Novel Role of Androgens in Mitochondrial Fission and Apoptosis

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Abstract

Androgen and androgen receptors (AR) play critical roles in the proliferation of prostate cancer through transcriptional regulation of target genes. Here, we found that androgens upregulated the expression of dynamin-related protein 1 (Drp1), which is involved in the induction of mitochondrial fission, a common event in mitosis and apoptosis. Clinical tissue samples and various prostate cancer cell lines revealed a positive correlation between Drp1 and AR levels. Treatment of androgen-sensitive cells with an AR agonist, R1881, and antagonist, bicalutamide, showed that Drp1 is transcriptionally regulated by androgens, as confirmed by an AR ChIP-seq assay. Live imaging experiments using pAcGFP1-Mito stably transfected LNCaP (mito-green) cells revealed that androgen did not induce significant mitochondrial fission by itself, although Drp1 was upregulated. However, when treated with CGP37157 (CGP), an inhibitor of mitochondrial Ca²⁺ efflux, these cells exhibited mitochondrial fission, which was further enhanced by pretreatment with R1881, suggesting that androgen-induced Drp1 expression facilitated CGP-induced mitochondrial fission. This enhanced mitochondrial fission was correlated with increased apoptosis. Transfection with dominant-negative (DN-Drp1, K38A) rescued cells from increased apoptosis, confirming the role of androgen-induced Drp1 in the observed apoptosis with combination treatment. Furthermore, we found that CGP reduced the expression of Mfn1, a protein that promotes mitochondrial fusion, a process which opposes fission. We suggest that androgen-increased Drp1 enhanced mitochondrial fission leading to apoptosis. The present study shows a novel role for androgens in the regulation of mitochondrial morphology that could potentially be utilized in prostate cancer therapy. Mol Cancer Res; 9(8): 1067–77. ©2011 AACR.

Introduction

Prostate cancer is a major cause of death among men in Western countries. Androgens, through their binding to the androgen receptor (AR), induce the proliferation of prostate cells and are responsible for the progression of the cancer (1, 2). Androgen ablation is the most commonly used therapy for prostate cancer. However, androgen ablation often leads to progression of the tumor toward androgen depletion–independent (ADI) status, which is more difficult to treat (3, 4). In most ADI prostate cancers, the AR is overexpressed and aberrantly activated despite the elimination of androgens (5).

Mitochondria have gained much attention for their paradoxical roles in cell survival and cell death (6). Mitochondrial morphology can be rapidly altered by mitochondrial fission and fusion in response to the physiologic requirements of a cell (7). Mitochondrial fission requires translocation of the cytoplasmic dynamin-related protein 1 (Drp1) to the mitochondria where it interacts with the outer mitochondrial membrane protein, Fission 1 or Fis1 (8–11). Drp1, via its GTPase activity, carries out the scission of the outer mitochondrial membrane (12, 13). Functional impairment of Drp1 results in aggregates of large, interconnected mitochondria characteristic of unfragmented mitochondria (14). Posttranslational modifications of Drp1 such as phosphorylation (14–16), ubiquitination (17), sumoylation (18, 19), and S-nitrosylation (20) affect the function of Drp1. During mitosis,
Cdk1/cyclin B mediates Drp1 phosphorylation at Ser-616 (16, 21) to promote mitochondrial fission. On the contrary, cyclic AMP-dependent phosphorylation of Drp1 at Ser-637 reduced mitochondrial fission (14, 15, 22). Thus, Drp1 function seems to be affected by the site of phosphorylation. In contrast to Drp1 function, mitofusin proteins 1 and 2 (Mfn1 and Mfn2, respectively) are essential for GTP-dependent mitochondrial fusion (7). A balance between mitochondrial fission and fusion events is necessary for normal functioning of the cell (23). Although mitochondrial fission (fragmentation) is a part of both mitosis and apoptosis, it is not clear why fission leads in one case to cell division (16) and in another to cell death (24–26). Therefore, a better understanding of the regulation of proteins involved in mitochondrial fission and fusion is critical.

The objective of this research was to examine the regulation of Drp1 and its role in mitochondrial fission in prostate cancer cells. Results presented here show that androgen regulated Drp1 expression at the transcriptional level. Androgen-induced expression of Drp1 and its phosphorylation at Ser-616 correlated with androgen-induced cell proliferation. However, when the cells were treated with CGP37157 (CGP), which is known to affect mitochondrial function by inhibiting a mitochondrial sodium–calcium exchanger to induce mitochondrial calcium overload, androgen-induced Drp1 facilitated a significant increase in mitochondrial fission and apoptosis. Thus, we show that androgen functions as a proapoptotic agent when mitochondrial function is disrupted. These results provide a novel alternative to induce apoptosis in prostate cancer cells by taking advantage of AR activation–induced Drp1 expression to sensitize prostate cancer cells to drugs that compromise mitochondrial function.

Materials and Methods

Reagents

The following mammalian expression plasmids were used: pAcGFP1-Mito (mito-green), a green fluorescent label for mitochondria (Clontech Laboratories, Inc.); and dominant-negative Drp1 (DN-Drp1, K38A; a kind gift from Alexander van der Bliek, University of California, Los Angeles, CA). Antibodies used were as follows: anti-Drp1 (BD Biosciences); anti-PSA and anti-AR (Santa Cruz Biotechnology Inc.); anti-Mfn1 (Novus Biologicals); anti-phospho-Drp1 (Ser-616), anti-COX IV, anti-mouse, and anti-rabbit IgG (Cell Signaling Technology); and anti-Mfn2, anti-β-actin, and anti-chicken IgG (Sigma-Aldrich) and anti-GAPDH and anti-Hsp90α (Chemicon International). The chemicals used were as follows: charcoal/dextran-stripped FBS (CSS) from Hyclone; the synthetic androgen methyl trienolone (R1881) from Perkin-Elmer Life Sciences; bicalutamide and cycloheximide from Sigma-Aldrich; chloro-benzothiazepin CGP37157 (CGP) from Calbiochem; and G418 from Cellgro. Scrambled siRNA was from Qiagen and siRNA for AR was from Dharmacon RNAi Technologies (part of Thermo Scientific).

Cell culture and treatment

Prostate cancer cell lines LNCaP, DU145, and PC3 were purchased from American Type Culture Collection (ATCC). LNCaP-derived C4-2 cells were a gift from Dr. Leland Chung, Cedars-Sinai Medical Center, Los Angeles, CA. Cells were maintained in RPMI 1640 (Hyclone) containing 9% FBS, 0.5% penicillin-streptomycin, and 0.1% fungizone. The CWR-R1 cells (provided by Dr. E. Wilson, University of North Carolina, Chapel Hill, NC) were grown in Richter’s Minimum Essential Medium (MEM). Nontumorigenic prostate epithelial cells (P69; a gift from Dr. Leland Chung) were maintained in T-medium (GIBCO-Invitrogen). VCaP cells (ATCC) were maintained in DMEM-GlutaMax medium (GIBCO-Invitrogen) containing 10% serum and treated in the same medium containing 5% CSS. Cells were maintained in CSS (5%)-containing steroid-free medium for 48 hours and treated with R1881 for 24 hours or the indicated periods. In combination experiments, bicalutamide was added 8 hours prior to R1881 treatment for 24 hours. Cycloheximide (50 μg/mL) was added 1 hour before and during R1881 treatment. CGP (50 μmol/L) was added after 24 hours of R1881 treatment for the indicated periods. Transfection with siRNA was carried out using HiPerFect reagent (Qiagen) for 48 hours. Stably transfected LNCaP cells expressing green fluorescent protein (GFP) in the mitochondria, pAcGFP1-Mito (mito-green), were generated by electroporation using the Electro Square Porator ECM-830 (BTX Inc.), followed by G418 (500 μg/mL) selection.

Protein extraction and Western blotting

Cell lysates were processed as described previously (27). For phosphorylation studies, phosphatase inhibitor cocktail 1 and 2 were added (Sigma-Aldrich) to the lysis buffer.

Quantitative real-time reverse transcription PCR

Total RNA was extracted using TRIzol (Invitrogen). cDNA was synthesized using a Reverse Transcription System (Promega Corporation) and was amplified by quantitative real-time reverse transcription PCR (qRT-PCR) using Mastercycler ep-realplex2 (Eppendorf) with the IQ SYBR Green Supermix (Bio-Rad Laboratories). Primers used in this study were as follows: Drp1: 5’-CCAAGG-TGCCCTGTAGGTGAT-3’ and 5’-CAGCAGTGACAG-CGAGGATA-3’; prostate-specific antigen (PSA): 5’-CATCAGGAA-CAAAAGCGTGA-3’ and 5’-AGCTGT-GGCTGACCTGAAAT-3’; glyceraldehyde-3-phosphate dehydrogenase (GAPDH): 5’-CGAGGATA-3’; and glyceraldehyde-3-phosphate dehydrogenase (GAPDH): 5’-CATCAGGAA-CAAAAGCGTGA-3’ and 5’-AGCTGT-GGCTGACCTGAAAT-3’. The cycle threshold (Ct) was normalized using the known Ct from the housekeeping gene GAPDH. To compare the relative levels of gene expression of Drp1 or PSA, ddCt values were calculated using gene expression from the untreated control cells and ddCt values were expressed as the real-fold increase in gene expression.
Chromatin immunoprecipitation
Chromatin immunoprecipitation (ChIP) was carried out as previously described (28). ChIP enrichment was tested on an aliquot of the isolated DNA by real-time (quantitative) PCR and the remainder was used for single-end Solexa library preparation.

ChIP-seq Solexa library preparation
Single-end Solexa sequencing libraries were prepared as previously described (28).

Sequence read analysis
As previously described (28), sequence reads were generated by the Illumina analysis pipeline version 1.3.4 and 1.4.0, and the ChIP-seq data (28) deposited in the Gene Expression Omnibus (GEO; both processed data files and raw data) at the following URLs: http://www.ncbi.nlm.nih.gov/gds?term=GSE28126 (processed data files) and http://www.ncbi.nlm.nih.gov/gds?term=SRA012454.1 (raw data files).

Illumina BeadArrays and analysis
Illumina BeadArrays were conducted using standard Illumina protocols as described previously (28). Autocorrelation analysis of Illumina gene expression data was conducted as described in the work of Massie and colleagues (28). The Illumina gene expression data (28) have been deposited on GEO at http://www.ncbi.nlm.nih.gov/geo.

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Mitochondrial morphology
Stably transfected mito-green LNCaP cells were maintained in androgen-depleted medium for 48 hours before R1881 treatment for 24 hours and/or CGP (50 μmol/L) for the last 1 hour. Cells were washed and suspended in HBSS (GIBCO-Invitrogen) and at least 200 cells were examined for mitochondrial fragmentation under a fluorescence microscope (Zeiss Axioskop: Carl Zeiss Imaging Inc.). Mito-green cells were also grown on cover slips and treated as above. Cells were fixed with 4% paraformaldehyde and then analyzed using LSM 510 confocal microscopy (Carl Zeiss). Using the Z-stack option, signals from different planes were obtained and combined to give a single picture (maximum intensity projection). We considered cells to be undergoing mitochondrial fission when more than 80% of mitochondria in the cell exhibited fragmentation.

Cell proliferation assays
Proliferation of LNCaP cells was measured using the Quick Cell Proliferation Assay Kit (BioVision). LNCaP cells (5 x 10^3 per well) seeded in 96-well microtiter plate were treated with bicalutamide (50 μmol/L) and/or R1881 (1 nmol/L). CGP (50 μmol/L) was added during the last 4 hours of treatment. Reagent from the kit was added and samples were read in a microplate reader. For S-phase analysis, cells were treated with 1 nmol/L R1881 and fixed overnight in 70% ethanol, stained with propidium iodide (20 μg/mL) and cells in S-phase were measured using flow cytometry.

Measurement of apoptosis
Total cell lysates (5 μg) were analyzed for apoptosis using an M30-Apoptosense kit (Peviva, Diapharma Group) as described previously (27).

Human prostate tissue
Deidentified coded tissue was obtained postsurgery from prostate cancer (n = 7) and hormone-refractory metastatic prostate cancer patients (n = 6) from the University of Michigan SPORE tissue bank, Ann Arbor, MI. Tissues were lysed in buffer containing 7 mol/L urea, 2 mol/L thiourea, 100 mmol/L DTT, 2% octyl glucoside, and EDTA-free protease inhibitor cocktail (Roche) and the proteins were separated by SDS-PAGE, transferred to membranes and immunoblotted for Drp1, PSA (positive control), or GAPDH (loading control).

Statistical analyses
Data are presented as means ± SEM. We compared group mean values, as appropriate, by Student’s unpaired 2-tailed t test or 1-way ANOVA with Tukey’s multiple comparison test (GraphPad Prism, La Jolla, CA). Significant differences were defined at ***, \(P \leq 0.001\); **, \(P \leq 0.01\); and *, \(P \leq 0.05\).

Results
Expression of Drp1 is correlated with the expression and function of AR in the prostate
Prostate cells undergo proliferation in response to androgens. Fragmentation or fission of mitochondria is a required step in the process of mitosis and requires the involvement of Drp1, a key regulator of mitochondrial fission. Analysis of prostate cells showed that the expression of Drp1 was highest in androgen-responsive CWR-R1 cells, followed by LNCaP and LNCaP-derived C4-2 cells (Fig. 1A). The lowest levels of Drp1 were found in androgen-independent PC3 and DU145 cells. To assess the mitochondrial content of these cell lines, we also examined the COX IV expression levels (Fig. 1A), which did not differ much in the cell lines. Densitometric analysis of the signals showed a correlation between the expression of Drp1 and of AR (correlation coefficient of 0.927) with a P value of 0.008 (Fig. 1B), suggesting a positive relationship between the expression of these 2 proteins. Furthermore, the expression of Drp1 mRNA was higher in cells expressing AR compared with AR-null cells (Fig. 1C). Analysis of tissue samples obtained postsurgery from prostate cancer patients showed higher Drp1 levels in tissues where AR is functional, as indicated by the presence of PSA (Fig. 1D). On the other hand, androgen-refractory metastatic tissue showed extremely low levels of Drp1 (Fig. 1D). These results show a positive correlation between the expression and levels of Drp1 and the expression/function of AR.

Androgen regulated Drp1 expression at the transcriptional level
As the levels of Drp1 correlated with the expression of AR, the role of androgens in the regulation of Drp1...
expression was investigated. Androgen-responsive LNCaP cells were maintained in androgen-depleted medium, which significantly reduced the levels of AR, Drp1, and PSA proteins (Fig. 2A; left). Treating these cells with the synthetic androgen, R1881, restored the protein expression of Drp1 and the positive control PSA (Fig. 2A, right), indicating that androgen increased the expression of Drp1 protein.

qRT-PCR analysis showed that treatment of LNCaP cells with R1881 increased the expression of Drp1 mRNA, suggesting that Drp1 is transcriptionally regulated by androgens (Fig. 2B, left). As expected, the mRNA levels of the positive control, PSA, were also upregulated in R1881-treated cells (Fig. 2B, right). The expression of Drp1 protein increased in an R1881 dose–dependent manner (Fig. 2C). Drp1 mRNA was also upregulated with increasing duration of R1881 treatment (Fig. 2D).

The specificity of the Drp1 response to androgens was confirmed using the antiandrogen bicalutamide. Bicalutamide inhibited androgen-induced Drp1 upregulation in a dose-dependent manner with a significant decrease at 50 μmol/L and a complete abrogation of androgen-induced Drp1 protein expression upon treatment with 80 μmol/L bicalutamide (Fig. 3A). Similar analysis of Drp1 mRNA showed decreased expression with increasing concentrations of bicalutamide (Fig. 3B). Furthermore, silencing AR using AR-specific siRNA resulted in Drp1 down-regulation in LNCaP cells (Fig. 3C). As the above results confirmed transcriptional regulation of Drp1 by androgens, experiments were carried out to determine whether the androgen-induced upregulation of Drp1 is mediated through an intermediary androgen-responsive protein. The expression of R1881-induced Drp1 mRNA was not altered when cells were treated with cycloheximide (Fig. 3D, left), suggesting that the regulation of Drp1 by androgens did not require de novo protein synthesis. Western blots confirmed that Drp1 protein expression did not increase with R1881 in cycloheximide-treated cells (Fig. 3D, right), indicating the effectiveness of cycloheximide treatment.

Presence of the AR-binding site in the Drp1 gene

To determine whether AR binds to an androgen response element (ARE) in the Drp1 promoter, we conducted ChIP analysis. The resultant ChIP screenshot data were uploaded in a .wig format into the UCSC genome browser and visualized against an hg18 reference genome. Analyzed data depicted the AR-binding region in the Drp1 gene (Fig. 4A, top). Tracks included RefSeq genes, ENCODE transcription factor binding site data, and publicly available AR binding site data (29) generated by the laboratory of Myles Brown in both an androgen-independent (abl_AR) and an androgen-dependent (LNCaP) cell line, as illustrated by black bars near the top of the screenshots. The .wig data appears in red and is generated by ChIP-seq analysis following immunoprecipitation of the AR in the LNCaP cell line as described in Materials and Methods. The identified binding site is within Drp1 and overlaps with AR sites in public data sets. Higher resolution of the same site depicted the bases where AR binds to Drp1 gene (Fig. 4A, bottom). The data predict an AR-binding site which is downstream of the RNA Pol II site at the start of the Drp1 (DNM1L) gene and is located at chr12: 32,724,000–32,725,500.

We also conducted Drp1 expression array plots by treating LNCaP cells with R1881 at different time points (Fig. 4B). Each plot represents a distinct BeadArray probe for the gene. Drp1 expression was clearly increased as a result of R1881 treatment in a time-dependent manner. TMPRSS2 was included as a positive control plot as a known androgen-regulated gene in LNCaP cells.

On the basis of the ChIP-seq analysis and expression BeadArray time course, we have confirmed that Drp1 is androgen regulated via an internal ARE located within a 5′ untranslated region of Drp1.
Regulation of Drp1 in androgen-sensitive and androgen-refractory cells

Treatment of another androgen-sensitive prostate cancer cell line, VCaP (30), with R1881 resulted in increased Drp1 expression, which was reduced in the presence of the antiandrogen bicalutamide (Fig. 5A), confirming our results obtained using LNCaP cells. Transition from androgen-dependent to ADI status is a critical problem in prostate cancer therapy. To determine the androgen regulation of Drp1 in ADI cells, C4-2 cells, which are known to have lost their dependence on androgen for proliferation (31, 32), were treated with R1881, which upregulated Drp1 protein (Fig. 5B). Treatment with bicalutamide reduced Drp1 expression, confirming the ability of ADI cells to respond to androgens. Similar experiments in AR-null DU145 and PC3 cells revealed no further increase in Drp1 protein with R1881 treatment, confirming the role of AR in mediating the upregulation of Drp1 (Fig. 5C and D).

Androgens facilitated mitochondrial fission induced by CGP

As Drp1 plays a critical role in mitochondrial fission, we hypothesized that its upregulation by androgens would affect mitochondrial morphology. To verify this hypothesis, LNCaP cells, stably transfected with a construct expressing GFP protein in the mitochondria, were treated with R1881 and mitochondrial fission was scored. Contrary to our expectation, mitochondria in androgen-treated cells remained filamentous as in controls (Fig. 6A), suggesting that the androgen-induced increase in Drp1 was not sufficient to induce significant mitochondrial fission. To confirm the occurrence of mitochondrial fission in these cells, cells were treated with CGP37157 (CGP), an inhibitor of mitochondrial Ca$$^2+$$ efflux, which induced mitochondrial fission in DU145, another prostate cancer cell line (9). As expected, CGP induced mitochondrial fission in LNCaP resulting in fragmented mitochondria, visualized as punctate structures (Fig. 6A). Surprisingly, CGP treatment in the presence of R1881 resulted in higher number of cells with fragmented mitochondria when compared with CGP treatment alone. These observations were confirmed by quantitative analysis of fragmented mitochondria (Fig. 6B). Therefore, we suggest that androgen-induced upregulation of Drp1 is not sufficient to induce mitochondrial fragmentation, but increased levels of Drp1 readily potentiated mitochondrial fission when an appropriate mitochondrial stimulus is provided.

Androgen affects the phosphorylation of Drp1 at Ser-616

As androgens are known to induce proliferation of prostate cells, the role of Drp1 in cell survival was investigated. As expected, R1881 treatment significantly increased the proliferation of LNCaP cells compared with vehicle-treated controls (Fig. 7A, left). The specificity of the response was confirmed by the ability of bicalutamide to reduce proliferation. Cell proliferation/viability was lower in CGP-treated cells even when the cells were also treated with R1881 (Fig. 7A, left). The effect of R1881 on proliferation was confirmed by flow cytometry,
Drp1 is an androgen-regulated gene. An increase in Drp1 levels in response to R1881, establishing that androgen increases Drp1 phosphorylation at Ser-616 (Fig. 7B). Because Drp1 levels increase in response to R1881, we hypothesized that androgen mediated CGP-induced apoptosis: mediation by Drp1

Androgens enhanced CGP-induced apoptosis: mediation by Drp1

As the known function of Drp1 is in mitochondrial fission concomitant with mitosis or cell death (23, 25), apoptosis was measured under our experimental conditions. CGP treatment for 18 hours resulted in a 3-fold increase in apoptosis as compared with controls (Fig. 7C). The apoptotic response to CGP was further enhanced (to 11-fold) in the presence of R1881. There was no significant apoptosis, as measured by caspase-cleaved cytokeratin 18, observed in the first 4 hours of CGP treatment, although mitochondrial fission was observed within 1 hour of CGP treatment, suggesting that mitochondrial fission is an early event compared with apoptosis (Fig. 7C). Transfection of a DN-Drp1 (K38A) plasmid reduced the apoptotic response in cells treated with both CGP and R1881 (Fig. 7D), indicating that the induction of apoptosis by combination therapy required Drp1 and further confirming that androgen via Drp1 facilitated CGP-induced apoptosis. However, we observed no increase in apoptosis when LNCaP cells overexpressed a wild-type Drp1 (WT-Drp1) plasmid under our treatment conditions (data not shown). This result suggested that the androgen-induced increase in Drp1 is necessary but not sufficient to enhance CGP-induced apoptosis. There may be other factors involved in the CGP-induced apoptotic response in the presence of androgen. Next, we examined the protein expression of Mfn1 and Mfn2, proteins that facilitate mitochondrial fusion, an opposing function relative to Drp1. Western blot analyses showed that Mfn1 expression was not affected by androgen alone. However, CGP treatment in the absence or presence of androgen significantly reduced the levels of Mfn1 (Fig. 7E). Similar analysis of Mfn2 did not show changes in expression with any treatment. These observations suggest that increased expression of Drp1, and perhaps a concomitant decrease in Mfn1, exaggerated CGP-induced mitochondrial fission and apoptosis.

Androgen depletion therapy is the mainstay of prostate cancer treatment. However, development of androgen-independent tumors is a major concern in patient care. Therefore, a more effective therapy would be an alternative to androgen ablation that still exhibits efficacy in inducing death of prostate cancer cells. In this article, we describe a mechanism to induce apoptosis in prostate cancer cells in the presence of androgens; indeed androgens facilitated the death of these cells.

Mitochondrial fission (fragmentation) is a common event in both cell proliferation and apoptosis. In resting cells, mitochondria are filamentous, whereas in cells undergoing mitosis or apoptosis, they are fragmented, appearing as punctate/pinhead-like structures. A key protein involved in mitochondrial fragmentation is Drp1. As androgens induce cell proliferation and are important in prostate cancer progression, we hypothesized that Drp1 may be androgen responsive. Indeed, our results confirmed a correlation between the expression of Drp1 and the expression and function of AR (PSA expression) in several prostate cell lines at both the mRNA and protein levels. Downregulation of AR using siRNA reduced the expression of Drp1, indicating androgen regulation of Drp1 expression. AR function and Drp1 expression also correlated significantly in androgen-refractory metastatic tissues, indicating that Drp1 is an androgen-regulated gene. An increase in Drp1
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Figure 4. ChIP-seq and Illumina BeadArray expression analyses confirmed the presence of an AR-binding site in the Drp1 gene. A, AR ChIP-seq data analysis showing .wig data of an AR-binding site located in the Drp1 gene. As denoted by "KLK2" in the figure, the sequence in red denotes only AR-binding sites that are enriched or more enriched than the binding site in kallikrein 2 (KLK2), a prostate-associated protease that activates PSA. The data predict an AR-binding site that is downstream of the RNA Pol II site at the start of the Drp1 (also known as dynamin-like protein 1 or DNM1L) gene, located at chr12: 32,724,000–32,725,500. B, LNCaP cells were treated with R1881 for different time periods and isolated mRNA was subjected to Illumina BeadArray analysis. The 2 left plots represent results obtained with 2 separate probes for Drp1. TMPRSS2 was analyzed as a positive control for an androgen-responsive gene. Open circles represent cells treated with androgen and blue circles with vehicle (EtOH). The analysis approach generates the autocorrelation factor (acf) for each gene as described previously (28).
mRNA and protein expression in androgen-treated cells and a decrease in Drp1 expression upon treatment with the androgen antagonist, bicalutamide, confirmed the ability of androgen to regulate Drp1 expression at the transcriptional level. Experiments using cycloheximide to inhibit the synthesis of proteins further confirmed that the increase in Drp1 mRNA due to androgen is not a response to other androgen-responsive proteins. Our ChIP-seq data confirmed the presence of an AR-binding site in the Drp1 gene. This AR-binding region was located almost 1 kb downstream of the transcription start site in an untranslated region of Drp1. Illumina BeadArray results confirmed transcriptional regulation of Drp1 by androgens. Thus, these results are significant, as this is the first report of the regulation of any gene associated with mitochondrial fission/fusion mechanisms by androgens.}

Initial experiments to explore the mechanisms involved showed an increase in the phosphorylation of Drp1 at Ser-616 in androgen-treated cells. The Drp1-Ser-616 site was selected for investigation as this amino acid is phosphorylated by Cdk1/cyclin B (16, 21). As cyclin B is an androgen-responsive gene (33), it is possible that phosphorylation of Ser-616 by R1881 is mediated through cyclin B, resulting in androgen-mediated proliferation of prostate cancer cells. Thus, androgens not only increase the levels of Drp1 with their effects on transcription but also seem to influence the function of Drp1 via an indirect androgen-responsive protein, ultimately leading to cell proliferation.

On the basis of the known function of Drp1 in mitochondrial fragmentation and apoptosis (23, 25), we expected that increased expression of Drp1 in androgen-treated cells would lead to mitochondrial fission. Surprisingly, despite the increase in Drp1 with their effects on transcription but also seem to influence the function of Drp1 via an indirect androgen-responsive protein, ultimately leading to cell proliferation.

On the basis of the known function of Drp1 in mitochondrial fragmentation and apoptosis (23, 25), we expected that increased expression of Drp1 in androgen-treated cells would lead to mitochondrial fission. Surprisingly, despite the increase in Drp1 with their effects on transcription but also seem to influence the function of Drp1 via an indirect androgen-responsive protein, ultimately leading to cell proliferation.

**Figure 5.** Androgens regulate Drp1 in androgen-sensitive and androgen-independent (VCaP and C4-2) but not in androgen-refractory, AR-negative (DU145 and PC3) prostate cancer cells. A, androgen-sensitive VCaP cells were treated with R1881 and bicalutamide, and total cell lysates were analyzed by Western blotting for Drp1 expression. B, ADI C4-2 cells were treated and analyzed (as in A). C and D, androgen-refractory, AR-negative DU145 (C) and PC3 cells (D) were treated with R1881 (10 nmol/L) for 24 hours. All cells were grown in steroid-depleted medium for 48 hours before R1881 treatment and analyzed (as in A).

**Figure 6.** Androgen facilitated mitochondrial fission induced by CGP. Stably transfected mito-green LNCaP cells were treated with R1881 (1 nmol/L) for 24 hours with or without CGP (50 μmol/L) for the last 1 hour, the cells were washed with HBSS and live cells were observed in a confocal fluorescent microscope. A, representative photographs of cells in each treatment group is shown, illustrating the shape of the mitochondria. These pictures are from confocal imaging (Zen software) using “Z-stack” option and signals from all the planes were considered using the “Maximum Intensity Projection” option. B, cells exhibiting more than 80% punctate (fragmented) mitochondria were counted and are presented as a percentage of the total number of cells counted. At least 200 cells were examined in each dish and values represent the means ± SEM from at least 3 separate experiments with *, P ≤ 0.05; **, P ≤ 0.01; ***, P ≤ 0.001.
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Figure 7. Androgen enhanced CGP-induced apoptosis: mediation by Drp1. A, androgen increased cell proliferation. LNCaP cells were treated with R1881 (1 nmol/L), bicalutamide (50 μmol/L), or CGP (50 μmol/L) as described above. Cell proliferation was measured using a Quick Cell Proliferation assay kit (BioVision). Data are expressed as means ± SEM, with the parentheses indicating the P values (n = 5; left). Right, cells were treated with R1881 (1 nmol/L) for 48 hours, fixed overnight with 70% ethanol, treated with RNase, and then stained with propidium iodide and analyzed using flow cytometry to determine the S-phase of the cell cycle (**, P ≤ 0.002; n = 3). B, androgens increased phosphorylation of Drp1-Ser-616. LNCaP cells were treated with R1881 (1 nmol/L) and CGP (50 μmol/L for 18 hours) as described earlier. Cell lysates were subjected to Western blot analysis and Drp1 phosphorylation at Ser-616 and total Drp1 determined. Bottom, the densitometric values for phospho-Ser-616 Drp1 levels were normalized to the levels of total Drp1 in the above blot. C, androgens increased CGP-induced apoptosis. LNCaP cells were treated with R1881 (1 nmol/L) for 24 hours. Cells were treated with CGP (50 μmol/L) for an additional 4 or 18 hours in the presence or absence of R1881. Cell lysates were used for an apoptosis assay using a M30-Apoptosense analyses kit. Results are presented as the percentage of apoptosis compared with the control (**, P ≤ 0.002; n = 3). D, DN-Drp1 reduced androgen-mediated CGP-induced apoptosis. LNCaP cells were transfected with DN-Drp1 and treated with R1881 (1 nmol/L) and CGP (50 μmol/L, for 18 hours) as described earlier. Cell lysates were analyzed for apoptosis using an M30-Apoptosense analysis kit. Results are presented as the percentage of the respective controls (**, P ≤ 0.01; n = 3). E, CGP induced a decrease in Mfn1. LNCaP cells were treated (as in B), and total cell lysates were analyzed for Mfn1 and Mfn2 levels.

(11, 34). However, the lack of mitochondrial fission in androgen-treated cells is not due to lack of response of the cells to undergo mitochondrial fission; treatment with CGP, either alone or in combination with androgen, induced mitochondrial fission. Overexpression of wild-type Drp1 in LNCaP cells also resulted in no further increase in androgen- or combination therapy–induced apoptosis (data not shown), further suggesting that the androgen-induced increase in Drp1 is not sufficient for the induction of apoptosis. Together with our findings that siRNA-mediated knockdown of Drp1 inhibited apoptosis induced by combination treatment with androgen and CGP, these results suggest that Drp1 is necessary but not sufficient for apoptosis. Clearly, an additional stimulus, such as mitochondrial calcium overload induced by CGP, is necessary to promote apoptosis in the presence of androgen-increased Drp1. Nevertheless, increased mitochondrial fission with combination therapy correlated with the induction of cell death, suggesting that the availability of higher levels of Drp1 in androgen-treated cells facilitated CGP-induced apoptosis. Thus, androgens seem to function as proapoptotic agents when combined with an agent that affects mitochondrial function. These data support earlier observations that androgens are proapoptotic under certain conditions such as upon Bax-mediated cell death (35) or treatment with phorbol 12-myristate 13-acetate (36) or with taxane (37).

In summary, we show a role for androgens in transcriptional and posttranslational regulation of Drp1, a key protein...
in the mitochondrial fission machinery. Furthermore, androgen-induced Drp1 facilitated mitochondrial fission and apoptosis in cells treated with CGP. These data suggest a novel proapoptotic mechanism of action of androgens and also address a fundamental question about the involvement of the mitochondria in proliferation and apoptosis.

**Disclosure of Potential Conflicts of Interest**

No potential conflicts of interest were disclosed.

**Acknowledgments**

The authors thank Arri Jain for her technical support, Asmaa Al-Enawy for her valuable suggestions during preparation of the manuscript, and Dr. Ana El-Romessy and her laboratory members for generously sharing their real-time PCR machine and other equipments.

**Grant Support**

This research was funded by VA merit awards to M.V. Kumar and W.B. Bollag and an award from the American Legion of Georgia to both M.V. Kumar and W.B. Bollag. This work is supported in part by the National Cancer Institute grants RO1CA133458-04, R03CA139849-01, and R1CA145444A to A. Sreekumar. A. Sreekumar was also supported by a Georgia Cancer Research Distinguished Scientist award.

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Received September 28, 2010; revised May 19, 2011; accepted June 23, 2011; published OnlineFirst July 1, 2011.

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