Increased UDP-glucuronosyltransferase Activity and Decreased Prostate Specific Antigen Production by Biochanin A in Prostate Cancer Cells

Xiao-Ya Sun, Cathie A. Plouzek, James P. Henry, Thomas T. Y. Wang, and James M. Phang

Laboratory of Nutritional and Molecular Regulation, National Cancer Institute–Frederick Cancer Research and Development Center, NIH, Frederick, Maryland 21702-1201

ABSTRACT

Our laboratory has characterized androgen metabolism in an androgen-responsive prostate cancer cell line (LNCaP) and showed that these cells accumulated intracellular testosterone primarily as glucuronidated metabolites. Using a cell-free assay with testosterone as substrate, we showed that LNCaP had UDP-glucuronosyltransferase (UDPGT) activity. Because dietary factors, such as flavonoids in soy products, may reduce the risk for hormone-dependent cancers, we studied the effects of flavonoids on testosterone-UDPGT activity. LNCaP cells were exposed to selected flavonoids for up to 6 days. The increase in UDPGT-specific activity was linear over this period. Of the compounds tested, biochanin A was the most potent, with increased activity at concentration range 0.5–50 μM. Activities were linear for time and protein and were unaffected by flavonoids added directly to the assay. Kinetics studies showed no change in Km for testosterone in the face of these large increases in specific activity. Cellular metabolism of testosterone reflected the increase in enzyme activity. Intact cells treated with biochanin A produced testosterone-glucuronide from testosterone at twice the rate of controls. The steroid form of the UDPGT transcript was expressed in LNCaP cells and was enhanced in biochanin A-treated LNCaP cells. Additionally, biochanin A markedly decreased prostate specific antigen (PSA) level against the effect of testosterone on PSA production. Biochanin A significantly decreased the testosterone-stimulated release of PSA, presumably because biochanin A increased UDPGT and increased the intracellular glucuronidation of testosterone. These studies suggest that the modulation of hormone metabolism by dietary factors may be important in the prevention and treatment of prostate cancer.

INTRODUCTION

It is well established that androgens (testosterone and its metabolites) play an important role in the normal growth and function of the prostate (1). Changes in androgen metabolism or responsiveness to androgens have been implicated in the formation of benign prostatic hypertrophy and prostate cancer (1).

The conjugation of steroid hormones with UDP-glucuronic acid is catalyzed by the enzyme UDPGT2 (EC 2.4.1.17; Ref. 2). This reaction, which converts steroids to more polar derivatives, not only inactivates but also expedites its elimination from tissues (3). Although conjugation of steroid hormones occurs primarily in the liver, it occurs also in peripheral tissues and may play a role in regulating androgen levels in the prostate. Our laboratory (4) and others (5) have characterized testosterone metabolism in an androgen-responsive prostate cancer cell line (LNCaP) and showed that these cells accumulated intracellular testosterone primarily as glucuronidated derivatives. Using a cell-free assay with testosterone as substrate, we showed that LNCaP prostate cancer cells had testosterone-UDPGT activity (4).

Because androgen levels in the prostate may be clinically important, the modulation of UDPGT and androgen metabolism by dietary factors is of considerable interest. Various studies have shown that consumption of soybean is associated with low incidence of prostate cancer in Asian populations (6). Flavonoids, a family of compounds plentiful in fruits and vegetables and especially in soy products, may reduce the risk for hormone-dependent cancers (7). We undertook the current studies to examine the possible effects of flavonoids on testosterone-UDPGT activity and on the metabolism of testosterone in prostate cancer cells. We found that UDPGT was induced by flavonoids in LNCaP cells, and this increase in UDPGT not only affected the conversion of testosterone to its conjugated glucuronide in intact LNCaP cells but also correlated with altered production of PSA, an androgen-responsive marker of prostate cancer (8).

MATERIALS AND METHODS

Chemicals. Radiolabeled ([3H]-1,2,6,7)testosterone was obtained from New England Nuclear (Boston, MA). Unlabeled testosterone, UDP-glucuronic acid, biochanin A, apigenin, kaempferol, quercetin, naringenin, and HPLC solvents were obtained from Sigma Chemical Co. (St. Louis, MO). Daidzein, genistein, galangin, formononetin, and prunetin were purchased from Indofine Chemical Co. (Somerville, NJ). Fresh stock solutions of various flavonoid compounds were prepared in DMSO at a concentration of 50 mm before each experiment.

LNCaP Cell Culture. The human prostatic carcinoma cell line LNCaP was obtained from the American Type Culture Collection (Rockville, MD). Cell culture components were purchased from Biofluids, Inc. (Rockville, MD). Cells were cultured (37°C, 5% CO2) in RPMI 1640 supplemented with 10% FBS, penicillin (100 U/ml) and streptomycin (100 μg/ml). Cells were passaged every 6–7 days by trypsinization, and the culture medium was changed every 2–3 days.

Cell Treatment for Enzyme Assay. LNCaP cells (1 × 106) were plated in 75-cm2 flasks in the presence of 25 mL of growth medium and allowed to attach for 2–3 days.

Preparation of Cell Homogenates for Enzyme Assay. Treated cells were harvested by scraping the cells into PBS, centrifuged, and resuspended in 1 mL of HBSS containing 25 mM HEPES buffer at pH 7.4 (9). The resuspended cells were disrupted by sonication, and the resultant homogenate was stored at -70°C. Protein concentrations for these homogenates were determined by Lowry's method (10) using BCA Protein Assay Reagent (Pierce, Rockford, IL).

UDPGT Assay. UDPGT activity was measured according to methods described previously (4). The reaction mixture contained: 1 μM [3H]testosterone, 4 μM unlabeled testosterone, 5 mM UDP-glucuronic acid, and 100 μM of homogenate protein obtained from LNCaP cells in a final volume of 1 mL (HBSS containing 25 mM HEPES buffer). After 1-h incubation at 37°C, the mixture was acidified with 25 μL of 1 N HCl for 15 min to release androgen steroids trapped by binding proteins (11). The samples were then neutralized with 1 mL of PBS and 5 μL of 10 n NaOH. Metabolites first were separated on C18 columns (Waters, Milford, MA) and then were analyzed on the Waters HPLC system with a Supelcosil 5 mm LC-18 reverse phase column (Supelco, Inc., Bellefonte, PA) (12). An isocratic mobile phase consisting of methanol:acetonitrile:water (15:45:40, v/v/v) for organic metabolites was used, whereas 35% acetonitrile, 0.1% trifluoroacetic acid in water at a flow rate of 1 mL/min
was used to identify polar metabolites. The effluent was fed into a FLO-ONE/Beta online radioactive flow detector (Radiomatic, Meriden, CT). The testosterone and conjugated testosterone were separated and identified by comparing the characteristic elution times with integrated standard peaks: testosterone and testosterone glucuronide. Characterization of metabolites in the HPLC eluant was also performed with a mass spectrometer as described by Henry et al. (4). UDPGT activity was expressed as testosterone-glucuronide formation in pmol/h/µg protein.

**Cellular Metabolism of Testosterone.** LNCaP cells (1 × 10^6) were plated on day 0, and biochanin A (5 µM) where indicated was added to the medium on day 1. This medium was replaced with or without biochanin A on day 3. On day 6 at the beginning of the experiment, growth medium was removed and substituted with 2 ml of fresh medium without serum or biochanin A. After an hour of equilibration, cells were exposed to 2 ml of fresh medium containing [3H]testosterone (92.1 Ci/mmol) and unlabeled testosterone so that the final concentration of testosterone was 31.2 nM. This concentration was chosen to approximate the total plasma testosterone concentration of 20 nM (13). Cells were incubated at 37°C. After incubation, aliquots of media were collected and acidified with HCl for 15 min and then neutralized. Metabolites of testosterone from aliquots of media were separated and analyzed on HPLC as described in UDPGT assay.

**Determination of UDPGT mRNA Levels.** Poly(A)^+ RNA from LNCaP cells treated with or without biochanin A was isolated using the Fast Track 2.0 kit (Invitrogen, San Diego, CA) directly from LNCaP cells. Poly(A)^+ RNA was separated by electrophoresis in 1.2% agarose gels containing 2.2 M formaldehyde (14) and transferred to Nitran membranes (Schleicher and Schuell, Keene, NH) by electroblotting. The poly(A)^+ RNA was then fixed to the membranes by UV cross-linking. The filters were hybridized at 60°C (riboprobes) in a buffer containing 50% (v/v) formamide, 8X Denhardt's solution, and a uniformly labeled antisense riboprobe or a random-labeled probe (10^6 cpm/ml). Riboprobes used for Northern hybridization were the cDNA encoding a steroid UDPGT isoform, UDPGT 3 (15), which based on the current nomenclature (16), is designated UGT2B15, and the phenolic UDPGT enzyme, UGT 1F (17), both generous gifts of I. S. Owens, J. K. Ritter, and F. Chen, and pTRI-cyclophilin (Ambion, Inc., Austin, TX; Ref. 18). Northern blots were washed as described previously (19).

**Determination of PSA Level.** LNCaP cells were grown under three different sets of media conditions in the presence or absence of biochanin A (5 µM) for 5 days: (a) in charcoal treated serum; (b) in regular serum; or (c) in regular serum with additional testosterone (0.1 µM). Then medium was collected, and the levels of PSA were determined immunoenzymatically according to the instruction provided by the supplier (Hybritech, Inc., San Diego, CA).

### RESULTS

**Stimulation of UDPGT Activity by Selected Flavonoids on LNCaP Cells.** Flavonoids are a large class of natural compounds that are found in a wide variety of fruits and vegetables. The selected flavonoids were apigenin, galangin, kaempferol, quercetin, naringenin, and quercetin, whereas the tested isoflavones were biochanin A, daidzein, formononetin, genistein, and prunetin. Structures of the selected flavonoids were shown in Fig. 1.

LNCaP cells were exposed to each of the selected flavonoids for 6 days. All of the selected flavonoids stimulated the activity of testosterone-UDPGT, which conjugates testosterone to testosterone-glucuronide. Fig. 2 illustrates the relative effect on testosterone-UDPGT by selected flavonoids at a concentration of 5 µM. The most effective compound was biochanin A (5 µM), which stimulated UDPGT activity about 10-fold.

**Increase of UDPGT Activity by Biochanin A on LNCaP Cells.** Of the flavonoid compounds tested, biochanin A was the most potent stimulator of testosterone-UDPGT activity in LNCaP cells. Therefore, we studied the effect of treatment time and concentration of biochanin A on testosterone-UDPGT activity.

LNCaP cells were exposed to biochanin A (5 µM) or vehicle for up to 6 days, then the enzyme activity of UDPGT from cell homogenates was measured using testosterone as a substrate. We observed a linear increase in UDPGT-specific activity by biochanin A in LNCaP cells, whereas there was no change in enzyme activity for control treatment over this period (data not shown). Testosterone-UDPGT activity stimulated by biochanin A in LNCaP cells was also increased with concentration of biochanin A. After 6 days of cultivation, biochanin A stimulated testosterone-UDPGT activity 3-fold at 1 µM and about 18-fold at 20 µM as compared with control (Fig. 3). The enzyme reaction mixture also was carried out either with varying amounts of homogenate or with varying incubation time to characterize the property of the enzyme reaction of testosterone-UDPGT activity. The UDPGT activity of LNCaP cells treated with biochanin A (5 µM) was linear for the amount of protein and incubation time (data not shown) in the enzyme assay. The activity of control cells was unaffected by biochanin A added directly to the assay (data not shown).

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**Isoflavones**

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![Fig. 1. Structure of selected isoflavones and flavonoids.](Image)
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Fig. 2. Increased activity of testosterone UDPGT in LNCaP cells treated with a variety of flavonoids. Cells were treated for 5 days with 5 \mu M of the respective isoflavone or flavonoid. The assay for UDPGT was as described. Briefly, 100 \mu l of homogenate containing about 200 \mu g of protein were incubated for 1 h with testosterone as substrate. Specific activity (pmol/h-\mu g protein) of testosterone UDPGT was calculated, and values normalized to control activity were assigned a value of one. \square, control; \bullet, values obtained with treatment by the indicated flavonoid. Values shown represent means from at least three experiments; bars, SE.

Fig. 3. Concentration effect of biochanin A on testosterone UDPGT activity. LNCaP cells were incubated with biochanin A at variable concentrations for 6 days. Testosterone UDPGT activity was determined as described in "Materials and Methods." Values shown represent means from at least three experiments; bars, SE.

Enzyme Kinetic Study of UDPGT Activity Stimulated by Biochanin A. Enzyme kinetic parameters for testosterone-UDPGT activity with biochanin A treatment were determined to further elucidate the action of biochanin A. The reaction mixtures containing homogenate obtained from LNCaP cells treated with or without biochanin A (5 \mu M), UDP-glucuronic acid, and a variable amount of testosterone as substrate were used to determine the kinetic parameters for UDPGT enzyme reaction. Enzyme kinetic studies analyzed with the Michaelis-Menten equation for testosterone-UDPGT activity in homogenate of cells treated with or without biochanin A are shown in Fig. 4. We found that \(K_m\) (testosterone as substrate) for biochanin A-treated cells was similar to control (\(K_m = 9.1 \pm 2.2 \mu M\) for control, \(K_m = 10.9 \pm 2.0 \mu M\) for biochanin A-treated). Whereas, there was a large increase in \(V_{max}\) when cells were treated with biochanin A (\(V_{max} = 17.1 \pm 1.2\) pmol/h-\mu g protein for control, \(V_{max} = 60.3 \pm 3.0\) pmol/h-\mu g protein for biochanin A-treated).

Increased Testosterone Metabolism by Biochanin A on LNCaP Cells. To correlate changes in enzymatic activity of testosterone-UDPGT to cellular metabolism of testosterone, we also examined conversion of testosterone to testosterone-glucuronide in intact cells. The cells treated with biochanin A (5 \mu M) produced testosterone-glucuronide at twice the rate of controls (Fig. 5). This increase of testosterone-glucuronide from the culture medium treated with biochanin A was consistent with the induction of UDPGT enzyme activity from cells treated with the same flavonoid.

Expression of UDPGT. Having shown that biochanin A can induce the UDPGT activity in LNCaP cells, we sought to determine whether this effect is mediated by changes in the level of UDPGT expression. Chen and coworkers (15) demonstrated that UGT2B15,
production, LNCaP cells were grown in charcoal-treated serum (to strip steroid hormones from serum) or in regular serum with or without additional testosterone (0.1 μM). Under these conditions, the accumulation of PSA was approximately linear with time for up to 5 days (data not shown). When testosterone increased in the media, we indeed observed increasing PSA with increasing testosterone concentrations in the growth media as shown in Fig. 7. PSA levels were three times higher in normal serum than in stripped serum, whereas PSA levels were 3.7 times higher in normal serum with additional testosterone than in stripped serum and 87% higher than in normal serum.

Having showed that biochanin A can induce enzyme activity of testosterone UDPGT and thereby augment inactivation of testosterone in intact cells, we then asked whether this reduction of testosterone affects the testosterone-dependent production of PSA. When LNCaP cells were treated with biochanin A, we found that PSA levels markedly decreased under all three different media conditions in the presence of biochanin A compared with control (Fig. 7). With biochanin A treatments, production of PSA decreased 70% in stripped serum or in normal serum and decreased about 40% in normal serum with additional testosterone. This treatment of cells markedly decreased the production of PSA in the presence or absence of testosterone, supporting the notion that biochanin A can affect the biological functions of testosterone. The presumed inactivation of testosterone underlying this decrease in PSA was supported by the finding that testosterone added at higher concentrations could overwhelm the effect of biochanin A treatment. This study indicated that biochanin A decreased PSA levels, which is functionally dependent on testosterone.

**DISCUSSION**

It is well established that androgens (testosterone and its metabolites) play an important role in the normal growth and function of the prostate (1). Changes in androgen metabolism or tissue responsiveness to androgens have been implicated in the formation of benign prostatic hypertrophy and prostate cancer (1). For overall androgen metabolism, conjugation with UDP-glucuronic acid is the major role which is localized in liver, prostate, and testis, selectively glucuronidates a single category of steroids. Northern blot analysis of LNCaP RNA probed with the UGT2B15 cDNA showed that the level of UDPGT transcript was significantly increased after 5 days of treatment with 5 μM biochanin A. As shown on the Northern blot in Fig. 6, the UGT2B15 probe hybridized to 2.1- and 2.3-kb transcripts, both of which were enhanced in the presence of biochanin A. Additionally, RNase protection assays indicated that all protected fragments in LNCaP cells were equally enhanced in the presence of biochanin A (data not shown).

To further investigate the regulation of expression of UDPGT in LNCaP cells by biochanin A, we studied the effect of biochanin A on mRNA expression of a phenolic UDPGT isoform, UGT1F (17). In contrast to the steroid form from UDPGT, the phenolic UDPGT was not detectable in LNCaP cells with or without treatment of biochanin A (data not shown).

**Decreased Production of PSA by Biochanin A.** Because biochanin A increased testosterone-UDPGT and thus inactivated testosterone, we asked whether it would affect testosterone-dependent functions in LNCaP cells. PSA is a single-chain glycoprotein used as a marker of prostate cancer (8). The ability of androgen to increase the production of PSA has been demonstrated in human tissue and in the LNCaP cell line (20). To ascertain the effect of testosterone on PSA which is localized in liver, prostate, and testis, selectively glucuronidates a single category of steroids. Northern blot analysis of LNCaP RNA probed with the UGT2B15 cDNA showed that the level of UDPGT transcript was significantly increased after 5 days of treatment with 5 μM biochanin A. As shown on the Northern blot in Fig. 6, the UGT2B15 probe hybridized to 2.1- and 2.3-kb transcripts, both of which were enhanced in the presence of biochanin A. Additionally, RNase protection assays indicated that all protected fragments in LNCaP cells were equally enhanced in the presence of biochanin A (data not shown).

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of inactivation and elimination. Although the greater part of steroid hormone-glucuronide conjugations are excreted into the feces, they also are present in plasma. Changes in plasma levels of steroid hormone glucuronides occur during puberty, in prostatic disease, and with anti-androgen therapy (21–23). More recent studies suggest that steroid conjugation, sulfation, and glucuronidation not only occurs in liver but also may play a role in androgen metabolism in peripheral tissues (24). Most studies using prostatic tissues or prostatic cells have focused on the production of organic (nonpolar) metabolites (25–27).

To our knowledge, there are only a few studies emphasizing glucuronidation in human prostate tissue or cultured prostate cells (28–31). Because conjugation mechanisms in the liver can use diverse substrates and is modulated by a variety of effectors, their presence and regulation in the prostate are of considerable interest.

Using LNCaP cells, a prostate cancer cell line that possesses androgen receptors and exhibits androgen-dependent proliferation (3), we showed that testosterone accumulates primarily as the glucuronidated metabolite. Using a cell-free assay with testosterone as substrate, we identified testosterone-UDPGT activity in cell-free homogenates of LNCaP cells and showed that the enzyme activity exhibited characteristics similar to that in liver. In an attempt to identify modulators of this activity, we tested a variety of dietary compounds including vitamins A, B, C, and D; trace elements (e.g., molybdate or selenium compounds); organic acids (e.g., fumaric acid); and antioxidants (e.g., butylated hydroxytoluene), all of which were without significant effect. But when flavonoids, compounds widely occurring in fruits and vegetables (32, 33), were tested, we found that the flavonoid compounds tested, biochanin A was the most potent.

To further elucidate the effect of flavonoids, we used biochanin A as a model and found that treatment with biochanin A resulted in increased enzyme activity of testosterone-UDPGT. From kinetics studies, in cell-free homogenates of treated cells, testosterone-UDPGT activity exhibited increased Vmax with no change in affinity for the substrate testosterone. This increase in UDPGT activity appeared to affect androgen metabolism in intact cells. The conversion of testosterone to its glucuronide was increased 2-fold in growth media from biochanin A-treated cells. More importantly, androgen target function in LNCaP cells was affected by this increased inactivation of testosterone by the increased testosterone-UDPGT activity. The production and release of PSA, the prostatic tumor marker (8), was shown to be testosterone dependent (20) and markedly decrease in biochanin A-treated cells. Although the effect of biochanin A on PSA may be explained by altering the level of androgen receptor, our preliminary studies actually showed a slight increase in binding sites of androgen receptor and androgen receptor protein (data not shown). To our knowledge, this is the first demonstration that a dietary factor (flavonoids) can exert its effect on a testosterone response pathway in a target tissue that results in altered response to the hormone in that target tissue.

Our studies showed that the increase in testosterone-UDPGT by biochanin A was mediated by direct or indirect mechanisms at the transcription level. In humans, a cDNA that encodes the steroid UDPGT isozyme, UGT2B15, was isolated from liver, and the corresponding mRNA was detected by Northern blot in the prostate (15). Interestingly, in LNCaP cells, mRNA for this isoform of UDPGT was detected but not in PC-3 cells, a prostatic androgen-independent cell line without measurable activity for formation of testosterone glucuronide (data not shown). In addition, the mRNA coding for the phenolic isomer of UDPGT was undetectable in LNCaP cells. Now that biochanin A has been shown to induce the steroid isomer of UDPGT in prostate cancer cells, it will be interesting to identify other natural and synthetic steroid hormones that are substrates for this enzyme and to characterize the specific molecular mechanisms underling its induction.

We propose that the present study adds another metabolic pathway for prostatic androgen metabolism that may be modulated with clinically relevant consequences. The previously established 5α-reductase pathway that catalyzes the reductive conversion of testosterone to dihydrotestosterone, a more potent androgen, is a current target in pharmacological modulation (34, 35). Proscar, inhibits 5α-reductase, thereby decreasing androgen-dependent growth of the prostate, and this drug is presently being tested in clinical trials (34, 35). We propose that an alternative strategy may be offered by the conversion and elimination of testosterone by its conversion to testosterone-glucuronide using biochanin A. The possibility that dietary factors or other chemopreventive agents can delay prostatic hypertrophy and carcinogenesis by modulation of testosterone-UDPGT is an attractive possibility and deserving of further studies.

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REFERENCES

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