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STEROID GLUCURONIDES: HUMAN CIRCULATORY LEVELS AND FORMATION BY LNCaP CELLS

ALAIN BÉLANGER,* MICHÈLE BROCHU, DANIEL LACOSTE, CAROLINE NOËL, FERNAND LABRIE, ANDRÉ DUPONT, LÉONELLO CUSAN, SIMON CARON and JEAN COUTURE

MRC Group in Molecular Endocrinology, CHUL Research Center and Laval University, Centre Hospitalier de l'Université Laval, 2705 Blvd Laurier, Ste-Foy, Québec, Canada G1V 4G2

Summary—We studied the relationship between circulating androsterone glucuronide, androstane-3α,17β-diol glucuronide and androstane-3β,17β-diol glucuronide concentrations and adrenal as well as testicular C-19 steroids in men. Among the three 5α-reduced steroid glucuronides, androsterone glucuronide is the predominant C-19 steroid glucuronide measured in plasma and its levels are markedly elevated compared to those of the non-conjugated steroid. The marked rise in testosterone during puberty was strongly correlated with the increase in both androsterone glucuronide and androstane-3α,17β-diol glucuronide, thus suggesting that testicular C-19 steroids are the main precursors of the steroid glucuronides. We also found that the presence of testicular androgen in plasma contributes to approx. 70% of plasma androsterone glucuronide and androstane-3α,17β-diol glucuronide. Our data suggest that the adrenal C-19 steroids remaining in circulation after castration in men are converted into potent androgen which are then glucuronidated by UDP-glucuronyltransferase. We also demonstrated that the human prostate cell line LNCaP is capable of converting to a large extent androstenedione into androsterone glucuronide. Our data further confirm that glucuronidation is a major pathway of steroid metabolism in steroid target tissues.

INTRODUCTION

There is accumulating evidence that the metabolism of testosterone in target tissue proceeds through a steroid reduction step with the formation of dihydrotestosterone and androstane-3α,17β-diol, and glucuronidation of the metabolites [1]. The data obtained by Moghissi *et al.* [2] and Mauvais-Jarvis *et al.* [3] suggest that androstane-3α,17β-diol glucuronide might be a good marker of testosterone transformation. Hence, the increase of 5α-reductase activity in the skin of both hirsute women with normal androgen levels and those with polycystic ovary syndrome was shown to be accompanied by a dramatic increase of plasma androstane-3α,17β-diol glucuronide [4].

Glucuronidation is the most important metabolic pathway by which lipophilic steroids are rendered more water-soluble; a process that usually enhances their rates of excretion. Conjugation with glucuronic acid is therefore important in regulating the levels of unconjugated steroids in tissues and, thus, their biological activities. The UDP-glucuronyltransferases (EC 2.4.1.17) that catalyze this process are found in many tissues, namely, liver, skin and prostate [5–7]. Evidence from enzymatic studies and cDNA cloning, has shown that there are several forms of this enzyme. Among the UDP-glucuronyltransferases isolated so far, two of these enzymes convert steroids; one glucuronidate testosterone, dihydrotestosterone and estradiol at position 17 and the second, glucuronidate etiocholanolone and androsterone at position 3 [8, 9].

The present study has been designed to extend our work done on steroid glucuronides in men. It was believed important to determine whether other steroid glucuronides, in addition to androstane-3α,17β-diol glucuronide, could be used as indicators of androgen metabolism in men. We compared circulating non-conjugated and steroid glucuronides in men during aging and, in patients with cancer of the prostate before and after castration. We also determined the influence of the plasma levels of sex hormone-binding protein (SHBG) on the levels of steroid glucuronides. Finally, the conversion of androstenedione into steroid glucuronides was studied using the LNCaP cancer cell line.

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*To whom correspondence should be addressed.

SUBJECTS AND METHODS

Participants

In the first study, aimed to determine the plasma steroid levels in men from infancy to adulthood, samples were drawn from 56 boys, aged 3–16 years (8/group in 2 year age levels) and from normal adult men ($n = 9$; 20–30 years old). The prepubertal and pubertal subjects had normal weight and height development. In the second study, 40 patients (aged 50–83 years) with histologically proven advanced prostatic cancer were used. Eighteen of which had been castrated in the year preceeding the study. In the last study, plasma steroid levels were determined in 14 vegetarian and 15 non-vegetarian (omnivorous) white caucasians of French Canadian origin ranging from 25–35 years of age.

Extraction procedure and chromatography on C-18 columns

Ethanol (5 ml) was added to 1 ml of plasma and centrifuged. The resulting pellet was further washed with 2 ml of ethanol and the two ethanol extracts were combined and evaporated under nitrogen. The C-18 columns (Amersham Canada, Oakville, Ontario, Canada) were conditioned by subsequently passing 10 ml of methanol, 10 ml of water and 10 ml of methanol–water (5:95, by vol; mixture A). The extracts were solubilized in 2 ml of mixture A and deposited on the C-18 columns. After washing the columns with 2 ml of mixture A, 5 ml of methanol–water (40:60, by vol) were added to elute the glucuronide and the sulfate derivatives. Addition of 5 ml of methanol–water (85:15, by vol) resulted in the elution of the non-conjugated steroids. Both fractions were evaporated with a Speed-Vac Evaporator (Savant Instruments, Armingdale, NY, U.S.A.)

Hydrolysis of the steroid conjugates

The residual pellet of the conjugate fraction was solubilized with 1 ml of 0.1 M phosphate buffer (pH 6.5). The steroid glucuronides were hydrolyzed with β-glucuronidase (Boeringer, Mannheim, Germany) for 72 h at 37°C with two daily additions of 4 U β-glucuronidase (studies with different amounts of enzyme indicated that maximal steroid release was achieved using 2 U enzyme). The steroids released were then extracted twice with ethyl ether, while the organic phase was evaporated and the residue stored for further analysis. The water phase was kept for the sulfate solvolysis. The solvolysis procedure was performed as follows: 0.1 ml of 12 M HCl and 5 ml of ethyl ether saturated with HCl were added to the water phase and the mixture was left to stand at room temperature overnight. After centrifugation, the organic was isolated and evaporated; the residue solubilized with 1 ml of 0.2 M phosphate buffer (pH 7.0) and further extracted twice with 5 ml of ethyl ether. The organic phase was evaporated and the residue stored for further analysis.

Chromatography on LH-20

The chromatography was performed as described previously [10, 11]. In brief, the non-conjugated steroids from the three fractions were solubilized in 1 ml of isooctane–toluene–methanol (90:5:5, by vol) and deposited on Sephadex LH-20 columns (Pharmacia, Uppsala, Sweden). Elution was performed by increasing the polarity of the organic solvent mixture and 5 fractions were collected. After deposition of steroids, 5 ml of isooctane–toluene–methanol (90:5:5, by vol) were eluted and discarded. The first fraction containing progesterone was eluted with 10 ml of the same mixture. After addition of 20 ml of isooctane–toluene–methanol (90:5:5, by vol) androsterone, androstenedione, dihydrotestosterone and dehydroepiandrosterone were collected. Isolation of testosterone, androstane-3α,17β-diol and androstane-3β,17β-diol was achieved by elution of another 20 ml of the solvent. Addition of 15 ml of the solvent mixture isooctane–toluene–methanol (70:15:15, by vol) caused the elution of androst-5-ene-3β,17β-diol.

Measurement of SHBG

Plasma SHBG levels were measured by direct immunoradiometric method using a commercial kit from Farmos Diagnostic (Turku, Finland). The limit of detection was 3 nmol/l and the intra- and interassay coefficients of variation were 3 and 7%, respectively. All samples were analyzed in the same assay.

Cell culture and androstenedione metabolism

LNCaP human prostate cancer cells were obtained from the American Tissue culture collection (Rockville, MD, U.S.A.) and cultured in RPMI-1640 medium supplemented with non-essential amino acid, 100 IU of penicillin/ml, 100 μg of streptomycin/ml and 10% (v/v) fetal bovine serum (Hyclone Logum, UT, U.S.A.) under a humidified atmosphere of 95% air CO_2 at 37°C. LNCaP cells were plated at a density of 5×10^6 cells/plate and 1 ml containing 120 pmol

tritiated androstenedione was added for a period of 24 h. Products of the metabolism of tritiated steroids were assayed as follows. Medium was extracted twice with 5 ml ethyl ether–acetone (1:1, by vol), the extract was evaporated to dryness. The C-18 columns were conditioned as described above. The residue were solubilized in 2 ml and deposited on the C-18 columns. After washing the columns with 2 ml of mixture A, 5 ml of methanol–water (40:60, by vol) were added to elute the glucuronide derivatives. Addition of 5 ml of methanol–water (85:15, by vol) resulted in the elution of the non-conjugated steroids. Both fractions were evaporated with a Speed-Vac Evaporator. The steroid glucuronides were hydrolyzed and analyzed on HPLC. The formation of tritiated metabolites was calculated by dividing the amount of radioactivity in the product peak by the sum of the radioactivity in all of the peaks recovered after chromatography, thus obtaining the percent conversion to that product. Percent conversion was expressed in picomoles of product formed from the known amount of precursor added.

Calculations

RIA data were analyzed using a program based on Model II of Rodbard and Lewald [12]. Statistical significance was measured according to the multiple-range test of Duncan–Kramer [13]. All RIA results are shown as the means ± SEM of duplicate determinations on individual samples.

RESULTS

Figure 1 shows that from the age of 3 to 15 years there was a progressive and parallel increase in both dehydroepiandrosterone sulfate and dehydroepiandrosterone plasma concentration (from 89 ± 31 and 0.39 ± 0.02 to 1115 ± 263 and 1.39 ± 0.11 ng/ml, respectively). Androstenedione and androst-5-ene-3β,17β-diol levels increased in the age group 11–12 years, corresponding to the period of puberty. In Fig. 2, it can be seen that the abrupt increase in testosterone at puberty was not accompanied by a parallel rise in dihydrotestosterone, androstane-3β,17β-diol and androsterone. On the contrary, plasma androstane-3α,17β-diol glucuronide and androsterone glucuronide levels were markedly affected by the change in circulating adrenal and testicular C-19 steroids (Fig. 3).

Figure 4(A) illustrates the plasma levels of non-conjugated testosterone, dihydrotestosterone, androstane-3α,17β-diol and androsterone

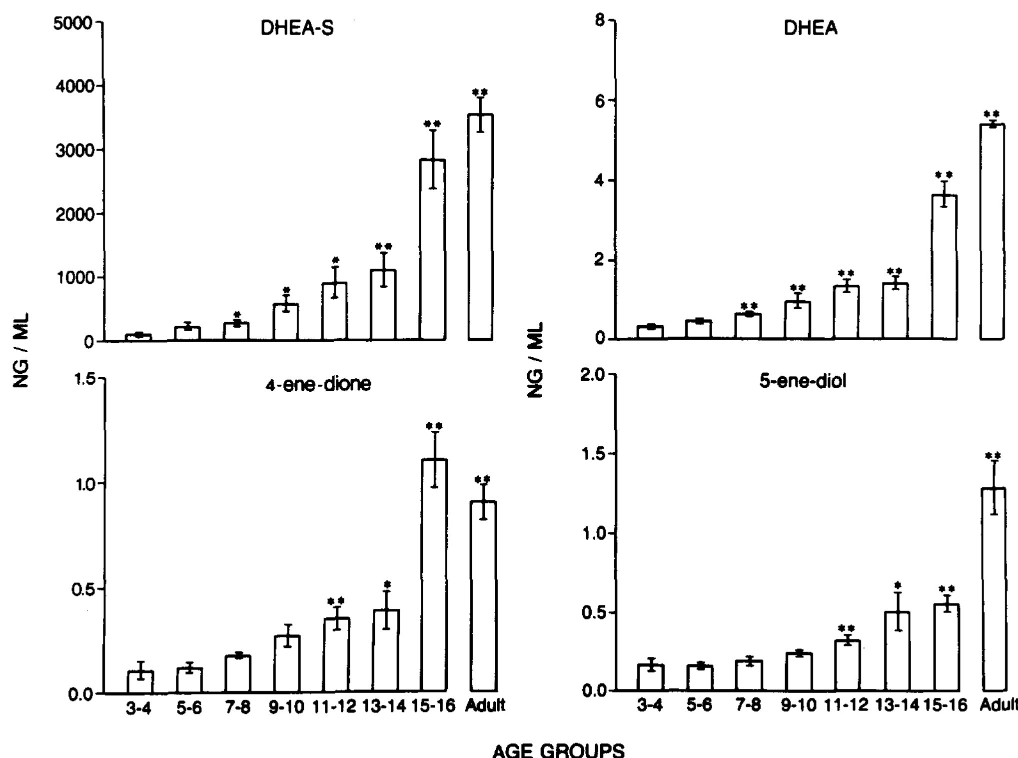

Fig. 1. Mean (±SEM) plasma dehydroepiandrosterone sulfate (DHEA-S), dehydroepiandrosterone (DHEA), androst-5-ene-3β,17β-diol(5-ene-diol) and androstenedione (4-ene-dione) concentrations in normal male subjects of increasing age. *$P < 0.05$; **$P < 0.01$ (vs group 3–4).

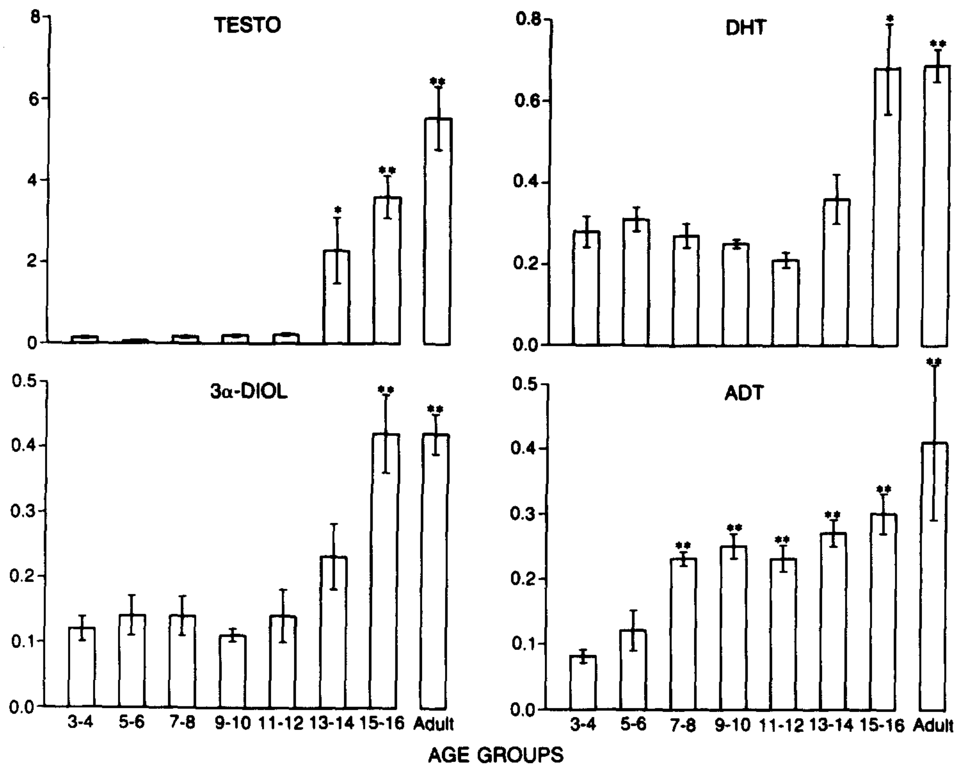

Fig. 2. Mean (±SEM) plasma testosterone (TESTO), dihydrotestosterone (DHT), androstane-3α,17β-diol(3α-diol) and androsterone (ADT) concentrations in normal male subjects of increasing age. $*P < 0.05$; $**P < 0.01$ (vs group 3–4).

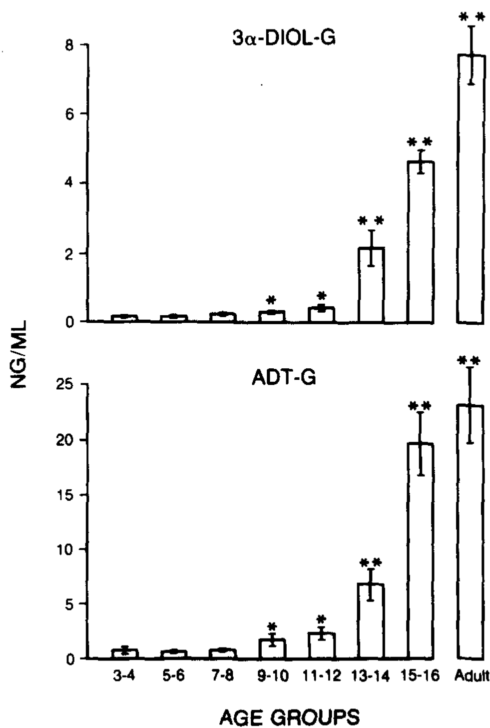

Fig. 3. Mean (±SEM) plasma androstane-3α,17β-diol glucuronide (3α-diol-G) and androsterone glucuronide (ADT-G) concentrations in normal male subjects of increasing age. $*P < 0.05$; $**P < 0.01$ (vs group 3–4).

in non-castrated and castrated men with prostatic cancer. Castration caused a decrease of >90% in plasma testosterone and dihydrotestosterone as well as a reduction of plasma androstane-3α,17β-diol and androsterone concentrations by approx. 25 and 40%, respectively. Figure 4(B) indicates that except for dihydrotestosterone glucuronide, which was not detected

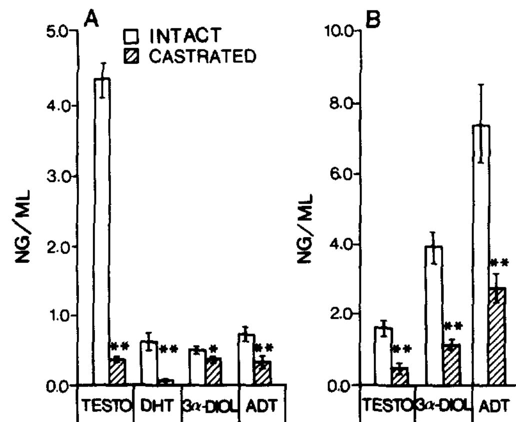

Fig. 4. Mean (±SEM) plasma testosterone (TESTO), dihydrotestosterone (DHT) androstane-3α,17β-diol(3α-diol) and androsterone (ADT) concentrations in intact and castrated patients with cancer of the prostate. A: non-conjugated steroid. B: glucuronide derivative. $**P < 0.01$.

Table 1. Plasma SHBG, androsterone glucuronide (ADT-G), androstane-3α,17β-diol glucuronide (3α-diol-G) and androstane-3β,17β-diol glucuronide (3β-diol-G) levels in 14 omnivorous (O) and 15 vegetarian (V) men during a 2-day period

	DAY 1		DAY 2	
	08.00 h	16.00 h	08.00 h	16.00 h
SHBG (nmol/l)				
V	34.5 ± 2.8*	ND[a]	36.4 ± 4.4*	ND
O	24.8 ± 1.7	ND	23.5 ± 2.9	ND
Steroid glucuronides (nmol/l)				
ADT-G				
V	69.0 ± 0.7*	66.0 ± 8.1**	66.7 ± 5.3*	60.7 ± 6.0**
O	44.6 ± 4.5	33.4 ± 5.0	43.9 ± 6.1	33.4 ± 3.2
3α-diolG				
V	11.1 ± 1.5*	10.9 ± 1.3*	13.4 ± 1.3*	11.9 ± 1.2
O	17.7 ± 2.3	16.6 ± 1.7	21.5 ± 3.0	14.3 ± 2.5
3β-diol-G				
V	1.9 ± 0.2**	2.0 ± 0.2**	2.4 ± 0.2**	2.3 ± 0.2**
O	4.0 ± 0.03	3.5 ± 0.3	4.25 ± 0.4	3.5 ± 0.3

*$P < 0.05$; **$P < 0.01$, significantly different from omnivorous group. [a]ND, not determined.

in non-castrated and castrated men, testosterone and 5α-reduced steroid glucuronide levels were markedly lowered in castrated patients. Specifically, mean testosterone glucuronide, androstane-3α,17β-diol glucuronide and androsterone glucuronide were diminished by approx. 60% in castrated patients.

We next studied the plasma steroid levels in vegetarian and omnivorous men. While the vegetarian and omnivorous subjects studied did not differ in their mean ages and height, the body mass index was significantly lower in the vegetarian group (data not shown). There were no differences in plasma dehydroepiandrosterone sulfate, testosterone, dihydrotestosterone and estradiol concentrations (data not shown). As shown in Table 1, significantly higher values in SHBG levels were however observed in the vegetarian group and, consequently, the free androgen index (FAI) calculated as 100 times the ratio testosterone/SHBG decreased by approx. 25%. Table 1 also shows a 25–50% lower plasma level of androstane-3α,17β-diol glucuronide and androstane-3β,17β-diol glucuronide in vegetarians compared to omnivorous.

Table 2 illustrates the results obtained while studying the metabolism of tritiated androstenedione by LNCaP cells in culture. Incubation of tritiated androstenedione for a period of 24 h caused a marked conversion of the precursor into androsterone glucuronide when analyzed using the C-18 columns and HPLC technique.

Table 2. Metabolism of tritiated androstenedione (120 pmol) by LNCaP cells incubated for 24 h

Steroid metabolites	Non-conjugated	Glucuronide derivatives
Androstenedione	42	—
Testosterone	6	2
Dihydrotestosterone	13	5
Androsterone	—	52

Analysis of radioactive steroids formed were carried out by C-18 columns and HPLC. Data were presented as pmol found in medium at the end of the period of incubation.

DISCUSSION

Previous results have clearly established that androstane-3α,17β-diol glucuronide concentrations in plasma are much higher than those observed for the non-conjugated steroid and suggest that the levels of this steroid glucuronide in plasma might be a good marker for androgen metabolism in target tissue. Our data confirm these observations and further demonstrate that, in adult men, the levels of androsterone glucuronide in plasma are also markedly elevated compared to those of the non-conjugated steroid. In fact, androsterone glucuronide levels exceed by 3-fold those observed for androstane-3α,17β-diol glucuronide and represent the predominant C-19 steroid glucuronide measured in plasma.

The slight increase in plasma non-conjugated testosterone metabolite levels observed between 9 and 15 years of age, as opposed to the 10- to 15-fold increase in dehydroepiandrosterone sulfate and testosterone in circulation, is indicative of the scant information obtained when only non-conjugated steroids are measured. The marked rise in testosterone during puberty was strongly correlated with the increase in both androsterone glucuronide and androstane-3α,17β-diol glucuronide, thus suggesting that testicular C-19 steroids are the main precursors of the steroid glucuronides. This is further confirmed by data obtained with castrated patients having cancer of the prostate. In fact, we noted that plasma testosterone levels in castrated men were lowered by 90% while there is a 63% reduction of androsterone glucuronide and a 72% reduction of androstane-3α,17β-diol glucuronide. This observation thus supports the hypothesis that the two 5α-reduced steroids are major metabolites of testicular steroids. In addition, our data demonstrate that the adrenal C-19 steroids remaining in circulation after castration are converted into potent androgen which are glucuronidated by UDP-glucuronyltransferase. We can also conclude that the presence of adrenal C-19 steroids in plasma contributes to approx. 40% of plasma androsterone glucuronide and androstane-3α,17β-diol glucuronide Using vegetarian and omnivorous subjects, we also demonstrated that the levels of SHBG and consequently, the FAI differs in both groups, the

omnivorous group having less SHBG in circulation. In agreement with the higher testosterone level available in the omnivorous group, we found a much higher level of plasma androstane-3β,17β-diol glucuronide and androstane-3α,17β-diol glucuronide, thus suggesting an enhancement of testosterone metabolism in the omnivorous group. Furthermore, a significant negative correlation was observed between plasma androstane-3α,17β-diol glucuronide and SHBG (data not shown), thus further confirming the role of free testosterone as a major source of androstane-3α,17β-diol glucuronide in men.

In addition to liver, UDP-glucuronyltransferase activity has been previously reported to be present in several tissues, namely prostate and skin. Recently, it was shown that MCF-7 glucuronidate estrogen at a very high rate of conversion [14]. In the present study, we demonstrated that the human prostate cell line LNCaP is capable of converting to a large extent androstenedione into androsterone glucuronide. These data further confirm that glucuronidation is a major pathway of steroid metabolism in steroid target tissues and has to be taken into account in the regulation of steroid concentrations.

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