Calreticulin Expression Is Associated with Androgen Regulation of the Sensitivity to Calcium Ionophore-induced Apoptosis in LNCaP Prostate Cancer Cells

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ABSTRACT

Calreticulin has been identified previously as one of the androgen-response genes in the prostate. The role of calreticulin in androgen action was studied using androgen-sensitive LNCaP and androgen-insensitive PC-3 human prostate cancer cell lines. Calreticulin appears to be a primary androgen-response gene in cultured LNCaP cells because androgen induction of calreticulin mRNA resists protein synthesis inhibition. Calreticulin is a high capacity intracellular Ca^{2+} binding protein, suggesting that calreticulin expression is likely to be associated with the intracellular Ca^{2+} buffering capacity that could regulate the sensitivity to cytotoxic intracellular Ca^{2+} overload. As expected, androgen protects androgen-sensitive LNCaP but not androgen-insensitive PC-3 cells from cytotoxic intracellular Ca^{2+} overload induced by Ca^{2+} ionophore A23187. To provide evidence for the role of calreticulin in reducing cytotoxic effect of Ca^{2+} influx in prostatic cells, we have shown that calreticulin antisense oligonucleotide down-regulates calreticulin protein level and significantly increases the sensitivity to A23187-induced apoptosis in both LNCaP and PC-3 cells. Furthermore, calreticulin antisense oligonucleotide reverses the androgen-induced resistance to A23187 in LNCaP cells. The above observations collectively suggest that calreticulin mediates androgen regulation of the sensitivity to Ca^{2+} ionophore-induced apoptosis in LNCaP cells.

INTRODUCTION

Androgen plays an important role in the progression of prostate cancer. Androgen ablation is still the front line treatment of metastatic prostate cancer. Unfortunately, androgen ablation therapy is only palliative, and patients eventually relapse with androgen-independent prostate cancer, which is responsible for the death of about 40,000 prostate cancer patients annually. There is a desperate need for new approaches for prevention and/or treatment of androgen-independent prostate cancer. Understanding the mechanism of androgen action is important for investigating the role of androgen in prostate cancer progression and may lead to the development of novel prostate cancer therapies.

Recently, we began to study the mechanism of androgen action in the prostate by identification and characterization of androgen-response genes in the rat ventral prostate model. Using a PCR-based cDNA subtraction method (1), we have searched extensively for androgen-response genes (2). One of the identified androgen-response genes encodes calreticulin (2). Both Northern and Western blot analyses showed that castration dramatically down-regulates the expression of calreticulin in the prostate, whereas androgen replacement rapidly up-regulates calreticulin mRNA and protein. The expression of calreticulin in the prostate is much more abundant than that in any other organs, and it appears that androgen regulates calreticulin expression in male sex accessory organs only. In situ hybridization and immunohistochemistry showed that calreticulin is an intracellular protein abundantly expressed in the glandular prostatic epithelial cells (3). These observations suggest that calreticulin could play an important role in androgen action in the prostate.

Calreticulin is a highly conserved multifunctional protein found in a wide range of species (4, 5). Calreticulin was initially identified as a major lumenal Ca^{2+} binding protein of endoplasmic reticula in nonmuscle cells (6). The human mature calreticulin consists of 400 amino acids and has a calculated molecular mass of 46.6 kDa (7). It contains one high-affinity and 25 low-affinity Ca^{2+}-binding sites (6, 7). Calreticulin contains a KDEL endoplasmic reticulum retrieval sequence at its extreme COOH terminus and a putative nuclear localization signal in the middle of the protein (6, 7). As a high capacity intracellular Ca^{2+}-binding protein, calreticulin can protect cells from cytotoxic intracellular Ca^{2+} overload (6). Down-regulation of calreticulin expression by antisense oligonucleotide treatment lowers the Ca^{2+} response to bradykinin and increases the sensitivity to ionomycin-induced cell death in neuroblastoma X glioma NG-108-15 cells (8). Conversely, overexpression of calreticulin increases the Ca^{2+} buffering capacity and protects HeLa cells against apoptosis (9). Also, marked reduction of calreticulin expression was observed prior to apoptosis in human leukemia HL-60 cells (10). These observations suggest that calreticulin has the potential to influence apoptosis via modulation of the intracellular Ca^{2+} levels.

The importance of intracellular Ca^{2+} elevation in apoptosis has been demonstrated in aggressive prostate cancer cell lines (11, 12). Agents such as ionomycin and thapsigargin cause sustained intracellular Ca^{2+} increase and lead to apoptosis. Overexpression of a Ca^{2+} binding protein, calbindin D, protects prostate cancer cells against intracellular Ca^{2+} elevation (12). Because calreticulin is a high-capacity Ca^{2+}-binding protein and its expression is androgen dependent and highly abundant in prostate epithelial cells, calreticulin is likely to have a role in buffering intracellular Ca^{2+} during androgen manipulation in both normal and cancerous prostatic epithelial cells.

We have chosen androgen-sensitive human prostate cancer cell line LNCaP as a model to investigate the role of calreticulin in androgen action, particularly the role in the regulation of the intracellular Ca^{2+} buffering capacity during androgen manipulation. The LNCaP is widely used for studying the mechanism of androgen action because it retains critical characteristics of a normal prostatic epithelial cell, including androgen-sensitive cell proliferation and production of PSA (13, 14). Thus, LNCaP appears to be an excellent model for investigating the effect of androgen on intracellular Ca^{2+} buffering and the role of calreticulin in androgen regulation of intracellular Ca^{2+} buffering. This report describes the interactions between androgen, calreticulin, and the sensitivity to Ca^{2+} ionophore-induced apoptosis in LNCaP cells.
MATERIALS AND METHODS

Materials. Agarose, chloroform, CsCl, DMSO, ethanol, EDTA, formamide, guanidine thiocyanate, NaeCl, SDS, Tris, xylene, and nylon membrane were from Fisher Biotech. PBS, penicillin/streptomycin, PBS solution, RPMI 1640, and trypsin/EDTA solution were from Life Technologies, Inc. 4-(2-aminoethyl)benzenesulfonyl fluoride, anisomycin, calcium ionophore A23187, cycloheximide, hydrogen peroxide, isopropyl-1-thio-β-D-galactoside, leupeptin, pepstatin, phenol, phenylmethylsulfonyl fluoride, and trypsin blue were from Sigma Chemical Co. ECL (enhanced chemiluminescence) kit and goat anti-rabbit secondary antibody were from Amersham. Synthesized androgen analogue mibolerone, [35S]methionine, and [3H]dCTP, and [35S]methionine were from DuPont NEN.

Oligonucleotide Synthesis. Calreticulin sense and antisense phosphorothioate oligonucleotides (18-mers and 21-mers) were synthesized by the Biotechnology Facility at Northwestern University Medical School. The 18-mer and 21-mer sequences were chosen according Liu et al. (8) and Leung-Hagestjeria et al. (15), respectively. The 18-mer is localized at the region coding for amino acids 83–88 (8), and the 21-mer is flanking the translation initiation region of the mRNA (15). The sequences of the oligonucleotides were as follows: 18-mer sense, 5'-GAGCGAACATCGACTG-3'; 18-mer antisense, 5'-ACAGTCGATTCTGTC-3'; 21-mer sense, 5'-CGGCCTCCATGCTGTACCC-3'; and 21-mer antisense, 5'-GGATAGCGCATGCGGCCC-3'.

RNA Isolation and Northern Analysis. Total RNA was isolated using the guanidinium/CsCl gradient method (16). Purified RNA samples were fractionated in a 1% agarose-formaldehyde gel. Ten μg of total RNA were loaded in each lane. After electrophoresis, RNA was transferred to a nylon membrane by capillary blotting and then cross-linked to the membrane by UV irradiation. Northern hybridization of the membrane was carried out at 42°C overnight in a 10% SDS (w/v) buffer containing 5× SSPE, 2× Denhart’s solution, 0.1% SDS, 0.1 mg/ml denatured salmon sperm DNA, and 50% formamide in the presence of human calreticulin DNA probes (IMAGE Consortium, EST: ze97a10.1) labeled by random priming. The membrane was then washed at room temperature with 1× SSC and 0.1% SDS for 20 min, followed by two 20-min washes at 65°C with 0.2× SSC and 0.1% SDS. The blot was exposed to X-ray film at −80°C.

Cell Culture. The human prostate cancer cell line LNCaP and PC3 were purchased from American Type Culture Collection. LNCaP and PC3 cells were cultured in RPMI 1640 with 10% FBS and 1% penicillin-streptomycin in 12-well plates at 37°C in 5% CO2 and 95% air to 60–70% confluency. FBS were cultured in RPMI 1640 with 10% FBS and 1% penicillin-streptomycin in 12-well plates. FBS were twice in PBS with 5 min each. Then the cells were rinsed twice in PBS for 5 min each. The endogenous peroxidase activity of the cells was quenched by incubating with 3% hydrogen peroxide in methanol for 30 min, followed by rinsing twice in PBS with 5 min each. Nonspecific binding sites were blocked by incubating with 2% goat serum in PBS at room temperature for 1 h. Cells were incubated with anti-calreticulin antibody (1:1500 dilution in the blocking solution) in a humidified box at 4°C overnight. The control group was incubated with a mixture of the primary antibody and the GST-calreticulin fusion protein. Cells were rinsed twice in PBS with 5 min each. Then the cells were processed as described in the Vectastain ABC kit from Vector Laboratories and developed in 3,3'-diaminobenzidine substrate (Vector Laboratories) for 5 min. After stopping the reaction in distilled water, the coverslips were dehydrated in an ethanol series, cleared in xylene, and dried overnight at room temperature. Finally, the coverslips were mounted with Permount. Pictures were taken using an Olympus VANOX-S camera and an Olympus AH-2 microscope.

RESULTS

Calreticulin Is a Primary Androgen-Response Gene in LNCaP Cells. To use the LNCaP cell line to study calreticulin function in androgen action, it is important to first characterize the expression of calreticulin in response to androgen in this androgen-sensitive cell line. Fig. 1A shows that calreticulin mRNA is regulated by androgen in LNCaP cells and androgen induction of calreticulin mRNA resists protein synthesis inhibition. PSA (20–22) was probed as a control for early androgen-response genes, and its induction partially resists protein synthesis inhibition (Fig. 1A). Glyceraldehyde-3-phosphate dehydrogenase was probed as a control for androgen-insensitive gene. The inhibition of protein synthesis by cycloheximide and anisomycin in cultured LNCaP cells was >97%, as assayed by measuring [35S]methionine incorporation (data not shown). Our observation indicates that calreticulin is a primary androgen-response gene in cultured LNCaP cells.

The androgen treatment time for inducing calreticulin mRNA in LNCaP cells was 36 h in the presence of CHX and anisomycin. This androgen induction time was chosen because the total RNA yield drops dramatically in LNCaP cells after 36 h in the presence of CHX. In fact, partial RNA degradation occurs within 36 h of the CHX

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treatment, as indicated by the smear of 18S and 28S rRNAs in methylene blue staining (Fig. 1A).

We have also studied the effect of androgen on calreticulin at the protein level. As expected, calreticulin protein is regulated by androgen in LNCaP cells (Fig. 1B).

Calreticulin Is Localized in an Endoplasmic Reticulum-like Structure in LNCaP Cells. Previous studies have showed that calreticulin is often colocalized with the membrane system of the endoplasmic reticulum (23). Fig. 2 shows the immunocytochemistry study of the intracellular distribution of calreticulin in LNCaP cells. The distribution in LNCaP is virtually identical to that in other cell types, such as a rat pigment epithelial cell line and dedifferentiated chick embryo cardiac myocytes (23), where calreticulin is predominantly localized in the endoplasmic reticulum. Thus, it appears that calreticulin is associated with the endoplasmic reticulum in LNCaP cells. However, we cannot rule out the possibilities that calreticulin may be associated with specialized areas of the endoplasmic reticulum and that a small amount of calreticulin may localize in areas other than endoplasmic reticulum.

Ca^{2+} Ionophore A23187 Induces Apoptosis in LNCaP and PC-3 Cells. It has been demonstrated previously that Ca^{2+} ionophore ionomycin induces apoptosis in prostatic cancer cells (11). It is expected, but has not been proven, that other Ca^{2+} ionophores such as A23187 also induce apoptosis. Although Ca^{2+} ionophore A23187 has been used frequently to induce cytotoxic intracellular Ca^{2+} influx, no experiment has been carried out to test whether A23187-induced cell death is apoptotic. Fig. 3A shows that A23187 induces internucleosomal DNA fragmentation, a hallmark of apoptosis, in both androgen-sensitive LNCaP and androgen-insensitive PC-3 prostate cancer cell lines. Thus, A23187 induces cell death via apoptosis. Because it is difficult to quantitatively determine cell death using DNA fragmentation assay, we also used the trypan blue staining method to measure cell death of LNCaP and PC-3 in response to A23187. Fig. 3B shows the dose-response curves of cell viability versus A23187 in LNCaP.
and PC-3 cells using trypan blue assay. More than 50% of the cells are dead in the presence of 5 μM or higher concentrations of A23187. Virtually all of the cells are dead in the presence of 20 μM A23187.

Calreticulin Antisense Oligonucleotides Down-Regulate Calreticulin and Enhances the Sensitivity to Ca2+ Ionophore in LNCaP and PC-3 Cells. To examine whether calreticulin is involved in the regulation of the sensitivity to Ca2+ ionophore in prostate cancer cells, we studied the effect of calreticulin antisense oligonucleotides on Ca2+ ionophore-induced cell death in LNCaP and PC-3 cells. Treatment with calreticulin antisense oligonucleotides significantly increases the sensitivity of LNCaP cells to Ca2+ ionophore A23187-induced cell death (Fig. 4A). The 21-mer pair of antisense and sense phosphorothioate oligonucleotides (see “Materials and Methods”) was used in the experiment. None of the oligonucleotides were toxic to LNCaP cells because treatment with oligonucleotides (10 μM) alone had no effect on LNCaP viability, as determined using trypan blue dye exclusion assay.

Fig. 3. The effect of Ca2+ ionophore A23187 on LNCaP and PC-3 cells. A, induction of DNA fragmentation by A23187 in LNCaP and PC-3 cells. Cultured cells were treated with (+) or without (−) 20 μM A23187 for 2 days before the DNA fragmentation assay. B, dose-response curves of A23187 concentration versus percentage of cell viability of LNCaP (solid line) and PC-3 (dotted line) as determined by trypan blue assay. Cultured cells were treated with various concentrations of A23187 for 2 days before the trypan blue assay. Values are the means of three independent measurements; bars, SD.

Fig. 4. The effect of antisense oligonucleotide on the sensitivity to Ca2+ ionophore A23187 and calreticulin expression in LNCaP (A and C) and PC-3 (B and D). A and B, the effect of A23187 at indicated concentrations on cell viability in the absence of oligonucleotides (control, □) or in the presence of either the antisense (■) or sense (□) 21-mer oligonucleotides. Values are the means of three independent measurements; bars, SD. C and D, Western blot analysis of calreticulin with mock treatment (M) in the absence oligonucleotides and in the presence of sense (S) or antisense (AS) oligonucleotides for the indicated number of days (d). An equal amount of protein extracts was loaded in each lane. The amount of protein extracts loaded in the gels was examined by staining the transferred nitrocellulose membrane with Ponceau-S.
staining. The treatment of sense oligonucleotides did not change the sensitivity of LNCaP cells to A23187. In contrast, the treatment with antisense oligonucleotides significantly enhanced the sensitivity of LNCaP cells to A23187. Antisense oligonucleotide treatment causes a 20–45% decrease in cell viability in the presence of A23187. The effect of calreticulin antisense oligonucleotides on PC-3 cells is very similar to the effect on LNCaP cells (Fig. 4B).

Antisense oligonucleotide treatment reduced calreticulin protein levels in both LNCaP and PC-3 cells (Fig. 4, C and D). Densitometric analysis shows that antisense treatment for 1 day caused a 2-fold decrease in calreticulin protein level, and antisense treatment for 2 days caused a 5-fold decrease (data not shown). The down-regulation of calreticulin protein by an antisense oligonucleotide in this experiment is comparable with that in other cell lines (8, 15).

It is important to point out that another pair of antisense and sense oligonucleotides, the 18-mer pair (see “Materials and Methods”), was also used in the experiment, and the results were virtually identical to that described above with the 21-mer pair. The fact that both pairs of calreticulin antisense and sense oligonucleotides had identical effects on calreticulin protein expression and the sensitivity to A23187 in cultured LNCaP cells indicates that antisense oligonucleotides are specific to calreticulin mRNA.

Androgen Regulates the Sensitivity of LNCaP but not PC-3 Cells to Ca\(^{2+}\) Ionophore A23187. To study the effect of androgen on intracellular Ca\(^{2+}\) buffering capacity, LNCaP cells were cultured in charcoal-stripped FBS for 1 day and then treated with a synthetic androgen analogue, mibolerone, at 0 or 10 nM for an additional 2 days before the addition of A23187. Up-regulation of calreticulin by androgen is expected to protect LNCaP cells from Ca\(^{2+}\) ionophore A23187-induced cytotoxic intracellular Ca\(^{2+}\) overload via enhancing the Ca\(^{2+}\) buffering capacity. Fig. 5A shows that mibolerone had a significant protective effect against A23187 in LNCaP cells. There are about 20–30% more cells alive in the whole population in the presence of 10 nM mibolerone. The effect of mibolerone is most striking in the presence of 20 \(\mu\)M A23187. In the absence of mibolerone, <5% of cells are alive when treated with 20 \(\mu\)M A23187 for 2 days. In contrast, in the presence of 10 nM mibolerone, 35% of LNCaP cells are still alive after 2 days of treatment with 20 \(\mu\)M A23187. The treatment of 10 nM mibolerone caused a 7-fold enhancement in cell viability in the presence of 20 \(\mu\)M A23187.

As a comparison, we also studied the effect of androgen on the sensitivity of Ca\(^{2+}\) ionophore-induced apoptosis in PC3, an androgen-insensitive human prostate cancer cell line. Fig. 5B shows that androgen had little or no effect on the sensitivity of PC-3 cells to A23187. Also, as expected, calreticulin expression is not influenced by mibolerone in PC-3 cells (result not shown).

Antisense Oligonucleotide Reverses the Androgen-induced Resistance to Ca\(^{2+}\) Ionophore in LNCaP Cells. To further investigate the relationship between androgen, calreticulin, and the sensitivity of LNCaP to Ca\(^{2+}\) ionophore A23187-induced cell death, we studied the effect of calreticulin antisense oligonucleotide on the sensitivity of LNCaP to A23187 either in the presence or absence of mibolerone.
(Fig. 6A). The protective effect of mibolerone on LNCaP cells was abrogated in the presence of calreticulin antisense oligonucleotide. As a control, the down-regulation of calreticulin protein by antisense oligonucleotide was demonstrated by Western blot (Fig. 6B). The cell viability of LNCaP to A23187 in the presence of both antisense oligonucleotide and mibolerone is higher than that in the presence of antisense oligonucleotide but absence of mibolerone. This difference in cell viability correlates with the difference in calreticulin protein level under these two treatment conditions. It appears that the more calreticulin protein is expressed, the higher percentage of viable cells can be detected.

**DISCUSSION**

The present experiments have studied the interplay between androgen, calreticulin, and the sensitivity to Ca^{2+} ionophore in LNCaP cells. Our results showed that androgen can regulate the sensitivity to Ca^{2+} influx-induced apoptosis, and calreticulin is involved in this androgen-dependent process in LNCaP cells. This observation suggests that calreticulin is likely to play an important role in modulating intracellular Ca^{2+} in prostatic epithelial cells in vivo during androgen manipulation.

The involvement of intracellular Ca^{2+} level in androgen action in prostatic epithelial cells was established previously by several elegant studies (24–26): (a) cell death induced by Ca^{2+} ionophore is indistinguishable from the cell death induced by castration in the prostate (26); (b) Ca^{2+} channel blockers can inhibit as much as 70% of the castration-induced increase in the rate of cell death in the prostate (24, 26); and (c) the channel blocker also inhibits the induction of Ca^{2+}-response genes in prostatic epithelial cells after castration (27). These observations suggest that castration induces Ca^{2+} influx through Ca^{2+} channels in prostatic epithelial cells because intracellular Ca^{2+} elevation could be inhibited by channel blocker. We have demonstrated that androgen regulates the sensitivity of LNCaP cells to Ca^{2+} ionophore A23187-induced apoptosis, suggesting that androgen could also modulate intracellular Ca^{2+} buffering capacity in prostatic epithelial cells. A combination of Ca^{2+} influx and reduction of Ca^{2+} buffering capacity could cause apoptosis very effectively in prostatic epithelial cells upon castration.

Androgen regulation of the sensitivity of cytotoxic Ca^{2+} overload is likely to be mediated through the expression of an androgen-response gene(s) because the androgen receptor is a ligand-dependent transcription factor. Calreticulin has the potential to mediate androgen regulation of the sensitivity to A23187 because it is an intracellular Ca^{2+}-binding protein encoded by a primary androgen-response gene and is abundantly expressed in prostatic epithelial cells in the rat model. The present study showed that in LNCaP cells calreticulin is also a primary androgen-response gene, and both calreticulin mRNA and protein are regulated by androgen, which agrees with our previous observations in the rat model (3).

The role of calreticulin in buffering intracellular Ca^{2+} was demonstrated previously by the observation that down-regulation of calreticulin by antisense oligonucleotides significantly increases the sensitivity of NG-108-15, a neuroblastoma X glioma cell line, to A23187-induced cytotoxic intracellular Ca^{2+} overload (8). The role of calreticulin in prostatic epithelial cells is likely to be similar to its role in other types of cells because calreticulin intracellular localization in LNCaP cells is virtually the same as its intracellular localization in other types of cells. The role of calreticulin in buffering intracellular Ca^{2+} in prostatic epithelial cells is further supported by the observation that down-regulation of calreticulin by antisense oligonucleotides significantly increases the sensitivity to A23187 in both LNCaP and PC-3 cells. Two different pairs of calreticulin sense and antisense oligonucleotides caused the same effect, indicating the antisense oligonucleotides are specific to calreticulin. Our study has also extended previous studies by demonstrating that A23187 induces cell death in LNCaP and PC-3 cells via apoptosis.

To further support the importance of calreticulin in androgen regulation in the sensitivity to A23187-induced apoptosis, we have demonstrated that calreticulin antisense oligonucleotide significantly inhibits the protective effect of androgen. This observation strongly suggests that calreticulin plays a major role in regulating the intracellular Ca^{2+} buffering capacity in LNCaP cells during androgen manipulation.

Although androgen induction of calreticulin in LNCaP cells is not as dramatic as the induction in the rat ventral prostate model, modest change in calreticulin level could have a profound effect on cells. For example, a 1.6-fold increase of calreticulin expression in mouse L fibroblast cell line increases intracellular Ca^{2+} storage and decreases store-operated Ca^{2+} influx (28). It is possible that androgen could have a more profound effect on intracellular Ca^{2+} buffering capacity in vivo because the response of calreticulin to androgen is very dramatic in the prostate in vivo.

Down-regulation of calreticulin does not appear to be sufficient to cause apoptosis in prostatic epithelial cells: (a) most epithelial cells in castrated prostate are still alive, although they express very low levels of calreticulin (3); (b) down-regulation of calreticulin in LNCaP cells by androgen deprivation or antisense oligonucleotides does not influence the viability of the cells; and (c) calreticulin also appears to be nonessential to the survival of other types of cells in culture. For example, homozygous knockout of the calreticulin gene in mouse embryonic stem cells does not influence the cell viability (29).

Although the calreticulin expression level has been shown to be associated with the sensitivity of LNCaP cells to Ca^{2+} ionophore, the mechanism of such an association remains unclear. Further characterization of calreticulin in LNCaP model may shed more light on the mechanism by which calreticulin regulates intracellular Ca^{2+} buffering and/or signaling in prostatic cells.

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