Implication of human UGT2B7, 2B15, and 2B17 in 19-norandrosterone metabolism

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INTRODUCTION

Nandrolone (19-nortestosterone) is an anabolic androgenic steroid commonly abused for doping purposes. Nandrolone is mainly metabolized in the liver into 19-norandrosterone prior to glucuronidation and excretion through urine over an extended period of time. Several UGTs (i.e., UGT2B7, UGT2B15, and UGT2B17) are thought to be the major enzymes responsible for conjugation of androgens in human. An in vitro study using recombinant enzymes expressed in insect cells showed that UGT1A4 and UGT2B7 are the two main enzymes responsible of 19-norandrosterone glucuronidation. However, the identity of the enzyme involved in nandrolone metabolism in vivo together with their relative contribution and regulation remain unknown. Inhibition assays using human liver microsomes (HLM) incubated with 19-norandrosterone and selective inhibitors confirmed that UGT2B7 and UGT2B15 are involved in 19-norandrosterone glucuronidation, since the presence of the specific UGT2B7 and UGT2B15 inhibitors gemfibrozil and valproic acid inhibited the 19-norandrosterone glucuronidation by 35 and 45%, respectively. HLM were genotyped for UGT2B15 D85Y, UGT2B7 H268Y, and the UGT2B17 deletion polymorphism. The glucuronidation activity on 19-norandrosterone was significantly higher in UGT2B15 DD than in the other UGT2B15 genotypes (p < 0.05). Moreover, human liver cancer HepG2 cells were exposed to androgens to determine if the transcriptional activity of the genes of interest was affected. Only UGT2B7 mRNA expression was significantly increased (1.8-folds) after incubation with nandrolone deconate. These results show that the UGT2B7 and UGT2B15 are involved in 19-norandrosterone glucuronidation and that the UGT2B15 polymorphism (D85Y) is the only UGT genetic variation that influences the glucuronidation activity. This could partly explain the inter-individual variation in 19-norandrosterone excretion.

Keywords: anabolic androgenic steroid, nandrolone, 19-norandrosterone, UGT2B7, UGT2B15
prostate cancer (Levesque et al., 1997; MacLeod et al., 2000). A deletion polymorphism of the UGT2B17 gene has been identified (Murata et al., 2003; Wilson et al., 2004) and correlated to glu-
curoconjugation of testosterone (Jakobsson et al., 2008). A lack
of this gene leads to a crucial decrease in urinary testosterone even after a testosterone injection (Schulze et al., 2008). However, there are no studies reporting UGT2B genetic variation and 19-NA glu-
curonidation activity.

The aim of this study was to identify the influence of UGT2B7, UGT2B15, and UGT2B17 genetic polymorphisms on 19-NA glu-
curonidation in order to better understand the large inter-
individual variation in nandrolone elimination. Moreover, we
investigated if supra-physiological doses of androgens affect the
gene expression of UGT2B enzyme genes in HepG2 cells.

MATERIALS AND METHODS
LIVER SAMPLES
Fifty human liver tissue samples were obtained from human donors to our human liver bank (approved by the Ethics Review
Board in Stockholm at Karolinska Institutet). The livers were
donated to our human liver bank (approved by the Ethics Review
Committee of the University of Stockholm). The livers were
homogenized in 50 mM potassium phosphate buffer (pH 7.4) and
stored at -80°C until use. The protein concentration was determined
at -80°C until use. The protein concentration was determined
according to Lowry et al. (1951).

GENOTYPING
Genomic DNA from human liver homogenate was obtained using QIAamp DNA Blood Mini Kit from Qiagen (Hilden, Ger-
many). UGT2B7 polymorphism (rs7439366) which alter the
amino acid sequence Histidine (H) to Tyrosine (Y) at position 268 were investigated by 5’nuclease activity method, using upper primer
AGCTGACGTATGGCIT hydrolysis probe 
and lower primer GGGTTTGCGAGGTTTGCAGTA.

The PCR reaction was carried out in 15 µL volume including 1–3 µL of genomic DNA, 2 × Taqman universal master mix, and run on an ABI 7500
Fast from Applied Biosystems. The PCR method consisted of an initial denaturation step at 95°C for 10 min followed by 40
cycles of denaturation for 15 s and annealing/elongation at 60°C
for 1 min. The UGT2B17 deletion polymorphism was performed by real-time PCR analysis as described elsewhere (Schulze et al., 2008).

GLUCURONIDATION ACTIVITY IN HUMAN LIVER MICROSOME AND
INHIBITION STUDY
Chemicals
Methanol, n-pentane, potassium dihydrogen phosphate (KH$_2$PO$_4$), potassium hydrogen phosphate (K$_2$HPO$_4$), natrium carbonate (Na$_2$CO$_3$), magnesium chloride (MgCl$_2$), and ethanethiol were provided by Merck (Darmstadt, Germany). Tris HCl, uridine 5’-diphospho-glucuronic acid (UDPGA) and natrium hydrogencarbonate (NaHCO$_3$) and ammonium iodide (NH$_4$I), methyltestosterone (Mct), gemfibrozil, valproic acid, and testosterone enanthate (TE) were provided by Sigma-Aldrich Chemie GmbH (Munich, Germany). β-glucuronidase from Escherichia coli (E. coli) was provided by Roche Diagnostics GmbH (Mannheim, Germany). N-methyl-N-(trimethylsilyl) trifluoroacetamide (MSTFA), were provided by Macherey-Nagel GmbH (Düren, Germany). Ultrapur water was obtained with a Milli-Q Reference Ultrapure Water Purification system equipped with a Q-Gard T1 purification pack and a BioPak Point-of-
Use ultrafilter from Millipore (Billerica, MA, USA). Nandrolone decanoate (19-NTD), 19-norandrosterone (19-NA), and 19-
norandrosterone glucuronide (19-NAG) were purchased from NMI (Pymble, Australia). Hecogenin acetate was provided by Tokyo Chemical Industry Co. (Tokyo, Japan).

SAMPLE PREPARATION
Human liver microsomes samples were thawed at 25°C and the
volume equivalent to a final protein concentration of 1.0 mg/mL
was spiked in a glass tube containing the 19-NA required for the
assay. The final reaction volume of 50 µL was obtained by addi-
tion of Tris HCl 50 mM pH 7.4 containing UDPG 20 mM
and MgCl$_2$ 50 mM. Inhibition assays were performed using specific
UGTs inhibitors, hecogenin acetate at 10 µM for UGT1A4 inhi-
bition, gemfibrozil 10 µM for UGT2B7, and valproic acid 1 mM
for UGT2B15. After incubation during the required time at 37°C
in a stirring bath, the reaction was stopped by addition of 2 mL
n-pentane. A liquid-liquid extraction (LLE) with two times 2 mL
n-pentane was performed to retrieve the free steroids. The organic
phase was collected in a new glass tube containing 100 ng MeT
as internal standard and evaporated to dryness under an air flow.
Steroids were finally derivatized at 60°C for 30 min with 50 µL
MSTFA/NH$_4$I/Ethanethiol (10:0:2:0.03, v/w/v) prior to injection onto GC-MS. The aqueous phase was treated by addition of 0.5 mL
steroid buffer 1 M pH 7.0 and 70 µL β-glucuronidase from
Es. coli before incubation 60 min at 50°C. Then, 200 mg of solid carbonato buffer [Na$_2$CO$_3$/NaHCO$_3$, 1:1 (w/w)] was added to
reach a pH of 8.5–9.0. A LLE was performed using two times
2 mL n-pentane. The organic layer containing the deconjugated
steroid was collected in a new glass tube containing 100 ng MeT
as internal standard and evaporated to dryness under an air flow.
Steroids were finally derivatized at 60°C for 30 min with 50 µL
MSTFA/NH$_4$I/Ethanethiol prior to injection onto GC-MS.

GC-MS ANALYSIS
The androgens quantification was performed on a gas chromatogra-
yph system Agilent 6890 (Palo Alto, CA, USA) equipped with a column Agilent HP-1 (17 m × 0.2 mm I.D., 0.11 µm) and coupled
to a mass analyzer Agilent 5973. A constant helium flow of
0.8 mL/min was applied on to the GC. The extracted sample (2 µL)
was injected in the split mode (1:10). The temperatures were set
at 250, 280, 150, and 230°C for the injector, transfer line, quadru-
pole, and ionization source, respectively. The GC oven temperature program was set as follow: hold 120°C for 1 min before gradi-
ent increase at 10°C/min to 200°C, then 2°C/min to 210°C, and
finally at 40°C/min to 310°C and maintained 2 min before the next
analysis. The ionization was performed at 70 eV. The compound identification was performed in the SCAN mode (m/z 50–500) with a scan time of 2.48 scan/s. The quantification was performed in the SIM mode with an analysis time of 30 ms/scan. Identity of 19-NA was controlled by the stability of the peak area ratios for m/z 405, 420, 315, 225, and 169. The ion with m/z 405 was corrected by MeT peak area (m/z 446) as internal standard and used for quantification.

UGT2B mRNA EXPRESSION IN HepG2 CELLS AFTER INCUBATION WITH ANDROGENS

Human liver cancer HepG2 cells from ATCC (Manassas, VA, USA) were cultured in MEM supplemented with 10% fetal bovine serum from the same provider and maintained in an incubator in humidified atmosphere at 37°C and 5% CO₂. Prior to androgen treatment, the HepG2 cells were split in 12-well plates and pre-incubated for 2–3 days. Nandrolone decanoate and TE were diluted in ethanol to 1 µM and added to the cells for 2 h prior to harvest using Trizol from Invitrogen (Carlsbad, CA, USA). The non-treated controls were incubated with vehicle only. The experiments were performed at eight independent times. Total RNA extraction from HepG2 cells was performed using 0.5 mL Trizol per well according to manufacturer’s instructions. RNA (0.5 µg) was reverse transcribed into cDNA with hexamer primer using first-strand cDNA synthesis kit from Invitrogen according to the manufacturer’s protocol and diluted 20 times in water.

The mRNA levels of UGT2B7, 2B15 and 2B17 in nandrolone treated HepG2s were determined by real-time PCR. Beta-actin (#4326315E) from Applied Biosystems was chosen as an endogenous housekeeping control gene. Quantitative real-time PCR was performed using the ABI 7500 Fast. Reaction mixtures contained SYBR green reaction mix from Kapa Biosystems (Woburn, MA, USA), UGTs primers from Cybergene for UGT2B7, UGT2B15, and UGT2B17 as described (Chouinard et al., 2006), 4 µL cDNA template in a total volume of 15 µL. Thermal cycling conditions included activation at 95°C for 10 min followed by 40 cycles of denaturation at 95°C for 15 s and annealing/elongation at 60°C for 1 min. Each reaction was performed in duplicate and no-template controls were included in each experiment. The untreated sample was employed as a calibrator and the delta CT-formula was used as described elsewhere (Schmittgen and Livak, 2008). The gene expression was quantified as the yield of the target gene relative to that of β-actin gene.

DATA ANALYSIS

Statistical analysis was performed using GraphPad Prism Software v 4.3 (San Diego, CA, USA). The genotype association analysis was calculated using one-way ANOVA and the mRNA expression levels in androgen and vehicle exposed cells were compared using Student’s t-test. Data are presented as mean ± SD.

RESULTS

HUMAN LIVER MICROSOMES ASSAYS – INHIBITION STUDIES

Less than 1.5% of free 19-NA was detectable in 19-NAG solutions, and 19-NAG remained stable in the buffer without HLM when warmed at 37°C for 100 min. No increase of 19-NA concentration was observed. The inhibition assay showed a significant inhibition of 19-NAG production by 35 ± 11% for UGT2B7 (p = 0.04) and 45 ± 12% for UGT2B15 (p = 0.03) specific inhibitors valproic acid and gemfibrozil (Figure 1). No significant inhibition was observed with the UGT1A4 inhibitor hecogenin.

GENETIC VARIATION AND 19-NA GLUCURONIDATION

UGT2B7 H268Y genotype was determined for 50 HLM prepared from our liver bank [YY (n = 12), YH (n = 25), HH (n = 13)]. The same HLM were incubated with 19-NA to determine their glucuronidation activity. No significant correlation between glucuronidation activity and UGT2B7 H268Y genotypes was revealed (Figure 2A). The same analysis was performed for UGT2B15 D85Y polymorphism. But due to problems during genotyping, only 42 of the HLM previously analyzed were genotyped [DD (n = 9), YD (n = 22), YY (n = 11)]. UGT2B15 DD homozygotes showed a significantly higher glucuronidation capacity than YD (p = 0.05) and YY (p = 0.02) subjects (Figure 2B). No significant difference in glucuronidation was observed between YD and YY genotypes. There was no significant difference between gender and UGTs glucuronidation activity.

Among the all HLM, nine microsomes of each UGT2B17 deletion polymorphism group (del/del, ins/del, ins/ins) were selected. No significant difference was reported between genotypes regarding glucuronidation activity (data not shown).

UGTs mRNA EXPRESSION IN HepG2 CELLS

UGT2B7 mRNA expression was significantly increased in human liver HepG2 after incubation at 37°C for 2 h with 1 µM nandrolone decanoate (p = 0.02) and TE (p = 0.04) compared to control HepG2s. The percentage of 19-norandrosterone glucuronide produced from 1 µM 19-norandrosterone in presence of UGTs specific inhibitors (hecogenin acetate 10 µM for UGT1A4, gemfibrozil 10 µM for UGT2B7, and valproic acid 1 mM for UGT2B15) in human liver microsomes (n = 5) presented as mean ± SD. Significant inhibition was found for the UGT2B7 and UGT2B15 specific inhibitors.
incubation with the vehicle only (Figure 3A). UGT2B15 mRNA expression was significantly increased only after incubation with TE ($p=0.01$) (Figure 3B).

**DISCUSSION**

The results of the inhibition study using UGT2B7 specific inhibitor gemfibrozil confirmed that this UGT is involved in the glucuronidation of the hydroxyl group in the $3\alpha$ position of 19-NA. This is in line with *in vitro* observations from other studies (Jin et al., 1997; Coffman et al., 1998; Gall et al., 1999). However, the specific inhibition of the UGT1A4 enzyme by hecogenin acetate did not affect the HLM ability to glucuronidate 19-NA, which was previously suggested (Kuuranne et al., 2003). Surprisingly, UGT2B15, known to be involved in the glucuronidation of the $17\beta$-OH position in androgens seems to be involved in 19-NA glucuronidation since its specific inhibitor, valproic acid, inhibited about 45% of the HLM glucuronidation activity.

The average glucuronidation activity in all tested HLM was of $84.5 \pm 10.7$ pmol/min/mg protein, that represents a glucuronidation of about 85% of the initial 19-NA added to the samples. This shows the high capacity of human liver microsome to conjugate 19-NA by glucuronidation. In addition to be excreted as glucuronides, 19-NA is also sulfated to some degree (Strahm et al., 2009) by cytosolic sulfotransferases.

A significantly higher glucuronidation activity was detected in individuals homozygous for the UGT2B15 D allele as compared to Y carriers. Information about the polymorphic variants of UGT2B15 activity toward androgens is conflicting. An early *in vitro* report indicated that the Y carriers show a higher activity than D carriers on dihydrotestosterone and androstane-3α, 17β-diol (Levesque et al., 1997), whereas another group reported that the D carriers have a higher glucuronidation activity than the Y carriers on the same androgens (Court et al., 2002).
No correlation with the polymorphism UGT2B7 H268Y was detected by us; which is in line with the results observed previously that UGT2B7 polymorphism did not influence the androstenedione glucuronidation in HLM (Bhasker et al., 2000) and the serum level of androstenedione glucuronide (Swanson et al., 2007). Androstenedione is similar to 19-NA except with a methyl in the 19 position. As expected, UGT2B17 deletion polymorphism did not affect the 19-NA glucuronidation since this enzyme prefers glucuronidation of steroids at 17β-OH position and consequently plays an important role in testosterone glucuronidation (Jakobsson et al., 2006). As reported elsewhere for other substrates (Gallagher et al., 2010; Ekstrom et al., 2012) no sex differences in 19-NA glucuronidation activity was observed. UGT2B7 mRNA expression was increased in HepG2 cells after incubation with 19-NT and TE whereas UGT2B15 mRNA expression was increased only after the incubation with TE. Our results indicate that androgens induce enzymes involved in its own elimination, which may further contribute to the inter-individual variability in disposition of 19-NA after the administration of nandrolone in healthy volunteers (Strahm et al., 2009) and in AAS abusers (Garevik et al., 2011). UGT2B17 on the other hand was not affected by androgen exposure of HepG2 cells. These results are opposite to what have been found in prostate cancer cells, where UGT2B15 and UGT2B17 has been shown to be down-regulated by androgens (Guillemette et al., 1997).

In conclusion, we show here that UGT2B7 and UGT2B15 are the main UGTs responsible for the glucuronidation of the main metabolite of nandrolone. However, only UGT2B15 D85Y polymorphism influences the glucuronidation of 19-norandrostenedione with an activity significantly higher for the DD carriers. This could partly explain the inter-individual variation in 19-norandrostenedione excretion, but a clinical study of nandrolone kinetics in healthy volunteers or studies in authentic nandrolone abusers would be required to confirm this hypothesis in vivo.

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