Testosterone metabolism by the rat gastrointestinal tract, *in vitro* and *in vivo*

M J G FARTHING, G P VINSON, C R W EDWARDS, AND A M DAWSON

From the Department of Gastroenterology and Endocrinology, St Bartholomew's Hospital, London, and the Department of Biochemistry, St Bartholomew's Medical College, London

SUMMARY We have shown previously that the capacity of the jejunal mucosa to oxidise testosterone to the weaker androgen, androstenedione, by the enzyme 17β -hydroxysteroid dehydrogenase (17β -HSD), is considerable. The present study extends these earlier observations by measuring 17β -HSD activity in different regions of the gastrointestinal tract, by investigating the potential for testosterone metabolism by slices and everted sacs of rat jejunum, and estimating the contribution of intestinal testosterone metabolites to circulating levels of plasma androgens, by portal vein sampling in the rat, *in vivo*. 17β -HSD activity in homogenates of gastric and duodenal mucosa was significantly higher than that in jejunum, and was also present in ileum and colon. In addition to androstenedione, slices and everted sacs of rat jejunum produced various metabolites, one of which was probably dihydrotestosterone. It was not, however, a major metabolite *in vivo*. It is suggested that 5α -reduction may be favoured *in vitro* by a lower oxidation-reduction potential resulting from tissue anoxia. The major portal vein metabolite was androstenedione, the same major metabolite produced by mucosal homogenates. We conclude that oxidation of testosterone is the major metabolic pathway in intestinal mucosa and the capacity of the gastrointestinal tract to reduce the potency of testosterone is considerable. Our findings suggest that the gut, rather than the liver, is responsible for the failure of oral testosterone to provide effective androgen replacement therapy. The qualitative difference in testosterone metabolism between *in vitro* and *in vivo* preparations emphasises the need for caution in the interpretation of similar *in vitro* experiments.

In a previous study we demonstrated that homogenates of rat and human jejunal mucosa can oxidise testosterone to the weaker androgen androstenedione.¹ This conversion was independent of gut bacteria. The enzyme responsible for this conversion, 17β -hydroxysteroid dehydrogenase (17β -HSD), was present at high specific activity and the overall capacity of the small intestine to perform this conversion appeared considerable. This suggested that the gut, like other non-endocrine tissues such as skeletal muscle,^{2,3} lung,⁴ and adipose tissue,^{5,6} may play a significant role in the peripheral metabolism of testosterone.

Previous reports⁷⁻¹¹ have indicated that mammalian intestinal mucosa is also capable of reducing testosterone in relatively minor amounts to a variety of 5α -reduced metabolites, including the more potent metab-

olite of testosterone found in androgen target organs,³ dihydrotestosterone.

The present study was designed to extend earlier observations of 17β -HSD activity in the gut, to investigate the potential for further metabolism of testosterone by slices and everted sacs of rat jejunum, and to estimate the possible contribution of intestinal testosterone metabolites to circulating levels of plasma androgens by continuous portal vein sampling *in vivo*, in the rat.

Methods

ANIMALS

Male Wistar rats bred in the animal house at St Bartholomew's Hospital Medical College, weighing 200-260 g, were used in the following experiments.

TISSUE PREPARATION

Homogenates of gastric, duodenal, mid-jejunal, mid-ileal, and colonic mucosa were prepared in Krebs-Ringer bicarbonate buffer (pH 7.4) with D-glucose (11

* Address for correspondence: Dr M J G Farthing, Tufts University School of Medicine, Department of Medicine, Division of Geographic Medicine, Box 22, 136 Harrison Avenue, Boston, MA 02111, USA.

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mM) as described previously for jejunum.¹ Protein concentration of homogenates was determined by the method of Lowry *et al.*¹⁷ Transverse slices and everted sacs of jejunum were prepared according to established techniques,^{18,14} and production of testosterone metabolites was expressed per 100 mg wet weight of intestine.

INCUBATION PROCEDURE

17 β -HSD assay

Mucosal homogenates from four male rats were incubated within 30 minutes of preparation in 25 ml conical flasks in Krebs-Ringer bicarbonate buffer with glucose (KRBG) with testosterone [$1,2,6,7\text{-}^3\text{H}$]-testosterone 1.0×10^6 cpm and unlabelled testosterone to produce a final substrate concentration of $4.0 \times 10^{-6}\text{M}$ — that is, greater than 10 times the apparent K_m ¹—and the cofactor for 17 β -HSD, NADP ($4.0 \times 10^{-4}\text{M}$) such that the rate of androstenedione production was proportional to the time of incubation and the protein content of homogenate. Incubations were performed for 20 minutes and the final protein concentrations of the homogenates were maintained between 0.5–1.0 mg/ml as established previously.¹ 17 β -HSD activity was expressed in pmol androstenedione per mg protein per minute. Control flasks containing substrate but no homogenate were carried through the entire procedure in all experiments. All 17 β -HSD assays were performed in duplicate and different mucosal homogenates from the same rat were assayed together. The reaction was stopped by immediate extraction with anhydrous diethyl ether.

Slices and everted sacs of jejunum

Testosterone ($4.0 \times 10^{-6}\text{M}$ including $1.0\text{--}2.0 \times 10^6$ cpm [^3H]-testosterone) in KRBG solution was incubated with freshly prepared slices or everted sacs of rat jejunum, without added cofactors, at 37°C and the medium was bubbled throughout the incubation with 95% O_2 /5% CO_2 . In everted gut sac experiments testosterone was added only to the mucosal medium. D-glucose concentration was determined in mucosal and serosal media of everted sacs by the glucose oxidase method^{15,16} and the development of a concentration gradient between these media¹⁴ was used as an index of metabolic integrity of the gut sac mucosa. Preliminary experiments with 54 jejunal sacs incubated for periods up to four hours showed that a concentration gradient for D-glucose was demonstrable after approximately 30 minutes and increased up to two hours. In the present studies, tissues were incubated for up to 60 minutes. Sacs were incubated in pairs, and were excluded from the study if they failed to achieve a D-glucose gradient appropriate to the time of incubation.

EXTRACTION AND SEPARATION OF METABOLITES

Incubation media and tissue samples after homogenisation were extracted in 10 volumes anhydrous diethyl

ether and the testosterone metabolites separated by Celite-propylene glycol column chromatography as described previously.¹ Column fractions were counted in a Packard Tri-carb liquid scintillation spectrometer (3380) either singly or in pooled groups corresponding to androgen peaks. Quantification of androgen metabolites was achieved by integration of the peaks of radioactivity obtained by column chromatography, subtraction of radioactivity in equivalent column fractions of control incubations without tissue, and expressing the values for each peak as a fraction of the total radioactivity. No correction was made for procedural losses in view of the consistency of quantitative recoveries of standard tritiated androgens reported previously.¹

ACID HYDROLYSIS OF STEROID CONJUGATES

The aqueous phase remaining after ether extraction of samples obtained from incubations of testosterone with everted jejunal sacs was subjected to acid hydrolysis by addition of concentrated hydrochloric acid to reduce the pH to less than 1.0. Samples were incubated at room temperature for 48 hours, re-extracted with 10 volumes diethyl ether, and subjected to further Celite column chromatography.

FURTHER IDENTIFICATION OF METABOLITES

Further evidence for the identity of androstenedione, the major testosterone metabolite produced by intestinal mucosal homogenates, was reported previously.¹

Fractions obtained by Celite column chromatography of mucosal and serosal media from everted jejunal sacs, after incubation with testosterone, were examined further by gas-liquid chromatography. Methods were developed from those used in other applications.¹⁷⁻¹⁹ The gas chromatography used was a Pye series 104, equipped with a ^6Ni electron capture detector (Voltage 47-60 V pulsed, pulse space 150 μS , pulse width $0.75 \pm 0.25 \mu\text{S}$). The column packing was XE-60 on Gas chrom Q support, at a loading of the stationary phase of about 0.3%. This packing was prepared according to the method of Rapp and Eik-Nes.²⁰ Columns (60 cm long and 0.4 cm internal diameter) were maintained at a temperature of 175°C and nitrogen was used as a carrier gas, at a flow rate of about 100 ml/minute. The temperature was selected on a trial and error basis to obtain maximal separation of a mixture of appropriate standard androgens. Steroids were chromatographed as the mono or diheptafluorobutyrate prepared as previously described.¹⁷⁻¹⁹

TESTOSTERONE METABOLISM AND ABSORPTION, IN VIVO

Rats were anaesthetised with pentobarbitone (20–25 mg/kg), a tracheostomy performed, and a fine polyethylene catheter (external diameter 0.61 mm) was passed

retrogradely, into the portal vein *via* an ileocaecal vein. A second identical catheter in an internal jugular vein enabled replacement blood transfusion during portal vein sampling. [1,2,6,7-³H]-testosterone ($15\text{--}20 \times 10^6$ cpm, approximately $5.0 \times 10^{-8}\text{M}$) in KRBG solution was introduced into a 30 cm closed loop of proximal jejunum which had been flushed with 0.9% sodium chloride (W/V) at 37°C.

In a preliminary study, during which the portal vein was sampled intermittently for 40 minutes, peak tritium activity was present at approximately five minutes. In subsequent experiments the portal vein was sampled continuously for 10 minutes, and the blood collected as five separate 1.0 ml samples, each covering a two minute period. Whole-blood was immediately extracted with 10 volumes diethyl ether. Aliquots of residual luminal fluid and gut tissue from the loop were also extracted, and the aqueous phase of all extracted samples was subjected to acid hydrolysis before re-extraction with diethyl ether. Metabolites were separated by Celite column chromatography.

Results

17 β -HSD ACTIVITY IN DIFFERENT REGIONS OF GASTROINTESTINAL TRACT

Figure 1 shows 17 β -HSD activity in different regions of rat gastrointestinal tract. Activity was significantly higher in gastric (502 ± 111 pmol/mg/min, mean $\pm$ SEM) and duodenal (595 ± 166 pmol/mg/min) mucosa than that of either jejunum or ileum (114 ± 20 and 29 ± 2 pmol/mg/min respectively; $P < 0.05$ by

Fig. 1 17 β -hydroxysteroid dehydrogenase activity in mucosal homogenates of rat stomach, duodenum, jejunum, ileum and colon (mean $\pm$ SEM, $n = 4$; Student's t test).

Student's t test). Activity in colonic mucosa (157 ± 25 pmol/mg/min) was significantly higher than that of ileum.

In one rat, 17 β -HSD activity was estimated throughout the small intestine in mucosal homogenates derived from 15 cm segments of intestine. Figure 2 confirms that 17 β -HSD activity is highest in the proximal small intestine and falls progressively until a small increase in activity in the distal ileum, which appears to anticipate the small but significant rise in colonic 17 β -HSD activity (Fig. 1).

TESTOSTERONE METABOLISM BY SLICES OF JEJUNUM

Figure 3 shows Celite column chromatograms of testosterone metabolites in the incubation media of control and experimental incubations and also of metabolites in jejunal slices. In addition to testosterone and its major metabolite, androstenedione, two other peaks were present. One was a major metabolite in the tissue and eluted identically with dihydrotestosterone, and a second more polar metabolite, denoted X, eluted after testosterone.

TESTOSTERONE METABOLISM AND TRANSPORT BY EVERTED SACS OF JEJUNUM

The same pattern of testosterone metabolites was found after incubation with everted rat jejunal sacs. Production of androstenedione, 'dihydrotestosterone', and X with respect to time, in mucosal and serosal media and in sac tissue is shown in Fig. 4.

Each point represents the mean of duplicate observations. Although androstenedione appeared as a

Fig. 2 17 β -hydroxysteroid dehydrogenase activity in mucosal homogenates prepared from 15 cm segments along the small intestine of a male rat.

Fig. 3 Celite column chromatograms after incubation of testosterone with and without (control) slices of rat jejunum for 60 minutes. The lower two experimental chromatograms show (^3H)-metabolites in the incubation medium and in jejunal slices. Columns were eluted sequentially with iso-octane (10 ml), 5% ethyl acetate (10 ml), 20% ethyl acetate (10 ml) and 30% ethyl acetate (10 ml) in iso-octane. The vertical lines indicate solvent changes.

major early metabolite in the mucosal medium (Fig. 4a), both 'dihydrotestosterone' and X were major metabolites in sac tissue (Fig. 4c). Both entered the serosal medium more rapidly and were present at higher concentrations than either testosterone or androstenedione (Fig. 4c). Neither testosterone nor any of its metabolites was transported into the serosal medium against a concentration gradient. After 60 minutes incubation, 1.5% of the total tritium activity in the mucosal medium and 10.3% in the serosal medium was recovered after acid hydrolysis and re-extraction and more than half of this was found to be testosterone.

Recovery of tritium activity (including that liberated by acid hydrolysis) at each time point of the 60 minute incubation was between 89.6% and 92.6%. All recoveries were therefore within the expected limits of this method, strongly suggesting that no other free or conjugated metabolites had been produced.

FURTHER IDENTIFICATION OF TESTOSTERONE METABOLITES BY GAS-LIQUID CHROMATOGRAPHY

Fractions corresponding to androstenedione, 'dihydrotestosterone', and X obtained by Celite column chromatography were subjected to gas-liquid chromatography. Peaks corresponding to monoheptafluorobutyrate of androstenedione and 'dihydrotestosterone' had retention times relative to testosterone diheptafluorobutyrate (retention time, 72 seconds) of 1.5 and 1.08 respectively, identical with their authentic standards. Peak X from the Celite column, however, resolved into four major peaks with relative retention times of 0.75, 0.92, 1.17, and 1.42. The first of these metabolites had the same retention time as authentic 5α -androstane- 3α , 17β -diol diheptafluorobutyrate, but the identity of the others is uncertain and requires further clarification.

TESTOSTERONE METABOLISM AND ABSORPTION, IN VIVO

The pattern of portal vein metabolites shown in Fig. 5 is a representative example of the three experiments performed. In the first sampling period (0–two minutes) the major metabolite was androstenedione and this accounted for 63.5% of the total tritiated metabolites in the portal blood at this time (Table 1). In contrast, only minor amounts of unaltered testosterone entered the portal circulation. Although other, as yet, unidentified metabolites (e, f, and g, Fig. 5) entered the portal vein during later sampling periods, androstenedione continued to predominate. A metabolite eluting identically with dihydrotestosterone was also present in the four to six minute sample but again this made a relatively minor contribution to the total tritium activity. A similar pattern of metabolites was found in the residual fluid in the gut lumen and in the gut tissue of the loop of jejunum (Fig. 6).

Fig. 4 Testosterone metabolites in the (a) mucosal medium, (b) serosal medium and (c) tissue of everted sacs of rat jejunum, with respect to time of incubation. Each point represents the mean of duplicate observations. T, testosterone; A, androstenedione, 'DHT', dihydrotestosterone; X, unidentified metabolite.

Table 1 [^3H]-Testosterone metabolites in portal blood, gut lumen and gut tissue after introduction of [^3H]-testosterone into a closed loop of proximal rat jejunum

Sampling period (min)	Portal blood			Jejunum			Tissue		
	% Total metabolites			% Total metabolites			% Total metabolites		
	% T	% A	% Others	% T	% A	% Others	% T	% A	% Others
0-2	4.4	63.5	32.1	—	—	—	—	—	—
2-4	4.1	46.2	49.7	—	—	—	—	—	—
4-6	3.3	42.7	54.0	—	—	—	—	—	—
6-8	3.5	48.3	48.2	—	—	—	—	—	—
8-10	5.5	47.7	48.4	17.5	43.4	39.1	14.7	57.1	28.2

T, testosterone; A, androstenedione.

Recovery of tritium activity before and after acid hydrolysis from portal blood, luminal fluid and gut tissue is shown in Table 2. The proportion of the total tritium activity liberated by acid hydrolysis was only 0.03% in portal blood, suggesting that the majority of tritium activity entering the circulation was free steroid. Although overall tritium recovery was low (38.5%), this can be accounted for by the fact that only 5% of the estimated portal blood flow was sampled — that is, 0.5 ml/min of approximately 10 ml/min.²¹ Applying this correction to the tritium recovery in portal blood, total recovery rises to 86%, which is within the expected range for our method of extraction and chromatography.

Table 2 Recovery of tritiated metabolites from portal blood, luminal fluid, and jejunal tissue

Metabolites	% Of total tritium activity			
	Portal blood	Jejunum Luminal fluid	Tissue	Total
Ether-extractable	2.5	6.5	23.9	32.9
Liberated by hydrolysis	0.03	1.3	4.3	5.6
Total	2.53	7.8	28.2	38.53

Discussion

17 β -HYDROXYSTEROID DEHYDROGENASE ACTIVITY

We have previously suggested that small intestinal 17 β -HSD may have a physiological role in the peripheral oxidative metabolism of testosterone in man and animals.¹ The present study confirms these earlier findings and in addition demonstrates high specific activities of 17 β -HSD in gastric and duodenal mucosa and moderate activity in colonic mucosa. The previous assessment of the overall capacity of the small intestine to oxidise testosterone to the weaker androgen androstenedione¹ is clearly an underestimate of the total capacity of the gastrointestinal tract. 17 β -HSD activity decreased with distal progression along the small intestine, an observation which is paralleled by the fall in UDP-glucuronyl transferase activity,²² the enzyme required for glucuronide synthesis. Conjugation of steroids and other substances prevents absorption and hastens excretion in the faeces, the clinical importance of which has been clearly shown for oestrogens.²³ A similar detoxifying or degrading role may also exist for intestinal 17 β -HSD, as it is capable of reducing the potency of testosterone and possibly oestradiol.

Fig. 5 Celite column chromatograms showing portal vein [^3H]-metabolites during three, 2-minute sampling periods after introduction of [^3H]-testosterone into a closed jejunal loop in rat, in vivo. T, testosterone; A, androstenedione; 'DHT', dihydrotestosterone; X, e f and g, unidentified metabolites.

Fig. 6 Celite column chromatograms of [^3H]-metabolites of testosterone in the lumen and gut tissue of a closed loop of rat jejunum. T, testosterone; A, androstenedione; 'DHT', dihydrotestosterone; X, unidentified metabolite.

5 α -REDUCTASE ACTIVITY

Observations on testosterone metabolism by slices and everted sacs of rat jejunum agreed with previous reports⁷⁻¹¹ which suggested that a variety of metabolites, including probably dihydrotestosterone are also produced by mammalian intestine. Experiments with everted gut sacs indicated that 'dihydrotestosterone' and the more polar metabolites, collectively designated as X, entered the serosal medium more rapidly than either testosterone or androstenedione, suggesting that these additional metabolites may be important contributors to circulating levels of plasma androgens. These findings conflict with those of Kreek *et al.*⁷ who found that 5 α -androstane-3 α , 17 β -diol glucuronide accumulated against a concentration gradient and was the major metabolite in the serosal medium of everted gut sacs. Contrary to the views of Schedl^{24, 25} it was proposed that glucuronidation was an important factor in the intestinal absorption of androgens, but, based on the results of acid hydrolysis, the present study has failed to confirm this. It should be noted that Kreek *et al.*⁷ used a much longer incubation period of two to three hours and the conjugate in the serosal medium was produced mainly in the last two hours of the incubation. This is a period when there is gross structural disorganisation of gut sac mucosa.²⁶

TESTOSTERONE METABOLISM, IN VIVO

Intestinal testosterone metabolism in the rat *in vivo* was qualitatively different from the findings in everted sacs, for, like the present and previous observations with mucosal homogenates,¹ the oxidised product androstenedione was the major metabolite in portal blood, gut tissue, and luminal fluid. Other metabolites were produced *in vivo*, but we found no evidence that these were absorbed preferentially, as was the case in experiments with everted gut sacs. The most likely explanation for this disparity between the findings *in vitro* and those *in vivo* is that reduction of testosterone was favoured in the *in vitro* tissue by a lower oxidation-reduction potential produced in relatively hypoxic tissue. The early appearance of metabolite 'dihydrotestosterone' in the serosal medium of everted gut sacs suggests that reduction was facilitated throughout the incubation and was not merely a feature of a deteriorating preparation during the final stages of the experiment. The relatively high concentration of 'dihydrotestosterone' found in the tissue would support this hypothesis.

It has been shown in erythrocytes²⁷ and liver²⁸ that oxidation of testosterone is favoured when the pH is above 7.4. Accumulation of the products of anaerobic metabolism such as lactic acid¹⁴ will inhibit this conversion. It is accepted that intestinal absorption of some substances — particularly drugs — is reduced *in vitro*,²⁹ but qualitative changes in metabolic pathways can clearly produce highly misleading results. This empha-

sises the need for caution in the interpretation of such *in vitro* studies.

CLINICAL IMPLICATIONS

A reversible abnormality of plasma androgens has been described in men with untreated coeliac disease.³⁰ It was suggested that impaired 5α -reduction of testosterone in the gut might account for this abnormality, particularly as at least 70% of plasma DHT is derived from peripheral conversion of testosterone.³¹ The present study failed to identify dihydrotestosterone as a major portal vein metabolite, which makes it highly unlikely that the intestine is an important source of circulating dihydrotestosterone. This contention is supported by a recent study in man.³²

The present study provides further evidence that intestinal mucosal 17β -HSD activity, rather than metabolism by the liver,³³ is the major factor leading to the testosterone's lack of oral potency.³⁴ Protection of the 17-position by addition of a methyl group to testosterone produces an orally active androgen³⁵ which is clearly no longer a substrate for 17β -HSD. This emphasises the importance of the enzyme 17β -HSD in determining the fate of orally ingested androgens and possibly oestrogens.

Intestinal 5α -reductase activity may contribute to the potency of a new orally active testosterone ester, testosterone undecanoate (TU)³⁶⁻³⁷ by its conversion to 5α -DHTU in the intestine. Plasma dihydrotestosterone levels were restored before testosterone levels in hypogonadal men receiving replacement therapy with testosterone undecanoate. In addition, 5α -reduction of testosterone to dihydrotestosterone may be necessary before testosterone can exert its stimulatory effects on intestinal epithelial cell proliferation^{38,39} and on the activity of certain mucosal glycolytic enzymes.⁴⁰

The present study confirms that the gastrointestinal tract has the potential to take an active role in peripheral androgen metabolism and may directly influence the plasma concentrations of some circulating androgens. In addition, the gut is likely to be an important factor determining the oral potency of androgens and possibly other steroids, as evidence is accumulating that the gut is also involved in the metabolism of oestrogens,⁴¹ progesterone,^{42,43} hydrocortisone,⁴⁴ and aldosterone.⁴⁵

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