Doxazosin induces apoptosis in PTEN-positive androgen-independent PC cells via inhibition of Akt activation

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KEY WORDS

doxazosin ◗ prostate cancer ◗ Akt ◗ PTEN ◗ AR ◗ Bcl-2

ABSTRACT

Introduction. Doxazosin, a selective α1-adrenoreceptor antagonist widely used in the management of benign prostatic hyperplasia (BPH), has been shown to induce apoptosis in androgen-independent (AI) and PTEN (phosphatases and tensin homolog)-negative prostate cancer (PC) cells. The objective of this study was to assess the effects of doxazosin on the growth of PC cells with a functional PTEN/PI3K/Akt pathway in relation to cell androgen sensitivity and Bcl-2 expression.

Material and methods. The DU145 cell line and two derivatives: 1) DKC9 [DU145 (Bcl2 +/+)] and 2) DAR19 [DU145 (AR+/+)] were used. The effect of doxazosin on i) cell proliferation and ii) Akt phosphorylation was measured using the MTT assay and Western blotting, respectively.

Results. Doxazosin caused significant concentration, but not time-dependent, decrease in cell viability. The threshold of sensitivity to the compound differed between the cell lines and was the lowest in the parental cell line. In DAR 19 cells, AR-mediated signaling further increased cell resistance to doxazosin. In all cell lines, doxazosin-induced apoptosis was rescued by EGF stimulation and, in DAR19 cells, by AR activation. Doxazosin reduced basal and EGF-/DHT-induced Akt phosphorylation.

Conclusions. This study demonstrates that doxazosin induces apoptosis in PTEN-positive AI PC cells, at least partially, via Akt deactivation and that over-expression of Bcl-2 or AR increases cell resistance to the drug. These results imply that translational potential of doxazosin depends on phenotypic characteristics of PC cells and provide evidence for limitations to its application in hormone refractory tumors.

INTRODUCTION

Although the mechanisms underlying PC evolution still remain poorly understood, a positive correlation between PC progression, loss of function of PTEN, and phosphorylation of Akt (pAkt) has been reported by a number of studies [1-3]. Akt is a protein kinase activated by various growth and survival factors and involves PI3-kinase (PI3K) [4], a well-established dominant survival mechanism in a wide spectrum of human cancers. In addition to PI3-K, Akt activity can be controlled via various phosphatases including PTEN. In particular, in PC, *PTEN* is thus far the most frequently mutated tumor suppressor gene [5, 6] where deletions, point mutations, and DNA methylation are reported to occur in almost 50% of hormone refractory tumors [7].

Progression of androgen-independent PC (AIPC, also called castrate or hormone resistant PC – HRPC) has also been associated with functional up-regulation of the Bcl-2 protein [8]. A number of reports, including our own work [9], demonstrated a link between Bcl-2 over-expression and PC radio- and chemo-resistance. In LNCaP cells, over-expression of Bcl-2 inhibited apoptosis induced by PTEN suggesting an association between the PI3K/Akt/PTEN signaling and Bcl-2 in the survival mechanism of PC cells [10].

Doxazosin, a selective α–1 adrenoceptor antagonist widely used in the management of benign prostatic hyperplasia (BPH), has been reported to induce apoptosis in PC cells. Investigations utilizing both androgen-sensitive and -insensitive PC cell lines have shown that the pro-apoptotic action of doxazosin is independent of α1-adrenoceptor action and appears to be independent of the androgen sensitivity of the cells [11]. The underlying mechanism involves activation of the TGF-β1-mediated signaling, but other pathways have also been implicated [12-14]. In particular, Shaw et al. have recently demonstrated that doxazosin–induced apoptosis of PC-3 cells carrying a mutated *PTEN* was, in part, attributable to the inhibition of Akt activation [15].

As an impact of interactions between the androgen receptor (AR) and PTEN–Akt signaling axes on PC progression has been well documented [16, 17], our study aimed to assess the effects of doxazosin on the growth of PC cells with a functional AR and PI3K/Akt/PTEN pathway. We used the DU145-derived *in vitro* model to examine a potential association between the apoptotic effect of doxazosin and Akt activation in relation to PC cell androgen sensitivity and Bcl-2 expression.

MATERIALS AND METHODS

Reagents and Antibodies

Doxazosin mesylate (dox) (Cardura) was kindly donated by Pfizer Ltd. (Sandwich, UK). Epidermal growth factor (EGF) and the MTT assay were purchased from Promega (UK) and dihydrotestosterone (DHT) from Sigma (UK). Rabbit anti-Akt and anti-pAkt (Ser473) were obtained from Cell Signaling (UK); mouse anti-AR from Biogenex (UK); mouse anti–PTEN from Neomarkers (USA); anti-mouse IgG Alexa Fluor from Invitrogen (UK) and horse biotin-conjugated anti-mouse IgG from Vector Labs (UK).

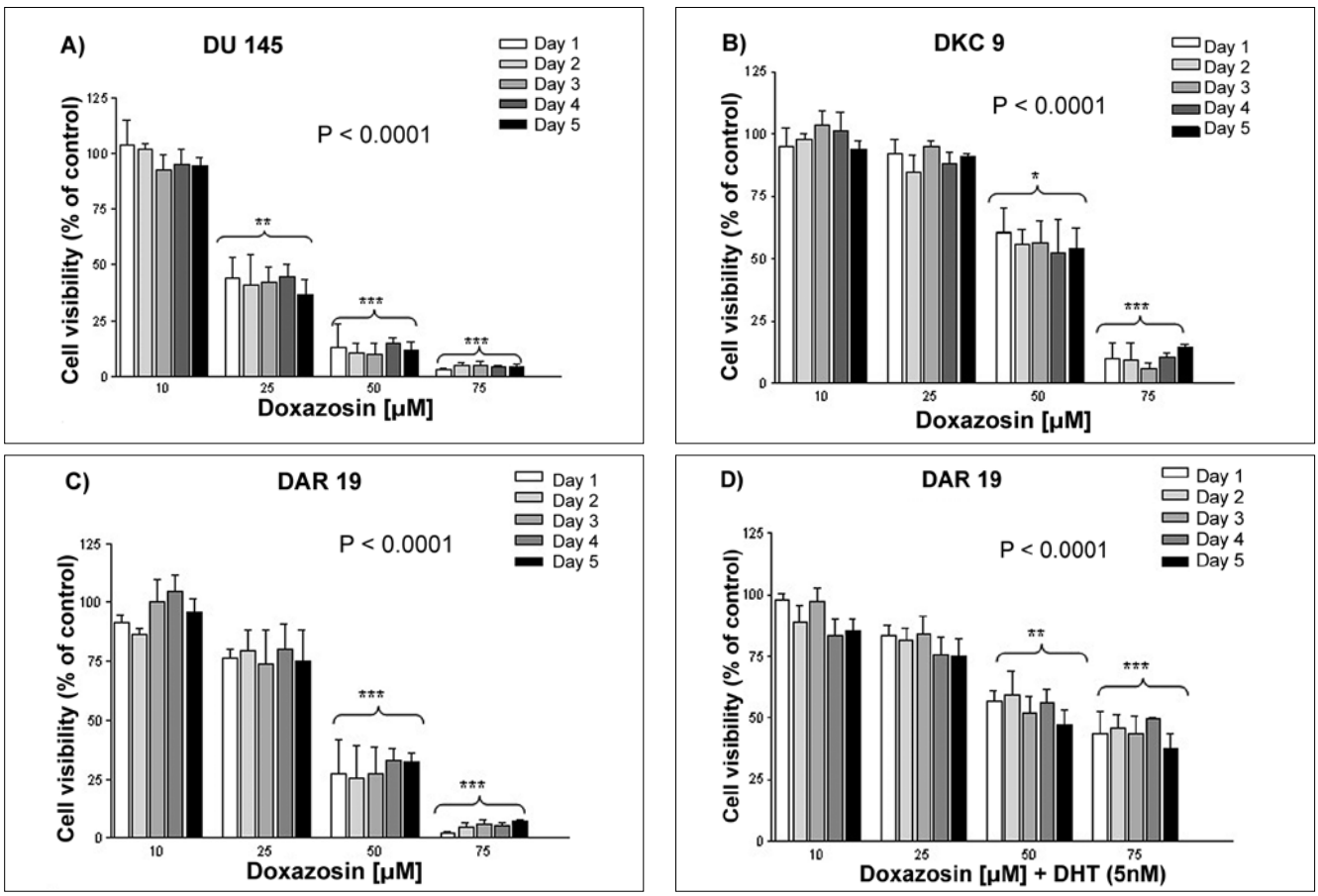

Fig. 1. Effect of doxazosin on growth of: A) -C) unstimulated cells: A) DU145; B) DKC9; and C) DAR19. Cells were seeded at a density of 104 cells/well into a 96-well plate and left to grow in complete DMEM for 24 hours. They were rendered quiescent by incubation with SF-DMEM for 24 hours and then treated in the absence of serum with doxazosin in ascending concentrations ranging from 10 μM to 75 μM. D) DAR19 cells stimulated with DHT. Cells were treated as above and on day 0 the medium was replaced with fresh SF-DMEM containing DHT (5nM) alone (untreated 0 μM dox control) or in combination with ascending concentrations (10-75 μM) of doxazosin. For all A) – D), cell numbers were determined prior to treatment (Day 0) and after 1, 2, 3, 4, and 5 days of incubation by a MTT Assay (Promega, UK). Data expressed as the average of at least three individual experiments are presented as percentage of Day 0 (control). A value of p <0.05 was considered to be statistically significant (statistical significance: * P <0.05, ** P <0.01, and *** P <0.001). For each cell line and concentration of doxazosin, the figures represent average values of data collected on consecutive days of treatment.

Cell lines

DU145, a human prostate adenocarcinoma cell line (ATCC, Rockville, MD), was grown in Dulbecco's Modified Eagles Medium (DMEM; Sigma-Aldrich, UK) containing 10% Fetal Bovine Serum (FBS) (Invitrogen, UK), 2 mM L-glutamine, and 1% Pen-Strep-Amphotericin solution (Gibco Life Technologies, U.K); further referred to as complete DMEM. Cells grown at 37°C and 10% CO_2 were routinely sub-cultured at a 1:3 ratio and re-fed daily with fresh medium.

Two stably transfected cell lines: 1) DKC9 (DU145 Bcl2 +/+) and 2) DAR19 (DU145 AR +/+) developed from the parental DU145 cell line [9, 18] were used in the study. Cells were grown in complete DMEM; the medium for i) DKC9 was supplemented with Puromycin (0.5 μg/ml; Sigma-Aldrich, UK) and ii) DAR19 with Zeocin (300 μg/ml; Invitrogen, UK).

Proliferation assay

Cells seeded into a 96-well plate (10^4 cells/well) were grown in complete DMEM for 24 hours. The medium was replaced with serum-free medium (SF-DMEM) and the cells were cultured for the next 24 hours. Doxazosin was then applied at ascending concentrations ranging from 10 μM to75 μM (day 0). To examine the effects of doxazosin on DHT- or EGF-stimulated cell proliferation, on day 0 the medium was replaced with fresh SF-DMEM containing EGF (50 ng/ml) [19] or DHT (5 nM) [18] alone or in combination with doxazosin (10-75 μM). The medium was refreshed every 24 hrs. Untreated cells (0 μMdox) were used as a control. Cell numbers were assessed for i) treatment with dox +/- DHT after 1, 2, 3, 4, and 5 days and ii) dox +/- EGF after 3 days of incubation using an MTT Assay, according to the manufacturer's instructions. Numerical data represent the average of three independent experiments performed in quintuplicate. Values were standardized to that of the control (100%) and presented as mean ± SEM.

Akt phosphorylation assay

Cells were grown to confluency in complete DMEM. The medium was then replaced with fresh SF-DMEM and cells were grown for the next 24 hours. Cells were treated with i) dox (50 μM; 24 hours) and ii), EGF (50 ng/ml; 20 min prior to harvesting), or iii) DHT (5 nM; 30 min prior to harvesting) alone or in combination. Cells were harvested and lysed at the end of the designated incubation time.

Western Blotting

Cells were lysed with cold cell lysis buffer [20 mM Tris-HCl, pH7.5, 150 mM NaCl, 1 mM Na_2EDTA. 1 mM EGTA, 1% (v/v) Triton, 2.5 mM sodium pyrophosphate, 1 mM β-glycerophosphate, 1 mM Na_3VO_4, 1 μg/ml leupeptin, 1 mM PMSF (Cell Signaling, Hitchin,

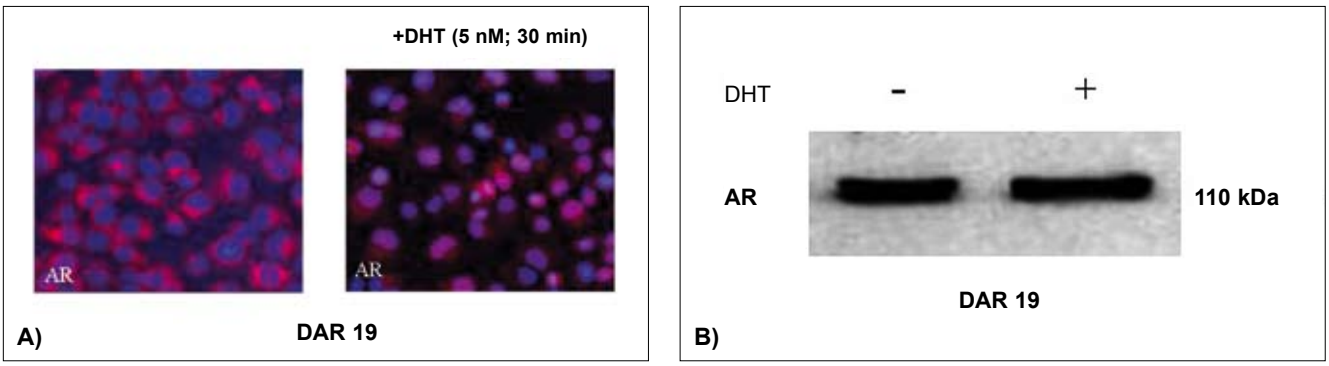

Fig. 2. AR expression in DAR19 cells. A) Immunocytochemistry: In the absence of DHT, AR expression is localized mainly to the cytoplasm. Treatment with 5 nM DHT results in the translocation of AR to the nucleus within 30 min. Nuclei stained with DAPI. B) Immunoblotting. A single 110 kD AR reactive band was observed in both lanes. Addition of 5nM DHT to the culture medium did not alter total AR protein content.

UK)] for 5 minutes on ice, then scraped and sonicated. Equal amounts of lysate proteins (30 µg) resolved by 10% SDS-PAGE were transferred to a nitrocellulose membrane and probed with rabbit anti-Akt (1:1000) and rabbit anti-pAkt (ser 473) (1:1000). Membranes incubated with HRP (horseradish peroxidase)-conjugated anti-rabbit antibodies (HRP; 1:5000, GE Healthcare UK) were visualized by enhanced chemiluminescence (Amersham Bioscience, USA). Blots were developed using the automatic Kodak film developer system (Kodak, UK).

Immunocytochemistry

Immunofluorescence for AR

Cells grown on chamber slides in DMEM supplemented with 10% charcoal stripped fetal bovine serum (csFBS; Invitrogen, UK) were stimulated with DHT (30 min; 5 nM) then fixed in methanol:acetone (1:1 v/v; -20°C) and immunostained for AR (1:10).

Immunocytochemistry for PTEN

Cells grown on chamber slides in complete DMEM were fixed in 4% paraformaldehyde and immunostained for PTEN. The anti-PTEN monoclonal antibody recognizes an epitope at the C-terminus of PTEN, which is lost in a range of human tumors [20]. Images were captured using a digital camera system (Nikon E400, Japan).

Statistical analysis

The statistical analysis was done using Prism software. One-way ANOVA (analysis of variance) and Dunnet's test were performed. A value of p <0.05 was considered to be statistically significant (statistical significance: * P <0.05, ** P <0.01, and *** P <0.001).

RESULTS

I. Effects of doxazosin on growth of Prostate Cancer cells

DU145 (the parental cell line) and its two derivatives: 1) DKC9 [DU145 (Bcl2 +/+)] and 2) DAR19 [DU145 (AR+/+)] were grown in SF-DMEM and treated with doxazosin in ascending concentrations of 10, 25, 50, and 75 µM, equivalent to those previously reported [21], for 24-120 hours. In all cell lines, doxazosin caused cell death and this effect was concentration-dependent (Fig. 1). The threshold of sensitivity to the compound differed between the cell lines (Fig. 1) and was the lowest in the parental cell line. In DU145 cells, decrease in cell viability was up to 41.5 ±6.23 % (P <0.01), 11.8 ± 8.75 % (P <0.001), and 4.00 ±1.09 % for 25 µM, 50 µM, and 75 µM respectively. The proportion of viable DKC9 cells treated with 50 µM and 75 µM was 63.99 ±9.63% (P <0.05) and 9.96 ±3.79% (P <0.001), respectively. DAR19 cells were also more resistant to the drug than control cells. Doxazosin caused a decrease in their viability at 50 µM and 75 µM to 28.93 ±9.641% (P <0.01) and 4.94 ±1.02% (P <0.001), respectively (Fig. 1). Longer incubation with the compound did not have any significant effect on cell growth (Fig. 1). These results suggest that both over-expression of Bcl-2 and restoration of androgen sensitivity increase resistance of the cells to the apoptotic activity of doxazosin.

In order to assess whether ligand-induced activation of AR can enhance this protective effect, DAR19 cells treated with doxazosin were stimulated with 5nM DHT. The conditions of DHT treatment (concentration and duration) were previously optimized and shown to induce translocation of AR to the nucleus in DAR19 cells within 30 min (Fig. 2) [18].

As illustrated in Figure 1D, incubation of DAR19 cells with DHT raised the threshold of cell sensitivity to doxazosin; at concentra-

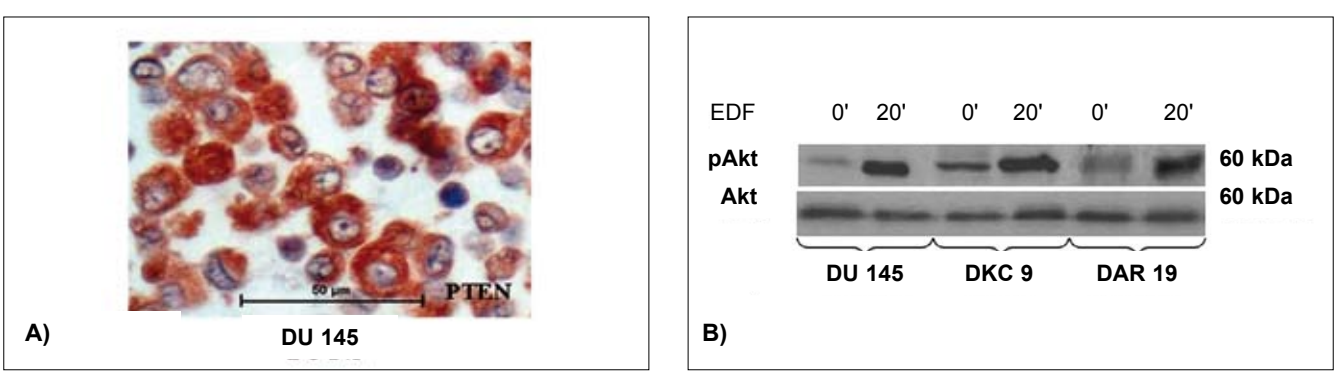

Fig. 3. A) Expression of PTEN in DU145 cells. PTEN expression is confined to the cytoplasm. All cells express PTEN, but the intensity of staining is heterogeneous. B) EGF – mediated pAkt phosphorylation. Parental DU145 cells and 2 derivatives DKC9 and DAR19 cells serum starved for 24 hours were treated with 50 ng/ml EGF for 20 min. Immunoblots probed with specific antibodies against pAkt (Ser473) and total Akt show increase in Akt phosphorylation in response to EGF in all cell lines.

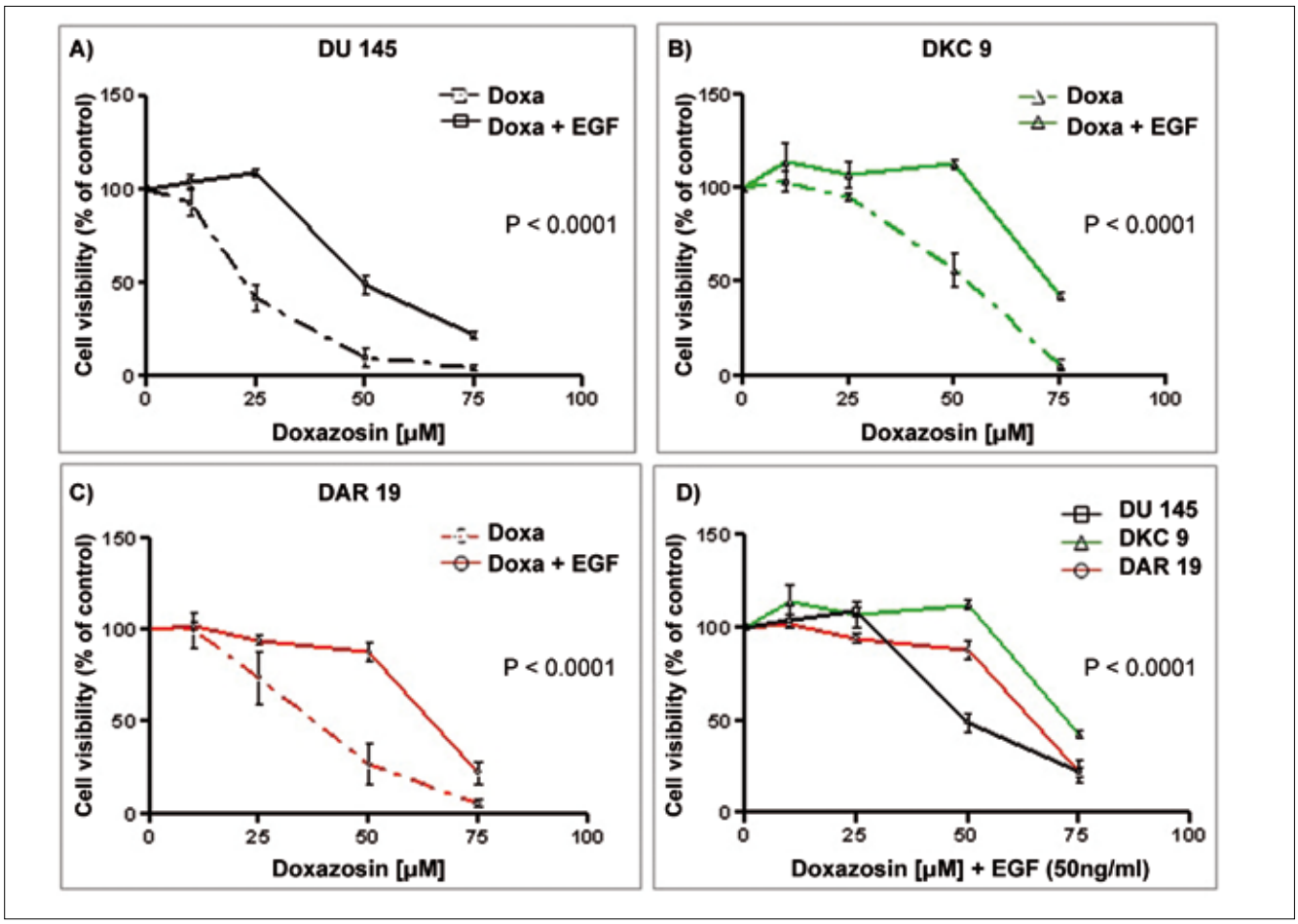

Fig. 4. Effects of doxazosin on growth of EGF-stimulated cells: A) DU145, B) DKC9, C) DAR19. Cells were seeded at a density of 104 cells/well into a 96-well plate and left to grow in complete DMEM for 24 hours. They were rendered quiescent by incubation with SF-DMEM for 24 hours and then treated with fresh SF- DMEM containing i) EGF (50 ng/ml) alone or in combination with doxazosin at concentrations of 10-75 μM (continuous line) or ii) doxazosin in concentrations of 10-75 μM (dotted line). Cell growth was assessed after 3 days of treatment by MTT assay. D) Combined growth curves of all 3 cell lines. Data expressed as the average of at least three individual experiments are presented as percentage of control (0 μM dox) values. P <0.05 was considered to be statistically significant (statistical significance: * P <0.05, ** P <0.01, and *** P <0.001).

tions of 50 μM and 75 μM, cell growth was rescued to 53.57 ±5.08% (P <0.01) and 43.84 ±5.82% (p <0.001), respectively. These results indicate that proliferation induced by AR-mediated signaling counteracts the apoptotic activity of doxazosin on androgen-sensitive PC cells. This protective effect was more pronounced than that seen in unstimulated Bcl-2-expressing PC cells.

As expected, DHT did not affect proliferation of neither DU145 nor DKC9 cells (not shown).

Effect of Doxazosin on Akt-mediated signaling in PC cells

DU145 is a PTEN positive cell line, expressing low basal level of activated Akt [22]. These cells also express EGFR [19] and stimulation with EGF (50 ng/ml) induces Akt phosphorylation (Fig. 3). Continuous EGF stimulation evokes a proliferative response of DU145 cells that reaches its maximum after 72 hours of stimulation [19].

A) Effects on EGF-induced PC cell proliferation

To examine a potential association between the apoptotic effect of doxazosin and growth promoting Akt activation, cells stimulated with EGF (50ng/ml) were treated with ascending concentrations of doxazosin (10 μM, 25 μM, 50 μM, and 75 μM). Cell proliferation was assessed in triplicate cultures after 72 hours of treatment [19].

Incubation with EGF attenuated the pro-apoptotic activity of doxazosin in all cell lines. At 50 μM and 75 μM of doxazosin, cell growth was rescued to A) 56.57 ±1.58% (P <0.01) and 22.41 ±2.13% (p <0.001), B) 112.5 ±2.43% (P >0.05) and 42.61 ±2.03% (P <0.01), and C) 87.84 ±5.16% (P >0.05) and 22.37 ±6.01% (P <0.01) in DU145, DKC9, and DAR19 cells respectively (Fig. 4).

B. Effects of doxazosin on Akt phosphorylation

To further investigate the mechanisms underlying the pro-apoptotic activity of doxazosin, cells were treated with EGF and doxazosin alone and in combination. Concentration and duration of doxazosin applications (50 μM for 24 hours) were chosen on the basis of results of the proliferation assays described above (all cell lines exhibited a significant decrease in cell viability – Fig. 1). As shown in Figure 5, doxazosin caused a decrease (~2 fold) in the levels of both basal and EGF-induced pAkt in all cells. This suggests that 1) the inhibitory effect of doxazosin on PC cell growth can be attributable (at least partially) to deactivation of Akt and 2) over-expression of Bcl-2 or AR significantly reduces this effect and increases resistance of androgen independent human PC cells to this compound.

C. Effects of doxazosin on DHT-induced Akt phosphorylation

A crosstalk between the PTEN/Akt pathway and AR-mediated signaling in PC cells has been well documented [23-25]. Androgens have been shown to activate the PI3K/Akt signaling pathway in

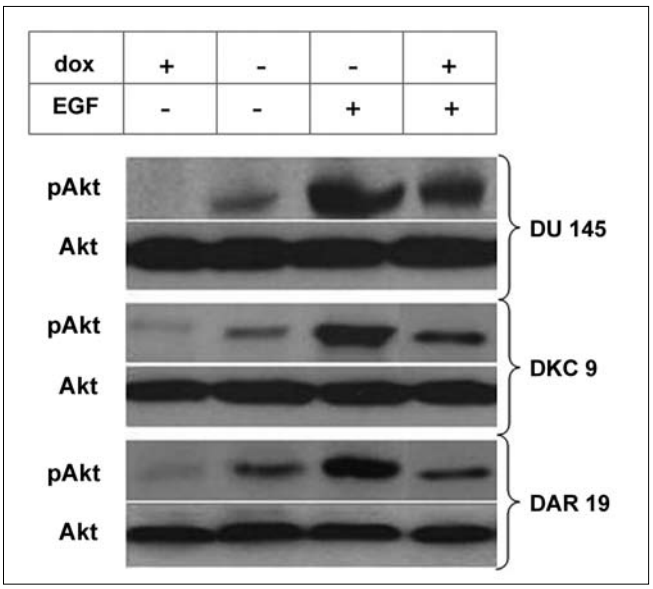

Fig. 5. Effects of doxazosin on EGF-stimulated Akt phosphorylation in DU145, DKC9, and DAR19 cells. Cells serum-starved for 24 hours were treated with dox (50 μM; 24 hours) and/or EGF (50 ng/ml; 20 min prior to harvesting). Blots were probed with antibodies to pAkt and Akt.

PC-3 cells only once they have been transfected with AR [26]. We have confirmed this observation and shown that DHT induced Akt activation in DAR19, but not in the parental DU145 cells (Fig. 6A). In order to examine whether activation of Akt contributes to the mechanisms underlying AR-mediated resistance of PC cells to doxazosin, DAR19 cells treated with doxazosin were stimulated with DHT (5 nM, 30 min) and expression of pAkt was assessed by Western blotting. The results show that doxazosin decreased DHT-induced expression of pAkt (Fig. 6B).

DISCUSSION

Several previous reports have associated the pro-apoptotic action of doxazosin with various PC cell phenotype characteristics. However, due to the heterogeneity of tumors and variability in PC progression, an accurate prediction of response to therapy calls for *in vitro* models with a wide spectrum of PC cell phenotypes. In this report, three isogenic PC cell lines were used for the first time to assess the effect and action of doxazosin on a PTEN-positive and androgen-sensitive PC cells.

Consistent with previous reports, doxazosin induced inhibition of PC cell growth. In all cell lines, this effect was concentration-dependant and reached plateau within the first 24 hrs of application. Longer incubation with the drug did not have any further effect on cell viability. In a recent study, Cal et al., used DU145 cells to demonstrate that doxazosin-induced apoptosis was time-dependent, peaking within 72 hours of application [27]. The discrepancies between these observations and our findings are likely to be due to the differences in the experimental conditions. Unlike Cal et al., our experiments were carried out under conditions of serum starvation thus allowing the drug to reach much faster intracellular saturation levels. Indeed, doxazosin has been shown to have a high affinity to serum proteins and, hence, has a lower potency in a serum-rich environment [28].

The threshold of cell sensitivity to the compound differed between the cell lines with the lowest being in the parental, AR-negative, and Bcl-2-negative cells. Restoration of androgen sensitivity increased resistance to doxazosin and this was further enhanced by activation of AR-mediated signaling. This suggests that an ability to activate survival mechanisms makes PC cells more resistant to external apoptotic stimuli activated by doxazosin. In contrast to our observation, Benning et al., have previously reported that the apoptotic activity of doxazosin is independent of the hormone sensitivity status of the cells [11]. Their study, however, was carried out in a PTEN–deficient LNCaP cell line. A strong link between AR–induced signaling and the PTEN–Akt axis in PC have been documented by a number of reports [16, 29]. For example, Akt is known to regulate the stability of the AR and acts synergistically with both AR-nuclear and nongenotropic signaling [30]. PTEN, on the other hand, has antagonistic interactions with AR by repressing the transcriptional activity of AR through the inhibition of Akt phosphorylation. Interestingly, ligand-activated AR protects PC cells from PTEN-induced apoptosis in an Akt-independent manner, thereby suggesting that the interaction between the AR- and PTEN-dependent signaling networks is even more complex [31]. These reciprocal interaction loops are modified and finely tuned by the hormonal status of the cell. Neither the role of PTEN nor its impact on the responsiveness of androgen-independent PC to therapy is fully understood. Little is also known about the interactions of doxazosin with pathways mediated by ligand activation of steroid receptors in hormone–regulated cancers in general. Hui at al. have recently demonstrated that in ER- and PTEN-positive breast cancer cells, doxazosin-induced apoptosis was less marked in the presence of estrogen [32]. Together with our results, these data would suggest that, in hormone-dependent and/or sensitive cancers, a cross-talk between various intracellular mechanisms, including the PI3K/Akt/PTEN and hormone receptors-mediated pathways, may decide on the ultimate responsiveness of tumor cells to the drug.

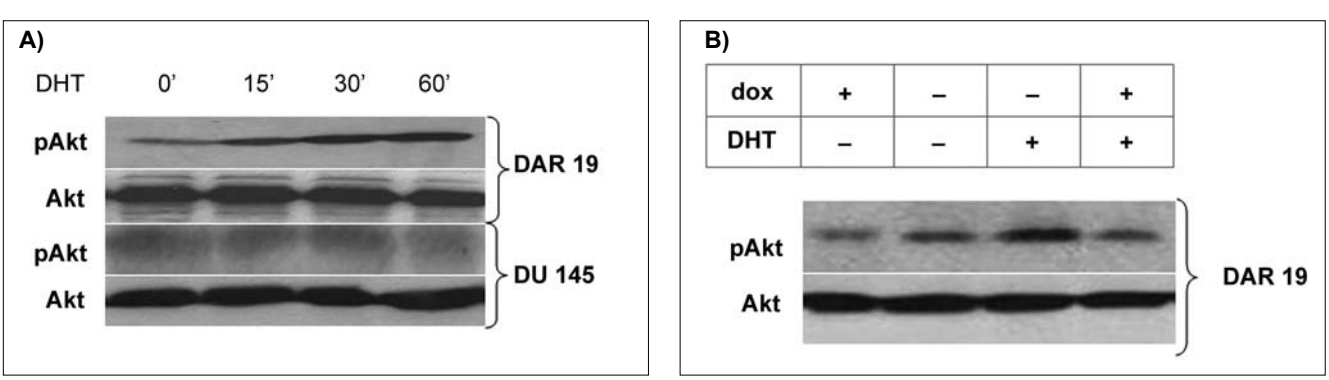

Fig. 6. A) DHT- mediated Akt phosphorylation DAR19 and parental DU145 cells serum-starved for 24 hours were treated with 5nM DHT for 0, 15, 30, and 60 min. Immunoblots probed with specific antibodies against pAkt (Ser473) and total Akt show an increase in Akt phosphorylation only in DAR19 cells. B) Effects of doxazosin on DHT-stimulated Akt phosphorylation in DAR19 cells. Cells serum-starved for 24 hours were treated with dox (50 μM; 24 hours) and/or DHT (5 nM; 30 min prior to harvesting). Blots were probed with antibodies to pAkt and Akt.

There is increasing evidence to suggest that doxazosin executes its action by affecting multiple intracellular signaling mechanisms [11-15, 33]. Our results showed that activation of EGFR-mediated signaling partially rescued all cell lines from doxazosin-induced apoptosis with a gradient of sensitivity similar to that seen in unstimulated cells. In addition, in all cell lines, doxazosin decreased EGF-induced Akt activation. The reduction of Akt phosphorylation by the drug has already been reported in PC-3 cells [15]. The authors demonstrated that although doxazosin inhibited Akt phosphorylation in these cells (androgen-independent and insensitive as well as PTEN-negative), it did not have any direct effects on the activity of any of the known kinases upstream of Akt, including PI3K. Since the compound did not affect the phosphorylation status of ERK (extracellular signal-regulated kinase), it has been suggested that its action might be specific to intracellular pathways interfering with other alternative intracellular Akt targeting mechanisms such as competition for ATP-binding or the death-receptor pathway [15]. It is also possible that this inhibitory effect is due to the substrate-specific phosphatase activity of doxazosin [34]. Whatever the exact mechanisms, our results together with the above findings indicate that Akt is one of the downstream targets and inhibition of its phosphorylation is likely to contribute to the final biological effect of doxazosin. In their recent study, Hui et al. demonstrated that doxazosin-induced apoptosis in a hormone-independent PTEN–positive MDA-MB-231 breast cancer cell line partially via the reduction of ERK phosphorylation [32]. While seemingly conflicting with reported data on PC-3 cells [15], these findings might suggest that the mechanism of doxazosin action is dependent on PTEN / hormonal status of the cells.

Bcl-2 has been shown to carry out survival functions against a range of apoptotic stimuli in a variety of cell systems. In PC, the *de novo* expression of Bcl-2 has been associated with progression to androgen-independence and up-regulation of this protein was often found in hormone refractory tumors [8]. Although the molecular mechanisms underlying the death suppressing activity of Bcl-2 in cancer cells are not fully understood, *in vitro* studies have consistently reported a link between the anti-apoptotic potential and cytotoxic chemoresistance of Bcl-2-expressing tumor cells [9, 35-37]. Furthermore, it has been recently demonstrated that Bcl-2 and doxazosin produced opposing effects on PC cell survival [14]. Our results confirm these observations and show that enforced expression of Bcl-2 partially rescues the cells from doxazosin-induced apoptosis but does not block the inhibitory effect of doxazosin on Akt phosphorylation. These data are consistent with previous studies and give support to the notion of a multifaceted antitumor effect of doxazosin that targets several cell survival pathways.

In summary, the results of our study show for the first time that the pro-apoptotic effect of doxazosin on PC cells is modulated by the hormonal status of the cells. Doxazosin is believed to hold a great promise for both the treatment and prevention of PC [38, 39], but its exact interactions with molecular mechanisms governing the intricate process that defines the balance between cell proliferation and death and, in particular, the functional PTEN/AR relationship are still not understood. Our findings, together with those already published, imply that a translational potential for doxazosin in PC depends on the phenotypic characteristics of the tumor and should be viewed as such to guide the selection of patients for specific therapeutic strategies.

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