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Calpain-mediated androgen receptor breakdown in apoptotic prostate cancer cells

Huanjie Yang¹, Shalini Murthy², Fazlul H. Sarkar¹, Shijie Sheng¹, G. Prem-Veer Reddy^{1,2,*}, and Q. Ping Dou^{1,*}

¹Barbara Ann Karmanos Cancer Institute, and Department of Pathology, School of Medicine, Wayne State University, Detroit, Michigan, USA

²Vattikuti Urology Institute, Henry Ford Hospital, Detroit, Michigan, USA

Abstract

Since androgen receptor (AR) plays an important role in prostate cancer development and progression, androgen-ablation has been the frontline therapy for treatment of advanced prostate cancer even though it is rarely curative. A curative strategy should involve functional and structural elimination of AR from prostate cancer cells. We have previously reported that apoptosis induced by medicinal proteasome-inhibitory compound celastrol is associated with a decrease in AR protein levels. However celastrol-stimulated events contributing to this AR decrease have not been elucidated. Here, we report that a variety of chemotherapeutic agents, including proteasome inhibitors, a topoisomerase inhibitor, DNA-damaging agents and docetaxel that cause cell death, decrease AR levels in LNCaP prostate cancer cells. This decrease in AR protein levels was not due to the suppression of AR mRNA expression in these cells. We observed that a proteolytic activity residing in cytosol of prostate cancer cells is responsible for AR breakdown and that this proteolytic activity was stimulated upon induction of apoptosis. Interestingly, proteasome inhibitor celastrol- and chemotherapeutic drug VP-16-stimulated AR breakdown was attenuated by calpain inhibitors calpastatin and N-Acetyl-L-leucyl-L-leucyl-L-methioninal. Furthermore, AR proteolytic activity pulled down by calmodulin-agarose beads from celastrol-treated PC-3 cells showed immunoreactivity to a calpain antibody. Taken together, these results demonstrate calpain involvement in proteasome inhibitor-induced AR breakdown, and suggest that AR degradation is intrinsic to the induction of apoptosis in prostate cancer cells.

Keywords

proteasome inhibitors; anticancer drugs; prostate cancer; apoptosis; cell death

Introduction

Androgen receptor (AR) is a physiological mediator of the development and function of male reproductive organs (Chang et al., 1995). Upon androgen binding, inactive AR is activated through dimerization and nuclear translocation, where it functions as a transcription factor to alter the expression of androgen responsive genes (Schaufele et al., 2005). AR plays an important role in the initiation and progression of prostate cancer by regulating cell proliferation, differentiation and apoptosis (Chang et al., 1995). Prostate

*Correspondence to: Q. Ping Dou, The Prevention Program, Barbara Ann Karmanos Cancer Institute, and Department of Pathology, School of Medicine, Wayne State University, 540.1 HWCRC, 4100 John R Road, Detroit, Michigan, 48201, U.S.A. Tel: 313-576-8301; Fax: 313-576-8307; E-mail: douq@karmanos.org, and G. Prem-Veer Reddy, Vattikuti Urology Institute, Henry Ford Health System, One Ford Place, 2D, Detroit, MI 48202. Phone: 313-874-5991; Fax: 313-874-4324; E-mail: preddy1@hfhs.org.

cancer has the highest incidence among malignancies in males, and is the second leading cause of cancer-related death in Western countries (Jemal et al., 2007). Early stages of prostate cancer can be effectively treated by androgen-ablation therapy through surgical and medical castration. However, most of these prostate cancer patients eventually relapse to a hormone-refractory state that no longer responds to androgen deprivation (Heinlein and Chang, 2004). AR appears to be a dominant factor in the transition from hormone-sensitive to hormone-refractory disease (Tamura et al., 2007). There is well-established evidence to show that the AR gene undergoes alterations such as amplification or mutation in hormone-independent cancers. As a result, these hormone-independent cancer cells are very sensitive to low or no androgen environments, and are responsive to a broad range of ligands such as growth factors, other steroid hormones, anti-androgens, etc. (Chen et al., 2004; Culig et al., 1994). It has also been reported that wild type AR can be activated by other signaling pathways in a ligand-independent manner (Culig, 2004; Unni et al., 2004). Furthermore, unliganded AR can bind the enhancer elements on the promoters of target genes and mediate their expression even in the absence of androgen, as seen with the prostate specific antigen (PSA) gene in androgen-independent prostate cancer cells (Jia et al., 2006). Therefore, finding effective means to eliminate AR from prostate cancer cells is essential for an effective treatment of prostate cancer.

AR is a member of the steroid receptor superfamily of ligand activated transcription factors. Like other members of this family, AR contains three domains: an NH₂-terminal domain (ATD), a central DNA binding domain (DBD), and a carboxy-terminal ligand binding domain (LBD). The DBD and LBD are separated by a hinge region containing a PEST (Proline-, glutamate-, serine-, and threonine-rich) motif (Lee and Chang, 2003). Degradation of AR *via* the ubiquitin-proteasome pathway has been suggested to occur at the putative PEST sequence located in the hinge region (Sheflin et al., 2000), and Akt/Mdm2 complex is responsible for AR phosphorylation that is required for ubiquitination and degradation (Lin et al., 2002). Early studies revealed that AR is degraded by a serine protease to generate ~30 kDa or ~41 kDa fragment containing the ligand binding domain (de Boer et al., 1987). Caspases are also reported to cleave AR with expanded polyglutamine repeats (Kobayashi et al., 1998; Wellington et al., 1998). We reported recently calcium-stimulated, calpain-mediated breakdown of AR to 31–34 kDa, ~50 kDa, and 75 kDa NH₂-terminal fragments in LNCaP prostate cancer cells (Pelley et al., 2006). An unknown neutral protease in the ventral prostate cytosol was shown to cleave AR to produce a fragment with similar size to ~50 kDa in the presence of serine protease inhibitor diisopropyl fluorophosphate (DFP) (Wilson and French, 1979). Later, calpain was reported to generate an 80 kDa truncated AR that appears to have elevated transcriptional activity (Libertini et al., 2007). Thus, the role of several of these proteases in generation of AR fragments and the biological significance of AR fragments generated by proteolytic cleavage in proliferation and/or viability of prostate cancer cells remain obscure.

Previously we reported that proteasome inhibitors caused depletion of AR protein in both androgen-dependent LNCaP cells and androgen-independent C4-2B cells (Chen et al., 2007; Yang et al., 2006; Yang et al., 2007; Yang et al., 2008). The observation that induction of apoptosis by proteasome inhibitors is accompanied by decreasing AR levels in AR-positive prostate cancer cells suggests that elimination of AR is intimately linked with apoptosis. To identify regulatory events contributing to the decrease in AR levels during proteasome inhibitor-induced apoptosis in prostate cancer cells, we examined AR expression at protein and mRNA levels following treatment with different proteasome inhibitors. Our observation that the dramatic decrease in AR protein upon treatment with proteasome inhibitors is not preceded by a corresponding decrease in AR mRNA led us to focus on AR protein stability. We attempted to identify protease(s) responsible for AR degradation in proteasome inhibitor-treated prostate cancer cells by using a novel *in vitro* AR degradation assay

involving recombinant human AR (rhAR) and PC-3 cell extracts, and intact LNCaP cells. Our results demonstrate calpain involvement in AR breakdown during proteasome inhibitor-induced apoptosis in prostate cancer cells.

Materials and Methods

Materials

PC-3 and LNCaP cell lines were purchased from American Type Culture Collection (Manassas, VA). Fetal bovine serum (FBS) was from Tissue Culture Biologicals (Temecula, CA). RPMI 1640, phenol red free RPMI 1640 medium, charcoal stripped FBS and SuperScript III first-strand system were purchased from Invitrogen Co. (Carlsbad, CA). B-DIM, a formulated DIM with higher bioavailability, was kindly provided by Dr. Michael Zeligs (BioResponse, Boulder, CO). Docetaxel was purchased from Aventis Pharmaceuticals (Bridgewater, NJ). Celestrol, withanferin A (WA), calpain inhibitors PD15060 and calpastatin, plasminogen activator inhibitor-1 (PAI-1), caspase-3 inhibitor III and monoclonal antibody against small subunit of calpain were purchased from Calbiochem, Inc. (San Diego, CA). VP-16, cisplatin, copper chloride, disulfiram (DSF), calpain inhibitor N-Acetyl-L-leucyl-L-leucyl-L-methioninal (ALLM), trans-Epoxy succinyl-L-leucylamido-(4-guanidin) butane (E64) or N-ethylmaleimide (NEM), phenylmethylsulfonyl fluoride (PMSF), N α -p-Tosyl-L-lysine chloromethyl ketone (TLCK), N-Tosyl-L-phenylalanine chloromethyl ketone (TPCK), leupeptin, aprotinin, calmodulin-agarose, trypsin inhibitor-agarose, and aprotinin-agarose were from Sigma (St. Louis, MO). All these reagents were prepared according to manufacture's instructions. Rabbit polyclonal antibody AR (N20) against N-terminus of human androgen receptor, goat polyclonal antibody against β -actin (C-11) and protein A-agarose were purchased from Santa Cruz (Santa Cruz, CA). High Pure RNA Isolation Kit was from Promega (Promega, WI). Mouse monoclonal antibody against human poly(ADPribose) polymerase (PARP) was from BIOMOL International LP (Plymouth Meeting, PA). Enhanced Chemiluminescence Reagent was purchased from Amersham Biosciences (Piscataway, NJ). Protein Assay Kit was purchased from Bio-Rad Laboratories (Hercules, CA). His-tagged recombinant human androgen receptor (rhAR) expressing baculovirus clone BV_AR10 was obtained from Magene Life Sciences Pvt. Ltd. (Hyderabad, India). rhAR protein was purified from BV_AR10-infected High Five insect cells using a standard procedure involving Ni-NTA column (Invitrogen, Carlsbad, CA).

Cell cultures

PC-3 and LNCaP cells were grown in RPMI 1640 medium supplemented with 10% fetal bovine serum, 100 units/mL of penicillin, and 100 μ g/mL of streptomycin and maintained at 37 °C and 5% CO₂. For steroid-depleted experiments, LNCaP cells were washed with PBS and cultured in RPMI 1640 without phenol red containing 10% charcoal stripped FBS for 3–4 days before the initiation of the experiment.

RNA isolation and RT-PCR

Total RNA was extracted from cells using High Pure RNA Isolation Kit according to the manufacturer's recommendations. Two μ g of total RNA, treated with DNase, were used for reverse transcription by using SuperScript III first-strand system. A total of 1 μ l of cDNA products was used as template for the subsequent PCR reactions using 200 nM gene specific primers. The primers for AR were 5'-CATCAAGGAAGCTCGATCGT and 5'-GAACTGATGCAGCTCTCTC. The glyceraldehyde-3-phosphate dehydrogenase (GAPDH) primers were 5'-TTGCAACTGTTTCTAGGACTTT and 5'-AGCATTGGGAAATGTTCAAGG. The PCR conditions were: 10 min at 95°C, 30 (for GAPDH) or 35 cycles (for AR); 1 min at 94°C; 1 min at 54°C; and 1 min at 72°C. Different PCR cycle numbers were tested for AR and GAPDH to ensure that the assay was in the

linear range of amplification. The resulting products were visualized by agarose gel electrophoresis followed by ethidium bromide staining.

Whole cell protein preparation and subcellular fractionation

Whole cell lysates were prepared in lysis buffer (50 mM Tris-Cl, pH 7.4/150 mM NaCl/ 0.5% NP-40) from cultured cells at 70–80% confluence, as described previously (Nam et al., 2001). For the subcellular fractionation, the cells harvested by scraper were suspended in ice-cold cell lysis buffer (10 mM HEPES, pH 7.9/10 mM KCL/0.1 mM EDTA/0.1 mM EGTA/1 mM DTT) and incubated on ice for 15 min. After addition of 10% NP-40 (3 µl for every 100 µl cell lysis buffer), the cell lysates were centrifuged at 12,000 rpm for 1 min and the supernatants were collected as cytosolic fractions. Nuclear pellet was suspended in ice-cold extraction buffer (20 mM HEPES, pH 7.9/0.4 M NaCl/1 mM EDTA/1 mM EGTA/ 1 mM DTT) and incubated on ice for 30 min with intermittent vortex. The nuclear extract was then subjected to centrifugation at 12,000 rpm for 5 min, and the supernatant was saved as nuclear fraction.

Western blot analysis

Proteins were subjected to PAGE gel separation and blots were detected by enhanced chemiluminescent (ECL) detection reagents (An and Dou, 1996).

In vitro AR breakdown assay

Purified recombinant human androgen receptor (rhAR) protein or AR present in whole cell extracts or nuclear fraction of LNCaP cells were used as substrates for *in vitro* AR degradation assay. Briefly, 1~2 ng purified rhAR protein or 20~40 µg of AR containing LNCaP cell extract was incubated at 37 °C for 1–3 h with 100 µg PC-3 cell nuclear or cytosolic fraction containing endogenous proteases in an AR breakdown buffer [10 mM Hepes, pH 7.4, 5 mM MgCl₂, 5 mM CaCl₂, 1 mM DTT supplemented with 5 mM ATP and ATP-regenerating system (0.1 mg/ml creatine kinase/100mM creatine phosphate)]. The *in vitro* reactions were stopped by addition of SDS loading buffer and boiling for 5 min. AR breakdown was analyzed by Western blotting to measure the level of residual AR in each incubation.

Protease inhibitor study *in vitro*

The cytosolic fractions from apoptotic PC-3 cells were pre-incubated with or without an indicated protease inhibitor for 20 min at 4 °C, followed by a co-incubation with rhAR (1~2 ng) in AR breakdown buffer. The reactions were then stopped and analyzed as described above.

Protease inhibitor study in cultured cells

Cultured LNCaP cells were pre-treated with or without an indicated protease inhibitor for 4 h, followed by a co-treatment with VP-16 for 16 h or Withaferin A (WA) for 20 h. The AR protein level was then analyzed by Western blot analysis using AR (N20) antibody.

Affinity separation of cellular AR proteolytic activity

PC-3 cells were treated with or without 5 µmol/L celastrol for 16 h to produce apoptotic or non-apoptotic cytosolic fraction, respectively. The supernatants (100 µg) were incubated overnight with calmodulin-, trypsin inhibitor-, aprotinin- or protein A-agarose beads that have been equilibrated with 0.01 M Tris-Cl, pH 7.4/0.1 M NaCl (for aprotinin-agarose or protein A-agarose), or 50 mM Tris-Cl, pH 7.5/0.1 M NaCl (for trypsin inhibitor-agarose), or 20 mM Tris-Cl, pH 7.4/4 mM MgCl₂/2 mM CaCl₂/ 10 mM KCl (for calmodulin-agarose). Agarose beads collected after several washes were then incubated with rhAR or LNCaP cell

extract containing AR protein. The reactions were stopped by boiling samples in SDS loading buffer for 5 min, followed by Western blot analysis using AR (N20) antibody.

Results

Proteolytic activity required for AR degradation is located in the cytosol

Previously, we reported that apoptosis-inducing natural products with proteasome-inhibitory activity, such as celastrol and withaferin A (WA), or copper-based proteasome inhibitors, such as clioquinol-copper, decreased AR protein levels (Chen et al., 2007; Pang et al., 2007; Yang et al., 2006; Yang et al., 2007). A similar decrease in AR protein levels was observed in LNCaP cells treated with a topoisomerase-inhibiting chemotherapeutic agent VP-16 for 16 to 24 hours (Fig. 1A). Interestingly, under the same treatment conditions, VP-16 had very little effect on AR mRNA synthesis as determined by semi-quantitative RT-PCR (Fig. 1B). We observed a similar decreasing effect of celastrol and WA on AR protein but not on AR mRNA in LNCaP cells (data not shown). Thus, the decrease in AR protein level in VP-16, celastrol or WA treated LNCaP cells is possibly due to its proteolytic degradation rather than the suppression of AR mRNA synthesis.

To test this possibility, we developed an *in vitro* AR degradation assay in which drug treated AR-negative PC-3 cell extracts (nuclear or cytosolic fractions) can be examined for the presence of AR proteolytic activity. In this assay either LNCaP nuclear AR (LNAR) or purified rhAR is incubated with PC-3 cell extracts for 3 hours at 37 °C, and the residual AR in the reaction mixture was determined by Western blot analysis. Using this assay, we observed that the cytosolic fraction, but not the nuclear fraction, isolated from celastrol-treated PC-3 cells contained proteolytic activity that is capable of degrading LNAR in a dose-dependent manner (Fig. 1C). We further confirmed the presence of AR proteolytic activity in the cytosolic fraction, but not in the nuclear fraction, by using purified rhAR as a substrate in AR-degradation assay. As shown in Fig. 1D, rhAR, like LNAR, was degraded only in the presence of cytosolic fraction but not nuclear fraction. These results suggest that AR is degraded mainly in the cytosol of celastrol-treated cells and that LNCaP cells do not contain endogenous nuclear protease inhibitors to prevent AR degradation. The ubiquitin-proteasome system that is responsible for the degradation of most intracellular proteins is also located mainly in the cytosol (Wilkinson, 1999). However, it is unlikely for proteasome system to be involved in AR degradation under these conditions since AR levels were decreased in cells treated with proteasome inhibitors (Yang et al., 2006; Yang et al., 2008).

AR proteolytic activity is stimulated in celastrol-induced apoptotic cells

In an attempt to determine whether the AR proteolytic activity is cell intrinsic or drug induced, we compared the rate of AR degradation in the presence of cytosolic fractions from celastrol treated (apoptotic) and control (non-apoptotic) PC-3 cells. As shown in Fig. 1E, whereas the cytosolic fraction from apoptotic cells degraded most of rhAR by 20 min, the degradation of rhAR in the presence of cytosolic fraction from non-apoptotic control cells was unnoticeable until 2 hours of incubation. These data suggest that the AR proteolytic activity exists under physiological conditions, but it is being stimulated by treatment conditions that lead to apoptotic cell death.

A variety of apoptosis-inducing chemotherapeutic agents stimulate AR degradation

To test the hypothesis that AR breakdown is stimulated by apoptosis in general, and not celastrol treatment in particular, we treated PC-3 cells with proteasome inhibitors celastrol (5 μ mol/L), DSF-Cu (10 μ mol/L), VP-16 (100 μ mol/L), cisplatin (100 μ mol/L), B-DIM (a formulated 3,3'-diindolylmethane, 50 μ mol/L), or docetaxel (10 nmol/L) to induce apoptosis. Morphological changes for the induction of apoptosis were monitored by light

microscopy (data not shown). The cytosolic fractions from each treatment were then incubated with AR-containing LNCaP cell lysates for 1 hour at 37 °C. Compared to the control (LNCaP cell lysate alone without an apoptotic cytosolic fraction), AR protein was completely depleted by addition of the apoptotic cytosolic fraction of PC-3 cells treated with celastrol, DSF-Cu, VP-16, B-DIM or docetaxel and partially depleted by that of cisplatin-treated cells (Fig. 2A), suggesting that the AR degradation in prostate cancer cells is stimulated by a variety of chemotherapeutic agents that induce apoptosis and not restricted to proteasome inhibitors.

We then determined the effect of these chemotherapeutic agents on endogenous AR levels in LNCaP cells. As shown in Fig. 2B, proteasome inhibitors celastrol and DSF-Cu completely depleted AR whereas VP-16, B-DIM and docetaxel partially depleted AR in LNCaP cells. Unlike under cell-free assay conditions (Fig. 2A), cisplatin treatment did not alter AR protein level in cultured cells (Fig. 2B). We further determined the apoptotic status of the cells in the same experiment by Western blot analysis of PARP. We observed PARP cleavage to a 65 kDa form (p65 PARP) under each treatment condition (Fig. 2B), indicating that the cells treated with different drugs were all in the late stages of apoptosis, although the intensity of cleaved PARP fragment varied among individual treatments.

AR degradation is associated with generation of a ~50 kDa fragment under apoptotic conditions

Since calpain is reported to generate AR fragments ranging in size from 31 kDa to 80 kDa (Pelley et al., 2006; Libertini et al., 2007), we examined whether AR degradation in apoptotic cells involves generation of intermediate AR fragments. We observed that most of AR in nuclear extracts of LNCaP cells grown in charcoal-stripped medium was of full length (112 kDa form) (Fig. 3A, Lane 1). By comparison, cytosolic fraction prepared from celastrol-treated apoptotic LNCaP cells contained only a ~50 kDa protein that was detectable with AR (N20) antibody (Fig. 3A, Lane 3). However, when full length AR-containing nuclear fraction was incubated with cytosolic fraction from celastrol-treated apoptotic cells, most of the full length AR in the incubation was converted to a ~50 kDa form (Fig. 3A, Lane 2). Thus AR breakdown in celastrol-treated apoptotic LNCaP cells is associated with the formation of a ~50 kDa AR fragment. The generation of ~50 kDa fragment as a byproduct of AR degradation is also implicated from our observation that there is a dose-dependent biphasic effect of celastrol on ~50 kDa AR fragment accumulation in cytosol of LNCaP cells (Fig. 3B). The cytosolic ~50 kDa AR fragment levels were at a high level in cells treated with 0.5 μ mole/L celastrol and its levels decreased as the celastrol concentration was increased from 1 μ mole/L to 5 μ mole/L. Interestingly, as the ~50 kDa AR fragment levels decreased in the cytosol there was a concomitant increase of its levels in the nuclear fraction of the cells treated with 1–5 μ mole/L celastrol (Fig. 3B). These results suggest that, although AR breakdown occurs primarily in the cytosol, the ~50 kDa AR intermediary breakdown product accumulated in cytosol of cells treated with high concentrations of celastrol are being translocated to the nucleus (see Discussion).

We then tested whether the ~50 kDa AR fragment generated during AR breakdown in celastrol-treated apoptotic cells in the present study is similar to the one reported to present in non-apoptotic LNCaP cells (Pelley et al., 2006). As shown in Fig. 3C, cell lysate prepared from exponentially growing LNCaP cells harvested by trypsinization, in which calpain-cleaved AR fragments could be detected, contained a ~50 kDa fragment that cross reacted with AR (N20) antibody (Fig. 3C, Lane 1). A similar analysis of cytosolic fraction prepared from celastrol-treated LNCaP cells showed that the AR fragment migrated to the same position as the one observed in trypsinized LNCaP cells (Fig. 3C Lane 2 vs 1). This observation suggests not only that the ~50 kDa fragments generated in apoptotic and non-

apoptotic cells are similar, but also that the generation of the ~50 kDa AR fragment in apoptotic cells may involve calpain.

Protease inhibitor evidence for calpain involvement in AR breakdown

To identify the activities of the protease(s) involved in AR breakdown during apoptosis, we examined the effects of different protease inhibitors, including calpain inhibitors, on the degradation of AR protein *in vitro* and in cultured cells. The degradation of rhAR by apoptotic (celastrol-treated) cytosolic fraction was completely inhibited by calpain inhibitors calpastatin (a calpain-specific peptide inhibitor) and ALLM, and by the cysteine protease inhibitor E64 (Fig. 4A). AR breakdown was partially inhibited by PMSF (which can inhibit both serine and cysteine proteases) (Fig. 4A). In contrast, the third calpain inhibitor PD15060, cysteine protease inhibitor NEM and serine protease inhibitors aprotinin, TLCK, TPCK, PAI-1 and leupeptin (which prefers to inhibit cysteine proteases but also inhibits serine proteases), failed to prevent AR degradation under cell-free conditions (Fig. 4A; see Discussion).

To determine whether these protease inhibitors could prevent apoptosis-induced breakdown of endogenous AR, we used VP-16 treatment, which also decreased AR protein levels (Fig. 1A). LNCaP cells were pre-treated with different protease inhibitors for 4 h, followed by 100 μ mole/L VP-16 treatment for additional 16 h. As shown in Figure 4B, compared to the control (DMSO treatment), VP-16-induced breakdown of AR was attenuated in cells pre-treated with calpastatin, an endogenous inhibitor of calpain (Fig. 4B). Calpain inhibitor ALLM, cysteine protease inhibitors E64 and PMSF also prevented AR breakdown to a varying extent (Fig. 4B). The other inhibitors including calpain inhibitor PD15060, cysteine protease inhibitor NEM, leupeptin, aprotinin, TLCK, TPCK and PAI-1 had very little protective effect on VP-16-induced AR breakdown in cultured LNCaP cells (Fig. 4B). Thus calpain seems to play an important role in AR degradation in apoptotic cells.

Since caspase-3 activation is an important step in apoptotic process, we tested whether caspase-3 plays role in AR breakdown in proteasome inhibitor-induced apoptotic cells. We studied the effect of caspase-3 inhibitor III on AR breakdown in cytosolic fraction of celastrol-treated apoptotic cells. As shown in Fig. 4C, a caspase-3 inhibitor had a very little protective effect on AR breakdown under these *in vitro* conditions. Similarly, pre-treatment of cells with caspase-3 inhibitor also failed to prevent intracellular AR breakdown in the medicinal proteasome-inhibitory compound WA-treated intact LNCaP cells (Fig. 4D). Therefore, caspase-3 does not seem to be responsible for AR breakdown in proteasome inhibitor-induced apoptotic cells.

Proteasome inhibitor-stimulated proteolytic activity responsible for AR breakdown binds to calmodulin

Both AR and calpain in exponentially growing LNCaP cells are reported to bind to calmodulin (CaM) under *in vitro* and *in vivo* conditions (Cifuentes et al., 2004; Pelley et al., 2006). Since protease inhibitor studies (Fig. 4) indicated calpain involvement in AR breakdown, we examined whether AR proteolytic activity in cytosolic fraction of celastrol-treated apoptotic PC-3 cells binds to CaM. The apoptotic cytosolic fraction prepared from celastrol-treated PC-3 cells or non-apoptotic cytosolic fraction from untreated cells were subjected to affinity chromatography using CaM-, aprotinin-, trypsin inhibitor- or protein A-agarose beads. The pulled down fractions were then incubated with AR-containing LNCaP cell extracts as source of AR substrate. As shown in Fig. 5A, both apoptotic and non-apoptotic cytosolic fractions pulled down by aprotinin-, trypsin inhibitor- and protein A-agarose beads lack AR proteolytic activity. In contrast, both apoptotic and non-apoptotic cytosolic fractions pulled down by CaM were able to degrade AR. Furthermore, CaM-

affinity proteins pulled down from the apoptotic cytosol fraction was relatively far more effective in degrading AR than those pulled down from non-apoptotic cytosolic fraction (Fig. 5A). These results are consistent with our previous report that calpain responsible for AR breakdown binds to CaM (Pelley et al., 2006), and suggest that CaM-bound calpain is activated in apoptotic cells to cause AR breakdown. This is also corroborated by our observation that CaM-bound proteins pulled down from apoptotic cytosolic fraction degraded rhAR and in the process generated a ~50 kDa fragment (Fig. 5B). Furthermore, Western blot analysis revealed the presence of ~28 kDa cleavage fragment of calpain small subunit in cytosol fraction of celastrol treated cells (Fig. 5B), suggesting calpain activation (Gao and Dou, 2000) under celastrol-induced apoptotic conditions. In addition, active calpain in cytosol fraction of celastrol-treated cells was pulled down by CaM-agarose beads (Fig. 5B) that contained AR proteolytic activity. Taken together, these observations provide evidence supporting calpain involvement in AR breakdown during apoptosis in prostate cancer cells.

Discussion

In the current study, we report that AR degradation is responsible for its elimination during apoptosis and that at least a calpain family member is involved in this phenomenon. The calpain-mediated AR proteolytic activity resides in the cytosol and generates ~50 kDa fragment as an intermediary byproduct of AR breakdown. The AR proteolytic activity induced by either proteasome inhibitor celastrol or VP-16 can be inhibited by calpain inhibitors calpastatin and ALLM in cell-free extracts and in intact cells, and can be pulled down by calmodulin-agarose beads.

Proteins with a short half-life are characterized by one or more regions rich in proline (P), glutamic acid (E), serine (S) and threonine (T), known as PEST regions (Dice, 1987). Calpain is one of the proteases that can recognize PEST sequences and further hydrolyze such proteins (Carafoli and Molinari, 1998). Previously we reported calpain involvement in breakdown of AR, which contains PEST sequences and binds to CaM, under physiological conditions (Pelley et al., 2006). A full understanding of the mechanism by which AR undergoes proteolytic breakdown is essential for developing effective strategies for elimination of AR leading to apoptosis and designing therapeutic strategies for the treatment of hormone refractory prostate cancer (Reddy et al., 2006; Tamura et al., 2007).

Apoptosis is a process in which a series of cellular proteases are activated to breakdown cellular constituent proteins. Most commonly activated proteases during apoptosis are caspases. Activated caspases are reported to cleave AR with extended polyglutamine tracts in neurodegenerative diseases (Kobayashi et al., 1998). However, in prostate cancer cells, stress-induced caspase-3 activation showed no relationship with AR breakdown in LNCaP cells (Pelley et al., 2006). In present study, a caspase-3/-7 inhibitor failed to block AR decrease brought about by chemotherapeutic agents that induce apoptosis (Fig. 4C, D), further confirming that caspase is not the enzyme responsible for AR depletion in apoptotic prostate cancer cells.

Resveratrol-mediated apoptosis of breast cancer cells involves a significant increase of intracellular calcium (Sareen et al., 2007), resulting in the activation of calcium dependent protease calpain. Calpain is one of the proteases that is activated at a very early stage of apoptosis (Daniel et al., 2003; Gao and Dou, 2000). Calpain-mediated Bax cleavage is also associated with drug-induced apoptosis (Wood and Newcomb, 1999; Wood et al., 1998; Gao and Dou, 2000). Therefore, in the current study, we focused on calpain for its role in AR degradation. This property is reflected in our finding that the apoptotic cytosolic fraction is more potent to breakdown AR than the non-apoptotic fraction (Fig. 1D).

It has been shown that, whereas caspase-3/-7 cleavage of PARP results in generation of the p85 PARP fragment (Lazebnik et al., 1994), calpain-mediated cleavage of PARP produces a ~65 kDa fragment (p65 PARP) (Pink et al., 2000). In present study, we observed p65 PARP formation suggesting calpain activation in cytosol of apoptotic cells treated with celastrol, DSF-Cu, VP-16, docetaxol, and other chemotherapeutic agents (Fig. 2B). We also observed that the proteolytic activity responsible for AR breakdown under cell-free conditions and in intact cells is associated with the cytosolic fraction, but not nuclear fraction, of apoptotic PC-3 or LNCaP cells (Fig. 1, and data not shown). This sub-cellular localization of AR proteolytic activity corresponds with the characteristics of calpain, which is reported to be a typical cytosolic cysteine protease (Nakagawa et al., 2001; Suzuki and Sorimachi, 1998). Since AR proteolytic activity was mainly in the cytoplasm and not in the nuclear compartment (Fig. 1), the presence of ~50 kDa AR fragment in the nucleus of cells treated with high concentrations of celastrol (Fig. 3B) suggests possible translocation of cytosolic AR fragments into nucleus of intact cells. Although, the well-known NLS located at the end of DBD and hinge region (around amino acids 617–633) (Zhou et al., 1994) may not be included in the ~50 kDa AR fragment generated by calpain cleavage (Pelley et al., 2006), it is possible that alternate NLS, present in the NH₂-terminal domain (ATD) of AR (Kaku et al., 2008), may play a role in translocation of AR fragment from cytosol to nucleus.

Since AR breakdown involves calpain (Pelley et al., 2006; Libertini et al., 2007), we anticipated that calpain inhibitors should prevent AR breakdown in apoptotic cells. We observed two calpain inhibitors, calpastatin and ALLM, inhibited AR breakdown both under cell-free conditions and in intact cells (Fig. 4). Calpastatin inhibited AR breakdown more effectively than ALLM. However, the third calpain inhibitor PD150606 was less effective in blocking AR degradation *in vitro* and in cells treated with VP-16 (Fig. 4). These observations are consistent with the different modes of action of these inhibitors on calpain activity. PD150606 is reported to inhibit calpains by interacting with the calcium-binding sites contained in the calmodulin-like EF-hand domain of the typical calpains (Suzuki et al., 2004), while ALLM acts at the active site of calpains (Carafoli and Molinari, 1998) and calpastatin can inhibit both the active site and the calmodulin-like domain of calpain (Suzuki et al., 2004). The fact that calpastatin exerted more inhibitory effect on AR breakdown than ALLM also suggest that in addition to typical calpain, atypical calpain without EF-hand might also be involved in AR breakdown. Although it has been reported that once calpain is activated it becomes resistant to the inhibitory action of calpastatin (Ray et al., 2003), calpastatin is known to complex with AR under *in vivo* and *in vitro* conditions (Pelley et al., 2006), and such AR-bound calpastatin under cell-free conditions may protect AR from proteolysis by celastrol-activated calpain (Fig. 4A). Other than calpastatin and ALLM, E64 and PMSF also prevented AR degradation to varying degrees (Fig. 4). This is because E64 inhibits cysteine proteases, which includes calpain, and PMSF inhibits a broad spectrum of serine and cysteine proteases (Lotem and Sachs, 1996; Zimmerman and Schlaepfer, 1982).

Taken together, the current study suggests that calpain in prostate cancer cells is activated upon induction of apoptosis by chemotherapeutic agents, and activated calpain is one of the proteases responsible for AR degradation. This finding should provide insights into regulatory events controlling AR protein level in prostate cancer cells, and lead to the development of effective strategies for elimination of AR and treatment of prostate cancer.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Literature Cited

- An B, Dou QP. Cleavage of retinoblastoma protein during apoptosis: an interleukin 1 beta-converting enzyme-like protease as candidate. *Cancer Res.* 1996; 56:438–442.
- Carafoli E, Molinari M. Calpain: a protease in search of a function? *Biochem Biophys Res Commun.* 1998; 247(2):193–203. [PubMed: 9642102]
- Chang C, Saltzman A, Yeh S, Young W, Keller E, Lee HJ, Wang C, Mizokami A. Androgen receptor: an overview. *Crit Rev Eukaryot Gene Expr.* 1995; 5:97–125. [PubMed: 8845584]
- Chen CD, Welsbie DS, Tran C, Baek SH, Chen R, Vessella R, Rosenfeld MG, Sawyers CL. Molecular determinants of resistance to antiandrogen therapy. *Nat Med.* 2004; 10:33–39. [PubMed: 14702632]
- Chen D, Cui QC, Yang H, Barrea RA, Sarkar FH, Sheng S, Yan B, Reddy GP, Dou QP. Clioquinol, a therapeutic agent for Alzheimer's disease, has proteasome-inhibitory, androgen receptor-suppressing, apoptosis-inducing, and antitumor activities in human prostate cancer cells and xenografts. *Cancer Res.* 2007; 67:1636–1644. [PubMed: 17308104]
- Cifuentes E, Mataraza JM, Yoshida BA, Menon M, Sacks DB, Barrack ER, Reddy GP. Physical and functional interaction of androgen receptor with calmodulin in prostate cancer cells. *Proc Natl Acad Sci U S A.* 2004; 101:464–469. [PubMed: 14695896]
- Culig Z. Androgen receptor cross-talk with cell signalling pathways. *Growth factors.* 2004; 22:179–184. [PubMed: 15518241]
- Culig Z, Hobisch A, Cronauer MV, Radmayr C, Trapman J, Hittmair A, Bartsch G, Klocker H. Androgen receptor activation in prostatic tumor cell lines by insulin-like growth factor-I, keratinocyte growth factor, and epidermal growth factor. *Cancer Res.* 1994; 54:5474–5478. [PubMed: 7522959]
- Daniel KG, Anderson JS, Zhong Q, Kazi A, Gupta P, Dou QP. Association of mitochondrial calpain activation with increased expression and autolysis of calpain small subunit in an early stage of apoptosis. *Int J Mol Med.* 2003; 12:247–252. [PubMed: 12851726]
- de Boer W, Bolt J, Kuiper GG, Brinkmann AO, Mulder E. Analysis of steroid- and DNA-binding domains of the calf uterine androgen receptor by limited proteolysis. *J Steroid Biochem.* 1987; 28:9–19. [PubMed: 3302538]
- Dice JF. Molecular determinants of protein half-lives in eukaryotic cells. *Faseb J.* 1987; 1:349–357. [PubMed: 2824267]
- Gao G, Dou QP. N-terminal cleavage of bax by calpain generates a potent proapoptotic 18-kDa fragment that promotes bcl-2-independent cytochrome C release and apoptotic cell death. *J Cell Biochem.* 2000; 80:53–72. [PubMed: 11029754]
- Heinlein CA, Chang C. Androgen receptor in prostate cancer. *Endocr Rev.* 2004; 25:276–308. [PubMed: 15082523]
- Jemal A, Siegel R, Ward E, Murray T, Xu J, Thun MJ. Cancer statistics, 2007. *CA Cancer J Clin.* 2007; 57:43–66. [PubMed: 17237035]
- Jia L, Shen HC, Wantroba M, Khalid O, Liang G, Wang Q, Gentzsch E, Pinski JK, Stanczyk FZ, Jones PA, Coetzee GA. Locus-wide chromatin remodeling and enhanced androgen receptor-mediated transcription in recurrent prostate tumor cells. *Mol Cell Biol.* 2006; 26:7331–7341. [PubMed: 16980632]
- Kaku N, Matsuda KI, Tsujimura A, Kawata M. Characterization of nuclear import of the domain-specific androgen receptor in association with the importin α/β and Ran-GTP systems. *Endocrinology.* 2008 Apr 17. [Epub ahead of print].
- Kobayashi Y, Miwa S, Merry DE, Kume A, Mei L, Doyu M, Sobue G. Caspase-3 cleaves the expanded androgen receptor protein of spinal and bulbar muscular atrophy in a polyglutamine repeat length-dependent manner. *Biochem Biophys Res Commun.* 1998; 252:145–150. [PubMed: 9813160]

- Lazebnik YA, Kaufmann SH, Desnoyers S, Poirier GG, Earnshaw WC. Cleavage of poly(ADP-ribose) polymerase by a proteinase with properties like ICE. *Nature*. 1994; 371:346–347. [PubMed: 8090205]
- Lee DK, Chang C. Endocrine mechanisms of disease: Expression and degradation of androgen receptor: mechanism and clinical implication. *J Clin Endocrinol Metab*. 2003; 88:4043–4054. [PubMed: 12970260]
- Libertini SJ, Tepper CG, Rodriguez V, Asmuth DM, Kung HJ, Mudryj M. Evidence for capain-mediated androgen receptor cleavage as a mechanism for androgen independence. *Cancer Res*. 2007; 67:9001–9005. [PubMed: 17909000]
- Lin HK, Wang L, Hu YC, Altuwaijri S, Chang C. Phosphorylation-dependent ubiquitylation and degradation of androgen receptor by Akt require Mdm2 E3 ligase. *Embo J*. 2002; 21:4037–4048. [PubMed: 12145204]
- Lotem J, Sachs L. Differential suppression by protease inhibitors and cytokines of apoptosis induced by wild-type p53 and cytotoxic agents. *Proc Natl Acad Sci U S A*. 1996; 93(22):12507–12512. [PubMed: 8901612]
- Nakagawa K, Masumoto H, Sorimachi H, Suzuki K. Dissociation of m-calpain subunits occurs after autolysis of the N-terminus of the catalytic subunit, and is not required for activation. *J Biochem*. 2001; 130:605–611. [PubMed: 11686922]
- Nam S, Smith DM, Dou QP. Ester bond-containing tea polyphenols potently inhibit proteasome activity in vitro and in vivo. *J Biol Chem*. 2001; 276:13322–13330. [PubMed: 11278274]
- Pang H, Chen D, Cui QC, Dou QP. Sodium diethyldithiocarbamate, an AIDS progression inhibitor and a copper-binding compound, has proteasome-inhibitory and apoptosis-inducing activities in cancer cells. *Int J Mol Med*. 2007; 19:809–816. [PubMed: 17390087]
- Pelley RP, Chinnakannu K, Murthy S, Strickland FM, Menon M, Dou QP, Barrack ER, Reddy GP. Calmodulin-androgen receptor (AR) interaction: calcium-dependent, calpain-mediated breakdown of AR in LNCaP prostate cancer cells. *Cancer Res*. 2006; 66:11754–11762. [PubMed: 17178871]
- Pink JJ, Wuerzberger-Davis S, Tagliarino C, Planchon SM, Yang X, Froelich CJ, Boothman DA. Activation of a cysteine protease in MCF-7 and T47D breast cancer cells during beta-lapachone-mediated apoptosis. *Exp Cell Res*. 2000; 255:144–155. [PubMed: 10694431]
- Reddy GP, Barrack ER, Dou QP, Menon M, Pelley R, Sarkar FH, Sheng S. Regulatory processes affecting androgen receptor expression, stability, and function: potential targets to treat hormone-refractory prostate cancer. *J Cell Biochem*. 2006; 98:1408–1423. [PubMed: 16619263]
- Ray SK, Hogan EL, Banik NL. Calpain in the pathophysiology of spinal cord injury: neuroprotection with calpain inhibitors. *Brain Res Rev*. 2003; 42:169–185. [PubMed: 12738057]
- Sareen D, Darjatmoko SR, Albert DM, Polans AS. Mitochondria, calcium, and calpain are key mediators of resveratrol-induced apoptosis in breast cancer. *Mol Pharmacol*. 2007; 72:1466–1475. [PubMed: 17848600]
- Schaufele F, Carbonell X, Guerbadot M, Borngraeber S, Chapman MS, Ma AA, Miner JN, Diamond MI. The structural basis of androgen receptor activation: intramolecular and intermolecular amino-carboxy interactions. *Proc Natl Acad Sci U S A*. 2005; 102:9802–9807. [PubMed: 15994236]
- Sheflin L, Keegan B, Zhang W, Spaulding SW. Inhibiting proteasomes in human HepG2 and LNCaP cells increases endogenous androgen receptor levels. *Biochem Biophys Res Commun*. 2000; 276:144–150. [PubMed: 11006097]
- Suzuki K, Hata S, Kawabata Y, Sorimachi H. Structure, activation, and biology of calpain. *Diabetes*. 2004; 53 Suppl 1:S12–S18. [PubMed: 14749260]
- Suzuki K, Sorimachi H. A novel aspect of calpain activation. *FEBS letters*. 1998; 433:1–4. [PubMed: 9738920]
- Tamura K, Furihata M, Tsunoda T, Ashida S, Takata R, Obara W, Yoshioka H, Daigo Y, Nasu Y, Kumon H, Konaka H, Namiki M, Tozawa K, Kohri K, Tanji N, Yokoyama M, Shimazui T, Akaza H, Mizutani Y, Miki T, Fujioka T, Shuin T, Nakamura Y, Nakagawa H. Molecular features of hormone-refractory prostate cancer cells by genome-wide gene expression profiles. *Cancer Res*. 2007; 67:5117–5125. [PubMed: 17545589]

- Unni E, Sun S, Nan B, McPhaul MJ, Cheskis B, Mancini MA, Marcelli M. Changes in androgen receptor nongenotropic signaling correlate with transition of LNCaP cells to androgen independence. *Cancer Res.* 2004; 64:7156–7168. [PubMed: 15466214]
- Wellington CL, Ellerby LM, Hackam AS, Margolis RL, Trifiro MA, Singaraja R, McCutcheon K, Salvesen GS, Propp SS, Bromm M, Rowland KJ, Zhang T, Rasper D, Roy S, Thornberry N, Pinsky L, Kakizuka A, Ross CA, Nicholson DW, Bredesen DE, Hayden MR. Caspase cleavage of gene products associated with triplet expansion disorders generates truncated fragments containing the polyglutamine tract. *J Biol Chem.* 1998; 273:9158–9167. [PubMed: 9535906]
- Wilkinson KD. Ubiquitin-dependent signaling: the role of ubiquitination in the response of cells to their environment. *J Nutr.* 1999; 129:1933–1936. [PubMed: 10539765]
- Wilson EM, French FS. Effects of proteases and protease inhibitors on the 4.5 S and 8 S androgen receptor. *J Biol Chem.* 1979; 254:6310–6319. [PubMed: 447715]
- Wood DE, Newcomb EW. Caspase-dependent activation of calpain during drug-induced apoptosis. *J Biol Chem.* 1999; 274:8309–8315. [PubMed: 10075737]
- Wood DE, Thomas A, Devi LA, Berman Y, Beavis RC, Reed JC, Newcomb EW. Bax cleavage is mediated by calpain during drug-induced apoptosis. *Oncogene.* 1998; 17:1069–1078. [PubMed: 9764817]
- Yang H, Chen D, Cui QC, Yuan X, Dou QP. Celastrol, a triterpene extracted from the Chinese "Thunder of God Vine," is a potent proteasome inhibitor and suppresses human prostate cancer growth in nude mice. *Cancer Res.* 2006; 66:4758–4765. [PubMed: 16651429]
- Yang H, Shi G, Dou QP. The tumor proteasome is a primary target for the natural anticancer compound Withaferin A isolated from "Indian winter cherry". *Mol Pharmacol.* 2007; 71:426–437. [PubMed: 17093135]
- Yang H, Landis-Piowar KR, Lu D, Yuan P, Li L, Reddy GP, Yuan X, Dou QP. Pristimerin induces apoptosis by targeting the proteasome in prostate cancer cells. *J Cell Biochem.* 2008; 103:234–244. [PubMed: 17541980]
- Zhou ZX, Sar M, Simental JA, Lane MV, Wilson EM. A ligand-dependent bipartite nuclear targeting signal in the human androgen receptor. Requirement for the DNA-binding domain and modulation by NH2-terminal and carboxyl-terminal sequences. *J Biol Chem.* 1994; 269:13115–13123. [PubMed: 8175737]
- Zimmerman UJ, Schlaepfer WW. Characterization of a brain calcium-activated protease that degrades neurofilament proteins. *Biochemistry.* 1982; 21:3977–3982. [PubMed: 6751383]

Abbreviations pertaining to the labeling of figures

ALLM	N-Acetyl-L-leucyl-L-leucyl-L-methioninal
Apop	Apoptotic cytosolic fraction
Apro	Aprotinin
AR	Androgen Receptor
BD	B-DIM
CaM	Calmodulin
Casp-3	Caspase 3
Cel	Celastrol
Cis	Cisplatin
Cyto	Cytosolic
Doc	Docetaxel
DSF-Cu	Disufiram-copper
E64	trans-Epoxy succinyl-L-leucylamido-(4-guanidin) butane

GAPDH	glyceraldehyde-3-phosphate dehydrogenase
Inh(s)	Inhibitor(s)
Leu	Leupeptin
LNAR	AR-containing nuclear fraction from LNCaP cells
NEM	N-ethylmaleimide
Non-Apop	Non-apoptotic cytosolic fraction
Nu	Nuclear fraction
PAI-1	Plasminogen Activator Inhibitor-1
PARP	poly(ADP-ribose) polymerase
PMSF	phenylmethylsulfonylfluoride
Pro A	Protein A
rhAR	recombinant human AR
Sm	Small subunit
TLCK	N α -p-Tosyl-L-lysine chloromethyl ketone
TPCK	N-Tosyl-L-pheylalanine choromethyl ketone
Try	Trypsin
VP	VP-16
WA	Withaferin A

Figure 1. Effect of VP-16 and celastrol on AR in intact cells and cell-free extracts

A: Effect of VP-16 on AR protein levels in LNCaP cells. Whole cell extracts prepared from LNCaP cells treated with 100 μmol/L VP-16 for indicated duration were subjected to Western blot analysis using AR (N20) antibody. β-actin was used as loading control. **B:** Effect of VP-16 on AR mRNA in LNCaP cells. AR mRNA expression during above treatment was determined by RT-PCR as described in Material and Methods. GAPDH was used as a control house keeping gene. **C:** AR proteolytic activity in cytosolic and nuclear fractions of celastrol-treated PC-3 cells. Cytosolic (Cyto) and nuclear (Nu) fractions (25 to 100 μg of protein) prepared from PC-3 cells treated with 5 μmole/L celastrol for 16 h were incubated with AR-containing nuclear fraction from LNCaP cells (LNAR) (25 μg of nuclear protein per incubation) for 3 h at 37 °C. AR protein remaining in individual incubation was then determined by Western blot analysis. Loading control β-actin levels showed an increase with increasing amount of PC-3 cytosolic and nuclear in the incubations 1 to 4 and 5 to 7, respectively. **D:** Effect of cytosolic and nuclear fractions from celastrol-treated PC-3 cells on purified recombinant human AR (rhAR). rhAR (2 ng) was incubated for 3 h at 37 °C with or without nuclear (Nu) or cytosolic (Cyto) fractions from celastrol-treated PC-3 cells. Residual rhAR in individual incubations was determined by Western blot analysis using AR (N20) antibody. **E:** Cytosolic fraction prepared from exponentially growing PC-3 cells treated with either the vehicle (non-Apop) or 5 μmol/L celastrol for 16 h (Apop) were incubated with rhAR (1 ng) for indicated duration and rhAR remaining in individual incubations was then determined by Western blot analysis using AR (N20) antibody. β-actin levels were used as loading control for cytosolic fractions.

Figure 2. Drug-induced AR proteolytic activity in cell-free extracts and intact cells

A: Cytosolic fractions prepared from PC-3 cells were treated with either 5 μ mol/L celastrol (Cel) or 10 μ mol/L DSF-Cu for 16 h, or 100 μ mol/L VP-16 (VP) or cisplatin (Cis) for 48 h, or 50 μ mol/L B-DIM (BD) or 10 nmol/L Docetaxel (Doc) for 72 h, were incubated with AR-containing whole cell lysates from untreated LNCaP cells for 1 h at 37 °C. AR remaining in the incubations was determined by Western blot analysis using AR (N20) antibody. β -actin was used as a loading control for cytosolic fractions. B: Whole cell extracts prepared from LNCaP cells treated with either vehicle DMSO (control) or 5 μ mol/L celastrol (Cel) for 16 h, 10 μ mol/L DSF-Cu for 2 h, 100 μ mol/L VP-16 (VP) or 100 μ mol/L cisplatin (Cis) for 48 h, 50 μ mol/L B-DIM (BD) or 10 nmol/L Docetaxel (Doc) for 72 h, were subjected to Western blot analysis using AR (N20) and PARP antibodies. The intact PARP is 116 kDa and cleaved PARP fragment is ~65 kDa. β -actin was used as loading control.

Figure 3. Generation of ~50 kDa fragment as a byproduct of AR degradation in apoptotic LNCaP cells

A: Effect of cytosolic fraction from celastrol-treated LNCaP cells on AR in the nuclear fraction of untreated (control) LNCaP cells. Nuclear fraction prepared from LNCaP cells cultured in charcoal-stripped medium for 4 days was incubated with cytosolic fraction prepared from 5 μ mol/L celastrol-treated LNCaP cells for 3 h at 37 °C. Nuclear fraction alone (Lane 1), nuclear fraction incubated with celastrol-treated cytosolic fraction (Lane 2), and cytosolic fraction alone (Lane 3) were subjected to Western blot analysis using AR (N20) antibodies. Conversion of full length (110 kDa) AR in nuclear fraction to a ~50 kDa fragment was seen when nuclear fraction was incubated with celastrol-treated cytosolic fraction (Lane 2). **B:** Effect of celastrol on AR in nuclear and cytosolic fraction of LNCaP cells. Nuclear and cytosolic fractions prepared from LNCaP cells treated with increasing concentrations of celastrol for 24 h were subjected to Western blot analysis using AR (N20) antibody. β -actin was used as a loading control. **C:** Comparison of AR proteolytic products formed in celastrol-treated cells with those in trypsinized LNCaP cells. Whole cell lysates of

exponentially growing LNCaP cells harvested by trypsinization (Lane 1) and cytosolic fraction of LNCaP cells treated with 5 $\mu\text{mol/L}$ celastrol for 3 h (Lane 2) were subjected to Western blot analysis using AR (N20) antibody.

Figure 4. Effect of various protease inhibitors on AR breakdown under cell-free conditions and in VP-16-treated intact cells

A: Effect of protease inhibitors on AR proteolytic activity in cytosolic fraction prepared from PC-3 cells treated with celastrol. Apoptotic cytosolic fractions (Apop) prepared from PC-3 cells treated with 5 μmol/L celastrol for 16 h to induce apoptosis were treated with indicated concentrations of selected protease inhibitors (Inhs) for 20 min at 4 °C, and then incubated with rhAR for 1 h at 37 °C. Residual AR in individual incubations was determined by Western blotting using AR (N20) antibody. B: Effect of protease inhibitors on VP-16-induced AR breakdown in intact LNCaP cells. LNCaP cells treated with or without (-Inh) PD15060 at 100 μmol/L, ALLM at 100 μmol/L, calpastatin at 10 μmol/L,

E64 at 100 $\mu\text{mol/L}$, NEM at 25 $\mu\text{mol/L}$, leupeptin (Leu) at 400 $\mu\text{mol/L}$, PMSF at 200 $\mu\text{mol/L}$, aprotinin (Apro) at 10 $\mu\text{mol/L}$, TLCK at 100 $\mu\text{mol/L}$, TPCK at 100 $\mu\text{mol/L}$ and PAI-1 at 100 nmol/L for 4 h were incubated in the presence of 100 $\mu\text{mol/L}$ VP-16 for 16 h. Vehicle (DMSO)-treated cells were used as control. Whole cell extracts prepared from control and VP-16 treated cells were subjected to Western blot analysis using AR (N20) antibody and β -actin was used as loading control. C: Effect of caspase-3 inhibitor on AR proteolytic activity in cytosolic fraction prepared from PC-3 cells treated with celastrol. Apoptotic cytosolic fraction (Apop) prepared from celastrol-treated cells as described above was treated with or without 2.5 $\mu\text{mol/L}$ caspase-3 inhibitor III (Casp-3 Inh) for 20 min at 4° C and then incubated with rhAR for 1 h at 37 ° C. Residual AR in individual incubations was determined by Western blot analysis using AR (N20) antibody. D: Effect of caspase-3 inhibitor on withaferin (WA)-induced AR breakdown in intact LNCaP cells. LNCaP cells were treated with 150 $\mu\text{mol/L}$ caspase-3 inhibitor III (Casp-3 Inh) for 4 h, and then incubated in the presence or absence of 10 $\mu\text{mol/L}$ WA for additional 20 h. Whole cell extracts prepared from WA-treated and untreated cells were subjected to Western blot analysis using AR (N20) antibody. β -actin was used as loading control (D).

Figure 5. AR proteolytic activity in cytosolic fraction from celastrol-treated apoptotic PC-3 cells binds to calmodulin (CaM)

Equal amount of cytosolic fractions (200 μ g) prepared from PC-3 cells treated without (non-Apop) or with (Apop) 5 μ mol/L celastrol for 20 hours were incubated overnight with calmodulin (CaM)-, aprotinin (Apro)-, trypsin inhibitor (Try Inh)- and Protein A (Pro A)-agarose beads, and the proteins pulled down with agarose beads as described in Materials and Methods were incubated with AR-containing LNCaP cell extracts (A) or rhAR (B) for 1 hour. Residual AR and cleaved calpain small subunit (Capain Sm) in the incubations were determined by Western blot analysis using AR (N20) and calpain small subunit antibodies.