by Osterman[7]. The lower electrode buffer consisted of 7% (w/v) NaOH in 70% (w/v) sucrose. On top of this was layered 0·5 ml of 65% (w/v) sucrose containing 1·5% (v/v) Ampholine (pH 5–8, LKB-Produkter AB, Bromma, Stockholm, Sweden). Above this was layered a linear 60–10% (w/v) sucrose gradient (10 ml) containing the sample and a linear gradient of 1·5–0·5% (v/v) Ampholine (pH 5–8). 0·5 ml of 5% (w/v) sucrose containing 0·5% (v/v) Ampholine was layered on top; the upper electrode buffer consisted of 1% (v/v) H_2SO_4. The apparatus was mounted in a cold room at +4°C and thoroughly chilled by the constant circulation of ice-cold water. An initial voltage of 100 V (2 mA) was applied and later adjusted to 800 V (0·6 mA). Focusing was conducted for 14 h. The contents of the apparatus were subdivided into 0·7 ml fractions which were assayed with respect to pH and radioactivity. Experiments with dextrane-coated charcoal were carried out as described by Beato and Feigelson[8]. The nature of the protein-bound radioactive metabolites recovered during various stages of the experiments were determined by thin-layer chromatography and radio–gas chromatography after extraction of radioactivity from the aqueous phases with 10 volumes of acetone–ethanol (1:1, v/v) at 37°C for 24 h. In some cases the thin-layer plates were scanned for radioactivity using a Berthold thin-layer scanner, model II (Berthold, Wildbad, Germany).

RESULTS AND DISCUSSION

Experiments on gonadal and hypophyseal regulation of liver metabolism

The findings on hepatic metabolism *in vitro* of 5α-androstane-3α,17β-diol, 4-androstene 3,17-dione and 5α-androstane-3α, 17β-diol 3, 17-disulphate and *in vivo* of corticosterone have shown the occurrence of three principal classes of enzyme activities with regard to the mechanisms regulating their activity: I. Enzymes irreversibly programmed or "imprinted" by androgens during the neonatal period and reversibly stimulated by androgens postpubertally; II. Enzymes reversibly inducible by androgens; and III. Enzymes primarily regulated by nongonadal factors and only slightly affected by androgens[9–13]. Some of these findings are summarized in Table 1.

In several experiments castrated female rats were shown to respond markedly less to treatment with testosterone propionate than castrated male rats with respect to liver enzyme activities[5]. It is reasonable to believe that this phenomenon is related to neonatal events since male rats castrated at 14 days of age responded about 2·5–3 times better to androgen administration than male rats castrated at birth[14].

In order to investigate further the mechanisms behind neonatal programming of liver enzyme activity levels and hepatic response towards androgenic treatment two principal approaches are presently being used. One approach involves studies on central regulation of liver enzymes whereas the other approach aims at defining intracellular handling of androgens in liver from male and female rats.

As can be seen from Table 2, hypophysectomy leads to an over-all masculinization of hepatic steroid metabolism in castrated female rats suggesting the existence of a hypophyseal feminizing factor that feminizes the basal masculine character of the hepatic sex-dependent enzyme activities. Castrated male rats are not affected by hypophysectomy with regard to hepatic enzyme levels. The masculinizing and feminizing effects on liver enzyme activities observed following treatment with testosterone propionate and estradiol

Table 1. Classification of steroid metabolizing enzymes in rat liver

	Enzyme activity	Androgenic effect (↑ = increase, ↓ = decrease)
I. Enzyme activities irreversibly programmed ("imprinted") by androgens neonatally and reversibly influenced by sex hormones postpubertally	2α-Hydroxylase (active on 5α-androstane-3α,17β-diol)	↑
	16α-Hydroxylase (active on 4-androstene-3,17-dione)	↑
	5α-Reductase (active on 4-androstene-3,17-dione)	↓
	5β-Reductase (active on 4-androstene-3,17-dione)	↑
	3β-Hydroxysteroid reductase (active on 4-androstene-3,17-dione)	↑
	17α-Hydroxysteroid reductase (active on 4-androstene-3,17-dione)	↑
	15β-Hydroxylase (active on 5α-androstane-3α,17β-diol 3,17-disulphate)	↓
II. Enzyme activities reversibly influenced by sex hormones	2β-Hydroxylase (active on 5α-androstane-3α,17β-diol)	↑
	18-Hydroxylase (active on 5α-androstane-3α,17β-diol)	↑
	6β-Hydroxylase (active on 4-androstene-3,17-dione)	↑
	3α-Hydroxysteroid reductase (active on 4-androstene-3,17-dione)	↓
III. Enzyme activities almost or completely androgen-independent	7α-Hydroxylase (active on 5α-androstane-3α;17β-diol and 4-androstene-3,17-dione)	
	17β-Hydroxysteroid reductase (active on 4-androstene-3,17-dione)	

benzoate, respectively, are not seen in hypophysectomized rats. The estrogen unresponsiveness characterizing these rats may be explained by the lost capacity to induce secretion of a hypophyseal feminizing factor. It may furthermore be speculated that the androgen unresponsiveness in hypophysectomized rats is due to the loss of a hypophyseal factor necessary for the action of androgens in the liver cell. Clearly the present findings call for a deeper knowledge of hypophyseal factors secreted in male and female rats. Present investigations where the influence of prolactin, FSH and LH upon liver enzyme activities and androgen responsiveness of liver are being studied give no indications that gonadotrophins are involved in hypophyseal regulation of hepatic enzyme levels.

Experiments on intracellular binding and transport of androgens in rat liver

The second type of approach currently being used in our laboratory involves search for a specific androgen receptor in liver cytosol possibly involved in intranuclear transport of androgens. When the ^{3}H-labelled cytosol obtained from castrated male rats was fractionated on a Sephadex G-25 column usually about 5–10% of the activity was recovered in the void volume. When the labelled cytosol was fractionated on a Sephadex G-100 column three main peaks of radioactivity were eluted (see Fig. 1). Peak C represented the unbound metabolites. It could be shown that peak B mainly consisted of protein-bound monosulphates whereas peak A was made up of protein-

Table 2. Effects of hypophysectomy on steroid metabolizing enzymes in liver from female rats, ↑ = increase, ↓ = decrease, — = no effect

I. Enzymes active on 5α-androstane-3α,17β-diol	
2α-Hydroxylase	↑
2β-Hydroxylase	↑
7α-Hydroxylase	↓
18-Hydroxylase	↑
II. Enzymes active on 4-androstene-3,17-dione	
6β-Hydroxylase	↑
7α-Hydroxylase	—
16α-Hydroxylase	↑
5α-Reductase	↓
5β-Reductase	↑
17α-Hydroxysteroid reductase	↑
17β-Hydroxysteroid reductase	—
3α-Hydroxysteroid reductase	↑
3β-Hydroxysteroid reductase	↑
III. Enzyme active on 5α-androstane-3α,17β-diol 3,17-disuphate	
15β-Hydroxylase	↓

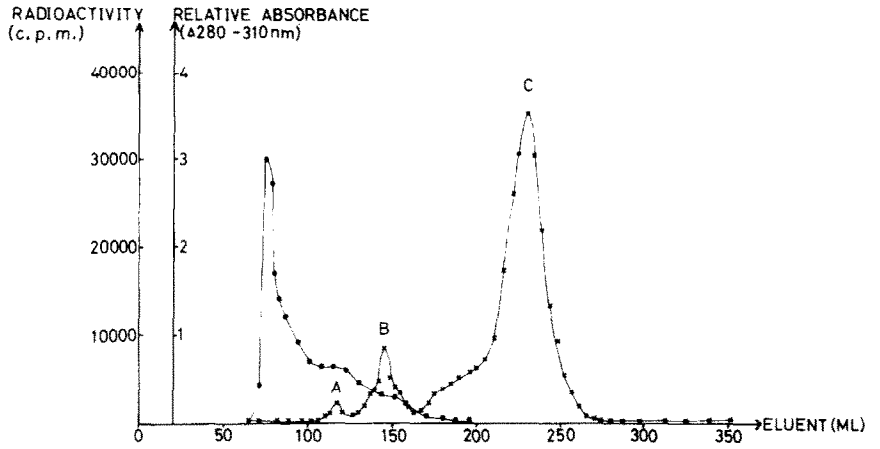

Fig. 1. Chromatography on Sephadex G-100 (Total volume: 250 ml) of liver cytosol from castrated male rats injected with [1,2,6,7-^{3}H]-testosterone using 0·01 M Tris–HCl buffer pH 7·4—0·01 M KCl–0·001 M EDTA as eluent. X–X–X–X = Radioactivity; O–O–O–O = Protein. For further explanation, see text.

bound unconjugated 4-androstene-3,17-dione. Treatment of peaks A, B and C with dextrane-coated charcoal resulted in elimination of practically all of peak C, about 80% of peak B and about 8% of peak A from the supernatant after centrifugation. When peak A—or the void volume from the Sephadex G-25 chromatography—was analysed by sucrose density gradient centrifugation the [^{3}H]-androstenedione-protein complex was shown to migrate with a sedimentation coefficient of about 10 S. Isoelectric focusing of the labelled complex indicated a pI of the protein of about 5.

In experiments where liver nuclei were prepared from rats injected with [^{3}H]-testosterone it was found that the nuclei specifically retained [^{3}H]-androstenedione. Furthermore, it was possible to extract a [^{3}H]-androstenedione-protein complex from the nuclei by 0·01 M Tris–HCl buffer pH 7·4—0·4 M KCl–0·01 M dithiothreitol–0·001 M EDTA solution that showed identical behaviour on a Sephadex G-100 column as the labelled cytosol complex. Furthermore, isolated liver nuclei from castrated rats (not given [^{3}H]-testosterone) were shown to concentrate specifically the [^{3}H]-androstenedione-protein complex when incubated with cytosol prepared from livers of rats given [^{3}H]-testosterone. The nuclear uptake of the [^{3}H]-androstenedione-protein complex was linear with time and concentration of nuclei. It could also be shown that the nuclear uptake was a saturable process.

Studies on steroid receptor proteins in liver are complicated by the extensive hepatic metabolism of steroids and by the large amount and variety of hepatic proteins. This is especially obvious in *in vitro* work where the added radioactive steroid is rapidly metabolized and bound to low affinity sites on unspecific proteins. Only preliminary results are so far available from *in vitro* labelling of androgen-binding proteins in hepatic cytosol but it seems as if testosterone is much more efficiently bound to the high affinity peak A protein than androstenedione. However, when the high affinity protein–androgen complex is isolated from the 20,000 *g* supernatant fraction after incubation of this fraction with [^{3}H]-testosterone the bound androgen has been identified as androstenedione. These results may indicate that, normally, testosterone is bound to a specific high affinity cytosol protein receptor and is then—still bound to the receptor molecule—immediately transformed into androstenedione which seems to be more firmly bound to the carrier protein than testosterone.

Based on these findings it seems reasonable to believe that the rat liver contains a specific receptor protein with high affinity for androstenedione. The physiological role of this receptor in androgenic regulation of liver enzyme activities is not clear at the present moment. It is obvious that central mechanisms exert a superior control of liver enzyme levels and it is possible that the hepatic androgen receptor participates in the control of other processes than the regulation of hepatic steroid metabolism.

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REFERENCES

1. Forchielli E. and Dorfman R. I.: *J. biol. Chem.* **223** (1956) 443–448.
2. Yates F. E., Herbst A. L. and Urquhart J.: *Endocrinology* **63** (1958) 887–902.
3. Conney A. H., Schneidman K., Jacobson M. and Kuntzman R.: *Ann. N.Y. Acad. Sci.* **123** (1965) 98–109.

4. Bergström S. and Gloor U.: *Acta chem. scand.* **9** (1955) 34–38.
5. Berg A. and Gustafsson J.-Å.: *J. biol. Chem.* **248** (1973) 6559–6567.
6. Gustafsson J.-Å. and Pousette Å.: *Biochemistry* **13** (1974) 875–881.
7. Osterman L.: *Sci. Tools* **17** (1970) 31–33.
8. Beato M. and Feigelson P.: *J. biol. Chem.* **247** (1972) 7890–7896.
9. Einarsson K., Gustafsson J.-Å. and Stenberg Å.: *J. biol. Chem.* **248** (1973) 4987–4997.
10. Gustafsson J.-Å. and Stenberg Å.: *J. biol. Chem.* **249** (1974) 711–718.
11. Gustafsson J.-Å. and Ingelman-Sundberg M.: *J. biol. Chem.* **249** (1974) 1940–1945.
12. Begue R.-J., Gustafsson J.-Å. and Gustafsson S. A.: *Eur. J. Biochem.* **40** (1973) 361–366.
13. Gustafsson J.-Å and Gustafsson S. A.: *Eur. J. Biochem.* **44** (1974) 225–233.
14. Gustafsson J.-Å. and Stenberg Å.: *J. biol. Chem.* **249** (1974) 719–723.

DISCUSSION

De Moor:

I cannot agree entirely with your conclusion, at least not for the 3β,5α derivatives of steroids. As published in 1968, in earlier work with Denef, we could show that these 3β,5α metabolites are formed in much larger quantities in adult male rats as compared with adult female rats and that the sexual difference disappears when the rats are castrated the first day of life or when female rats are injected the first day of life with testosterone. In the first experiment both rats become female as far as liver metabolism is concerned and in the second experiment they both behaved in a masculine way. Besides these experiments, I have three new bits of evidence to add to this paper of Dr. Gustafsson. We have recently studied the metabolism of testosterone and dihydrotestosterone in hepatoma tissue culture cells. Both in hepatoma cells derived from a male rat and in cells derived from a female rat, large amounts of 3β,5α derivatives were formed. Another bit of evidence: Denef has a paper in print in *Endocrinology* where he shows conclusively, as you did, that after hypophysectomy, both in castrated male rats and castrated females, livers of both these animals make the same large amounts of 3β,5α derivatives; they both acted in a masculine way. But in the same paper Denef, in another experiment, has implanted pituitaries under the capsule of the kidney in hypophysectomized rats. These animals with the pituitary independent from the hypothalamic region have livers which always behave in a feminine way. They don't produce any more 3β-hydroxy derivatives. So my conclusion is that liver metabolism of steroids is actively feminized by an hypophyseal factor repressed in normal adult males by some hypothalamic factor.

Gustafsson:

Thank you Dr. de Moor. Contrary to what you say I think your data confirm what we have presented. We have also shown that the microsomal 3β-hydroxysteroid dehydrogenase is imprinted at birth using androstanedione as a substrate (Gustafsson J. A. and Stenberg A.: *J. biol. Chem.* **249** (1974) 711–718). Also your results with hypophysectomized animals are in good agreement with ours (Gustafsson J. A. and Stenberg Å.: *Endocrinology* in press).