A Gonadotropin-Releasing Hormone-II Antagonist Induces Autophagy of Prostate Cancer Cells

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Abstract

Gonadotropin-releasing hormone-I (GnRH-I) is known to directly regulate prostate cancer cell proliferation. However, the role of GnRH-II in prostate cancer is unclear. Here, we investigated the effect of the GnRH-II antagonist trptorelix-1 (Trp-1) on growth of PC3 prostate cancer cells. Trp-1 induced growth inhibition of PC3 cells in vitro and inhibited growth of PC3 cells xenografted into nude mice. FITC-N3, an FITC-conjugated Trp-1 analogue, was largely present in the mitochondria of prostate cancer cells, but not in other cells that are not derived from the prostate. Trp-1–induced PC3 growth inhibition was associated with decreased mitochondrial membrane potential and increased levels of mitochondrial and cytosolic reactive oxygen species (ROS). Growth inhibition was partially prevented by cotreating cells with N-acetyl cysteine, an antioxidant. Cytochrome c release and caspase-3 activation were not detected in Trp-1–treated cells. However, Trp-1 induced autophagosome formation, as seen by increased LysoTracker staining and recruitment of microtubule-associated protein 1 light chain 3 to these new lysosomal compartments. Trp-1–induced autophagy was accompanied by decreased AKT phosphorylation and increased c-Jun NH2-terminal kinase phosphorylation, two events known to be linked to autophagy. Taken together, these data suggest that Trp-1 directly induces mitochondrial dysfunction and ROS increase, leading to autophagy of prostate cancer cells. GnRH-II antagonists may hold promise in the treatment of prostate cancer.

Introduction

Prostate cancer is the most common malignancy in men and the second leading cause of cancer-related deaths in western countries (1). In early stages of prostate cancer, androgen deprivation is useful for suppression of tumor growth. However, this initial regression in growth is followed by resistance to androgen deficiency, resulting in aggressive and metastatic tumors that are fatal (2). For this reason, development of effective drugs for treatment of androgen-independent prostate cancer is of utmost importance.

Both agonists and antagonists of gonadotropin-releasing hormone-I (GnRH-I) are effective treatments for benign prostatic hyperplasia and androgen-dependent prostate cancers (3). These GnRH analogues down-regulate or antagonize the GnRH receptor in the pituitary, thus causing a substantial decrease in serum gonadotropin and androgen levels (4–6). Although these pituitary-mediated GnRH actions are useful only for androgen-dependent prostate cancer, GnRH-I also has direct actions on both androgen-dependent and androgen-independent prostate cancer cells. GnRH-I agonists directly inhibit epidermal growth factor–induced or insulin-like growth factor–induced prostate cancer cell proliferation (7, 8) and directly induce apoptosis of serum-deprived [0.1% fetal bovine serum (FBS) containing media] cancer cells. The latter effect may be mediated by the GnRH-I receptor, which triggers Gt-mediated activation of apoptotic signaling proteins, including c-Jun NH2-terminal kinase (JNK; refs. 9, 10).

Like GnRH-I, GnRH-II functions in human reproductive and immune tissues (11–14). Little is known, however, about the functional significance of GnRH-II in prostate cancer. GnRH-II is found to be more potent than GnRH-I in inhibiting human endometrial and ovarian cancer cell proliferation (15). GnRH-II produced by human T cells induces laminin receptor gene expression and cell migration (16). Our laboratory and other groups have identified GnRH-II receptors in a variety of nonmammalian species, as well as in monkey (17–19). In humans, GnRH-II receptor genes are localized to chromosomes 1 and 14. Although mRNAs for these genes are expressed in a variety of tissues, including the brain, they are thought to be nonfunctional pseudogenes due to the introduction of a premature stop codon (20, 21). The absence of a functional human GnRH-II receptor has led to the proposal that the actions of GnRH-II are mediated by the GnRH-I receptor (22). However, some pharmacologic studies support the existence of a functional GnRH-II receptor that is distinct from the conventional GnRH-I receptor in structure. For instance, GnRH-II–induced laminin receptor gene expression is not blocked by the GnRH-I antagonist cetrorelix (16). In addition, GnRH-II retains the ability to inhibit proliferation of ovarian cancer cells in which GnRH-I receptor has been knocked down (15).

The involvement of a novel protein in GnRH-II action is further supported by our recent observation that radiolabeled GnRH-II is able to bind a variety of human prostate cancer cells (23). This binding is displaced by unlabeled GnRH-II, but not by GnRH-I. Photoaffinity labeling with [125I]azidobenzoyl-D-Lys5]GnRH-II revealed that GnRH-II specifically bind to a protein with a mass of 80 kDa, around 30 to 40 kDa larger than the GnRH-I receptor (9, 24). Recently, we developed a novel GnRH-II antagonist, known as trptorelix-1 (Trp-1; refs. 25, 26). This antagonist is able to induce...
prostate cancer cell death, whereas the Gnrh-I antagonist cetrorelix fails to do so (23). However, the mechanism underlying the Trp-1–induced prostate cancer cell death has been poorly understood. Here, we investigated the growth inhibitory effects of Trp-1 on PC3 human androgen-independent cells in vivo and examined the possible mechanism of cell death through mitochondrial dysfunction followed by reactive oxygen species (ROS) production and autophagy of prostate cancer cells.

Materials and Methods

Gnrh analogues. The Gnrh-II analogues Trp-1 [Ac-o-Nal(2)-t-Phe(4c)-d-Pal(3)-Ser-tyr-d-Cit-tPyr-Pro-o-Ala-NH2], N1 [Ac-o-Nal(2)-t-Phe(4c)-d-Pal(3)-Ser-tyr-d-Arg-tPyr-Pro-Pro-Gly-NH2], N3 [Ac-o-Nal(2)-t-Phe(4c)-d-Pal(3)-Ser-tyr-d-Lys-tPyr-Pro-Pro-Gly-NH2], N4 [Ac-o-Nal(2)-t-Phe(4c)-d-Pal(3)-Ser-tyr-d-Lys-tPyr- Pro-Gly-NH2], N5 [Ac-o-Nal(2)-t-Phe(4c)-d-Pal(3)-Ser-tyr-d-Cit-tPyr-Pro-Pro-Ala-NH2], N6 [Ac-o-Nal(2)-t-Phe(4c)-d-Pal(3)-Ser-tyr-d-Cit-tPyr-Pro-Pro-Ala-NH2], FTTc-N3 [Ac-o-Nal(2)-t-Phe(4c)-d-Pal(3)-Ser-tyr-d-Cit-Leu-Pro-o-Ala-NH2], and FTTc-N3 [Ac-o-Nal(2)-t-Phe(4c)-d-Pal(3)-Ser-tyr-d-Cit-Leu-Arg-Pro-o-Ala-NH2] was obtained from ASTA Medica AG.

Cell culture. PC3 and LNCap cells were obtained from American Type Culture Collection and cultured at 37°C in RPMI 1640 containing 10% heat-inactivated FBS, 100 units penicillin, and 100 μg/mL streptomycin. Other cells, such as DU145, ALVA51, MCF7, DLD, Wi38, HeLa, αt3, and GH3GII cells, were cultured at 37°C in DMEM containing 10% FBS.

Cell viability. Cells were seeded in 12-well plates in triplicate (1 × 10^4 per well) and cultured in DMEM containing 0.1% or 5% FBS. After 24 h, cells were treated with the indicated concentration of Trp-1 solubilized in 0.1% DMSO or Trp-1–related compound every 24 h for 3 d. After treatment, cells were washed with PBS, dissociated with trypsin/EDTA, and resuspended in 500 μL complete growth media. Cell suspensions were incubated with trypan blue (0.4%), and dye-excluding cells were counted under an inverted optical microscope (Olympus).

In vivo growth of prostate cancer cells. Six-week-old female athymic (BALB/c–nuf) mice were obtained from Harlan. Mice were housed under pathogen-free conditions in individually ventilated caging system (Techniplast) with γ-irradiated laboratory chow (LabDiet, PMI) and sterilized water ad libitum. All animal experiments and procedures were performed after receiving approval from the Institutional Animal Care and Use Committee of Clinical Research Institute in Seoul National University Hospital (Association for Assessment and Accreditation of Laboratory Animal Care–accredited facility). PC3 and LNCap cells were obtained from American Type Culture Collection and cultured at 37°C in RPMI 1640 containing 10% heat-inactivated FBS, 100 units penicillin, and 100 μg/mL streptomycin. Other cells, such as DU145, ALVA51, MCF7, DLD, Wi38, HeLa, αt3, and GH3GII cells, were cultured at 37°C in DMEM containing 10% FBS.

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Subcellular localization of FITC-N3. PC3, DU145, HeLa, DLD, MCF7, Wi38, and αt3 cells were plated on poly-l-lysine–coated glass coverslips in 12-well plates. After 2 d, the cells were treated at 37°C with 66 nmol/L Mitotracker (Molecular Probes) for 30 min and then with 10 μmol/L of FITC-N3 for 10 min. Cells were fixed with 4% paraformaldehyde and mounted. Nuclei were counterstained with Hoechst 33342 (10 μg/mL; Molecular Probes). Fluorescence labeling was visualized under an LSM510 confocal laser microscope (Carl Zeiss).

Measurement of intracellular ROS. ROS were measured using the fluoro probes, 2′,7′-dichlorofluorescin diacetate (DCF-DA; Sigma) and Mitosox (Molecular Probes). Approximately, 8 × 10^4 cells were plated on 60-mm dishes, cultured in 5% FBS RPMI media, and treated with the indicated agent for 3 d. After treatment, cells were incubated in the dark with 50 μmol/L DCF-DA or 0.5 μmol/L Mitosox for 30 min at 37°C. Cells were resuspended in 500 μL 0.2% SDS and transferred to a 96-well plate in duplicate. DCF-DA fluorescence was measured by spectrophotometer (GENios Plus, TECAN) according to the manufacturer’s instructions. Fluorescence was normalized to the number of viable cells. DCF-DA and Mitosox fluorescence was also analyzed using a FACScalibur flow cytometer (Becton Dickinson).

Measurement of mitochondrial membrane potential by fluorescence-activated cell sorting. Mitochondrial membrane potential was measured using 5,5′,6′,6′-tetrachloro-1,1′,3′,3′-tetratetraethylbenzimidazolylcarbocyanine iodide (JC-1; Molecular Probes). In healthy cells, JC-1 forms aggregates within mitochondria, generating red fluorescence. However, during mitochondrial depolarization, JC-1 monomers diffuse throughout the cell, producing green fluorescence. Stock solutions of JC-1 (2.5 mg/mL) were prepared in DMSO. After treatment with the indicated agents for 3 d, cells were incubated in PBS containing JC-1 (2.5 μg/mL) for 20 min at room temperature. Cells were washed and resuspended in 500 μL PBS. Fluorescence was then measured using the FACScalibur system.

Autophagosome formation. PC3 cells were incubated with 50 nmol/L LysoTracker Red DND-99 (Molecular Probes) for 30 min at room temperature to label lysosomes. In addition, cells were transfected with vector encoding yellow fluorescence protein (YFP)–fused light chain 3 (LC3) to monitor lysosomal recruitment of LC3. The YFP-LC3 construct was generated by cloning LC3 cDNA into EcoRI-cleaved and Sal-I-cleaved YFP-CL. LC3 cDNA was generated by reverse transcription–PCR using the primer set 5′-CCGGATATCTACATGCGCCGTTTGGT-3′ and 5′-CCCCTCGACCTTACAAAGCCGAGTTTCTG-3′. After YFP-LC3 transfection, PC3 cells were treated with Trp-1 for 3 d. Fluorescent-labeled LC3 and lysosomes were then visualized under a confocal microscope.

Western blot analysis. PC3 cells (5 × 10^5) were plated in 100-mm dishes and treated with 10 μmol/L Trp-1 for 3 d. Cells were harvested and incubated in buffer A [10 mmol/L HEPES (pH 7.9), 10 mmol/L KCl, 0.5 mmol/L DTT, 0.5 mmol/L phenylmethylsulfonyl fluoride] for 30 min on ice. The cells were disrupted with a Dounce homogenizer using 20 strokes and centrifuged at 5,000 × g for 10 min to collect the cytosolic fraction. The resulting pellets were resuspended in cold buffer A, briefly sonicated thrice, and centrifuged at 5,000 × g for 10 min. The resulting supernatant, which contained a crude preparation of mitochondrial proteins, was then collected. Proteins (20 μg) were electrophoresed on 12% SDS-polyacrylamide gels and transferred to polyvinylidene difluoride membranes (Millipore). The membranes were blocked with 3% bovine serum albumin and incubated with one of the following primary antibodies: cytochrome c (Phar-mingen), caspase-3 (Phar-mingen), Akt (Santa Cruz Biotechnology), phosphorylated Akt (Cell Signaling Technology), phosphorylated mTOR (Cell Signaling Technology), mTOR (Cell Signaling Technology), phosphorylated S6 ribosomal protein (Cell Signaling Technology), S6 ribosomal protein (Cell Signaling Technology), JNK (Cell Signaling Technology), phosphorylated JNK (Cell Signaling Technology), extracellular signal-regulated kinase (ERK; Cell Signaling Technology), phosphorylated ERK

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924 www.aacrjournals.org
(Cell Signaling Technology), or β-actin (Sigma). Membranes were then incubated with appropriate horseradish peroxidase–conjugated secondary antibody, and protein signals were detected using an enhanced chemiluminescence system (Amersham Biosciences).

**Immunocytochemistry.** PC3 cells (4 × 10^4) were plated on glass coverslips in six-well plates and treated with 10 μmol/L Trp-1 for 3 d. Cells were incubated in 66 nmol/L MitoTracker (Molecular Probes) for 30 min to label mitochondria and then fixed with 4% paraformaldehyde for 10 min. The cells were permeabilized with PBST (0.1% Triton X-100) for 10 min, blocked in PBS containing 10% goat serum for 30 min, incubated with cytochrome c antibody (Pharmingen) for 3 h, and incubated with Alexa 594–conjugated antimouse antibody for 1 h. Cells were stained with either Alexa 488–conjugated donkey anti-rabbit IgG or Alexa 594–conjugated antimouse antibody. Cells were then stained with Hoechst 33342 (1 g/mL) for 5 min, mounted onto slides, and observed under a laser scanning confocal microscope (LSM510, Carl Zeiss).

**Statistical analysis.** Data were analyzed using PRISM4 software (GraphPad). Group means were compared using Student's t test or one-way ANOVA followed by Bonferroni’s multiple comparison test. P < 0.05 was accepted as significant.

**Results**

**Prostate cancer cell–specific growth inhibition by Trp-1 in vitro.** PC3 cells were treated with 10 μmol/L Trp-1 in culture media with different concentrations of FBS. When cells were cultured in low concentrations (0.1%, 0.5%, 1.0%, and 2.0%) of serum, Trp-1 induced 80% to 95% growth inhibition of PC3 cells within 3 days. However, increasing serum concentration up to 5% significantly retarded growth inhibition of PC3 cells. Treatment of Trp-1 for 5 days induced 43% and 22% growth inhibition of PC cells in 5% and 10% serum condition, respectively (Fig. 1A). This result suggests that the effect of Trp-1 is highly dependent on serum concentration in culture media. To determine prostate cancer cell–specific effect of Trp-1, various cancer cell lines were treated with 10 μmol/L Trp-1 under 0.1% (Fig. 1B) and 5% (Fig. 1C) serum conditions. Treatment with Trp-1 for 2 days under 0.1% serum condition drastically inhibited the growth of prostate cancer cells, LNCaP, PC3, ALVA41, and DU145 cells, but not the growth of MCF7 (breast cancer) and HeLa (cervical cancer) cells (Fig. 1B). Treatment with Trp-1 for 3 days under 5% serum condition significantly inhibited the growth of PC3 and DU145 cells by >50% (Fig. 1C). However, growth of HeLa, DLD (colon cancer), MCF7, and WI38 (lung fibroblast) cells was unaffected by Trp-1 (Fig. 1C). In PC3 cells, the growth-inhibiting effects of the GnRH-II antagonist cetrorelix and the Trp-1–derived analogues N1 to N7 were also investigated. Treatment of PC3 cells with N3 for 2 days under 0.1% serum condition inhibited growth (~93%; Fig. 1D), indicating that substitution of D-Lys of Trp-1 for D-Cit does not affect growth-inhibiting activity. N4 and N5 inhibited PC3 growth by ~40%, whereas cetrorelix, N1, N2, N6, and N7 only had a marginal effect on growth (Fig. 1D).

**Effect of Trp-1 on in vivo tumor growth.** Nude mice bearing PC3-derived tumors were treated with various concentrations of Trp-1 for 21 consecutive days. Treatment with 0.001 mg Trp-1 decreased tumor volume by 35%, 0.01 mg Trp-1 by 52%, and 0.1 mg Trp-1 by 60% (compared with vehicle-treated group; Fig. 2A). Body weight did not vary significantly among the control and Trp-1–treated groups (data not shown). To exclude the possible involvement of ovarian factors in tumor growth, we examined the effects of Trp-1 and cetrorelix on PC3 cell growth in ovariectomized nude mice. Trp-1 induced a significant growth retardation of the tumor, but cetrorelix failed to do so (Fig. 2B). To determine whether Trp-1 acts specifically on tumor cells, we analyzed the tissue distribution of FITC-conjugated N3 (0.1 mg). One day after administration, FITC-N3 had accumulated in the PC3 tumor (Fig. 2C). No FITC-N3 was detected in the heart, intestine, pituitary, or spleen, whereas slight amounts of FITC-N3 were

**Figure 1.** Trp-1 inhibits prostate cancer cell growth. A, PC3 cells were incubated in RPMI media containing indicated concentrations of FBS in the presence or absence of 10 μmol/L Trp-1 for indicated time. Number of viable cells was measured under an inverted optical microscope. B, LNCaP, PC3, ALVA41 (ALVA), DU145, MCF7, and HeLa cells were incubated under 0.1% serum condition and treated with 10 μmol/L Trp-1 or DMSO for 2 d. C, PC3, DU145, HeLa, DLD, MCF7, and WI38 cells cultured under 5% serum condition were treated with 10 μmol/L Trp-1 or DMSO for 3 d. D, effect of Trp-1 (10 μmol/L) and its analogues (N1, N2, N3, N4, N5, N6, N7, and cetrorelix) on PC3 viability. PC3 cells were incubated under 0.1% serum condition. Data are expressed as percentage of viable cells relative to the DMSO-treated control. Columns, means of quadruplicate determinations, independent at least twice; bars, SE. Representative data are shown. *, P < 0.05 versus control.
detected in the kidney and liver (Fig. 2C). Histologic and blood chemistry analyses were performed to determine if the Trp-1 treatment was toxic to the latter two tissues. Liver tissue from mice treated with Trp-1 exhibited no histopathologic abnormalities (data not shown). In addition, serum levels of alkaline phosphatase, aspartate aminotransferase, alanine aminotransferase, blood urea nitrogen, creatinine, and total bilirubin did not differ between Trp-1–treated and control mice (data not shown). Thus, long-term Trp-1 treatment has negligible toxicity on the liver and kidney.

**Mitochondrial accumulation of FITC-N3.** Analysis of the subcellular distribution of FITC-N3 (1 μmol/L) in PC3 and DU145 cells revealed that, after a 10-minute treatment, FITC-N3 was primarily present in the mitochondria, as seen by overlap between FITC and MitoTracker dye (Fig. 3). FITC-N3 also accumulated in α3T cells, a mouse pituitary cell line that expresses the GnRH-I receptor type. In these cells, FITC-N3 appeared as cytoplasmic punctae that did not colocalize with mitochondria (Fig. 3). FITC-N3 also accumulated in mitochondria of PC3 and DU145 cells but was scarcely detected in HeLa, CV-1, and WI38 cells. Like FITC-N3, FITC-conjugated GnRH-II accumulated in the mitochondria of PC3 and DU145 cells but was barely detectable in HeLa, CV-1, and WI38 cells. FITC-conjugated GnRH-II also accumulated in mitochondria of ALVA41 prostate cancer cells. In GH3 rat pituitary tumor cells expressing monkey GH3GII (GnRH-II receptor; ref. 26), FITC-GnRH-II was primarily localized outside the mitochondria (Supplementary Fig. S7A). Mitochondrial accumulation of FITC-GnRH-II was greatly decreased when PC3 cells were preincubated with unlabeled GnRH-II, Trp-1, or N3 for 1 h before FITC-GnRH-II treatment. Preincubation of these cells with cetrorelix did not decrease the FITC-GnRH-II accumulation (Supplementary Fig. S7B).

Isolation of N3-binding proteins by affinity chromatography yielded a mixture of proteins with masses of ~80 kDa. Liquid chromatography-electrospray ionization-tandem mass spectrometry identified these proteins to be glucose-regulated protein 75 (GRP75; 75 kDa), tumor necrosis factor receptor-associated protein 1 (TRAP-1; 80 kDa), and hydroxyacyl-CoA dehydrogenase/3-ketoacyl-CoA thiolase/enoyl-CoA hydratase (trifunctional protein) α subunit (HADHA; 82 kDa; Supplementary Fig. S8). All three proteins are found in the mitochondria, in good agreement with the mitochondrial localization of the GnRH-II analogues.

**Mitochondrial damage and ROS induction in Trp-1–treated cells.** Mitochondrial dysfunction and the resultant increase in ROS formation are thought to promote cell death through induction of apoptotic signaling or autophagy (27, 28). The finding that FITC-N3 accumulated in the mitochondria prompted us to investigate whether Trp-1 may induce prostate cancer cell death by altering mitochondrial function. As shown in Fig. 4A, a 3-day treatment of PC3 cells with Trp-1 decreased red fluorescence intensity, indicative of a decrease in mitochondrial membrane potential (ΔΨm). The geometric mean of green fluorescence intensity also increased by 30% in cells treated with Trp-1. Trp-1 treatment also increased cytoplasmic H2O2 levels in a dose-dependent manner, as measured by fluorescence of a fluoroprobe, DCF-DA (Fig. 4B). Trp-1 treatment also increased mitochondrial O2− levels, as determined by the fluorescence of MitoSOX (Fig. 4C). The geometric mean fluorescence intensities were significantly (63%) increased by Trp-1, but not by cetrorelix (Fig. 4C). To determine whether this increase in ROS was responsible for PC3 cell growth inhibition, we tested the effect of the antioxidant N-acetyl cysteine (NAC) on the viability of Trp-1–treated cells. Pretreatment of PC3 cells with NAC...
suppressed Trp-1–induced increases in ROS levels (data not shown). Trypan blue dye exclusion assays revealed that NAC alone did not alter the number of viable cells. However, pretreatment with NAC partially suppressed Trp-1–induced cell death (Fig. 4D).

**Trp-1 does not induce cytochrome c release or caspase-3 activation.** Cytochrome c release from mitochondria is an early event during caspase-dependent cell death. Cytochrome c then interacts with the cytoplasmic proteins Apaf-1 and pro-caspase-9 to form the apoptosome, which activates caspase-3 and initiates the

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**Figure 3.** Subcellular localization of N3 in prostate cancer cells. Confocal images of FITC-N3 (green) and MitoTracker (red) fluorescence in PC3, DU145, HeLa, DLD, MCF7, WI38, and αT3 cells. Scale bars, 10 μm.

**Figure 4.** Trp-1 decreases mitochondrial membrane potential and increases ROS. A, flow cytometric analysis of JC-1–stained PC3 cells treated with DMSO or 10 μmol/L Trp-1. Red fluorescence (y axis) indicates intact mitochondrial membrane potential, and green fluorescence (x axis) indicates mitochondrial membrane potential collapse. B, effect of Trp-1 on intracellular H₂O₂ levels, as determined by mean DCF-DA fluorescence. *, P < 0.05; **, P < 0.001 versus control. C, effect of Trp-1 and cetorelix (Cet) on mitochondrial O₂⁻ levels, as determined by flow cytometric analysis of MitoSOX fluorescence. The representative flow cytometric profiles (gray area for cetorelix-treated or Trp-1–treated group; top) and the geometric mean (GM) fluorescence intensity (bottom) ± SE of four independent experiments were presented. ***, P < 0.001 versus control. D, effect of NAC (100 μmol/L) on viability of Trp-1 (10 μmol/L)–exposed PC3 cells. a, P < 0.01 versus control; b, P < 0.001 versus Trp-1.
mitochondria were largely aggregated in the perinuclear area by microscopy. Trp-1–exposed cells had shrunken nuclei, and their decrease in mitochondrial cytochrome c was observed (33). Western blot analysis revealed that cytosolic cytochrome c could be detected (Fig. 5B). Cytochrome c release was also examined using laser scanning confocal microscopy. Trp-1–exposed cells had shrunken nuclei, and their mitochondria were largely aggregated in the perinuclear area (Fig. 5C). No cytochrome c release was, however, observed (Fig. 5C).

Trp-1 induces autophagy. Because mitochondrial cytochrome c release and caspase activation were not observed after Trp-1 exposure, we investigated whether Trp-1 may inhibit cell growth by inducing autophagy. Treatment of PC3 cells with increasing concentrations of Trp-1 resulted in a progressive increase in LysoTracker staining, which had a punctuate appearance (Fig. 6A). To provide additional support for Trp-1–induced autophagy, we transfected PC3 cells with plasmid encoding YFP-tagged microtubule-associated protein 1 LC3. LC3 is a well-established marker of autophagosome formation (30). During autophagy, LC3 proform is cleaved into a soluble form, known as LC3-1. LC3-1 is further processed to a membrane-bound form, LC3-II, which is recruited onto autophagosomes. Autophagosomes engulfing organelles then fuse with lysosomes and mature into the autolysosomes. YFP-LC3 was spread throughout untreated PC3 cells but appeared as punctuate forms in Trp-1–treated cells (Fig. 6B). These YFP-LC3 punctae largely overlapped with LysoTracker staining, indicating formation of autolysosome in Trp-1–treated cells. These autophagosome-like structures were absent in PC3 cells cotreated with 3-methyladenine (3-MA), an autophagy inhibitor (Fig. 6B). Because autophagy is associated with altered levels of protein kinases, such as AKT and microtubule-associated protein kinases (31, 32), we analyzed levels of phosphorylation of autophagy-related proteins. Trp-1 decreased AKT phosphorylation. This decreased phosphorylated AKT levels was accompanied with decreased protein levels of mTOR (mammalian target of rapamycin) and S6 proteins. Trp-1 greatly increased JNK phosphorylation, a cell stress marker, although it did not alter phosphorylation of ERK (Fig. 6C).

Discussion

GnRH-I is able to inhibit growth of sex steroid-regulated cancer cells, including prostate cancer cells, not only by decreasing gonadotropin and sex steroid levels but also by directly activating cell death pathways in these cells (9, 10, 15). The finding that GnRH-II has functions in the human reproductive and immune systems (11–16, 33) intimates that GnRH-II may exert similar effects in human prostate cancer. The present study shows that Trp-1 exerts a growth inhibitory effect on androgen-independent PC3 cells in vitro and in vivo. Importantly, this growth inhibitory effect seems to be highly specific for prostate cancer, as Trp-1 does not inhibit the growth of breast, cervical, lung, and colon cancer cells. Accordingly, FITC-N3 largely accumulated in the xenografted PC3 cells, but not in other tissues such as the heart, liver, kidney, intestine, spleen, and pituitary. No adverse effects of long-term Trp-1 treatment have been observed in the xenografted mice, suggesting that Trp-1 can be used for the treatment of androgen-independent prostate cancer cells.

Whereas the growth inhibitory effect of the GnRH-II analogues in steroid-related cancer cells is evident, the mechanism underlying GnRH-II antagonist-mediated growth inhibition of these cells is poorly understood. The GnRH-I receptor–mediated signaling pathway in cancer cells is quite complicated and is distinct from GnRH-I receptor–mediated pathways in the pituitary. In the pituitary, GnRH-I receptors are preferentially coupled to G11, resulting in activation of phospholipase C and protein kinase C signaling pathways (34). In cancer cells, GnRH-I receptors are linked to G10, although these cells have functional G11/phospholipase C machinery (23). In addition, in serum-deprived cancer cells, GnRH-I agonists may activate JNK-mediated signaling and concomitantly reduce phosphatidylinositol 3′-kinase (PI3K)/AKT signaling (9). The finding that GnRH-I antagonists have the same antiproliferative effects in
the steroid-regulated cancer cells as GnRH-I agonists adds to the complexity of this system (15, 35).

Participation of the GnRH-I receptor in GnRH actions in cancer cells is also under debate. Knockdown of the GnRH-I receptor in ovarian and endometrial cancer cells abrogates the antigrowth effect of GnRH-I, but not that of GnRH-II agonist (15). Similarly, GnRH-I receptor-independent growth inhibition has been observed in GnRH-II–treated human T cells (16). GnRH-II may achieve this effect via two possible mechanisms. First, GnRH-II may act through a GnRH-II receptor complex. The human GnRH-II receptor gene encodes a partial peptide that interacts with other molecules, such as the GnRH-I receptor, to form a GnRH-II–responsive complex, which mediates the effects of GnRH-II in steroid-regulated cancer cells. Indeed, Eicke and colleagues (36) showed GnRH-II receptor–like antigenicity in the human placenta and reproductive organ cancers. Alternatively, it can be proposed that GnRH-II acts through an uncharacterized GnRH receptor that is pharmacologically distinct from the conventional GnRH-I receptor (23). Cytoplasmic accumulation pattern of FITC-N3 in prostate cancer cells differs from that observed in αT3 cells expressing the type-I GnRH receptor. In αT3 cells, FITC-N3 was primarily present as punctae in the cytoplasm, with partial overlap in the mitochondria. Thus, it can be postulated that, in prostate cancer cells, FITC-N3 enters through a prostate cancer cell membrane–specific mechanism, which is distinct from GnRH receptor–mediated endocytosis. Using photoaffinity labeling, we have previously identified a putative 80-kDa protein in human prostate cancer cells that specifically binds to GnRH-II (23). The discovery that GRP75, TRAP-1, and HADHA bind the GnRH-II analogue N3 and are all ~80-kDa mitochondrial proteins is in good agreement with our previous observation (23). This finding is further supported by the fact that FITC-N3 largely accumulated in the mitochondria of prostate cancer cell lines. However, this finding does not fully explain the mechanism by which FITC-N3 penetrates the outer membrane of prostate cancer cells, as we failed to characterize a plasma membrane protein as a GnRH-II antagonist binding protein. One reason for this failure is likely that interaction between the GnRH-II antagonist and a protein in the plasma membrane of prostate cancer cells is highly reversible.

Mitochondrial proteins participate in a host of processes, including mitochondrial calcium homeostasis, ROS formation, and electron transport (28, 31, 37, 38). Disturbance of these mitochondrial functions is capable of triggering cell death through activation of either apoptotic or autophagic pathways (27, 28, 38). The mitochondrial accumulation of GnRH-II analogues in prostate cancer cells is consistent with the hypothesis that Trp-1 inhibits mitochondrial function, leading to growth inhibition and cell death. In accord with this idea, Trp-1 significantly decreased mitochondrial membrane potential and increased mitochondrial and cytoplasmic ROS levels. The ability of NAC to restore cell growth in the presence of Trp-1 shows that ROS partly mediate the antigrowth effects of Trp-1. Although mitochondrial dysfunction is often associated with mitochondrial cytochrome c release and subsequent caspase-3 activation (29), neither event was observed in

Figure 6. Trp-1 induces autophagy of prostate cancer cells. A, dose-dependent effect of Trp-1 on LysoTracker Red labeling of PC3 cells. B, images of YFP-LC3 and LysoTracker Red fluorescence in PC3 cells treated with DMSO or Trp-1 (10 μmol/L) ± 3-MA (10 mmol/L). C, Western blot analysis of autophagy-regulating proteins in DMSO-treated or Trp-1–treated PC3 cells. Scale bars, 100 μm.
Trp-1–exposed cells. Thus, Trp-1–induced cell growth inhibition is likely independent of caspase activation. Rather, Trp-1 is more likely to induce autophagic cell death based on the finding that this analogue increases LysoTracker staining and the recruitment of YFP-LC3 into the lysosomal membrane.

Autophagy is characterized by sequestration of bulk cytoplasm and organelles in autophagic vesicles, which are eventually degraded by the lysosomal system. This process is activated by nutrient starvation and promotes cellular adaptation and survival in response to stress (29, 39). Excess autophagy, on the other hand, leads to cell death (29). A large body of data shows that some malignant cell types undergo autophagic cell death in response to anticancer agents (28, 40). This process occurs in the absence of caspase activation and is often accompanied by alterations in the AKT and JNK signaling pathways (31, 41). Activation of the PI3K/AKT pathway switches on survival pathways, leading to inhibition of apoptosis and activation of the autophagy antagonist mTOR (31). PC3 cells are PTEN-null, resulting in the constitutive activation of PI3K/AKT pathway (41). In this study, Trp-1 strongly suppressed AKT phosphorylation and reduced protein levels of mTOR and S6 ribosomal protein in PC3 cells, an effect that may promote autophagy of PC3 cells. Recently, caspase-independent JNK activation has been reported to contribute to autophagic cell death induced by tumor necrosis factor-α and chemicals (32, 42). Consistent with this finding, Trp-1–induced autophagic changes were associated with JNK phosphorylation. However, the mechanism underlying JNK-mediated autophagic cell death is not yet known.

Recently, Fister and colleagues (43) showed that GnRH-II antagonists, similar in structure to Trp-1 (i.e., AG-1, AG-2, and AG-3), induced human endometrial and ovarian cancer cell death through an apoptotic process associated with mitochondrial membrane disruption and caspase-3 activation. However, no evidence of autophagic cell death was provided in these cells. How similar compounds are able to activate different cell death pathways is not clear. Rotenone and TTFA, the mitochondrial electron-transport-chain inhibitors, induce apoptosis in primary mouse astrocytes by autophagic death in transformed HEK 293 and U87 cells (28). Thus, cell death pathways may be cell type–dependent whereas additional studies will be necessary to clarify this differential activation of cell death pathways.

In summary, our data clearly show that the GnRH-II antagonist Trp-1 exerts a growth inhibitory effect on prostate cancer cells in vitro and in vivo. Accumulation of FITC-conjugated GnRH-II analogue in the mitochondria of the prostate cancer cells suggests that Trp-1 may directly act on the mitochondria to disrupt mitochondrial function, leading to increase ROS production and autophagy of prostate cancer cells. This novel mechanism underlying prostate cancer growth inhibition supports the use of GnRH-II antagonists in treating prostate cancer.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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attachment site to Cys residue in N-terminal segment.


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