Androgens Markedly Stimulate the Accumulation of Neutral Lipids in the Human Prostatic Adenocarcinoma Cell Line LNCaP*

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ABSTRACT

Microscopic evaluation of LNCaP cells stained with the lipophilic dye Oil red O revealed that androgens induce a marked stimulation of lipid droplet accumulation. As determined by quantitative analysis of the Oil red O extracted from the stained cells, stimulatory effects of the synthetic androgen R1881 became apparent at concentrations as low as 10^{-11} M. Maximal induction (15-fold) was reached at 10^{-8} M. Increases were observed 2 days after hormone addition and were maximal 6 days later. Accumulation of lipid droplets was also induced by mibolerone (another synthetic androgen) and by the natural androgens testosterone and dihydrotestosterone. In agreement with the aberrant ligand specificity of the mutated androgen receptor in LN- CaP cells, stimulation of lipid accumulation was also apparent after treatment with progesterone and estradiol. Cortisol and the synthetic glucocorticoid dexamethasone were ineffective. The androgen antagonist Casodex (bicalutamide) abolished the stimulatory effect of R1881, further supporting the involvement of the androgen receptor. In agreement with this conclusion, no changes in lipid accumulation were observed after androgen treatment of the androgen receptor-negative prostate tumor lines PC-3 and DU-145. To investigate the nature of the lipids affected by androgens, lipid extracts were analyzed by TLC, complemented with enzymatic lipid analyses. Androgens were shown to have major effects on the content of triglycerides and cholesterol esters (30- and 7-fold stimulation, respectively), the two main classes of lipids stained by Oil red O. Phospholipid and cholesterol contents were increased by a factor of 2. Incorporation studies with [2-14C]acetate revealed that androgens caused a major stimulation of 2,14C incorporation into triglycerides and cholesterol esters (11- and 13-fold, respectively), suggesting that androgens act at least in part at the level of lipid synthesis. Taken together, these findings indicate that androgens, besides affecting proliferation and protein secretion, also markedly stimulate the production and accumulation of neutral lipids, revealing a novel interesting aspect of androgen regulation of LNCaP cells. (Endocrinology 137:4468-4474, 1996)

Received April 30, 1996.
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* This work was supported by a grant from Geconcerteerde Onderzoeksaak van de Vlaamse Gemeenschap, Grant 3.0048.94 from the Belgian National Fund for Scientific Research, and a grant from the Vereniging voor Kankerbestrijding.
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SINCE THE Nobel prize-winning findings of Huggins and Hodges more than 50 yr ago (1), androgens have been known to play a major regulatory role in the biology of human prostate cancer. Still today, most medical therapies known to play a major regulatory role in the biology of prostate cancer were effective, at least in part, by androgen manipulation. Inhibition of androgen action or removal of androgen from the body is currently the mainstay of therapy by castration or the use of anti-androgens. The androgen receptor antagonist Casodex (bicalutamide) abolished the stimulatory effect of androgens on lipid accumulation in the androgen receptor-negative PC-3 and DU-145 prostate tumor lines. This finding is in agreement with the aberrant ligand specificity of the mutated androgen receptor in LNCaP cells (3, 4) and in epithelial cells of the rat prostate (9), which prompted us to investigate whether androgens, besides affecting cell proliferation and protease secretion, also influence the metabolism of lipids in LNCaP cells. Here, using simple methods, such as histochemical

binding inhibitor/acyl-coenzyme A (CoA)-binding protein (DBI/ACBP) (3, 4). This gene encodes a 10-kDa multifunctional polypeptide that is, for instance, involved in the regulation of various aspects of lipid metabolism. DBI/ACBP has been shown to induce the synthesis of medium chain acyl-CoA esters by goat mammary gland fatty acid synthetase (5). It is thought to bind acyl-CoA-esters (hence the name acyl-CoA-binding protein, ACBP) (5), thereby attenuating the acyl-CoA inhibition of enzymes such as acetyl-CoA carboxylase (involved in the synthesis of fatty acids for fatty acid synthesis) and protecting long chain acyl-CoA esters against hydrolysis (6). In addition, DBI/ACBP was found to extract acyl-CoA from phosphatidylycholine membranes, to mediate intermembrane acyl-CoA transport, and to donate activated fatty acids for β-oxidation or triglyceride synthesis (7). Independently from the previous observations, DBI/ACBP has been shown to facilitate the translocation of cholesterol to the inner mitochondrial membrane during steriodogenesis (8). Our finding that the expression of DBI/ACBP is controlled by androgens in LNCaP cells (3, 4) and in epithelial cells of the rat prostate (9) prompted us to investigate whether androgens, besides affecting cell proliferation and protein secretion, also influence the metabolism of lipids in LNCaP cells. Here, using simple methods, such as histochemical
staining with Oil red O, we explored the effect of androgens on the accumulation of neutral lipids in LNCaP cells.

Materials and Methods

Cell culture

The human prostatic adenocarcinoma cell line LNCaP, originally established by Horoszewics (2), was obtained from the American Type Culture Collection (Rockville, MD). Cultures were trypsinized twice weekly and were maintained in RPMI 1640 medium supplemented with 10% FCS, 3 mM L-glutamine, 100 μg/ml streptomycin, and 100 U/ml penicillin (Life Technologies, Gaithersburg, MD). To study the effects of steroids, cells were plated in dishes and cultured in medium containing 5% dextran-coated charcoal-stripped FCS (CT-FCS), prepared as described by Leake et al. (10). Steroids were dissolved in ethanol and added to the cultures. Final ethanol concentrations did not exceed 0.1%. Control cultures received similar amounts of ethanol only. Natural steroids and dexamethasone were obtained from Sigma Chemical Co. (St. Louis, MO). Mibolerone and R1881 (methyltrienolone) were purchased from DuPont-New England Nuclear (Dreieich, Germany). Casodex (bicalutamidine) was kindly provided by Zeneca Pharmaceuticals (Manchester, UK) and is a trademark of Zeneca Ltd.

Oil red O staining

LNCaP cells were plated in 60-mm dishes (3 x 10⁵ cells/dish) in medium containing 5% FCS. After 2 days, the medium was changed to RPMI 1640 with 5% CT-FCS. Cells were incubated without (control) or with hormones for 3–4 days unless otherwise indicated. Cultures were washed with PBS and fixed for 1 h in a 5% formaldehyde solution in PBS. After washing with PBS, cells were incubated for 15 min in an Oil red O solution, prepared as described by Ramirez-Zacarias et al. (11). For photography, cells were counterstained during 1 h with a 1% methyl green solution in 0.02 M citrate buffer, pH 5.8. For quantitative analysis, cells were plated in sextuple. Three plates were fixed and stained with Oil red O as described above. After washing with PBS, cell-bound Oil red O was extracted in 1 ml 2-propanol, and absorbance was measured at 510 nm. The absorbance of similarly treated plates, but without cells, was taken as blank and subtracted from the experimental values. The
Analysis and quantitation of cellular lipids

To study the nature of the lipids affected by androgens, LNCaP cells were incubated in medium containing androgen-stripped serum and were treated with various concentrations of R1881. Control cultures (C) received ethanol vehicle only. After 4 days, cells were fixed and stained with Oil red O as described in Material and Methods. Cell-bound Oil red O was extracted with 2-propanol, and absorbance was measured spectrophotometrically at 510 nm. Absorbance values were corrected for differences in cellular DNA content and were expressed relative to the values of the control condition. Results shown represent the mean ± SD of incubations performed in triplicate.

Veldhoven, P. P., and G. P. Mannaerts, to be published). Total glycerides, present in lipid extracts, and mono-, di-, and trilinoleins were determined as follows using a commercial serum triglyceride kit from Sigma Chemical Co. After TLC separation, the corresponding spots were scraped, transferred to glass tubes and extracted with 2 ml of chloroform-methanol (1:1, vol/vol). After a centrifugation step to pellet the silica, an aliquot of the supernatant (containing up to 50 nmol glycerides) or aliquots of total lipid extracts or of a standardized triolein solution were transferred to another set of tubes and mixed with 20 µl 20% (wt/vol) Thesit (polyoxyethylene-9-lauryl ether dissolved in chloroform) before drying. The dried oily residues were dissolved in 50 µl water, followed by the addition of 450 µl reconstituted triglyceride reagent (Sigma). Reaction mixtures were incubated for 20 min at 30 °C and read at 540 nm. Readings were corrected by appropriate blanks. Despite the presence of surface active compounds in the Sigma reagent, the kit, which was intended to measure serum triglycerides, did not work on dried standards or lipid extracts. Fortifying the extracts or standards with Thesit before drying allowed subsequent action of the lipase on the formed mixed micelles to proceed smoothly and to completion. In our hands, mono-, di-, and triglycerides were completely hydrolyzed to glycerol (unpublished data).

Incorporation of [2-14C]acetate into lipids

LNCaP cells were plated in six-well plates (3 × 10⁵ cells/well) in medium containing 5% CT-FCS. After 3 days, medium was changed, and three wells of each plate received R1881 at a final concentration of 10⁻¹⁰ M. The other wells were treated with ethanol vehicle. Two days later, medium was changed again, and fresh hormone was added. [2-14C]Acetate (57 mCi/mmol; 1 μCi/well; Amersham International, Aylesbury, UK) was added to all wells, and incubation was continued for 24 h. Cells were washed with PBS and trypsinized. Lipids were extracted as described above, and the radioactivity of aliquots was measured by scintillation counting. [2-14C]Acetate incorporation into specific lipids was visualized after separation of lipids by TLC and autoradiography.
more prominent lipids were scraped. Incorporation of radioactive label was quantitated by scintillation counting. Independent six-well plates were used to measure the DNA content.

Results

Effects of steroids on the accumulation of lipid droplets in LNCaP cells

To evaluate the effect of androgens on the accumulation of lipids, LNCaP cells grown in the presence or absence of androgens were fixed and stained with Oil red O, a dye that is soluble in lipids and suitable for the histochemical staining of neutral lipids such as triglycerides and cholesterol esters (11, 16). In a first series of experiments, the synthetic androgen R1881 was chosen because, unlike the natural androgen 5α-dihydrotestosterone, it is metabolically stable. As observed microscopically, LNCaP cultures maintained in the presence of 10⁻⁸ M R1881 for 4 days displayed a marked accumulation of Oil red O-positive lipid droplets compared to androgen-deprived cultures (Fig. 1). Accumulation of lipid droplets was heterogeneous, with some cells staining more intensely than others. In the presence of androgens, not only was the percentage of Oil red O-positive cells increased, but the number of lipid droplets per cell was also dramatically higher than that in control cells. These effects were accompanied by a major change in the morphology of the cells. Cells exposed to R1881 were overall bigger in size and round in appearance (Fig. 1). Spectrophotometric determination of Oil red O extracted from cells treated with different concentrations of R1881 and for different periods of time allowed a more detailed and quantitative analysis of the observed effects of R1881. A significant stimulation of Oil red O accumulation was apparent at R1881 concentrations as low as 10⁻¹¹ M (Fig. 2). At a concentration of 10⁻¹⁰ M, the concentration that optimally stimulates proliferation of the cells (17), R1881 induced a 3- to 4-fold increase. Higher concentrations of R1881 further increased the accumulation of lipids, with a maximal stimulation of 7- to 15-fold at 10⁻⁸ M, a concentration that maximally stimulates differentiated function such as the secretion of prostate-specific antigen (17). At these concentrations, lipid accumulation became apparent 2 days after the addition of the hormone and reached a maximum 1 day later (Fig. 3). Accumulation of Oil red O was also induced by mibolerone, another synthetic androgen, and by the natural androgens testosterone and 5α-dihydrotestosterone (Fig. 4). Cells exposed to progesterone or estradiol also displayed increased Oil red O staining. Cortisol and dexamethasone were without effect. The observed steroid specificity is typical for androgen-regulated functions in LNCaP cells and is explained by the mutation in the androgen receptor in these cells, resulting in increased affinity of the androgen receptor for progestins and estrogens (18). The involvement of the androgen receptor is further evidenced by inhibition of the effects of R1881 by the androgen antagonist Casodex. In analogy with the inhibition of R1881-stimulated cell proliferation (Esquenet, M., unpublished observations), 10⁻³ M Casodex was used to efficiently block the effects of 10⁻¹⁰ M R1881 (Fig. 5). At these concentrations, Casodex reduced R1881-stimulated lipid accumulation to levels similar to those with Casodex treatment alone. No Oil red O staining was observed in the androgen receptor-negative prostate tumor cell lines DU-145 and PC-3, and in these lines androgen treatment did not induce measurable lipid accumulation.

Identification and quantification of lipids affected by androgens

To investigate the nature of the lipids affected by androgens, lipid extracts from LNCaP cells grown in the presence or absence of 10⁻⁸ M R1881 during 4 days were analyzed by TLC. In agreement with our results obtained by staining with Oil red O, marked changes were observed in the amount of neutral glycerolipids and cholesterol esters (Fig. 6). The contents of phospholipids and cholesterol were barely changed. To quantify the effects of androgens, the cellular levels of specific lipids were determined. As Table 1 illustrates, the contents of triglycerides and cholesterol esters expressed per µg DNA were increased 33- and 7-fold, respectively, after exposure to R1881. Also, mono- and diglyceride levels tended to be elevated, but exact quantitation was not possible because the levels in untreated controls were near the limit of detection. In agreement with the observed change in morphology of the cells, androgens also caused a slight (2-fold) stimulation of phospholipid content.
Effects of androgens on de novo synthesis of lipids in LNCaP cells

To explore the possibility that the increased accumulation of triacylglycerol and cholesterol esters was due to androgen-induced changes in the de novo synthesis, LNCaP cells exposed to $10^{-8}$ M R1881 for 2 days were incubated with [2-$^{14}$C]acetate for 24 h. Lipid extracts were prepared, and incorporation of labeled acetate was measured by scintillation counting. As Table 2 shows, overall [2-$^{14}$C]acetate uptake and incorporation into lipids were increased 2-fold after androgen treatment. Specific incorporation into various classes of lipids was determined by scintillation counting of lipid spots separated by TLC. R1881 treatment induced an 11- and a 13-fold stimulation of $^{14}$C incorporation in triglycerides and cholesterol esters, respectively (Table 2). Effects on phospholipids were only moderate (1.8-fold).

Discussion

Making use of Oil red O staining and quantitative enzymatic lipid determinations, we demonstrated that androgens, besides affecting cell proliferation and protein secretion, cause a marked (up to 15-fold) stimulation of the accumulation of neutral lipids in the human prostatic adenocarcinoma cell line LNCaP. Incorporation studies with labeled acetate suggest that these effects are at least in part related to androgen-induced increases in lipid synthesis.

Several lines of evidence indicate that the observed effects of androgens are mediated by the androgen receptor. 1) Accumulation of lipid droplets, as observed microscopically after staining with Oil red O and as measured quantitatively after extraction of Oil red O, was noticed after treatment of the cells with synthetic agonists (R1881 and mibolerone) as well as with natural androgens (testosterone and dihydrotestosterone). 2) The observed steroid specificity reflected the specificity of the mutated androgen receptor in LNCaP cells. 3) The effects of androgens could be neutralized by the antiandrogen Casodex. 4) No androgen-induced effects on lipid accumulation were observed in the androgen receptor-negative prostate cell lines PC-3 and DU-145.
ANDROGEN STIMULATION OF LIPID ACCUMULATION IN LNCaP CELLS

The nature of the accumulating lipids was analyzed by TLC and enzymatic lipid determination. In agreement with the staining specificity of Oil red O, these techniques revealed that the major classes of lipids affected by androgens were triglycerides (a 33-fold increase) and cholesterol esters (a 7-fold increase), typical neutral storage products found in lipid droplets. Incorporation studies with labeled acetate demonstrated that LNCaP cells have the capacity to synthesize these lipids de novo. The finding that the incorporation of labeled acetate is stimulated by androgens strongly suggests that the observed increase in neutral lipid accumulation is at least in part the result of stimulatory effects on enzymes or regulatory proteins involved in lipogenesis. Preliminary experiments indeed suggest that androgens stimulate the expression and activity of fatty acid synthase, one of the key enzymes involved in lipogenesis (Swinnen, J. V., unpublished observations). Interestingly, using antisera recognizing fatty acid synthase, this enzyme has been shown to be highly expressed in selected prostate cancers, and its use as a potential progression marker has been proposed (19, 20). As mentioned previously, we recently reported that androgens up-regulate the expression of DBI/ACBP, a multifunctional polypeptide involved in the regulation of several aspects of lipid metabolism. Whether the observed changes in DBI/ACBP may contribute to the androgenic effects seen on lipid accumulation in LNCaP cells remains to be investigated. Also, additional effects at the level of lipid uptake and/or turnover cannot be excluded at the present time. Whether the observed androgen-induced accumulation of neutral lipid droplets is a peculiarity of LNCaP cells or whether this phenomenon may be generalized to other normal or cancerous prostatic epithelial cells remains to be explored. In this respect it is worth mentioning that the normally differentiated human prostate secretes not only proteins and citrate, but also significant amounts of various lipids (21, 22). In agreement with this differentiated function of prostate tumor cells divert energy otherwise lost by accumulation of lipid droplets might represent a means by which prostate tumor cells divert energy otherwise lost by the secretion of citrate.

Taken together, the finding that androgens markedly stimulate the production and accumulation of lipids in LNCaP cells uncovers a novel and interesting aspect of the androgen regulation of these cells and provides a sensitive parameter of androgen action that can be followed using fast, inexpensive, and simple methods.

Acknowledgment

The technical assistance of R. Vanderhoydonc is kindly acknowledged.

References

12. Labarca C, Paigen K 1980 A simple, rapid, and sensitive DNA assay procedure. Anal Biochem 102:244-249
13. Van Veldhoven P, Bell RM 1988 Effect of harvesting methods. Growth con-
ANDROGEN STIMULATION OF LIPID ACCUMULATION IN LNCaP CELLS


