Androgen Responsiveness of the Liver of the Developing Rat

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(Received 16 April 1974)

The activities of the hepatic microsomal 2α -, 2β -, 7α - and 18-hydroxylase systems active on 5α -[4- ^{14}C]androstane- 3α , 17β -diol were studied in male and female rats which had been castrated at birth and at the age of 7, 13, 21, 27, 34, 43 and 55 days, treated for 5 days with 2 mg of testosterone propionate/kg body weight and killed 6 days after castration. The 7α -hydroxylase system was affected very little by androgen treatment at all stages during development. On the other hand it was found that the rat liver passed through three phases during development with respect to androgen responsiveness as judged by changes in the activities of the 2α -, 2β - and 18-hydroxylase systems: a first phase (from the neonatal period up to about 19 days of age) with a relative androgen unresponsiveness in both male and female rats, a second phase (from about 27 to about 33 days of age) when male and female rats responded equally well to androgens and a final phase (from about 40 days of age) with a successively decreasing androgen responsiveness in female rats but with a retained responsiveness in male rats. The hypothesis is presented that neonatal imprinting of the liver by testicular androgen(s) determines the development and degree of androgen responsiveness of liver tissue in the rat.

Berg & Gustafsson (1973) have shown that post-pubertally castrated female rats are relatively resistant towards androgen treatment with regard to several microsomal hydroxylase activities whereas post-pubertally castrated male rats respond promptly by showing increased activities of the same enzymes. This sexual difference is probably attributable to a different hormonal influence in male and female rats in early life, since it has also been demonstrated that testicular secretion products, probably androgen(s), imprint the liver during the postnatal period so that it responds differently to androgens in adult life than if neonatal androgenic influence does not occur (Gustafsson & Stenberg, 1974). To understand more about the mechanisms regulating the androgen responsiveness of liver enzyme activities it was decided to study the effect of androgens on hepatic enzyme activities in male and female rats during development from birth until adulthood. One of the most suitable substrates for studies on sexual differentiation of hepatic metabolism in rats is 5α -androstane- 3α , 17β -diol, since the microsomal fraction of rat liver contains several androgen-responsive hydroxylase systems active on this steroid (Berg & Gustafsson, 1973). The present paper describes the influence of testosterone propionate treatment on the hepatic microsomal 2α -, 2β -, 7α - and 18-hydroxylase systems active on 5α -androstane- 3α , 17β -diol in male and female rats of various ages.

Materials and Methods

Reference compounds

Several of the hydroxylated steroids used in this investigation were kindly supplied by colleagues from their collections: 5α -androstane- 2β , 3α , 17β -triol, 5α -androstane- 2β , 3β , 17β -triol (Dr. M. Wolff, San Francisco, Calif., U.S.A.), 5α -androstane- 3α , 16α , 17β -triol, 5α -androstane- 3β , 16α , 17β -triol (Dr. F. Galletti, Milan, Italy), 3β , 17β -dihydroxy- 5α -androstane-7-one (Dr. B. P. Lisboa, Hamburg, Germany) and 3α , 18 -dihydroxy- 5α -androstane-17-one (Dr. D. K. Fukishama, New York, N.Y., U.S.A.). Reduction with NaBH_4 (Gustafsson & Sjövall, 1968) of 3α , 18 -dihydroxy- 5α -androstane-17-one yielded 5α -androstane- 3α , 17β , 18 -triol. 5α -Androstane- 3β , 17β , 18 -triol was prepared by oxidation of 5α -androstane- 3α , 17β , 18 -triol with purified 3α -hydroxy steroid oxidoreductase from *Pseudomonas testosteroni* by the method of Hurlock & Talalay (1957). The 17β , 18 -dihydroxy- 5α -androstane-3-one (the identity of which was confirmed by gas chromatography-mass spectrometry) thus obtained was reduced with NaBH_4 in methanol to 5α -androstane- 3β , 17β , 18 -triol (Gustafsson & Lisboa, 1969). NaBH_4 reduction of 3β , 17β -dihydroxy- 5α -androstane-7-one yielded 5α -androstane- 3β , 7α , 17β -triol (about 80% of the reaction products) and 5α -androstane- 3β , 7β , 17β -triol (about 20%).

4-Androstene-3,17-dione and 3 α - and 3 β -hydroxy-5 α -androstane-17-one were kindly donated by Dr. J. Babcock, Upjohn Co., Kalamazoo, Michigan, U.S.A. 5 α -Androstane-3 α (and 3 β),17 β -diol and 17 β -hydroxy-5 α -androstane-3-one were purchased from Sigma Chemical Company (St. Louis, Mo., U.S.A.). 5 α -Androstane-3,17-dione was prepared from 3 β -hydroxy-5 α -androstane-17-one by chromic acid oxidation in acetone (Gustafsson & Sjövall, 1968).

Radioactive steroids

4-[4-¹⁴C]Androstene-3,17-dione (specific radioactivity, 210 μ Ci/mg) was purchased from The Radiochemical Centre (Amersham, Bucks., U.K.). 5 α -[4-¹⁴C]Androstane-3 α ,17 β -diol was prepared from 4-[4-¹⁴C]androstene-3,17-dione as described previously (Berg & Gustafsson, 1973). Before incubation with rat liver microsomes the 5 α -[4-¹⁴C]androstane-3 α ,17 β -diol was diluted with unlabelled 5 α -androstane-3 α ,17 β -diol (the purity of which was assayed by g.l.c.) to yield a specific radioactivity of 3.0 μ Ci/mg.

Animal experiments

Male and female rats of the Sprague-Dawley strain were castrated at birth and at the age of 7, 13, 21, 27, 34, 43 and 55 days, and were given daily intramuscular injections of 2mg of testosterone propionate/kg body weight for 5 days. The steroid was administered in propylene glycol; control rats received this vehicle only. Each experimental group consisted of at least four animals and separate incubations were carried out with liver microsomal preparations from each of these rats except in experiments with rats castrated at birth and 7 days of age where livers from several rats were pooled to provide sufficient material for homogenization.

The animals were killed by cervical dislocation. The liver was excised quickly, chilled and weighed. Liver homogenates, 20% (w/v), were prepared in a modified Bucher medium (Bergström & Gloor, 1955), pH 7.4, with a Potter-Elvehjem homogenizer equipped with a loosely fitting pestle. The homogenate was centrifuged at 20000 g for 15 min. The microsomal fraction was obtained by centrifuging the 20000 g supernatant fluid at 105000 g for 60 min. The microsomal fraction was resuspended in the homogenizing medium in a volume corresponding to that of the 20000 g supernatant fluid from which it had been isolated, and was homogenized with a loosely fitting pestle. The protein concentration of the microsomal fraction was determined by the method of Lowry *et al.* (1951), with bovine serum albumin as standard.

Incubations with 5 α -[4-¹⁴C]androstane-3 α ,17 β -diol

The incubations with 5 α -[4-¹⁴C]androstane-3 α ,17 β -

diol were carried out for 10 min at 37°C as described previously (Berg & Gustafsson, 1973). The conditions under which the incubations were performed were tested and the conversions obtained were found to be linear with respect to time and enzyme concentration. The incubations were terminated by the addition of 20 vol. of chloroform-methanol (2:1, v/v). The precipitate was filtered off and 0.2 vol. of aq. NaCl (0.9%, w/v) was added. The chloroform phase was collected and the solvent was evaporated. The residue was dissolved in 0.5 ml of chloroform-methanol (2:1, v/v) and was applied on precoated silica gel plates (250 μ m). The extracts were developed five times in the solvent system ethyl acetate-cyclohexane (3:2, v/v). The thin-layer plates were radioautographed with an exposure time of 6 days. The radioactive zones on the t.l.c. plates were determined exactly from the X-ray film and were scraped off, eluted with methanol and measured for radioactivity in a Packard liquid-scintillation spectrometer model 3003, with Instagel (Packard Instrument Co. Inc., 2200 Warrenville, Ill., U.S.A.) as scintillator liquid.

Gas chromatography-mass spectrometry analysis

The methanol extracts of the radioactive zones pooled from the corresponding incubations were evaporated to dryness, silylated and analysed by gas chromatography-mass spectrometry using an LKB 9000 instrument. A 1.5% SE-30 column was used. Retention times (t_R) were calculated relative to 5 α -cholestane. Mass spectra were recorded on magnetic tape by using the incremental mode of operation and were treated in an IBM 1800 computer (Reimendal & Sjövall, 1972). A compound was considered identified if it had the same mass spectrum and behaviour in g.l.c. as the reference compound.

Statistical analysis

Student's *t* test was used and the significance level was set at 0.05.

Results

After incubation of 5 α -[4-¹⁴C]androstane-3 α ,17 β -diol with the microsomal fraction of rat liver there were total conversions of 20–30% of the substrate. Details of the identifications of hydroxylated metabolites of 5 α -androstane-3 α ,17 β -diol formed during incubation with liver microsomes from intact and castrated male and female rats after various kinds of treatment have been given in previous papers (Berg & Gustafsson, 1973; Einarsson *et al.*, 1973a,b). The following metabolites were found in the present experiment: 5 α -androstane-2 α ,3 α ,17 β -triol;

Fig. 1. Activity of the hepatic microsomal 2 α -hydroxylase system on 5 α -androstane-3 α ,17 β -diol in male and female rats castrated at birth and at 7, 13, 21, 27, 34, 43 and 55 days of age and treated with testosterone propionate in propylene glycol (T) or propylene glycol only (P)

The animals were killed 6 days after castration. The enzyme activity is expressed as nmol of 5 α -androstane-2 α ,3 α ,17 β -triol formed/10min per mg of protein; +1 S.D. is indicated by vertical lines on the top of the bars.

Fig. 3. Activity of the hepatic microsomal 18-hydroxylase system on 5 α -androstane-3 α ,17 β -diol in male and female rats castrated at birth and 7, 13, 21, 27, 34, 43 and 55 days of age, treated with testosterone propionate in propylene glycol (T) or propylene glycol only (P)

The animals were killed 6 days after castration. The enzyme activity is expressed as nmol of 5 α -androstane-3 α ,17 β ,18-triol and 5 α -androstane-3 β ,17 β ,18-triol formed/min per mg of protein; +1 S.D. is indicated by vertical lines on the top of the bars.

Fig. 2. Activity of the hepatic microsomal 2 β -hydroxylase system on 5 α -androstane-3 α ,17 β -diol in male and female rats castrated at birth and at 7, 13, 21, 27, 34, 43 and 55 days of age and treated with testosterone propionate in propylene glycol (T) or propylene glycol only (P)

The animals were killed 6 days after castration. The enzyme activity is expressed as nmol of 5 α -androstane-2 β ,3 α ,17 β -triol formed/10min per mg of protein; +1 S.D. is indicated by vertical lines on the top of the bars.

3 β ,17 β ,18-triol. The 2 α -, 2 β -, 7 α - and 18-hydroxylase activities were estimated as the amounts of 2 α -, 2 β -, 7 α - and 18-hydroxylated metabolites formed respectively. In some cases, e.g. in the case of 5 α -androstane-3 β ,17 β ,18-triol, it is possible that the hydroxylated metabolites were formed by hydroxylation of derivatives of the substrate rather than by hydroxylation of the substrate itself. Experience has shown, however, that reproducible values of the enzyme activities in question are best obtained by adding the amounts of all hydroxylated metabolites formed (Berg & Gustafsson, 1973; Einarsson *et al.*, 1973a,b; Gustafsson & Stenberg, 1974).

Fig. 1 demonstrates how the 2 α -hydroxylase activity is affected by testosterone propionate treatment during different phases of development. On the whole, rats below 19 days of age (first phase) were not affected very much by androgen administration. The only exceptions were female rats aged 19 days, which were characterized by a drop in enzyme activity after androgen treatment. At the age of 27 days, however, both male and female rats responded to androgen by an increased 2 α -hydroxylase activity and this was also true for male and female rats aged 33 days (second phase). At this age, the increase in 2 α -hydroxylase activity was about 2–2.5 times

5 α -androstane-2 β ,3 β ,17 β -triol; 5 α -androstane-3 α ,7 α ,17 β -triol; 3 α ,7 α -dihydroxy-5 α -androstane-17-one; 7 α ,17 β -dihydroxy-5 α -androstane-3-one; 5 α -androstane-3 α ,17 β ,18-triol and 5 α -androstane-

after androgen treatment. A third phase in the development of liver 2α -hydroxylase responsiveness towards androgens was observed in rats above 40 days of age. Although male rats continued to respond well to testosterone propionate by an increased 2α -hydroxylase activity, female rats became successively less responsive. At the age of 61 days, androgen-treated female rats showed an increase in 2α -hydroxylase activity of only 9% whereas the same enzyme activity in male rats increased by 123%.

Figs. 2 and 3 show how the 2β - and 18-hydroxylase activities respectively were affected by testosterone propionate treatment during different periods of development. It is evident that these two enzyme activities resembled the 2α -hydroxylase activity with respect to androgen sensitivity and that the same three principal developmental phases in responsiveness were seen.

A different type of regulation characterized the 7α -hydroxylase activity. Generally, this enzyme activity was not affected by androgen treatment to any large extent except in rats 19 and 27 days of age. Female rats and 19-day-old male rats performed 7α -hydroxylation less efficiently after androgenic treatment whereas 27-day-old rats of both sexes reacted by increased 7α -hydroxylase activities after the same hormone treatment.

Discussion

The present study has demonstrated that the androgen unresponsiveness that is characteristic of hepatic enzyme activities in adult female rats begins to develop between 33 and 40 days of age after a period of equally high androgen responsiveness in female and male rats. Male rats display a relatively constant androgen responsiveness from 19 days of age and onwards as is apparent from the 100–200% increases in 2α -, 2β - and 18-hydroxylase activities after testosterone propionate treatment. In the neonatal period neither male nor female rats respond significantly to androgens with respect to hepatic enzyme activities.

The small effects of testosterone administration on hepatic hydroxylase activities during the neonatal and postnatal period is perhaps somewhat surprising in view of the long-term effects on hepatic steroid metabolism of neonatal androgen exposure (DeMoor & Denef, 1968; Kraulis & Clayton, 1968; Einarsson *et al.*, 1973b). During this period androgens may affect the mechanisms of the liver cell that are responsible for regulation of enzyme activities later in life without affecting these enzyme activities at the actual time of interaction with the liver cell.

The postpubertal decrease in androgen responsiveness is pre-determined by neonatal hormonal events since neonatally castrated male rats show

the same degree of androgen unresponsiveness when adult with respect to hepatic enzyme activities as do female rats, whereas male rats castrated at 14 days of age respond normally to androgen treatment when adult (Gustafsson & Stenberg, 1974). Imprinting processes effectuated by testicular secretion product(s) at birth thus appear to programme the postpubertal development of hepatic androgen responsiveness.

The biochemical mechanisms responsible for the described events remain to be elucidated. Principally, they may be related to (a) changed androgen metabolism, especially with reference to pathways leading to biologically active metabolites, (b) changed concentrations of liver cytoplasmic and/or nuclear androgen receptor proteins, and (c) changed expression of genetic information.

The second explanation deserves special attention. Vertes & King (1971) have shown that treatment of female rats with testosterone propionate shortly after birth decreases the uptake of oestradiol in the hypothalamus and uterus at 60 days of age. Tuohimä & Niemi (1972) on the other hand, have shown that feminization of newborn male rats with the anti-androgen, cyproterone, results in increased oestradiol binding in the hypothalamus. Finally, Kato *et al.* (1971) have presented evidence for the age-dependent development of oestrogen receptors in the rat hypothalamus. In view of these findings it appears quite possible that neonatal imprinting could determine the development and degree of androgen responsiveness of liver tissue in the rat by influencing the concentrations of cytoplasmic and/or nuclear receptor proteins.

This work was supported by the Swedish Medical Research Council (grant no. 13X-2819) and Carl-Bertel Nathhorsts Vetenskapliga Stiftelse.

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