Doxazosin Induces Apoptosis of Benign and Malignant Prostate Cells via a Death Receptor–Mediated Pathway

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Abstract
Quinazoline-based α1-adrenoceptor antagonists such as doxazosin and terazosin have been previously shown to induce apoptosis in prostate cancer cells via an α1-adrenoceptor–independent pathway, involving activation of transforming growth factor-β1 (TGF-β1) signaling. In this study, the molecular events initiating this apoptotic effect were further investigated in vitro using the human androgen-independent prostate cancer cells PC-3 and the human benign prostate epithelial cells BPH-1. Quantitative microarray assays were done in PC-3 and BPH-1 cells after treatment with doxazosin (25 μmol/L, 6 and 24 hours) to identify the early gene changes. Transient changes in the expression of several apoptosis regulators were identified, including up-regulation of Bax and Fas/CD95 and down-regulation of Bcl-xL and TRAMP/Apo3. Moreover, there were significant changes in the expression pattern of signaling components of the extracellular matrix such as integrins α2, α5, β1, and β3. Western blot analysis revealed activation of caspase-8 and caspase-3 within the first 6 to 12 hours of treatment with doxazosin in both PC-3 and BPH-1 cells. Doxazosin-induced apoptosis was blocked by specific caspase-8 inhibitors, supporting the functional involvement of caspase-8 in doxazosin-induced apoptosis. The effect of doxazosin on recruitment of Fas-associated death domain (FADD) and procaspase-8 to the Fas receptor was examined via analysis of death-inducing signaling complex formation. Doxazosin increased FADD recruitment and subsequent caspase-8 activation, implicating Fas-mediated apoptosis as the underlying mechanism of the effect of doxazosin in prostate cells. These results show that doxazosin exerts its apoptotic effects against benign and malignant prostate cells via a death receptor–mediated mechanism with a potential integrin contribution towards cell survival outcomes. (Cancer Res 2006; 66(1): 464-72)

Introduction
Dysfunctional apoptotic programming leading to loss/suppression of apoptosis has been causally implicated in prostate cancer development and progression; thus, the apoptotic process becomes a promising therapeutic target for the effective elimination of prostate cancer cells (1). The two major apoptotic pathways are the mitochondrial dependent pathway and the death receptor pathway (2). In response to specific signals (such as DNA damage), the mitochondrial pathway occurs via the release of cytochrome c and formation of the apoptosome, a multiprotein complex containing Apaf-1, cytochrome c, and caspase-9. Activation of caspase-9 triggers a cascade of effector caspsases and subsequent apoptotic cell death. The receptor-mediated pathway is activated at the cell membrane by receptors such as Fas/CD95 and TRAIL/ Apo3, which on ligand binding initiate recruitment of the tumor necrosis factor (TNF) receptor–associated death domain (TRADD), Fas-associated death domain (FADD), and procaspase-8 to form the death-inducing signaling complex (DISC). The Fas/Fas ligand signaling pathway has been implicated in the progression and chemoprevention of prostate cancer (3–5). Fas can be activated by molecules besides Fas ligand or anti-Fas antibody, and various chemotherapeutic agents have been shown to enhance apoptosis of androgen-independent human prostate cancer cells via activation of the Fas signaling pathway (6–8). Activation of the caspase cascade mediates apoptosis in prostate cancer cells in response to a number of cytotoxic agents as well as transforming growth factor β (TGF-β; refs. 9–11).

α1-Adrenoceptor antagonists, such as quinazoline-based compounds doxazosin and terazosin, are clinically effective in the relief of benign prostatic hyperplasia (BPH) symptoms via their ability to selectively antagonize the α1a-adrenoceptors (12). Recent experimental and clinical evidence, however, indicates that induction of prostate epithelial and smooth muscle cell apoptosis by doxazosin and terazosin is one of the molecular mechanisms contributing to the overall long-term clinical efficacy of these medications in improving lower urinary tract symptoms in BPH patients (13). Suppression of prostate growth proceeds via an α1-adrenoceptor–independent mechanism by activation of latent apoptotic machinery by effector (Smad) activation of TGF-β1 signaling and IκBα (14, 15). More recent evidence established the ability of doxazosin to induce anoikis in prostate epithelial and endothelial cells (16). Additional signaling mechanisms involving disruption of cell attachment to the extracellular matrix (ECM) and subsequent induction of anoikis have been functionally implicated (16) in a molecular cross-talk of the cell death actions of quinazolines; the molecular signaling of this apoptotic effect, however, has yet to be defined.

In this study, we hypothesize that doxazosin induces prostate cancer cell apoptosis by affecting cell migration/anoikis via the Fas receptor pathway. Our results, based on cDNA array analysis and temporal dissection of the events leading to the apoptotic outcome of benign and malignant human prostate cells, show the ability of doxazosin to activate the Fas-mediated apoptotic pathway.

Materials and Methods
Cells Lines and Transfections
The human androgen-independent prostate cancer cell line PC-3 was obtained from the American Type Tissue Culture Collection (Rockville, MD).
PC-3 cells were routinely cultured in RPMI 1640 purchased from Invitrogen (Carlsbad, CA) containing 10% fetal bovine serum (Invitrogen) and antibiotics. The nontumorigenic benign human prostatic epithelial cells BPH-1 (derived from human prostate epithelium of benign pathology; a generous gift from Dr. Simon W. Hayward, Department of Urological Surgery, Vanderbilt University Medical Center, Nashville, TN; ref. 17) were cultured in RPMI 1640 (Invitrogen) containing 10% fetal bovine serum and antibiotics.

Human prostate cancer cells PC-3 were transfected with a dominant-negative form of FADD (DN-FADD) made with PCR primers (5'-CCAAGCT-TATGGACGACTGGAGCGGG and 5'-AAGGATCCACCGCGAACG- CAGCGGCC) flanking the region encoding 117 to 208 amino acids and using the FADD cDNA as template. This region contains the death domain that binds to the clustered receptor death domain but does not contain the death-effector domain and therefore cannot bind procaspase-8. The HindIII/BamHI restriction sites were used for cloning DN-FADD into the expression vector pcDNA3.1/Hyrgo. Empty vector was used to obtain control cells resistant to hygromycin.

Wild-type (WT)-FADD was cloned into a modified pcDNA3 expression vector (Invitrogen) in which a hemagglutinin (HA) epitope tag (YPYDVPDYA) had previously been placed downstream of the cytomegalovirus promoter/enhancer (pcDNA3 HA-FADD). In addition, an AU1 epitope (DTYRYI)-tagged FADD and mutants were made with PCR primers encoding the epitope and using the FADD cDNA as template (pcDNA3 AU1-FADD). Empty vector was used to obtain control cells resistant to G418. FuGENE6 reagent kit (Roche, Basel, Switzerland) was used for transfection and cells were selected in the presence of hygromycin (DN-FADD) or G418 (WT-FADD).

Reagents
Doxazosin mesylate (Cardura) was a gift from Pfizer Pharmaceuticals (New York, NY). Collagen type I was purchased from Sigma-Aldrich Co. (St. Louis, MO). Fibronectin and z-IETDfmk caspase-8 inhibitor were purchased form BD Biosciences (Bedford, MA). Recombinant human vascular endothelial growth factor was purchased from R&D Systems, Inc. (Minneapolis, MN). The polyclonal anti-Bax, monoclonal anti-caspase-3 and caspase-8, and polyclonal anti-FADD were purchased from Cell Signaling Technology (Beverly, MA). The monoclonal anti-TRADD was purchased from BD Biosciences International (Temecula, CA). The monoclonal anti-Fas antibody was purchased from StressGen Biotechnologies Corp. (Victoria, British Columbia, Canada).

RNA Isolation and Analysis
Total RNA was extracted from treated and untreated prostate cells by TRIzol (Invitrogen). cDNA was labeled from total RNA (4 μg) with biotin-16-dUTP and the GEArrayTM AmpoLabelling-LPR Kit (SuperArray, Frederick, MD) based on the protocol of the manufacturer. The labeled cDNA was then hybridized overnight to GEArray Q Series Human Cancer PathwayFinder, GEArray Q Series Human ECM and Adhesion Molecule, GEArray Q Series Human Apoptosis, or GEArray Q Series Human NFκB Signaling Pathway gene arrays (SuperArray) according to the protocol of the manufacturer. Signal detection was achieved by exposure to CDP-Star alkaline phosphatase chemiluminescent substrate (SuperArray).

Data analysis was done using a UVP Bioimaging System (Upland, CA) which converted the images into numerical data. Raw signal intensities were corrected for background by subtracting the signal intensity of the average

**Figure 1.** Effect of doxazosin on cell viability and apoptosis of malignant and benign prostate epithelial cells. Subconfluent cultures of PC-3 and BPH-1 cells were exposed to increasing concentrations of doxazosin (0-35 μmol/L) and cell death was determined using the MTT assay (A) or stained with Hoechst (B); apoptotic cells were visualized and counted as described in Materials and Methods. Points, mean of three independent experiments done in duplicate; bars, SE. *, P < 0.01; **, P < 0.001, compared with the control.
between two sets of housekeeping genes, β-actin and glyceraldehyde-3-phosphate dehydrogenase, or a blank. The normalized signals were used to determine the relative fold change of particular transcripts before and after treatment.

### Apoptosis and Cell Viability Assay Evaluation

**Hoechst staining.** Cells were plated on six-well plates at $5 \times 10^4$ per well and at subconfluency were treated with increasing concentrations of doxazosin (1, 10, and 25 μM/L) in the presence or absence of the caspase-8 inhibitor z-IEETDfmk. After 24 and 48 hours of treatment, cells were fixed with 4% (w/v) paraformaldehyde (Sigma) and stained with 10 μg/mL Hoechst 33342 (B2261; Sigma) in the presence of 0.1% Triton X-100 (Sigma) as previously described (1). Cells were visualized using a Zeiss Axiosvert S100 fluorescence microscope (Thornwood, NY) with a UV filter (356 nm) and those cells containing fragmented nuclei were designated apoptotic ($> 100$ magnification). The apoptotic index was determined by counting three random fields in duplicate wells per treatment group. Each experiment was done in two independent experiments.

**Trypan blue exclusion assay.** Subconfluent cultures of cells were exposed to increasing concentrations of doxazosin (0–25 μM/L) and cell viability was determined after 24 hours via the trypan blue exclusion assay (14). Values from three independent experiments (done in duplicate) are expressed as mean percent of cell viability relative to untreated cultures.

### 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay.

Subconfluent cultures of cells were exposed to increasing concentrations of doxazosin (0–35 μM/L). After treatment, the medium was replaced with 250 μL of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT; 1 mg/mL; Sigma) and incubated at 37°C for 3 hours. After 2 hours, the MTT was removed and replaced with 250 μL DMSO and incubated overnight at 37°C. The absorbance of the DMSO-crystal solution was read at 540 nm in a microplate reader (Bio-Tek Instruments, Winooski, VT). The percent change in cell viability was determined by comparing the absorbance values. Numerical data represent the average of two independent experiments done in triplicate.

**Measurement of Caspase-8 Activity**

The caspase-8 activity was determined using the colorimetric assay kit (Chemicon International) as described by the protocol of the manufacturer. Cultures of malignant (PC-3) and benign (BPH-1) prostate epithelial cells ($2 \times 10^5$), treated at subconfluency with doxazosin (25 μM/L) for 24 and 48 hours were lysed in cell lysis buffer and equivalent amounts of protein in each sample were incubated with the assay mixture containing IETD-pNA for 2 hours at 37°C. The absorbance was read at 405 nm in a microplate reader (Bio-Tek Instruments). Fold increase in activity was determined by comparing the absorbance values. Numerical data represent the average of two independent experiments done in duplicate.

**Western Blot Analysis**

Cultures of PC-3 and BPH-1 cells treated with doxazosin (25 μM/L) for 0, 6, 12, or 24 hours were lysed in radioimmunoprecipitation assay (RIPA) buffer [150 mM NaCl, 50 mM Tris (pH 8.0), 0.5% deoxycholic acid, 1% NP40 with 1 mM phenyl methyl-sulfonyl fluoride]. The total protein concentration in each sample was quantified by bicinchoninic acid (BCA) protein assay kit (Pierce, Rockford, IL) and protein samples (30 μg) were subjected to SDS-PAGE and transferred to Hybond-C membranes (Amersham Pharmacia Biotech, Piscataway, NJ). Membranes were blocked with 5% dry milk in TBS-T (TBS containing 0.05% Tween 20) for 1 hour at room temperature and were subsequently incubated overnight at 4°C with antibodies against caspase-3 or caspase-8, Akt, or phosphorylated Akt (Cell Signaling Technology). After incubation with the respective primary antibody, membranes were exposed to species-specific horseradish peroxidase–labeled secondary antibodies (room temperature). Signal detection was achieved with SuperSignal West Dura Extended Duration Substrate (Pierce) and visualized using a UVP Imaging System. All bands were normalized to β-actin expression (Oncogene Research Products, La Jolla, CA) and fold changes in protein expression were determined on the basis of β-actin loading control.

**Immunoprecipitation**

Benign and malignant prostate epithelial cells were treated with doxazosin (25 μM/L) for 0, 6, 12, or 24 hours and were subsequently

### Table 1. Gene expression in PC-3 and BPH-1 cells in response to doxazosin (6 hours)

<table>
<thead>
<tr>
<th>Genebank</th>
<th>Gene name</th>
<th>Fold change</th>
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<tr>
<td>NM_001160-1</td>
<td>Apaf-1</td>
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<tr>
<td>L22474</td>
<td>Bax</td>
<td>11.8</td>
</tr>
<tr>
<td>Z23115</td>
<td>Bcl-x</td>
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</tr>
<tr>
<td>NM_004346</td>
<td>caspase-3</td>
<td>–1.6</td>
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<tr>
<td>NM_001228</td>
<td>caspase-8</td>
<td>–2.5</td>
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<tr>
<td>NM_004049</td>
<td>FADD</td>
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</tr>
<tr>
<td>X63717</td>
<td>Fas/CD95</td>
<td>4.3</td>
</tr>
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<td>NM_002392</td>
<td>Mdm2</td>
<td>–2.1</td>
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<tr>
<td>NM_003894</td>
<td>RIP</td>
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<td>U75285</td>
<td>survivin</td>
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<td>NM_005658</td>
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<td>NM_004620</td>
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NOTE: ND, not detected.

### Table 2. Effect of doxazosin on ECM/adhesion gene expression in PC-3 prostate cancer cells

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<th>Fold change</th>
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<td>Z13009</td>
<td>E-cadherin</td>
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<td>catenin β1</td>
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<td>AF062343</td>
<td>catenin δ1</td>
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<td>X02761</td>
<td>fibronectin-1</td>
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<td>X17033</td>
<td>integrin α2</td>
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<td>integrin α3</td>
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<td>integrin αV</td>
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<td>integrin β1</td>
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<td>integrin β4</td>
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<td>JQ5633</td>
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<td>MUC-18</td>
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</tr>
<tr>
<td>M83248</td>
<td>osteopontin</td>
<td>7.3</td>
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<tr>
<td>NM_000442</td>
<td>PECAM-1</td>
<td>–2.6</td>
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<tr>
<td>M30640</td>
<td>E-selectin</td>
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<td>NM_002006</td>
<td>P-selectin</td>
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</tr>
<tr>
<td>NM_003249</td>
<td>TSP-1</td>
<td>–1.4</td>
</tr>
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</table>
lysed in RIPA buffer. Total protein concentration was quantified by BCA protein assay kit (Pierce) and protein samples (30 μg) were incubated overnight at 4 ℃ with the specific antibody. Lysates were then incubated for 3 hours with Protein G Plus/Protein A agarose beads (30 μL; Oncogene). Beads were washed five times with RIPA buffer, subjected to SDS-PAGE, and blot transferred to nitrocellulose membranes (Amersham Pharmacia Biotech).

**Statistical Analysis**

One-way ANOVA was done using the StatView statistical program to determine the statistical significance between values. All data are presented as mean ± SE. *P* < 0.05 was considered statistically significant.

**Results**

Doxazosin causes a loss of cell viability and induces apoptosis in malignant and benign prostate epithelial cells. Doxazosin leads to a dose-dependent loss of PC-3 (14) and BPH-1 cell viability after 24 hours of treatment as determined by MTT assay (Fig. 1A). A similar profile of loss of cell viability was obtained using the trypan blue exclusion assay (data not shown). The apoptotic nature of this effect was documented using the Hoechst stain in benign prostate epithelial cells exposed to doxazosin. As shown in Fig. 1B, there was a significant increase in the number of apoptotic cells at 24 and 48 hours of exposure to the drug (as identified by fragmented nuclei).

Doxazosin-induced gene expression profile of BPH-1 and PC-3 cells. In an attempt to identify the genes differentially expressed in response to doxazosin in benign and malignant prostate epithelial cells, a gene expression array analysis was done after treatment of cells with doxazosin (25 μmol/L). Table 1 summarizes the data of the gene transcription profile of genes known to play a critical role in apoptosis. After a 6-hour treatment with doxazosin, several genes were significantly up-regulated in PC-3 cells: Bax, Bcl-xL, FADD, and Fas. No detectable levels of FADD mRNA were observed in BPH-1 cells whereas Fas mRNA was up-regulated in response to doxazosin treatment. The gene expression pattern was different between the two cell lines in response to doxazosin (Table 1).

Considering that doxazosin might elicit a potential anoikis effect (6), we did an array analysis of genes functionally involved in cell adhesion, migration, and ECM attachment on PC-3 cells. The results summarized in Table 2 revealed a considerable reduction in mRNA levels of integrins α2, α5, β1, β3, β5, and β6 whereas there was an increase in mRNA levels for integrins α2 and β3. Also shown in Table 2 are data indicating alterations in gene expression for several molecules involved in cell adhesion. Major changes were detected for E-cadherin, carcinomaembryonic antigen, β-catenin, platelet/endothelial cell adhesion molecule 1 (PECAM-1), laminins, selectins, and thrombospondin-1 (TSP-1).
Driven by the findings indicating that doxazosin up-regulates the mRNA of the proapoptotic Bax, we subsequently investigated the ability of doxazosin to regulate additional key apoptosis players of the mitochondrial pathway. Western blot analysis revealed a 2.5- and 3-fold increase in Bax protein levels in response to doxazosin in PC-3 cells at 12 and 24 hours, respectively. Furthermore, in BPH-1 cells there was a 2.5-fold increase in Bax after 24 hours of doxazosin treatment compared with untreated cells (Fig. 2A). This temporally correlates with the observed changes in mRNA expression.

Caspase-8 activation by doxazosin. Western blotting indicates an increase in the levels of activated caspase-8 at 6 hours following doxazosin treatment in BPH-1 cells (Fig. 2B). For the PC-3 cells, this caspase activation was observed after 12 hours (Fig. 2B). After 6 hours of treatment, PC-3 cells show an increase in FADD protein levels whereas BPH-1 cells show no change, consistent with our gene array data (Fig. 2B). There were no major changes in c-FLIPL protein expression in prostate cell lines. Furthermore, on stimulation with doxazosin (25 μmol/L), both PC-3 and BPH-1 cells exhibited a significant increase in caspase-8 activity compared with control (Fig. 2C).

The data shown in Fig. 3 indicate that there was a significant suppression of apoptosis in the presence of the caspase-8 inhibitor z-IETDfmk in both the benign and malignant prostate cells.

Doxazosin induces caspase-3 activation and death receptor and adaptor protein complex formation. We have previously reported that doxazosin treatment of malignant prostate cells causes activation of caspase-3 (15). To further dissect the molecular pathway of apoptosis triggered by doxazosin in benign prostate epithelial cells, caspase-3 activation was determined by Western blot analysis. Following caspase-8 activation, cleavage of full-length caspase-3 was seen in BPH-1 cells after 12 hours of doxazosin treatment (Fig. 4A). Interestingly, there was no change in TRADD protein expression following treatment with doxazosin (data not shown).

To determine if caspase-8 activation was a result of Fas activation and/or increased FADD recruitment, their complex formation was analyzed. Immunoprecipitation analysis showed an increase in FADD and caspase-8 interactions in both PC-3 and BPH-1 cell lines following treatment with doxazosin (Fig. 4B). Moreover, there was an increased association of FADD and Fas following treatment of both cell lines (Fig. 4B).

Doxazosin causes FADD-dependent apoptosis. Prostate cancer cells PC-3 were stably transfected with either a WT-FADD or a DN-FADD. High levels of WT-FADD and DN-FADD were detected in the PC-3 transfectants by Western blot analysis (data not shown). Four clones from each PC-3 WT-FADD and DN-FADD were selected to determine the effects doxazosin on cell viability. Doxazosin treatment results in a dose-dependent decrease in cell viability in PC-3 WT-FADD transfectants (Fig. 5A). In contrast, there were no significant changes in cell death of PC-3 DN-FADD transfectants compared with parental cells in response to doxazosin (Fig. 5B). As shown in Fig. 5C, overexpression of WT-FADD in prostate cancer cells resulted in a significant increase in doxazosin-induced apoptosis in the four cloned transfectants whereas PC-3 cells expressing the DN-FADD exhibited a reduced apoptotic response to doxazosin (18). Figure 6 shows a decrease in Akt phosphorylation with no apparent change in Akt protein levels in response to doxazosin (25 μmol/L) in PC-3 cells following normalization to actin. In contrast, PC-3 DN-FADD transfected cells show no change in the levels of phosphorylated Akt in response to doxazosin as determined by densitometric analysis of total Akt/pAkt.

Discussion

Apoptosis targeting coincides with the goal of successfully treating patients with advanced prostate cancer. A large number of human tumors exhibit down-regulation of, or insensitivity to,
Interestingly enough, benign prostate cells seem to be in vitro. cell viability and enhanced apoptosis of benign prostate cells data indicate that doxazosin also causes a significant loss of concentrations comparable with the therapeutic doses (13). The present and human prostate clinical specimens at intracellular concentrations documented in benign and malignant prostate epithelial cells through the death receptors (19–21).

The role of Akt signaling in regulating death receptor signaling is not understood. We have previously shown that exposure to doxazosin causes a decrease in Akt phosphorylation (18). Mechanistically, Akt activation promotes the degradation of IκB, which is involved in the apoptotic action of doxazosin in prostate cancer cells (15). In other cellular systems, such as T lymphocytes, blocking Akt signaling has been shown to increase caspase-8 activity, ultimately resulting in Fas-dependent apoptosis (34). Moreover, canstatin inhibits Akt activation and induces Fas-dependent apoptosis in endothelial cells (35). Taken together, this evidence strongly supports Akt signaling as a critical downstream target of the death receptor–mediated apoptotic pathway.

Figure 4. Effect of doxazosin on caspase-3 activation and DISC formation. A, BPH-1 cells were treated with doxazosin (25 μmol/L) for the time periods indicated and cell lysates (30 μg of protein) were subjected to Western blotting. The antibody showed activation of caspase-3 at 12 and 24 hours. B, benign and malignant prostate epithelial cells were exposed to doxazosin (25 μmol/L) for 6, 12, and 24 hours and DISC formation was determined using immunoprecipitation assays. Specific antibodies and conditions are described in Materials and Methods. Following treatment, PC-3 and BPH-1 cells show increased association between molecules involved in the DISC.

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proapoptotic molecules such as Fas and FADD or up-regulation of antiapoptotic molecules like FLIP, suggesting that these cells possess regulatory mechanisms blocking the death signal received through the death receptors (19–21).

Induction of apoptosis in response to doxazosin is well documented in benign and malignant prostate epithelial cells and human prostate clinical specimens at intracellular concentrations comparable with the therapeutic doses (13). The present data indicate that doxazosin also causes a significant loss of cell viability and enhanced apoptosis of benign prostate cells in vitro. Interestingly enough, benign prostate cells seem to be more sensitive to the apoptotic effect of doxazosin than tumor cells (16).

The data from the gene array analysis revealed that a modest change in down-regulation in caspase-8 mRNA in both cells lines after treatment is in agreement with recent evidence indicating no major changes in the mRNA levels of caspase-8 during the period preceding the onset of apoptosis (22). Doxazosin treatment of benign and malignant prostate cells resulted in cleavage and activation of caspase-8, and caspase activation parallels the decreased cell viability in a time-dependent manner, indicative of a Fas-receptor pathway for apoptosis induction. In a similar temporal pattern to malignant prostate epithelial cells (15), benign prostate cells exhibited cleavage of caspase-3 after 12 hours of treatment.

Caspase-8 activation is preceded by its association with FADD and their subsequent recruitment by Fas. In addition, all human prostate cancer cell lines express Fas, but most are resistant to apoptosis induced by an agonistic anti-Fas antibody (23). Some Fas-resistant cell lines will go through apoptosis by concurrent incubation with a Fas antibody and a protein synthesis inhibitor or by sensitizing the cells with chemotherapeutics (24, 25). This would support the notion that some chemotherapeutic agents can sensitize tumor cells to Fas-mediated apoptosis (25, 26). A number of anticancer drugs activate the death receptor pathway via enhancement of Fas and Fas ligand expression (27). We found an increase in both Fas mRNA and protein levels with no change in Fas ligand mRNA expression in either cell line after treatment with doxazosin, consistent with reported evidence that Fas ligand is not required for drug-induced apoptosis because apoptosis is not suppressed by inhibition of Fas/Fas ligand interaction (27, 28).

One could argue that FADD expression may contribute to the sensitivity of Fas activation in prostate cancer cells, as shown in tongue carcinoma cells in response to carboplatin (29). Moreover, an increase in FADD and procaspase-8 expression has been shown in colon carcinoma cells on treatment with cisplatin, doxorubicin, or mitomycin C (30, 31). In prostate cancer cells, we show an increase in FADD mRNA and protein expression as well as an increased DISC formation in response to doxazosin. In agreement with our data, other groups have shown similar increases in FADD and DISC formation in tumor cells following drug treatment (32).

FADD protein has been shown to interact with caspase-8 via its death-effector domain and blocking proteolytic activation of caspase-8 at the DISC prevents apoptosis (33). Considering this evidence, we examined the functional consequences of WT-FADD overexpression and the expression of DN-FADD (lacking death-effector domain) on doxazosin-induced apoptosis of prostate cells. PC-3 cells overexpressing WT-FADD exhibited a dramatic increase in their apoptotic response to doxazosin whereas cells expressing DN-FADD have a significant decrease in doxazosin-mediated apoptosis. This further confirms our hypothesis that doxazosin-induced apoptosis occurs via increased FADD protein levels and DISC formation.

The role of Akt signaling in regulating death receptor signaling is not understood. We have previously shown that exposure to doxazosin causes a decrease in Akt phosphorylation (18). Mechanistically, Akt activation promotes the degradation of IκB, which is involved in the apoptotic action of doxazosin in prostate cancer cells (15). In other cellular systems, such as T lymphocytes, blocking Akt signaling has been shown to increase caspase-8 activity, ultimately resulting in Fas-dependent apoptosis (34). Moreover, canstatin inhibits Akt activation and induces Fas-dependent apoptosis in endothelial cells (35). Taken together, this evidence strongly supports Akt signaling as a critical downstream target of the death receptor–mediated apoptotic pathway.
A mutant form of FADD would potentially uncouple caspase signaling and block apoptosis. Indeed, the present data indicate no changes in Akt activation in doxazosin-treated DN-FADD clones correlating with the apoptotic response compared with the untreated controls. A similar pattern has been found in another experimental system of apoptosis in staurosporine treated cells expressing a DN-FADD (36). Moreover, apoptosis induction and detachment from ECM can be blocked by expression of a DN-FADD mutant with concurrent treatment with various chemotherapeutic agents (31, 37, 38). Studies by other investigators have also shown that cisplatin and camptothecin induce Fas ligand–independent aggregation of death receptors and recruitment of FADD to death receptors (37). Further experiments are required to examine the possible cross-talk between Akt and FADD.

In endothelial cells, Fas/Fas ligand interaction, Fas-FADD complex formation, and caspase-8 activation have been implicated as the precursor to anoikis, and inhibition of any of these events blocks anoikis (39). As shown in the present study, doxazosin-mediated apoptosis may also rely on changes in integrin expression at the cell surface that may cause anoikis. Considering that anoikis-resistant cells are able to detach from the primary tumor without undergoing apoptosis, acquisition of anoikis resistance becomes a critical step in the metastatic process.
Overexpression of a number of mediators involved in cell-matrix and cell-cell anchorage can provide resistance to anoikis, as well as the tumor suppressor genes PTEN and p53, or overexpression of oncogenes such as ras, raf, and src (40). The Fas-receptor signaling pathway and bel-2 overexpression have also been intimately implicated in anoikis (37, 41).

In summary, the present study indicates that doxazosin treatment of benign and malignant prostate epithelial cells leads to a significant increase in DISC formation and subsequent apoptosis via caspase-3 activation. The apoptotic effect of doxazosin provides a molecular basis for therapeutic targeting of apoptosis via caspase-3 activation. The apoptotic effect of doxazosin-treated PC-3 and PC-3 DN-FADD1 cells show no change.

Figure 6. Reduction in Akt phosphorylation by doxazosin. Western blot analysis was applied to cell lysates from doxazosin-treated PC-3 and PC-3 DN-FADD1 cells as described in Materials and Methods. Following treatment, PC-3 cells show a decrease in Akt phosphorylation whereas PC-3 DN-FADD1 cells show no change.

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