Differential Effects of Dehydroepiandrosterone and Testosterone in Prostate and Colon Cancer Cell Apoptosis: The Role of Nerve Growth Factor (NGF) Receptors

Vasileia Anagnostopoulou,* Iosif Pediaditakis,* Saad Alkahtani, Saud A. Alarifi, Eva-Maria Schmidt, Florian Lang, Achille Gravanis, Ioannis Charalampopoulos, and Christos Stournaras

Departments of Biochemistry (V.A., C.S.) and Pharmacology (I.P., A.G., I.C.), University of Crete Medical School, GR-71003 Heraklion, Greece; Foundation of Research and Technology (Institute of Electronic Structure & Lasers [IESL]-Foundation of Research & Technology-Hellas [FORTH]) (I.P., A.G.), 71110 Heraklion, Greece; Department of Zoology (S.A., S.A.A.), Science College, King Saud University, Riyadh, 11352 Saudi Arabia; and Department of Physiology (E.-M.S., F.L., C.S.), University of Tubingen, Tubingen, 72076 Germany

Tumor growth is fostered by inhibition of cell death, which involves the receptiveness of tumor to growth factors and hormones. We have recently shown that testosterone exerts proapoptotic effects in prostate and colon cancer cells through a membrane-initiated mechanism. In addition, we have recently reported that dehydroepiandrosterone (DHEA) can control cell fate, activating nerve growth factor (NGF) receptors, namely tropomyosin-related kinase (Trk)A and p75 neurotrophin receptor, in primary neurons and in PC12 tumoral cells. NGF was recently involved in cancer cell proliferation and apoptosis. In the present study, we explored the cross talk between androgens (testosterone and DHEA) and NGF in regulating apoptosis of prostate and colon cancer cells. DHEA and NGF strongly blunted serum deprivation-induced apoptosis, whereas testosterone induced apoptosis of both cancer cell lines. The antiapoptotic effect of both DHEA and NGF was completely reversed by testosterone. In line with this, DHEA or NGF up-regulated, whereas testosterone down-regulated, the expression of TrkA receptor. The effects of androgens were abolished in both cell lines in the presence of TrkA inhibitor. DHEA induced the phosphorylation of TrkA and the interaction of p75 neurotrophin receptor with its effectors, Rho protein GDP dissociation inhibitor and receptor interacting serine/threonine-protein kinase 2. Conversely, testosterone was unable to activate both receptors. Testosterone acted as a DHEA and NGF antagonist, by blocking the activation of both receptors by DHEA or NGF. Our findings suggest that androgens may influence hormone-sensitive tumor cells via their cross talk with NGF receptors. The interplay between steroid hormone and neurotrophins signaling in hormone-dependent tumors offers new insights in the pathophysiology of these neoplasias. **(*Endocrinology* 154: 2446–2456, 2013)**

Hormonal microenvironment has a strong influence in the growth of various cancers, defining the necessary balance between factors that act to facilitate cell survival and those that inhibit cell proliferation and lead to apoptosis. Indeed, the close interplay between steroid hormones and growth factors control the auto/paracrine regulation of proliferation, invasiveness, and apoptosis of many tumor cells (1–3). Specifically in hormone-depen-

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* V.A. and I.P. contributed equally to this work.

Abbreviations: Bcl-2, B-cell lymphoma 2 protein; Ct, threshold cycle; DHEA, dehydroepiandrosterone; E2, estradiol; FACS, fluorescence-activated cell sorting; FITC, fluorescein isothiocyanate; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; HAS, human albumin serum; iAR, intracellular AR; IB, immunoblot; IP, immunoprecipitation; mAR, membrane androgen receptor; NGF, nerve growth factor; PI3K/Akt, phosphoinosidite 3-kinase/protein kinase B; RhoGDI, Rho protein GDP dissociation inhibitor; RIP2, receptor interacting serine/threonine protein kinase 2; Trk, tropomyosin-related kinase.

dent prostate cancer, androgens testosterone and dehydroepiandrosterone (DHEA) hold a key role in regulating cancer cells proliferation and tumorogenicity. In the case of colon cancer, estradiol (E2) and vitamin D were shown to prevent cancer initiation (4). Furthermore, the expression of membrane androgen receptors (mARs) is also detected, suggesting an important role of androgens (5). It is of note that postmenopausal hormone replacement therapy reduces the risk of colon cancer among women (6), and DHEA has been proposed as a potential protective agent against colorectal cancer (7, 8).

DHEA is the most abundant, naturally occurring steroid hormone in humans. Many cell types express the necessary enzymatic machinery to metabolize DHEA to testosterone, DHT, and then 5a-androstane-3b, 17b-diol or aromatize it to E2. Several studies indicate that DHEA may enhance cancer-promoting activities in several prostate cancer cell lines acting as agonist or antagonist for the intracellular AR (iAR) (9–11), a major regulatory player in prostate cancer (12, 13). Moreover, the estrogenic metabolites of DHEA, 5a-androstane-3b, 17b-diol (3b-Adiol) and E2 bind to estrogen receptors but not to AR (14). Despite the aforementioned influence of DHEA on AR and estrogen receptor, no specific receptor has been identified for DHEA. In the last 30 years, many studies described and characterized several membrane receptors as potential low-affinity DHEA-binding sites, including many neurotransmitter receptors (15, 16) acting on multiple organs beside the nervous system (17).

The other main component of tumor microenvironment, which complements the local hormonal milieu and participates in the control of tumor growth, is the autocrine and paracrine production of specific growth factors and their receptors. Important members of such proteins with trophic activities are neurotrophins (nerve growth factor [NGF], brain-derived growth factor, and neurotrophin-3 and neurotrophin-4/5). Besides the well-described role of neurotrophins in neuronal development and maintenance (18, 19), new insights are now reported involving neurotrophins in tumor biology of both central and peripheral tissues. They bind specifically and with high affinity to a large family of cell-surface receptors called tropomyosin-related kinases (Trks) (NGF binds to TrkA, brain-derived growth factor (BDNF) to TrkB, and neurotrophin-3 to TrkC), which posses an intrinsic, ligand-controlled tyrosine-kinase activity. Trk receptors regulate diverse functions in normal cells (18, 19) and have a crucial role in oncogenesis (20, 21). All neurotrophins and their immature isoforms (proneurotrophins) bind as well to the less investigated $p75^{NTR}$ receptor, a member of the TNF receptor superfamily: $p75^{NTR}$ binds all neurotrophins with lower affinity, whereas on the contrary, it consists the high-affinity receptor for the proneurotrophins. Trks and $p75^{NTR}$ receptors have been strongly associated to proliferation, cell survival, or death of a variety of cancer cells (20–22).

Different members of neurotrophins are expressed during cancer progression, suggesting their involvement in cell proliferation, anoikis protection, and malignancy. However, the role of NGF and its receptors in cancer is far to be clear (21). The importance of NGF in cancer cells is unraveled through its identification and purification from a sarcoma and its biological titration using PC12 tumoral cells. Recently, TrkA receptors were detected in human colon carcinoma biopsies, whereas $p75^{NTR}$ receptors were purified from a human melanoma cell line (23–26). It is of note that NGF and its receptors have strong prognostic value for pancreatic cancer (27, 28).

Interestingly, normal prostate was shown to be richest sources of peripheral NGF (29): prostatic smooth muscle stromal cells produce NGF but lack its receptors. In contrast, epithelial prostate cells express both NGF receptors (TrkA and $p75^{NTR}$). It appears that stromal cell-derived NGF interacts in a paracrine manner with TrkA and $p75^{NTR}$ receptors on the adjacent epithelial cells (29). Expression of $p75^{NTR}$ is decreased during tumor progression, an observation raising the possibility that $p75^{NTR}$ may play a role as a tumor suppressor in the prostate gland (29). In contrast, Trk receptors appear to be involved in malignant progression. Indeed, their levels appear to increase with increasing malignancy of prostate cancer: inhibition of autocrine Trk signaling via the small Trk inhibitor induces the apoptotic death of malignant prostate cells in rodents (30), whereas neutralizing antineurotrophin antibodies effectively decrease the growth of prostate tumor xenografts in nude mice (31).

Testosterone is a main regulator of apoptotic process, both in prostate (32–34) and colon cancer (5, 35, 36) apoptotic responses. Regulation of the apoptotic machinery in prostate and colon cancer cells by testosterone occurs rapidly and is initiated at the plasma membrane level through specific membrane-binding sites not involving the classical cytoplasmic AR (33–35, 37). These effects are controlled through the regulation of prosurvival kinases phosphoinosidite 3-kinase/protein kinase B (PI3K/Akt) and small GTPases like RhoA (33, 37). Furthermore, testosterone governs death-related proteins Bcl-2-associated death promoter and Caspase-3 through regulation of actin cytoskeleton reorganization and the transcription factor nuclear factor kappa B (34). On the other hand, DHEA protects rat pheochromocytoma PC12 cells against apoptosis (38) via an acute but transient sequential phosphorylation of the prosurvival MAP kinase kinase/ERK and SH2-containing protein/PI3K/Akt kinases, the induction

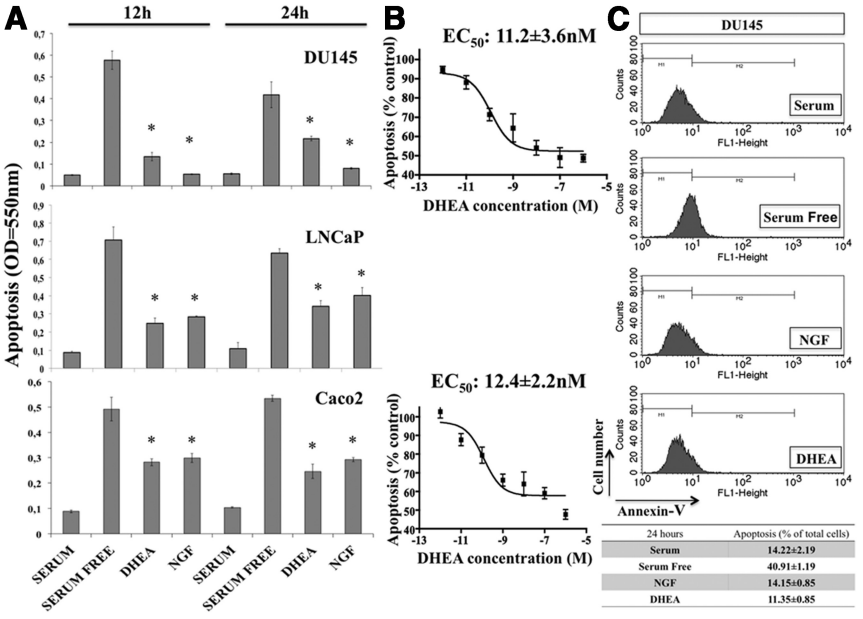

Figure 1. DHEA and NGF inhibit serum deprivation-induced apoptosis in prostate and colon cancer cells. Prostate (DU145 and LNCap) and colon (Caco2) cancer cell lines were treated for 12 or 24 hours in serum-starved conditions in the presence or absence of 10^{-7}M DHEA or 100-ng/mL NGF. Apoptosis was evaluated with the APOPercentage assay (A) measuring OD at 550 nm after lysing the cells, or by FACS analysis (C) for DU145 cells measuring Annexin-V positive cells. In addition, reduction of apoptosis from DHEA was measured for several concentrations of DHEA, ranging from 10^{-12}M up to 10^{-6}M (B). The mean OD measured at 550 nm ± SE of 3 independent experiments performed in triplicates was compared with OD ± SE of the untreated control (serum free) cells. Results are presented in bars as OD arbitrary units (*, $P < .01$). FACS analysis shows the percentage of Annexin-V positive cells compared with control (serum) after measuring 10 000 cells. The data shown are averages ± SE derived from 3 independent experiments.

of cAMP response element-binding protein/nuclear factor kappa B transcription factors, and the subsequent transcriptional regulation of antiapoptotic B-cell lympoma 2 protein (Bcl-2)/Bcl-xL proteins (39). These effects of DHEA are mediated through specific membrane-binding sites (40) and cytoskeletal mobilization (41). The DHEA-induced prosurvival signaling pathways are at least partly mediated by NGF TrkA receptor. We have recently reported that DHEA binds to TrkA and $p75^{NTR}$ receptors of neurotrophin-activating intracellular survival pathways in neuronal cells (42). However, the interplay between androgens and neurotrophins in regulating the apoptotic machinery in tumor cells remains unknown.

Based on these findings, in the present study, we examined the potential role of NGF receptors in the effects of androgens in prostate and colon cancer cell fate, using the prostate (DU145) and colon (Caco2) cancer cell lines. Our findings suggest that 1) DHEA activates both TrkA and $p75^{NTR}$ receptors, in contrast to ineffective testosterone, and 2) testosterone enhances cell apoptosis in both cancer cell types, inhibiting the prosurvival effects of NGF and DHEA and antagonizing their ability to activate TrkA and $p75^{NTR}$. Our results indicate that the intratumoral hormonal microenvironment may play a critical role in tumor progression also via a paracrine cross talk with locally produced neurotrophins.

Materials and Methods

Cell cultures

The LNCaP human prostate cancer cells of epithelial morphology, derived from metastatic site (left supraclavicular lymph node), and DU145 (human prostate cancer cells), derived from metastatic site brain carcinoma. Caco2 human colon cancer cells of epithelial morphology, derived from colorectal adenocarcinoma. LNCaP and DU145 human prostate cancer cell lines are cultured in RPMI 1640 (Invitrogen-Gibco, Carlsbad, California) supplemented with 25mM HEPES, 2mM L-glutamine, 10% fetal bovine serum (Biochrom KG, Berlin, Germany), and 1% antibiotics. Caco2 human colon cancer cells are cultured in DMEM (Invitrogen, Carlsbad, California) supplemented with 25mM HEPES, 4.5-g/L glucose, L-glutamin, and 10% fetal bovine serum (Biochrom KG) and 1% antibiotics. DU145 and Caco2 cells were transfected with the appropriate plasmids (receptor interacting serine/threonine protein kinase 2 [RIP2] and Rho protein GDP dissociation inhibitor [RhoGDI] and RhoGDI) by using TurboFect (catalog no. R0531; Thermo Scientific, Rockford, Illinois) according to manufacturer's instructions. Transfected cells were typically used on the second day after transfection.

Plasmids, antibodies, and proteins

Plasmids to express RIP2 and RhoGDI were previously described (42). Anti-RIP2 was from Enzo Life Sciences (catalog no. ADI-AAP-460; Farmingdale, New York), anti-RhoGDI from Sigma (catalog no. R3025; St Louis, Missouri), and mouse monoclonal antibody to rat $p75^{NTR}$ anti-$p75^{NTR}$ (for immunoprecipitation [IP]) from Millipore (catalog no. MAB365R; Bedford, Massachusetts). NGF was purchased from Millipore (catalog no. 01-125).

Measurement of apoptosis

APOPercentage apoptosis assay

This assay is based on the staining of the apoptotic cells undergoing the cellular membrane flip-flop event when phosphatidylserine is translocated to the outer leaflet. After staining according to the manufacturer's instructions, the amount of dye bound was released from the labeled cells into the solution, and the concentration was measured at a wavelength of 550 nm using a color filter microplate colorimeter. DU145 and Caco2 cells

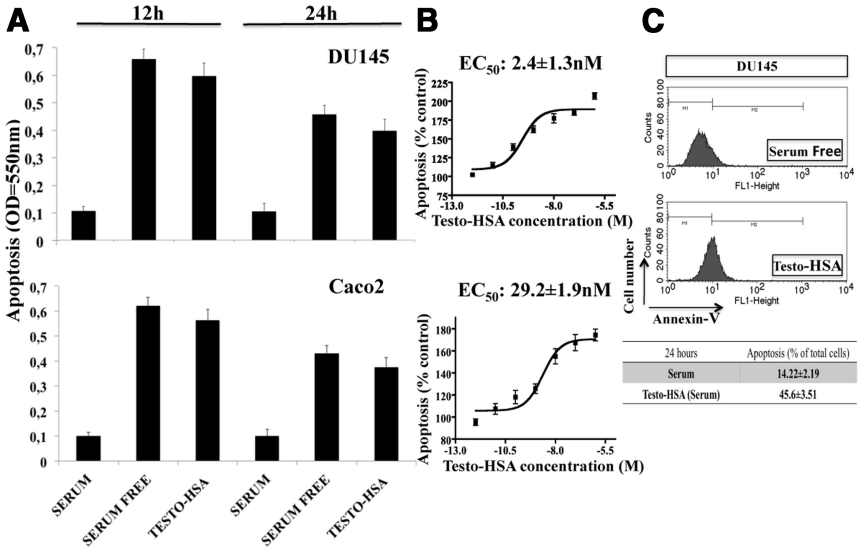

Figure 2. Testosterone induces apoptotic cell loss in prostate and colon cancer cells cultured in serum supplement medium. Prostate (DU145) and colon (Caco2) cancer cell lines were treated for 12 or 24 hours in serum-supplemented or serum-starved conditions in the presence or absence of 10^{-7}M testosterone conjugated with HSA (TESTO-HSA) to ensure the membrane-impermeable effects. Apoptosis was evaluated with the APOPercentage assay (A) measuring OD at 550 nm after lysing the cells, or by FACS analysis (C) for DU145 cells measuring Annexin-V positive cells. In addition, induction of apoptosis from testosterone-HSA was measured for several concentrations of DHEA, ranging from 10^{-12}M up to 10^{-6}M (B). The mean OD measured at 550 nm ± SE of 3 independent experiments performed in triplicates was compared with OD ± SE of the untreated control (serum supplemented) cells. Results are presented in bars as OD arbitrary units (*, $P < .01$). FACS analysis shows the percentage of Annexin-V positive cells compared with control (serum) after measuring 10 000 cells. The data shown are averages ± SE derived from 3 independent experiments.

were cultured in 96-well plates for the APOPercentage Apoptosis Assay (Biocolor Ltd, Belfast, Ireland). Cells were incubated with 100-ng/mL NGF and 10^{-7}M DHEA in serum-free conditions for 24 hours. All cell treatments in the presence of 10^{-7}M testosterone-human albumin serum (HSA) for 12 and 24 hours were performed in the presence of serum.

Fluorescence-activated cell sorting (FACS) analysis

Annexin-V-fluorescein isothiocyanate (FITC) is used to quantitatively determine the percentage of cells within a population that are actively undergoing apoptosis (early apoptosis vs late apoptosis that is detected with the APOPercentage assay). It relies on the property of cells to lose membrane asymmetry in the early phases of apoptosis. In apoptotic cells, the membrane phospholipid phosphatidylserine is translocated from the inner leaflet of the plasma membrane to the outer leaflet, thereby exposing phospholipid phosphatidylserine to the external environment. Cells that stain positive for Annexin-V-FITC are undergoing apoptosis. DU145 and Caco2 cells were cultured in 12-well plates for FACS analysis Annexin-V-FITC Apoptosis Detection kit I (BD Pharmigen TM, San Diego, California). The former detection assays has been previously described (42) to assess apoptosis.

Detection of membrane receptors using FACS analysis

Cells were pelleted and incubated with 50 μL of the primary antibodies against TrkA or $p75^{NTR}$ receptors for 30 minutes over ice. Afterwards, cells were washed 3 times with PBS, and 50 μL of the secondary (antirabbit-R-phycoerythrin conjugated and antimouse-fluorescein conjugated) antibodies were added according to the protocol. Then, cells were washed twice with ice-cold PBS and resuspended in 500-μL PBS. Cells were analyzed within 1 hour by flow cytometry using a FACS Apparatus (BD Biosciences, San Jose, California). The determination of NGF receptors (namely, TrkA and $p75^{NTR}$), expression levels were calculated vs negative control (naive HEK293 cells).

IP and Western blotting

DU145 and Caco2 cells were incubated with 10^{-7}M DHEA, 100-ng/mL NGF in the presence of serum free and testosterone-HSA 10^{-7}M in the presence of serum, washed twice with ice-cold PBS, and suspended in 500-μL cold lysis buffer containing 1% Nonidet P-40, 20mM Tris (pH 7.4), and 137mM NaCl, supplemented with protease and phosphatase inhibitors. Cleared lysates were preadsorbed with protein A-Sepharose beads (Amersham, Piscataway, New Jersey) for 1 hour at 4°C and IP with anti-TrkA (TrkA, catalog no. 06-574; Millipore) overnight at 4°C. Protein A-Sepharose beads were incubated with the lysates for 2–4 hours at 4°C with gentle shaking. For immunoblot (IB) analysis, the beads were suspended in sodium dodecyl sulfate-loading buffer and separated by SDS-PAGE. Proteins were transferred to nitrocellulose membranes and blotted with the antibodies: TrkA (dilution 1:1000) (phospho-TrkA, catalog no. 9141; Cell Signaling, Beverly, Massachusetts, and TrkA, catalog no. 06-574; Millipore) and $p75^{NTR}$ (1:1000) (catalog no. G3231, Promega, Madison, Wisconsin), and secondary antibodies: horseradish peroxidase-conjugated antirabbit IgG (dilution 1:5000) and horseradish peroxidase-conjugated antimouse IgG (dilution 1:2000). Then, the membranes were exposed to Kodak X-Omat AR films (Kodak, New York, New York). A PC-based Image Analysis program was used to quantify the intensity of each band (Image Analysis, Inc, Carleton Place, Ontario, Canada).

Quantitative RT-PCR of TrkA receptor

Total RNA was isolated from DU145 cells using TRIzol (Invitrogen). The cDNA was synthesized from equal amounts (300 ng) of total RNA with a random hexamer primer from a Thermoscript RT-PCR system kit (Invitrogen), according to instructions. The quantitative RT-PCR was performed by using the ViiA 7 Real-Time PCR System (Applied Biosystems, Foster City, California). Briefly, the 15-μL reaction mixtures contained 7.5 μL of SYBR Green PCR Master Mix (Bioline, Taunton, Massachusetts), 0.5 μL of each specific oligonucleotide primer, and 1 μL of nondiluted first strand cDNA synthesized from 300 ng of

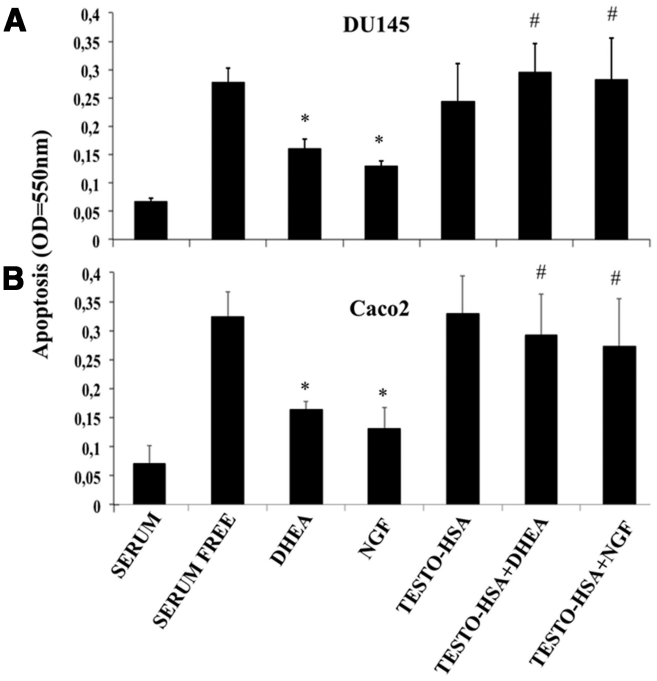

Figure 3. Priming of prostate and colon cancer cells with testosterone blocks prosurvival effects of DHEA or NGF. Prostate (DU145) and colon (Caco2) cancer cell lines were treated for 24 hours in serum-starved conditions in the presence or absence of 10^{-7}M DHEA, testosterone-HSA (TESTO-HSA), or 100-ng/mL NGF or simultaneously with 10^{-5}M testosterone-HSA and 10^{-7}M DHEA or 100-ng/mL NGF (testosterone was used in excess when treated with DHEA or NGF, because its binding ability for NGF receptors was found to be 10-fold less than DHEA). Apoptosis was evaluated with the APOPercentage assay (A) measuring OD at 550 nm after lysing the cells. The mean OD measured at 550 nm ± SE of 3 independent experiments performed in triplicates was compared with OD ± SE of the untreated control (serum free) cells. Results are presented in bars as OD arbitrary units (#, $P < .01$).

total RNA. The conditions for quantitative RT-PCR amplification were set up as: 95°C for 10 minutes, 40 cycles at 95°C for 15 seconds, and 64°C for 1 minute, after the melt curve thermal conditions. For human TrkA, the sense primer was 5′-TCCGCCTCCATCATGGCTGCCTT-3′ and antisense primer was 5′-CCCAAACTTGTTTCTCCGTCCACA-3′. For human glyceraldehyde 3-phosphate dehydrogenase (GAPDH), the sense primer was 5′-ACCACAGTCCATGCCATCAC-3′ and antisense primer was 5′-TCCACCACCCTGTTGCTGTA-3′. Dissociation curves were obtained for each amplified product at the end of amplification. Each individual sample was run in triplicate and the average critical threshold cycle (Ct) used for data analysis. The Ct values of target genes were normalized by the Ct value of internal control (human GAPDH gene).

Results

DHEA and NGF decrease, whereas testosterone increase, apoptosis in prostate and colon cancer cells

Our previous work has shown that testosterone exerts potent regulatory effects on prostate and colon cancer cell apoptosis (32–37). Based on recent findings showing strong expression of NGF in prostate cancer and the interaction of androgens DHEA and testosterone with NGF receptors to control neuronal cell apoptosis, we explored their potential interactions in controlling apoptosis of prostate cancer cells DU145 and LNCaP, as well as colon cancer cells Caco2. DU145 cells are negative for iAR (43, 44) and express only the mAR (33), whereas LNCaP cells express both mAR and iAR. DHEA and NGF significantly reduced serum deprivation-induced apoptosis in all 3 cancer cell types (Figure 1), quantitated with the APOPercentage assay (Figure 1A) (apoptosis was reduced from 0.587 ± 0.053 to 0.142 ± 0.0016 or 0.059 ± 0.002 after treatment for 12 hours with DHEA or NGF, respectively; $n = 3$, $^*P < .01$), and by flow cytometry analysis (FACS) for DU145 cells (Figure 1C). The antiapoptotic effect of DHEA was dose dependent (Figure 1B) with an EC_{50} at nanomolar concentrations (EC_{50}: 11.2 ± 3.6nM and 12.4 ± 2.2nM in DU145 and Caco2 cells, respectively). In parallel, we cultured DU145 and Caco2 cells in the presence of membrane-impermeable testosterone conjugated to HSA protein in serum supplement conditions, shown to induce apoptosis in these cancer cell lines. In line with previous reports (5, 33, 34), testosterone increased apoptosis of DU145 and Caco2 cells after 12 and 24 hours of treatment, quantitated either by the APOPercentage assay (Figure 2A) or by FACS analysis (Figure 2C). Testosterone increased cell death in a dose-dependent manner, with an EC_{50} of 2.4 ± 1.3nM and 29.2 ± 1.9nM for in DU145 and Caco2 cells, respectively (Figure 2B).

Priming of prostate and colon cancer cells with testosterone blocks the antiapoptotic effects of DHEA and NGF

We have previously shown that testosterone antagonizes the prosurvival effects of DHEA in neuronal cells, blocking its binding to NGF receptors (40). We further tested whether this antagonizing effect is also relevant in tumor cells. Indeed, priming of cells with testosterone-HSA (10^{-6}M) effectively reversed the prosurvival effects of DHEA at 100nM or of 100-ng/mL NGF in both, prostate DU145 or colon Caco2 tumor cells (Figure 3).

NGF receptors are expressed in DU145 and Caco2 cells

Prostate cancer cells are secreting growth factors like NGF, which stimulate proliferation and facilitate the escape from cell death (25, 26). Accordingly, we tested the expression of NGF receptors, TrkA and $p75^{NTR}$, in prostate and colon tumor cells. Western blot analysis (Figure 4B) and fluorescence staining (FACS analysis [Figure 4A]

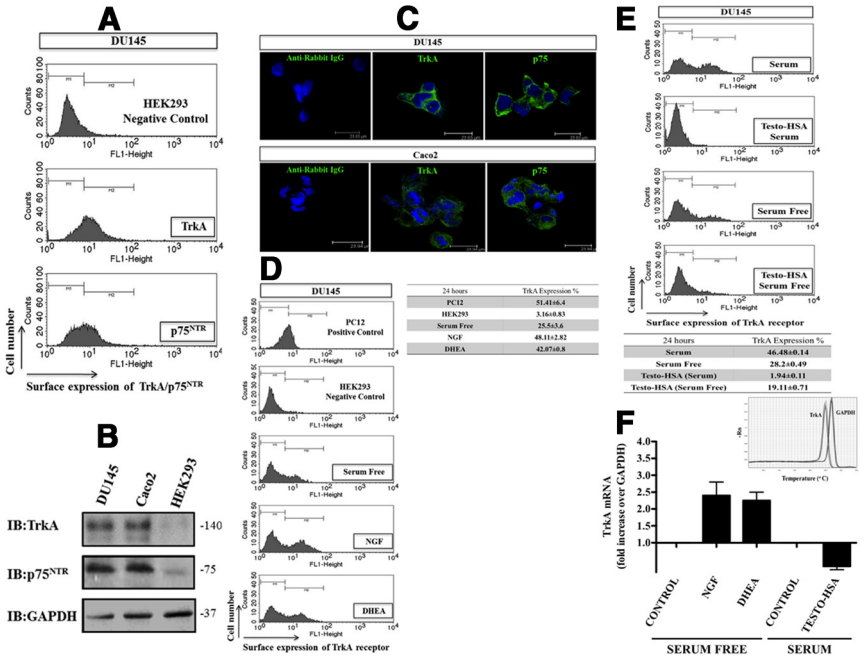

Figure 4. Both prostate and colon cancer cell lines express TrkA and p75^{NTR} receptors, DHEA and NGF up-regulate and testosterone down-regulates TrkA receptor expression levels in DU145 cells. FACS analysis show the expression of TrkA and p75^{NTR} receptors in DU145 cells compared with unstained HEK293 cells (A). IB analysis using specific antibodies for TrkA and p75^{NTR} proteins confirms expression of both receptors in DU145 and Caco2 cells. Naive HEK293 cells were used as negative control (B). Immunostaining of DU145 or Caco2 cells for TrkA (labeled green) or p75^{NTR} (labeled green) and DAPI staining (blue) show membrane-located receptors (C). Treatment for 24 hours with 10^{-7}M DHEA or 100-ng/mL NGF in serum-starved conditions resulted in up-regulation of TrkA receptors (D) to equal levels as in PC12 cells that are known to express TrkA and differentiate towards neurons upon NGF treatment. Treatment for 24 hours with 10^{-7}M testosterone-HSA (TESTO-HSA) in serum-supplemented medium resulted in marked reduction of TrkA expression (from 46.48 ± 0.14% to 1.94 ± 0.11% of TrkA-positive cells after the measurement of 10 000 cells, n = 3), whereas in serum-starved conditions, TrkA positive cells were 28.2 ± 0.49% before and 19.11 ± 0.71% after treatment with testosterone-HAS. The data shown are averages ± SE derived from 3 independent experiments (E). Finally, by performing real-time PCR for TrkA and GAPDH genes, we evaluated the effects of DHEA, NGF, and testosterone on the expression levels of TrkA mRNA. In the presence of serum, testosterone down-regulates (0.8-fold decrease) the mRNA levels of TrkA, whereas DHEA and NGF up-regulate (2.5-fold increase) the mRNA levels of TrkA in the absence of serum (F).

or immunocytochemically [Figure 4C]) showed that both cell lines strongly express both NGF receptors. The expression levels of p75^{NTR} receptor were not affected by a 24-hour exposure to DHEA or testosterone (data not shown). However, protein expression of TrkA was significantly induced after treatment of DU145 cells with DHEA or NGF for 24 hours (Figure 4D). On the contrary, exposure of DU145 cells to testosterone, either in serum-supplemented or in serum-free conditions, decreased the expression levels of TrkA receptor (Figure 4E). It is of note that in the presence of serum, testosterone showed a stronger decrease of TrkA expression. This observation may explain why the proapoptotic effects of testosterone are shown only under serum-supplemented conditions (33, 34). Additionally, we measured the expression levels of TrkA mRNA, using real-time PCR. NGF and DHEA significantly increased the levels of TrkA mRNA, whereas testosterone decreased it (Figure 4F). These findings suggest that androgens and NGF control TrkA levels both at the transcriptional and translational level. Down-regulation of prosurvival TrkA receptor decreases the ability of cancer cells to respond to growth factors and thus to effectively maintain survival signaling. In parallel, endogenous ligands of TrkA receptor, like mature NGF or its precursor pro-NGF (a high-affinity ligand for p75^{NTR} receptor), may exert their proapoptotic actions through prodeath receptor p75^{NTR}.

The effects of DHEA and testosterone on apoptosis are not mediated by NGF

Cancer cells produce various growth factors, supporting tumor proliferation and migration in an autocrine, paracrine manner. Because prostate cancer is known to express endogenous NGF (29), we tested the effects of DHEA and testosterone in DU145 and Caco2 cells, in the presence of a specific immune-neutralizing antibody against NGF. The survival effects of DHEA or the proapoptotic actions of testosterone in DU145 (Figure 5A) or Caco2 (Figure 5B) cells were not significantly affected by the presence of anti-NGF (DHEA decreased serum-induced apoptosis from 0.252 ± 0.016 in serum-free conditions to 0.07 ± 0.002 and from 0.342 ± 0.042 in the presence of anti-NGF to 0.14 ± 0.016 in the cotreatment with DHEA, n = 3). The antibodies against NGF had no effects on serum-deprived conditions (Figure 5, A and B, columns under "serum free"), suggesting that under these conditions, NGF regulation of apoptosis and survival reached plateau levels. Furthermore, treatment of cells with DHEA exerted a strong antiapoptotic effect, not reversed in the presence of anti-NGF, suggesting that the prosurvival effects of DHEA are not dependent upon the presence of endogenous NGF.

On the contrary, in serum-supplemented conditions (Figure 5, A and B, columns under "serum"), where endogenous NGF and other growth factors effectively supported survival and proliferation of tumor cells, anti-NGF induces apoptosis in both cancer cell lines. Testosterone-

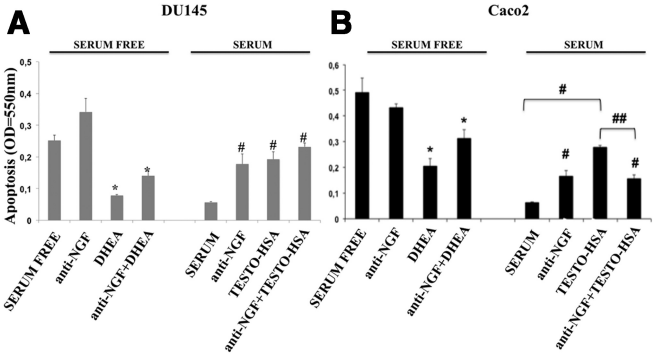

Figure 5. Effects of anti-NGF neutralizing antibody. Prostate (DU145) (A) and colon (Caco2) (B) cancer cell lines were treated for 24 hours in serum-supplemented or serum-starved conditions in the presence or absence of 10^{-7}M DHEA or testosterone-HSA (TESTO-HSA) and containing or not a polyclonal rabbit anti-NGF-neutralizing antiserum (at 1:500 dilution, AB1526; Millipore). Apoptosis was evaluated with the APOPercentage assay measuring OD at 550 nm after lysing the cells. The mean OD measured at 550 nm ± SE of 3 independent experiments performed in triplicates was compared with OD ± SE of the control (serum free for DHEA, serum supplement for testosterone-HSA, anti-NGF alone for DHEA or testosterone plus anti-NGF) cells. Results are presented in bars as OD arbitrary units (#, *, $P < .01$ vs untreated cells, ± anti-NGF, respectively; ##, $P < .01$ vs testosterone-HSA).

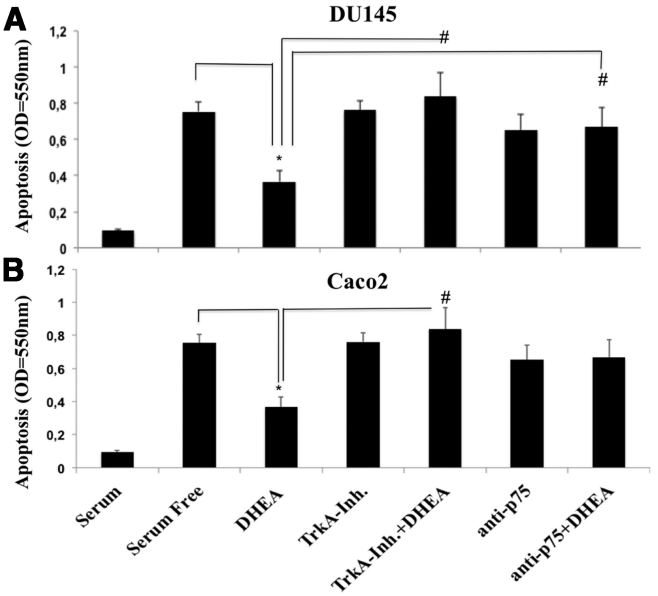

Figure 6. Effects of TrkA and $p75^{NTR}$ inhibitors. Prostate (DU145) (A) and colon (Caco2) (B) cancer cell lines were treated for 24 hours in serum-supplemented or serum-starved conditions in the presence or absence of 10^{-7}M DHEA or testosterone-HSA and containing or not a chemical inhibitor for TrkA (TrkA inhibitor, catalog no. 648450; Calbiochem) or the blocking antibody for $p75^{NTR}$ (anti-$p75^{NTR}$, catalog no. MAB365R; Millipore). Apoptosis was evaluated with the APOPercentage assay measuring OD at 550 nm after lysing the cells. The mean OD measured at 550 nm ± SE of 3 independent experiments performed in triplicates was compared with OD ± SE of the untreated control (serum free for DHEA, serum free plus TrkA inhibitor or anti-$p75^{NTR}$ for DHEA plus each inhibitor, respectively) cells. Results are presented in bars as OD arbitrary units (*, $P < .01$ vs serum free alone, #, $P < .01$ vs TrkA or $p75^{NTR}$ inhibitor alone in serum-free conditions).

HSA exerts antiapoptotic effects under serum-supplemented conditions, most probably acting as a NGF receptor antagonist and blockade of the effects of endogenous NGF. In DU145 cells, exposure of cells to the combination of anti-NGF with testosterone had similar effects as each agent alone. On the contrary, in Caco2 cells, the combination of anti-NGF antibody with testosterone resulted in apoptosis similar to anti-NGF, and less than testosterone alone, indicating that stoichiometry of TrkA and $p75^{NTR}$ receptors is critical to their effects on apoptosis.

Inhibitors of NGF receptors affect the effects of androgens on apoptosis

To test whether TrkA and $p75^{NTR}$ receptors mediate the effects of DHEA or testosterone in prostate and colon cancer cell apoptosis, we used a specific TrkA inhibitor and a neutralizing antibody against $p75^{NTR}$. Both inhibitors had no effect given alone and did not alter the proapoptotic effects of testosterone in serum-supplemented DU145 or Caco2 cells (data not shown). On the contrary, the use of NGF receptor inhibitors significantly affected the antiapoptotic effects of DHEA. Specifically, TrkA and $p75^{NTR}$ inhibitors blocked the antiapoptotic effects of DHEA in DU145 cells (Figure 6A), suggesting that both receptors are mediators of prosurvival actions of DHEA, similarly to neural cells (42). TrkA inhibitor blocked the antiapoptotic effects of DHEA in Caco2 colon cancer cells. However, the $p75^{NTR}$ inhibitor was ineffective in reversing the antiapoptotic effects of DHEA. These findings suggest a differential role of 2 NGF receptors in cancer cell apoptosis. TrkA receptor appears to be the key regulator of cell survival in both cancer cell lines, whereas $p75^{NTR}$ holds a role only in DU145 cells.

Androgens affect NGF receptors signaling

TrkA is autophosphorylated after binding to its ligand (NGF) to its dimer form, inducing postreceptor prosurvival signaling. Autophosphorylation of TrkA has been also shown to be ligand independent (45), leading in that case to cell death. We performed co-IP studies (Figure 7A) or Western blotting in whole-cell lysates (Figure 7B) to test the ability of DHEA or testosterone to induce phosphorylation of TrkA in DU145 and Caco2 cells, cultured in serum-free conditions. Treatment for 15 minutes of DU145 and Caco2 cells with DHEA (100nM) of both cancer cell lines induced phosphorylation of TrkA receptor (Figure 7, A and B). On the contrary, testosterone was ineffective in inducing TrkA phosphorylation (Figure 7, A and B). We also explored the potential ability of testosterone to reverse the effects of DHEA on TrkA phosphorylation. The combination of testosterone with DHEA or NGF fully reversed the ability of the latter to induce TrkA phosphorylation in both cell lines (Figure 7, A and B). We

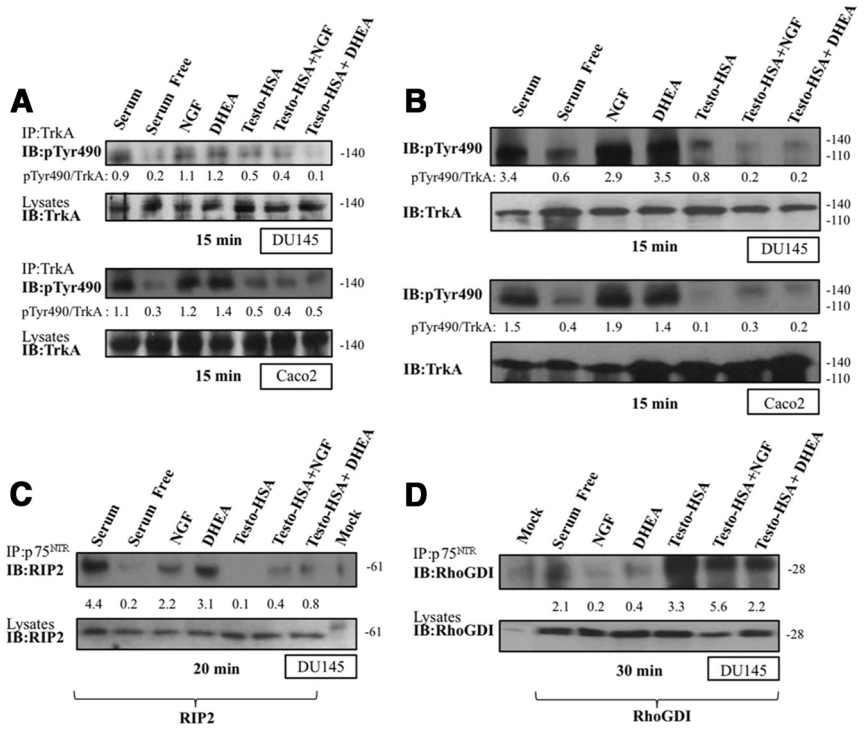

Figure 7. Androgens signaling through TrkA and p75^{NTR} receptors. DU145 cells or Caco2 cells were exposed for 15 minutes to 100nM DHEA or testosterone-HSA or 100-ng/mL of NGF, or they were priming with 1μM testosterone-HSA (TESTO-HSA) and after treatment with DHEA or NGF. Then, cell lysates were IP with anti-TrkA and analyzed by Western blotting, using phospho-specific TrkA antibodies (pTyr490) (A). Serum-deprived DU145 cells or Caco2 cells were incubated for 15 minutes with 100nM DHEA or 100-ng/mL of NGF, or simultaneously with 1μM testosterone-HSA, and cell lysates were analyzed with Western blotting, using specific antibodies against the phosphorylated and total forms of TrkA receptor (B). DU145 cells were transfected with the plasmid cDNAs of each one of the effectors RIP2 (C) or RhoGDI (D). Transfectants were exposed for 20 minutes for RIP2 assays or 30 minutes for RhoGDI to 100nM DHEA or testosterone-HSA or 100-ng/mL of NGF, or simultaneously with 1μM testosterone-HSA, and lysates were IP with p75^{NTR}-specific antibodies and then IB with antibodies against RIP2 (C) or RhoGDI (D).

also tested the effects of DHEA and testosterone on the recruitment of p75^{NTR} intracellular interactor, RIP2 protein, in DU145 cells. As shown in Figure 7C, DHEA recruited RIP2 protein on p75^{NTR} receptor, mimicking NGF. Again, testosterone was ineffective in recruiting RIP2 to p75^{NTR} receptor (Figure 7C). Priming of cells with testosterone-HSA resulted in blocking the recruitment of RIP2 on the receptor by DHEA or NGF (Figure 7C). Moreover, we tested the ability of DHEA and testosterone to induce the dissociation of p75^{NTR} receptor from another of its interactors, RhoGDI, known to control the activity of RhoA. Both NGF and DHEA induced the release of RhoGDI protein from p75^{NTR} (Figure 7D). In that case too, testosterone was unable to promote RhoGDI dissociation. When DU145 cells were treated with the combination of testosterone with DHEA or NGF, the release of RhoGDI induced by the 2 latter was blocked, suggesting that testosterone acts as an antagonist of p75^{NTR} receptor activation, as in the case for TrkA receptor.

The aforementioned interactions of steroids DHEA and testosterone on both NGF receptors, TrkA and p75^{NTR}, and their sequential effects on prostate and colon cancer cell fate are illustrated on Figure 8.

Discussion

Androgens hold a central role in prostate and colon cancer biology (46), participating in the complex local interactions of these tumors (3, 47). DHEA is an important source of androgens, which, when metabolized by the prostate cells, contribute significantly to the amount of DHT present in the prostate (11–13). It is of note that elevated levels of DHEA or its sulfate ester DHEA-sulfate in young adults are associated to low incidence of androgen-dependent tumors. Thus, DHEA may play a protective role in young prostate. The decline of DHEA with aging may contribute to prostate cancer progression associated with advanced age (48). Prostate cancer cell lines like PC3, DU145, and LNCap, with different proliferative/invasive behavior, are expressing TrkA and p75^{NTR} receptors. TrkA expression is significantly higher in AR-negative compared with AR-positive cells, and their expression is related to the invasive capacity/malignancy of prostate cancer cells (49).

The last 2 decades, the mode of action of steroid hormones was enriched with the description of steroid membrane-initiated effects, related also to actions in cancer cells (50–52). We have previously studied the effects of testosterone in prostate and colon cancer cell apoptosis mediated through the activation of specific membrane-binding sites (5, 53) and the subsequent regulation of the prosurvival PI3K/Akt pathway (34, 35), apoptotic proteins of Bcl-2 family, Bad (34, 35), and the reorganization of actin cytoskeleton through specific small GTPase-governed signaling, including cell division protein 42 homolog/Ras-related C3 botulinum toxin substrate 1 (Cdc42/Rac1) and RhoA/B (33, 37).

In the present study, we show that DHEA is an effective antiapoptotic factor, reversing the serum deprivation-induced apoptosis in prostate cancer cells (DU145 and LN-

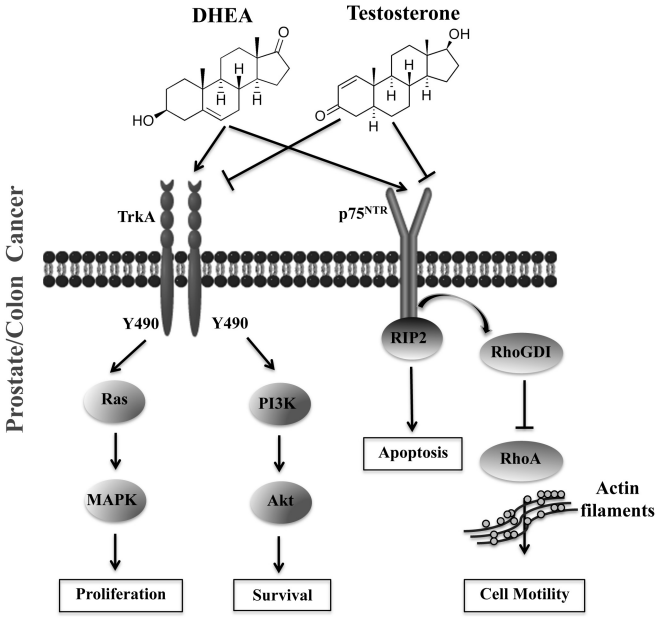

Figure 8. Schematic illustration of the effects of androgens in prostate and colon cancer cells via TrkA and $p75^{NTR}$ receptors. DHEA induces the phosphorylation of TrkA receptor on specific tyrosine residues and subsequently activates Ras-MAPK and PI3K/Akt signaling, regulating cell survival or proliferation of prostate or colon cancer cells. Additionally, DHEA activates the death receptor, $p75^{NTR}$, leading to recruitment of RIP2 protein or release of RhoGDI, which in turn blocks RhoA, thus inducing rearrangement of actin filaments and controlling cell motility. On the contrary, testosterone acts as a DHEA and NGF antagonist, blocking DHEA- or NGF-mediated activation of NGF receptors. The final outcome of these interactions is an orchestrated regulation of cancer cell apoptosis.

CaP cell lines) as well as in colon cancer cells (Caco2 cell line). NGF appears to exert similar antiapoptotic actions in both prostate and color cancer cells. On the contrary, exposure of prostate DU145 and colon Caco2 cancer cells to testosterone totally blocked the protective effects of both DHEA and NGF. These findings suggest that testosterone acts as an antagonist of DHEA and NGF. Similar antagonist effects of testosterone on DHEA and NGF actions were recently described in neuronal cells, mediated by NGF receptors, TrkA and $p75^{NTR}$ (42). Moreover, testosterone was unable to activate postreceptor signaling of both TrkA and $p75^{NTR}$ receptors. These findings support the hypothesis that testosterone may inhibit cancer cell growth by antagonizing the proliferative, antiapoptotic effects of endogenous factors, such as DHEA or NGF, in the case of prostate and colon cancer cells. It is of note that testosterone inhibits DHEA- or NGF-induced activation of both NGF receptors, reversing DHEA- or NGF-driven phosphorylation of TrkA or recruitment of RIP2 and release of RhoGDI from $p75^{NTR}$ receptors (Figure 7). Testosterone seems also to inhibit autophosphorylation of TrkA in the absence of its ligands (45), a ligand-independent activation of TrkA receptor and promotion of survival and growth of cancer cells. The effects of testosterone in the inactivation of RhoGDI may explain the previously described effects of this steroid in RhoA activation: RhoGDI interacts intracellularly with the $p75^{NTR}$ receptor and is released after binding of NGF (or DHEA), leading to the subsequent RhoA inactivation (54). Testosterone, by blocking the release of RhoGDI from the $p75^{NTR}$ receptor, indirectly keeps active the RhoA protein, promoting apoptosis. This assumption is in line with previous findings, showing rapid RhoA activation in testosterone-treated DU145 cells and regulating both the early actin reorganization and the late proapoptotic response (33).

The dose-response effects of DHEA and testosterone in the prevention or induction of apoptosis of cancer cells (Figures 1B and 2B) suggests a receptor-mediated effect. DHEA and NGF up-regulated, whereas testosterone decreased, the expression of the TrkA receptor, especially under serum-supplemented conditions (no changes were observed for $p75^{NTR}$). These data partially explain the inability of testosterone to induce apoptosis in serum-free conditions (where levels of TrkA are low and apoptosis plateaued). Testosterone was shown to decrease the expression of TrkA without affecting $p75^{NTR}$ levels, resulting in a lower TrkA/$p75^{NTR}$ ratio, favoring thus apoptosis. These findings are in full agreement with those reported in the pheochromocytoma PC12 cell clone, nnr5, expressing only the $p75^{NTR}$ receptor. In nnr5 cells, DHEA or NGF induce apoptosis. However, transfection of nnr5 cells with the TrkA cDNA reconstitutes the prosurvival actions of both agents (42).

In addition, blocking of endogenous NGF with specific immune-neutralizing antibody resulted in a statistically significant increase of apoptosis only under serum-supplemented conditions, without affecting apoptosis under serum-free conditions, where the absence of growth factors has already driven survival to its limits (Figure 5). The presence of anti-NGF did not affect the effects of DHEA on survival of DU145 and Caco2 cells. Additionally, blocking of NGF did not influence the proapoptotic effects of testosterone, which had similar potency with the anti-NGF on DU145 cells but was more effective in Caco2 cells. These findings suggest that endogenously produced NGF may affect apoptosis of cancer cells.

We further addressed the importance of both NGF receptors as mediators of the effects of DHEA or testosterone in DU145 and Caco2 cell apoptosis. Specific TrkA or $p75^{NTR}$ inhibitors did not modify the apoptotic effects of testosterone (data not shown). However, TrkA inhibition fully blocked the protective effects of DHEA in both cancer cell lines, whereas $p75^{NTR}$ receptor inhibition showed differential effects. In DU145 cells, it abolished the protective effects of DHEA, but it was ineffective in Caco2 cells, in which blockade of $p75^{NTR}$ was not sufficient to reverse DHEA-induced

cytoprotection. These differential effects support the hypothesis that $p75^{NTR}$ receptors possess different roles in the 2 tumor cell types, In prostate cancer, the receptor is necessary for TrkA-dependent protection by DHEA. On the other hand, in colon cancer cells, TrkA activation is sufficient to mediate the antiapoptotic effects of DHEA, whereas activation of $p75^{NTR}$ by DHEA signals for other cellular processes, such as differentiation or migration of cancer cells. Further studies are needed to address these hypotheses.

Future experiments using in vivo models for specific types of prostate or colon cancer (for a review, see Ref. 55) would provide additional information on the intracrinology of androgens and neurotrophins in these tumors. By using xenografts and carcinogen-induced tumors (eg, neuroblastomas) that express high levels of these receptors, we could use the small, highly lipophilic steroids as DHEA or testosterone (or synthetic analogs of these molecules that are deprived of any metabolic process to other steroids) (56) to examine tumor growth or repression. More specifically, rat R3327 AT 6.3 and H (57) in vivo models or transgenic mice for prostate cancer (58) could be subjected to DHEA- or testosterone treatment to evaluate steroids effects on prostate cancer in vivo. Finally, our work on NGF receptors structure and function (59, 60), with the construction and functional characterization of mutated receptors, could be proven useful for deciphering the multiple signaling pathways that are implicated in cancer properties and thus to develop more selective therapeutic methods for cancer prevention.

In conclusion, our findings implicate for the first time NGF receptors as mediators of the effects of androgens in prostate and colon cancer cell fate, suggesting that the intratumor hormonal microenvironment may play a critical role in tumor progression. The paracrine interactions of androgens with locally produced NGF may define tumor cell fate. The interplay between steroid hormone and neurotrophin signaling in hormone-dependent tumors offers new insights in the pathophysiology of these commonly met neoplasms.

Acknowledgments

Address all correspondence and requests for reprints to: Ioannis Charalampopoulos, Department of Pharmacology, University of Crete Medical School, Voutes, GR-71003 Heraklion, Greece. E-mail: gharalab@med.uoc.gr.

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