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Regulation of citrate metabolism by androgen in the LNCaP human prostate carcinoma cell line

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Citrate production and accumulation are characteristic physiological functions of the prostate gland that are regulated by testosterone and prolactin. Results reported here show that treatment of LNCaP cells with dihydrotestosterone (DHT) resulted in increased intracellular citrate and increased citrate accumulation in the medium. Moreover, DHT also caused an increase in both mitochondrial aspartate aminotransferase (mAAT) activity and the steady state level of pmAAT (precursor) mRNA. Androgen treatment increased the rate of citrate oxidation by LNCaP cells as it does in rat ventral prostate which suggests that DHT increased aconitase activity in LNCaP cells. The results reported here are consistent with the operation of the glutamate-aspartate-citrate pathway that we described for rat ventral prostate. In addition, these results provide the first evidence that androgen responsive functions associated with citrate metabolism are retained in LNCaP cells. In addition, and more important, these results suggest that the more aggressive PC-3 carcinoma cell line has a higher rate of citrate oxidation than the less aggressive LNCaP cell line. This could have significant implications for our understanding of the relationship between alterations in prostate citrate metabolism and expression of the malignant phenotype.

Keywords: PC-3; prostate cancer; aspartate aminotransferase; testosterone; dihydrotestosterone

Introduction

The prostate gland is unique in its ability to accumulate extraordinarily high levels of citric acid. This capability is a function of the prostate epithelium and is regulated by testosterone and prolactin (Costello & Franklin, 1991a). While it is well established that prostate citrate levels are extremely high in benign prostatic hyperplasia (BPH) and low in prostate cancer (Costello & Franklin, 1991a), most information concerning regulation of prostate citrate production has been derived from studies on rat ventral prostate. Studies with rat ventral prostate and pig prostate have established a glutamate-aspartate-citrate pathway for citrate synthesis (Franklin & Costello, 1984). However, these metabolic relationships have not been established for human prostate. In addition, since malignant prostate epithelial cells undergo an apparent metabolic transformation from citrate-producing to citrate oxidizing cells, we compared citrate oxidation in the less well differentiated, fast growing PC-3 cell line with the more differentiated, slower growing LNCaP cell line.

Both cell lines were established from metastatic lesions of human prostate cancer (Kaighn *et al.*, 1979; Horoszewicz *et al.*, 1983). LNCaP cells contain high affinity androgen receptors in the cytosol and nuclear fractions and functional differentiation is preserved. The cells are hormonally responsive since androgens stimulate growth and secretion of prostate specific products (Horoszewicz *et al.*, 1983; Young *et al.*, 1991; Lin *et al.*, 1992). Therefore, although these cells do not represent normal human prostate, they provide an appropriate model for studies on the mechanism of androgen regulation of citrate production in prostate. PC-3 cells, on the other hand, do not express androgen receptor (Tilley *et al.*, 1990) and show anchorage-independent growth (Kaighn *et al.*, 1979). PC-3 cells have a greatly reduced dependence upon serum for growth and do not respond to androgens nor epidermal growth factor or fibroblast growth factor (Kaighn *et al.*, 1979). Therefore, PC-3 cells represent a hormone independent, less differentiated phenotype compared to the LNCaP cell line.

In the present work we investigated the hormonal and metabolic relationships associated with citrate production in prostate derived cells. These studies were undertaken to determine (1) whether these human prostate derived cells accumulate citrate in the medium in response to androgen treatment, and if so to determine (2) whether androgen treatment increased expression of mAAT, a key enzyme required for citrate synthesis in ventral prostate and pig prostate. We also compared medium citrate accumulation and citrate oxidation by LNCaP and PC-3 cells to ascertain whether these parameters correlate with the degree of differentiation characteristic of these prostate cell lines.

Results

Citrate production and accumulation are unique characteristics of prostate epithelial cells. We have reported that testosterone stimulates citrate accumulation by both rat ventral prostate organ cultures and pig prostate primary cultures (Franklin *et al.*, 1984; Costello *et al.*, 1988). Since LNCaP cells are an androgen sensitive cell line, we wanted to determine if androgens would increase citrate production in these cells. In these experiments, we used cultures of LNCaP cells grown to 80% confluence in complete growth medium and then transferred to medium containing 2% charcoal treated serum for 18-22 h before exposure to 5α-dihydrotestosterone (DHT). Figure 1 shows that incubation with DHT resulted in a dose dependent accumulation of citrate in the medium over a 24 h period. DHT at a concentration of 10^{-10} M resulted in a significant increase in medium citrate. The results also showed that treatment with DHT resulted in a significant increase in intracellular citrate (Figure 2). Consequently, it is clearly established that DHT stimulates net citrate production by LNCaP cells.

In earlier studies we showed that testosterone regulates the synthesis of OAA via regulation of mAAT biosynthesis in rat ventral prostate and in pig prostate epithelial cells (Franklin *et al.*, 1987, 1990). Therefore, it was important to determine if an increase in mAAT was associated with DHT stimulation of net citrate production by LNCaP cells. To determine the effect of DHT on mAAT activity in LNCaP cells, we incubated cells with various concentrations of DHT for 3 h. The results (Figure 3) show that mAAT activity increased in a dose dependent manner. We also collected the medium and measured the medium citrate concentration in the same cul-

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tures. Medium citrate concentration also increased in response to increasing concentrations of DHT (data not shown). Testosterone stimulates expression of the pmAAT gene in rat ventral prostate and pig prostate (Franklin *et al.*, 1987, 1990). Consequently, we determined the effect of DHT on the steady state level of mAAT mRNA in LNCaP cells.

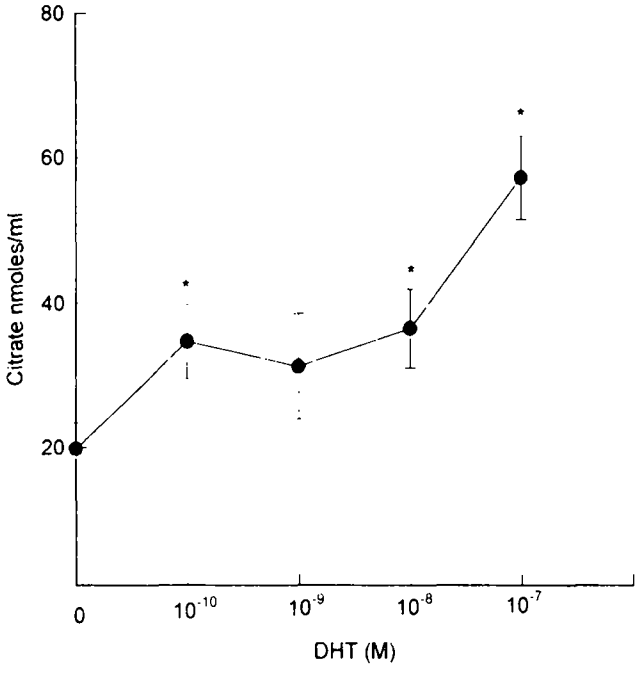

Figure 1 Effect of DHT on citrate accumulation by LNCaP cells. LNCaP cells were incubated for 24 h in RPMI 1640 medium supplemented with 2% charcoal-treated fetal bovine serum and various concentrations of DHT or an equal volume of vehicle. After DHT treatment, medium was collected, deproteinized by addition of 0.1 volume of 80% TCA and assayed for citrate. Value are mean ± SEM for three samples at each concentration. *Statistically different at $P < 0.05$ when compared to 0 DHT concentration

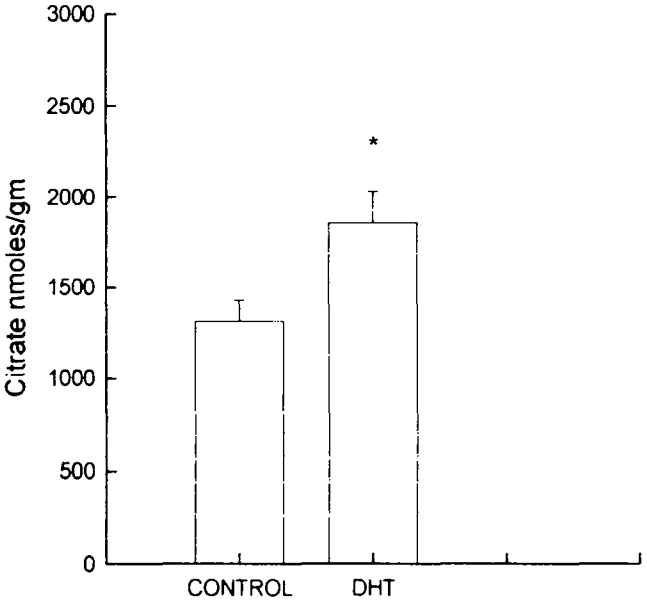

Figure 2 Effect of DHT on intracellular citrate concentration of LNCaP cells. LNCaP cells were incubated overnight in RPMI 1640 medium supplemented with 2% charcoal-treated fetal bovine serum and 5 nM DHT or an equal volume of vehicle. After DHT treatment the cells were collected by centrifugation, the medium removed, the inner wall of the tube dried and the cell pellets weighted. The pellets were resuspended in 7% TCA and assayed for citrate. Values are mean ± SEM for quadruplicate samples. *Statistically different at $P < 0.05$ when compared to control

As little as 10^{-10} M DHT resulted in an eight-fold increase in mAAT mRNA after exposure to DHT for 3 h (Figure 4). Induction of mAAT mRNA was maximal at this concentration, and higher concentrations of DHT did not further increase mAAT mRNA. Bands for 28S ribosomal RNA show a slight difference in loading for the 10^{-9} M DHT treatment, however, the different in RNA load does not account for the difference in mAAT mRNA between DHT treatment and control. The effect of DHT on mAAT activity and mAAT mRNA suggests that regulation of mAAT biosynthesis is a major action of androgen in the regulation of net citrate production by LNCaP cells.

A major aspect of citrate accumulation in prostate is the limited rate of citrate oxidation characteristic of prostate epithelial cells (Costello *et al.*, 1976; Costello & Franklin, 1981). However, although this limited citrate oxidation rate is essential for citrate accumulation, and testosterone increases citrate accumulation, testosterone treatment also stimulates citrate oxidation by rat ventral prostate cells (Franklin *et al.*, 1986). Therefore, we determined whether DHT would affect citrate oxidation in LNCaP cells. Table 1 shows $^{14}CO_2$ production by LNCaP cells incubated with 1,5-^{14}C labeled citrate. The results show that DHT significantly increased $^{14}CO_2$ production and thus citrate oxidation by these cells.

Citrate synthesis and accumulation are differentiated functions of prostate epithelial cells. In addition, it is well esta-

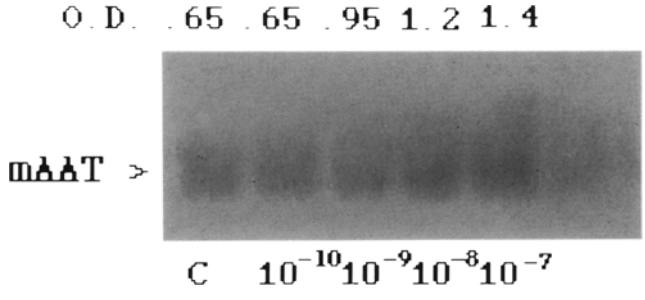

Figure 3 Effect of DHT on mAAT activity in LNCaP cells. LNCaP cells were incubated in RPMI 1640 medium without serum and various concentrations of DHT for 3 h. mAAT was separated by electrophoresis of 80 μg total protein extract from each sample on a 1% non-denaturing agarose gel. mAAT activity was detected by AAT activity staining in the gel. The results are representative of experiments repeated three times

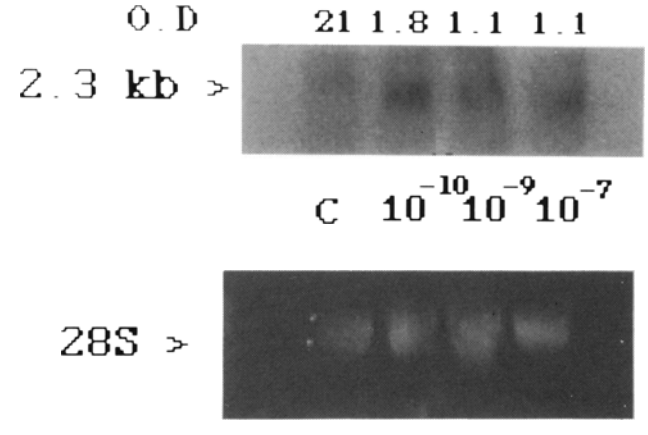

Figure 4 Effect of DHT on mAAT mRNA steady state level in LNCaP cells. LNCaP cells were incubated with various concentrations of DHT for 3 h. After DHT treatment the cells were collected and RNA extracted. Equal amounts (20 μg) of total RNA were separated by electrophoresis on formaldehyde denaturing agarose gels and blotted to nitrocellulose filters. The blots were probed with rat mAAT cDNA. Bands for 28S ribosomal RNA are presented as loading controls. The figure is representative of experiments repeated two times with similar results

blished that LNCaP cells represent a more differentiated phenotype than PC-3 cells. Therefore, we compared the rate of citrate oxidation and citrate accumulation by LNCaP cells with citrate oxidation and accumulation by PC-3 cells. Figures 5 and 6 show citrate oxidation and medium citrate accumulation by LNCaP and PC-3 cells. Oxidation was determined at two concentrations of cells to ensure that substrate was not limiting and to control for possible differences in cell number. Results in Figure 5 show that the rate of citrate oxidation by PC-3 cells was significantly greater than that by LNCaP cells. Moreover, the rate of citrate oxidation increased proportionally as the number of cells was increased. Figure 6 shows LNCaP cells accumulated significantly more citrate in the medium than PC-3 cells. Based on these studies, LNCaP cells would be characterized as citrate producing, low citrate oxidizing prostate cells, whereas PC-3 cells are characterized as citrate-oxidizing, non-citrate producing cells.

Table 1 Effect of DHT on citrate oxidation by LNCaP cells

	DPM/1 × 10⁶ cells Control	*DPM/1 × 10⁶ cells DHT*
Experiment 1	669.9 ± 44.4	986 ± 128*
Experiment 2	752 ± 17	935 ± 68*
Experiment 3	521 ± 81	979 ± 80*

LNCaP cells were incubated in RPMI 1640 medium containing 10 mM citrate and 0.1 μci 1,5^{14}C-citrate. Citrate oxidation was measured as $^{14}CO_2$ production from 1,5^{14}C citrate. The cells were exposed to 5 nM DHT or vehicle for 4 h before the oxidization assays. Values are DPM/1 × 10^6 cells. Control and DHT groups are triplicate samples in each experiment. *Statistically different at $P<0.05$ when compared to control

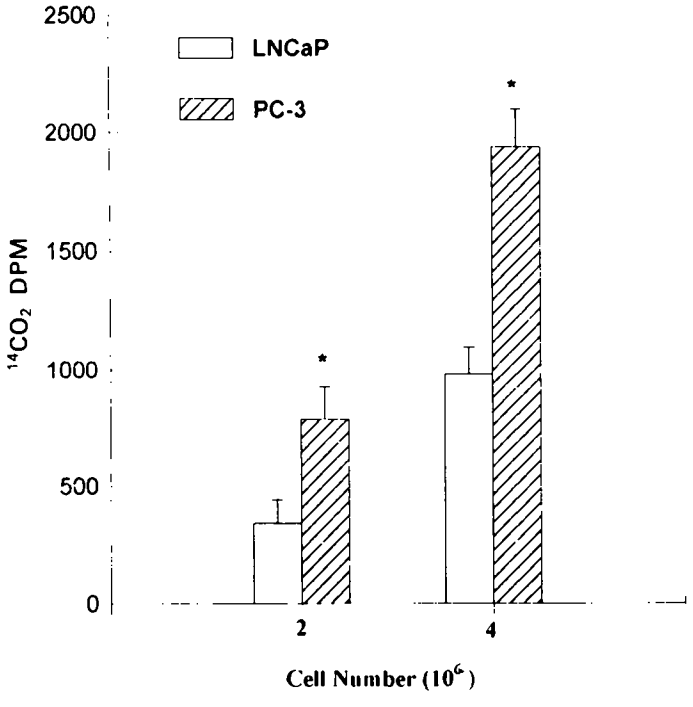

Figure 5 Comparison of citrate oxidation by LNCaP and PC-3 cells. LNCaP and PC-3 cells were suspended in RPMI 1640 medium without serum and counted by hemacytometer. An equal number of cells were placed in reaction flasks and incubated with 1,5 ^{14}C-citrate in 10 mM citrate for 2 h. CO_2 was collected and counted. Citrate oxidation rates are expressed as $^{14}CO_2$ DPM/2 h. Oxidation rates were determined at two concentrations of cells to ensure that substrate was not limiting. Values are mean ± SEM of triplicate flasks from three separate experiments ($n = 9$). *Statistically different at $P<0.05$ when compared to LNCaP cells

Discussion

The major physiological functions of the prostate in many mammals, including man, are the accumulation and secretion of an extremely high concentration of citrate. Although the function of citrate in semen is not well established, that the prostate is the major source of seminal citrate is established. Studies using rat ventral prostate and pig prostate revealed that the metabolic pathway for citrate accumulation involves citrate synthesis from aspartate and pyruvate and decreased citrate oxidation due to a limited mitochondrial aconitase activity (Costello & Franklin, 1991b). Aspartate via mAAT is the major source of OAA and glucose via pyruvate oxidation in the major source of acetyl CoA. Thus mAAT, PDH and m-aconitase are key enzymes in the pathway of net citrate production. We previously showed with rat ventral prostate and pig prostate epithelial cells that testosterone stimulates all three of these enzymes (Costello *et al.*, 1994; Franklin *et al.*, 1982, 1984, 1986). In this report we show that DHT stimulates mAAT and probably m-aconitase in LNCaP cells also. Thus we have shown an effect of androgen on these three key enzymes and citrate production in rat ventral prostate, pig prostate and now LNCaP cells. Therefore, it seems likely that the same pathway for citrate accumulation also functions in normal human prostate.

Metabolic studies using human prostate epithelial cells are complicated by the heterogeneous nature of the human prostate. Several epithelial cell types are present and not all of these are citrate producing cells. Therefore, one cannot be assured of which cell type is being studied when using fresh tissue. In addition, the availability of fresh normal human prostate tissue is limited. LNCaP cells, on the other hand, are a human prostate cancer cell line derived from a metastatic lesion. Therefore, these cells represent a single clone of epithelial cell. Although LNCaP cells are not normal human prostate epithelial cells, they retain their androgen responsiveness and many differentiated prostate epithelial cell functions. Therefore, these cells are a reasonable model with which to study citrate related metabolism and androgen responses of prostate epithelium.

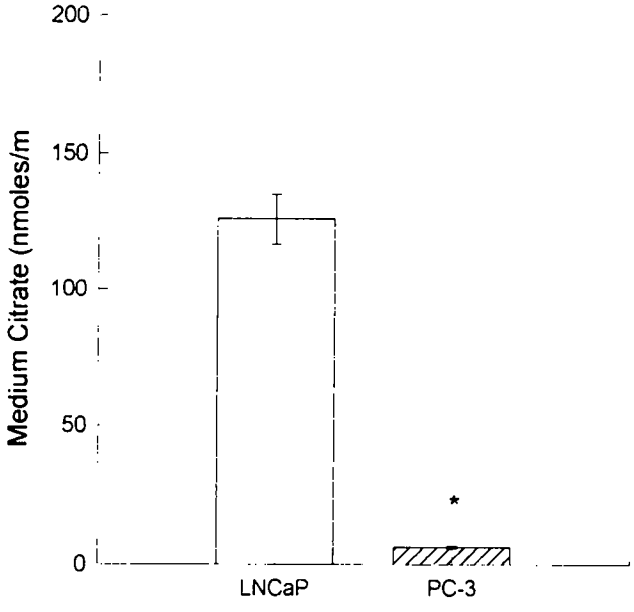

Figure 6 Comparison of 24 h medium citrate accumulation by LNCaP and PC-3 cells. LNCaP and PC-3 cells were incubated in RPMI 1640 medium containing 10% FBS overnight. Medium was collected, deproteinized with 80% TCA and assayed for citrate. Values represent means ± SEM of triplicate flasks from three separate experiments ($n = 9$). *Statistically different at $P<0.001$ when compared to LNCaP cells

Under the culture conditions used in these studies, LNCaP cells accumulated citrate intracellularly and secreted citrate into the medium. Cells were cultured in RPMI 1640 medium which contains aspartate and glutamate. When cells were cultured in DMEM medium, which does not contain these two amino acids, much less citrate accumulated (data not shown). In addition, treatment of LNCaP cells with DHT resulted in a dose dependent increase in citrate. The response was similar to that reported by others for PSA (Lee *et al.*, 1995). There was an increase in citrate both intracellularly and in the medium, as well as an increase in the rate of citrate oxidation. Thus, under conditions of increased citrate accumulation at the same time that citrate oxidation was also increased, there must have been an increase in net citrate synthesis to account for the citrate accumulation. Therefore, these results are consistent with the operation of the glutamate-aspartate-citrate pathway that we described for rat ventral prostate (Franklin & Costello, 1984). Consequently, these results suggest that the metabolic pathway that accounts for the unique physiological function of citrate production and accumulation in rat ventral prostate and pig prostate is also present in citrate producing human prostate epithelial cells.

Basal citrate production by LNCaP cells was stimulated by DHT in these studies. This is a significant observation since it establishes that the androgen responsiveness reported for PSA and prostatic acid phosphatase in these cells (Young *et al.*, 1991; Stephenson *et al.*, 1992) is also present with regard to citrate metabolism. Since DHT increased citrate production, we investigated the effect of DHT on mAAT activity, a key enzyme responsible for citrate synthesis by rat ventral prostate and pig prostate. DHT increased mAAT activity in a dose dependent manner. Moreover, when citrate production and mAAT activity were measured in the same cells, DHT increased both mAAT activity and citrate production with a similar time course. Thus, the mechanism of the DHT effect on net citrate production probably involved stimulation of mAAT activity.

We previously reported that testosterone increased mAAT and the level of pmAAT (precursor mAAT) mRNA in rat ventral prostate (Franklin *et al.*, 1987, 1990). Furthermore, testosterone had no effect of the level of cAAT, the cytosolic isoform of the enzyme, nor on the level of mitochondrial malic dehydrogenase in rat ventral prostate (Franklin *et al.*, 1987). Therefore, the effect on mAAT is specific. Northern blot analysis in the studies reported here shows that DHT increased the pmAAT mRNA level in LNCaP cells also. Although we did not test the specificity of the response, based on results in rat ventral prostate, we believe that this response in LNCaP cells is also specific. The androgen receptor in LNCaP cells contains mutations which effect the steroid binding domain and alters the steroid specificity of the receptor (Romero *et al.*, 1993; Selva *et al.*, 1993). However, since we used DHT, a non aromatizable androgen is these studies, the possibility that estrogen was responsible for the effects we observed is eliminated. Therefore, as we reported for animal prostate cells, these results are consistent with androgen regulation of pmAAT expression in human prostate cells.

Prostate epithelial cells display limited citrate oxidation (Costello & Franklin, 1991b). Furthermore, the limited citrate oxidation is the result of limited aconitase activity (Costello *et al.*, 1976). However, citrate oxidization can be increased by testosterone (Harkonen *et al.*, 1982; Franklin *et al.*, 1986). We observed that testosterone increased prostate mitochondrial citrate oxidation and, correspondingly increased the m-aconitase activity of ventral prostate (Franklin *et al.*, 1986). In these studies, we show that testosterone stimulates citrate oxidation by LNCaP cells. Consequently, these results suggest that testosterone stimulates m-aconitase activity in LNCaP cells as it does in rat ventral prostate cells, and that the increased m-aconitase activity results in increased citrate oxidation.

Although the LNCaP and PC-3 cell lines have been used as models to study prostate carcinogenesis, very little is known concerning citrate related metabolic aspects of these cells. While growth requirements and hormone responses of these cell lines have been well characterized, almost no studies exist on citrate production, the most characteristic feature of prostate epithelial cells. We have previously emphasized that citrate production is a unique metabolic and functional relationship that is essential for the characterization and differentiation of prostate epithelial cells (Costello & Franklin, 1991a). We have proposed that prostate epithelial cells can be characterized as citrate producing cells that have a low rate of citrate oxidation, or as non-citrate producing cells that have a high, non-limiting rate of citrate oxidation. Results reported here demonstrate that LNCaP cells and PC-3 cells are different with regard to citrate metabolic relationships, though both are derived from prostate metastatic cancer. LNCaP cells are citrate-producing cells whereas PC-3 cells are non-citrate producing (i.e. citrate oxidizing) cells. Loss of citrate accumulating capability is an established characteristic of prostate cancer. The loss of citrate accumulating capability represents the loss of a differentiated function of prostate epithelial cells. Results of the comparison of citrate oxidation by LNCaP and PC-3 cells reported here are consistent with this concept. PC-3 is a more aggressive, faster growing and less well differentiated cell line than LNCaP (Kaighn *et al.*, 1979; Horoszewicz *et al.*, 1983; Passaniti *et al.*, 1992). Consequently, the higher citrate oxidation rate and lower citrate accumulation are consistent with a loss of differentiated function by PC-3. LNCaP cells express a number of differentiated functions and remain androgen responsive. PC-3 cells, on the other hand, represent less well differentiated cells that have progressed to a state of androgen independence and a more aggressive phenotype. Thus, loss of the capability to accumulate citrate and increased citrate oxidation represent a less well differentiated phenotype and may represent metabolic changes that accompany progression to hormone independence.

Materials and methods

Cell culture

LNCaP and PC-3 cells were obtained from the American Type Culture Collection. LNCaP at passage 19 and PC-3 at passage 16. Cells were routinely cultured in medium supplemented with 10% fetal bovine serum (FBS), 100 U/ml penicillin, 100 μg/ml streptomycin and 0.25 μg/ml amphotericin in a water saturated atmosphere of 95% air 5% CO_2 at 37°C. LNCaP cells were cultured in RPMI 1640 medium, PC-3 cells were cultured in DMEM medium supplemented with 20 mg/L L-aspartate. Cells were subcultured by gentle dispersion in a solution of trypsin (0.05%)-EDTA (0.02%) and inoculated into new 75 sq cm plastic flasks. The cultures were allowed to reach confluence with media changes at 2–3 day intervals. In all experiments confluent monolayer cultures of LNCaP cells in 75 sq cm flasks were first changed to RPMI 1640 medium containing 2% charcoal-treated FBS and incubated overnight. After 18–22 h, the medium was replaced with the same medium containing various concentrations of DHT. Cells were collected using a plastic policeman and washed with phosphate buffered saline (PBS) by centrifugation at 800 *g* for 5 min at room temperature.

Citrate accumulation

After DHT treatment for the indicated times, medium and cells were collected by centrifugation. Medium was removed, the inner wall of the tube dried, the cell pellets weighted and resuspended in 7% TCA. The medium was deproteinized by addition of 0.1 volume of 80% TCA. Medium citrate and cellular citrate were assayed as previously described (Costello & O'Neill, 1969).

mAAT activity

In experiments where mAAT activity was measured after hormone treatment, cells were collected by centrifugation, resuspended in PBS and sonicated. The sonicated suspension was centrifuged and an aliquot of the supernatant removed for protein determination (Bradford, 1993). mAAT in the supernatant was separated by loading equal amounts of total protein on 1% non-denaturing agarose gels. The gels were electrophoresed at 60 milliamps constant current for 1.5–2.0 h. mAAT activity was detected by mAAT activity staining in the gel as previously described (Franklin *et al.*, 1987). The results are representative of experiments that were repeated at least three times.

Northern hybridization

LNCaP cells cultured as described above were treated with various concentrations of DHT for 3 h. After DHT treatment cells were collected and total RNA extracted by the acid guanidinium thiocyanate procedure (Chomczynski & Sacchi, 1987). Equal amounts of total RNA (20 μg) were separated by formaldehyde denaturing agarose gel electrophoresis and transferred to nitrocellulose membranes. The nitrocellulose membranes were hybridized as previously described (Franklin *et al.*, 1987) with a ^{32}P labeled mAAT probe (Mattingly *et al.*, 1987). Autoradiographs were scanned using a densitometer.

Citrate oxidation

We measured citrate oxidation by incubating LNCaP and PC-3 cells with ^{14}C labeled citrate as we have previously described (Franklin *et al.*, 1986). Briefly, LNCaP cells were grown to 80% confluence in RPMI 1640 medium containing 10% FBS. The medium was then changed to RPMI 1640 medium containing 2% FBS and incubated overnight. After 18–22 h, cells were collected as described above and suspended in RPMI 1640 medium without serum. The cell suspension was divided into two aliquots containing equal numbers of cells. DHT (5 nM) or an equal volume of vehicle was added and the cells incubated at 37°C for 4 h. After the 4 h incubation one ml aliquots of the cell suspension were removed from the control and DHT treated cells and incubated with 1,5-^{14}C-citrate (0.1 μci/ml) in 15 ml flasks fitted with serum stoppers and plastic center wells. The center wells contained 0.2 ml of phenethylamine/methanol (1:1) to trap released CO_2. Cells were incubated, with shaking, in a Dubnoff metabolic shaker for 2 h at 37°C to generate $^{14}CO_2$. After incubation, 1 ml of 0.5 N HCl was injected into the reaction flasks and $^{14}CO_2$ collected for 1 h. The center wells were removed, the phenethylamine/methanol placed in counting vials with 5 ml of counting fluid and counted in a liquid scintillation counter.

In experiments where citrate oxidation by LNCaP cells was compared to citrate oxidation by PC-3 cells. LNCaP and PC-3 cells were cultures in RPMI 1640 and DMEM media respectively. Both media contained 10% FBS. After cells reached confluence, they were collected as described above and resuspended in RPMI 1640 medium without serum. Cells were counted using a hemocytometer and an equal number of cells were placed in 15 ml reaction flasks fitted with serum stoppers and plastic center wells. CO_2 was collected and counted as described above. Citrate oxidation is presented as $^{14}CO_2$ DPMs per 2 h incubation.

Statistical analysis

Analysis of the difference between the means of DHT dose response shown in Figure 1 was determined by one way ANOVA and Bonferroni *t* test for multiple comparisons against a single control (0 DHT). All other analysis of the differences between means of control groups and treatment groups were determined by student's *t* test. Unless otherwise indicated, the results represent experiments that were repeated at least three times with similar results.

Acknowledgements
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