Antagonists of growth hormone-releasing hormone (GHRH) reduce prostate size in experimental benign prostatic hyperplasia

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Edited by Patricia K. Donahoe, Massachusetts General Hospital, Boston, MA, and approved January 10, 2011 (received for review December 16, 2010)

Growth hormone-releasing hormone (GHRH), a hypothalamic polypeptide, acts as a potent autocrine/paracrine growth factor in many cancers. Benign prostatic hyperplasia (BPH) is a pathologic proliferation of prostatic glandular and stromal tissues; a variety of growth factors and inflammatory processes are inculpated in its pathogenesis. Previously we showed that potent synthetic antagonists of GHRH strongly inhibit the growth of diverse experimental human tumors including prostate cancer by suppressing various tumoral growth factors. The influence of GHRH antagonists on animal models of BPH has not been investigated. We evaluated the effects of the GHRH antagonists JMR-132 given at doses of 40 μg/d, MIA-313 at 20 μg/d, and MIA-459 at 20 μg/d in testosterone-induced BPH in Wistar rats. Reduction of prostate weights was observed after 6 wk of treatment with GHRH antagonists: a 17.8% decrease with JMR-132 treatment; a 17.0% decline with MIA-313 treatment; and a 21.4% reduction with MIA-459 treatment ($P < 0.05$ for all). We quantified transcript levels of genes related to growth factors, inflammatory cytokines, and signal transduction and identified significant changes in the expression of more than 80 genes ($P < 0.05$). Significant reductions in protein levels of IL-1β, NF-κβ/p65, and cyclooxygenase-2 (COX-2) also were observed after treatment with a GHRH antagonist. We conclude that GHRH antagonists can lower prostate weight in experimental BPH. This reduction is caused by the direct inhibitory effects of GHRH antagonists exerted through prostatic GHRH receptors. This study sheds light on the mechanism of action of GHRH antagonists in BPH and suggests that GHRH antagonists should be considered for further development as therapy for BPH.

chronic prostatic inflammation | prostatic hypertrophy | prostatic cell death | rodent benign prostatic hyperplasia model

The hypothalamic neuropeptide growth hormone-releasing hormone (GHRH) stimulates the secretion of growth hormone (GH) from the anterior pituitary gland upon binding to its receptor (GHRH-R) (1). In turn, GH stimulates the production of insulin-like growth factor 1 (IGF1), a major anabolic growth factor and a potent mitogen for many cancers (2). GHRH and its pituitary-type receptor as well as its truncated receptor splice variants (SV) are expressed in various normal human tissues including prostate, kidney, lung, and liver (3) and on many human cancer cell lines and tumors (1). Pituitary-type GHRH-R and SV1 appear to mediate effects of GHRH and its antagonists on tumors (4). GHRH itself acts as an autocrine/paracrine growth factor in human cancers (1, 5), including prostate (6).

To develop therapies for cancer, our laboratory has synthesized GHRH antagonists with high antiproliferative activity in numerous experimental cancer models (1). The inhibitory effect of these analogs is exerted in part by indirect endocrine mechanisms through the suppression of GHRH-evoked release of GH from the pituitary, which in turn results in the inhibition of the hepatic production of IGF1 (7). Direct mechanisms involved in the main antitumor effects of GHRH antagonists appear to be based on blocking the action of autocrine GHRH on tumors and inhibition of autocrine IGF1/2 (1, 7). Recent studies also indicate that GHRH antagonists reduce generation of reactive oxygen species (8), which cause damage to prostatic stroma and epithelium (9). GHRH antagonists inhibit the growth of androgen-independent human prostatic cancers and also numerous other cancers xenografted into nude mice and suppress tumoral growth factors EGF, FGF2, IGF1, IGF2, and VEGF-A (1, 10, 11).

Benign prostatic hyperplasia (BPH) is a progressive hyperplasia of prostatic glandular and stromal tissues. BPH is an age-related disease and is present in 20% of 40-y-old men and in 70% of 60-y-old men (12). Currently, there is no completely effective treatment for BPH. Medical therapies consist of α-adrenergic blockers, which lower adrenergic tone, and 5α-reductase inhibitors, which decrease levels of dihydrotestosterone (DHT). In some patients surgery, mostly transurethral resection of the prostate, is the only effective intervention (13). New therapies are clearly needed.

Despite the enormous burden of BPH on public health, its pathogenesis is incompletely understood. Hyperplastic growth in BPH has been ascribed to an imbalance between androgen/estrogen signaling (14), tissue remodeling in the aging prostate (9), chronic inflammation (15), stem cell defects (16), overexpression of stromal and epithelial growth factors (17), hypoxia (18), epithelial–mesenchymal transition (19), and other obscure factors.

A model of BPH in male rats can be produced by repeated injections of testosterone (20). This model has been adapted for several studies (21, 22), including our own (23). Because the mechanism of prostate growth is complex and heterogeneous in different species, and the testosterone-induced models of BPH show an epithelial hyperplasia (21), the androgen-induced models of BPH have limitations. Alonso-Magdalena et al. (19) proposed that BPH is not a proliferative disease of the stroma but rather is an accumulation of mesenchymal-like cells derived from the prostatic epithelium and the endothelium. Alonso-Magdalena's description of human BPH as predominantly of epithelial origin supports the use of a testosterone-induced model of BPH with predominant epithelial hyperplasia.

Author contributions: F.G.R., A.V.S., N.L.B., and L.S. designed research; F.G.R., M.N., K.S., M.Z., I.V., R.P., G.H., and L.S. performed research; A.V.S. and M.Z. contributed new reagents/analytic tools; F.G.R., M.N., K.S., I.V., R.P., G.H., and L.S. analyzed data; and F.G.R., A.V.S., and N.L.B. wrote the paper.

Conflict of interest statement: A.V.S. and N.L.B. are listed as co-inventors on the patent applications on GHRH antagonists filed by the University of Miami. However, this article deals with an experimental study on BPH, which is purely an academic project.

This article is a PNAS Direct Submission.

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This article contains supporting information online at www.pnas.org/lookup/suppl/doi:10.1073/pnas.1018086108/-/DCSupplemental.

This study estimated the therapeutic effect of the GHRH antagonists JMR-132, MIA-313, and MIA-459 using a testosterone-induced rat model of BPH (23). The therapeutic effect of the 5α-reductase 2 (5AR2) inhibitor finasteride was estimated also. We investigated the mechanisms of action of GHRH antagonists, including their in vivo effects on the expression levels of GHRH, GHRH-R and its splice variant SV1, 5AR2, α1A-adrenoreceptor (α_{1A}-AR), androgen receptor (AR), IL-1β, cyclooxygenase 2 (COX-2), and NF-κβ in rat prostates. Quantitative PCR arrays for growth factors, inflammatory cytokines, and signal transduction genes were performed as well as analysis of the effect of GHRH antagonists on cell division and apoptosis.

Results

Expression of GHRH-R, SV1, and GHRH. Protein and mRNA for GHRH-R and for GHRH and the protein of splice variant SV1 of GHRH-R were detected in rat prostate (Fig. 1 *A* and *B*). Levels of prostatic GHRH-R protein were significantly increased after testosterone-enanthate (TE) treatment as compared with control ($P < 0.01$; protein signal intensity values are shown in Fig. S1).The GHRH antagonist JMR-132 and finasteride significantly elevated GHRH-R protein levels compared with TE-treated controls ($P < 0.05$ and $P < 0.01$, respectively) (Fig. 1*B* and Fig. S1). Radioligand binding assays revealed a single class of high-affinity binding sites for GHRH in rat prostate with a dissociation constant (K_d) of 4.13 ± 0.09 nM and a mean maximal receptor binding capacity (B_{max}) of 313.0 ± 25.9 fmol/mg membrane protein. The number of receptors for GHRH in rat BPH tissues induced by TE was significantly ($P < 0.01$) increased to 540.7 ± 50.1 fmol/mg membrane protein. Receptor K_d was unchanged (4.02 ± 0.20 nM). No significant changes were found in K_d or B_{max} values in rat BPH tissues after treatment with finasteride or the GHRH antagonists JMR-132, MIA-313, and MIA-459, compared with TE-induced rat BPH tissues. Further, protein expression of GHRH-R encoded by SV1 was quantified in rat prostates by Western blot (Fig. 1*B* and Fig. S1). Expression of GHRH mRNA and protein was elevated after treatment with TE, whereas GHRH antagonists and finasteride significantly suppressed expression of prostatic GHRH mRNA and protein levels compared with TE-induced BPH (Fig. 1 *A* and *B* and Fig. S1).

Immunohistochemical Confirmation of the Expression of GHRH-R Protein. Immunohistochemical analyses revealed that expression of GHRH-R is confined to the cytoplasm and luminal membrane of prostatic acinar cells in rat (Fig. 1*C*).

Reduction of Prostate Size by GHRH Antagonists. Body weights of rats when killed were not affected by treatment with any GHRH antagonist plus TE or by treatment with finasteride plusTE compared with TE treatment alone (Table 1). Corn oil-injected control prostates weighed 234.9 ± 16.7 mg/100-g rat; whereas in TE-treated controls prostates were enlarged by 55.5% to 365.4 ± 20.3 mg/100-g rat ($P < 0.001$) (Table 1). The GHRH antagonists JMR-132 at 40 μg/d, MIA-313 at 20 μg/d, and MIA-459 at 20 μg/d significantly lowered prostate weights by 17.8%, 17.0%, and 21.4%, respectively, compared with TE-treated controls ($P < 0.05$) (Table 1). These reductions in prostate weight were superior to the nonsignificant 14.43% reduction obtained with finasteride at 0.1 $mg \cdot kg^{-1} \cdot d^{-1}$ (Table 1). In addition, GHRH antagonists significantly decreased prostatic DNA content (Table 1). Testicular weights did not change after treatment with GHRH antagonists (Table 1).

Effect of GHRH Antagonists on 5AR2, α_{1A}-AR, and AR. There were no significant changes in levels of prostatic 5AR2 protein in TE-induced BPH. The GHRH antagonists JMR-132, MIA-313, and

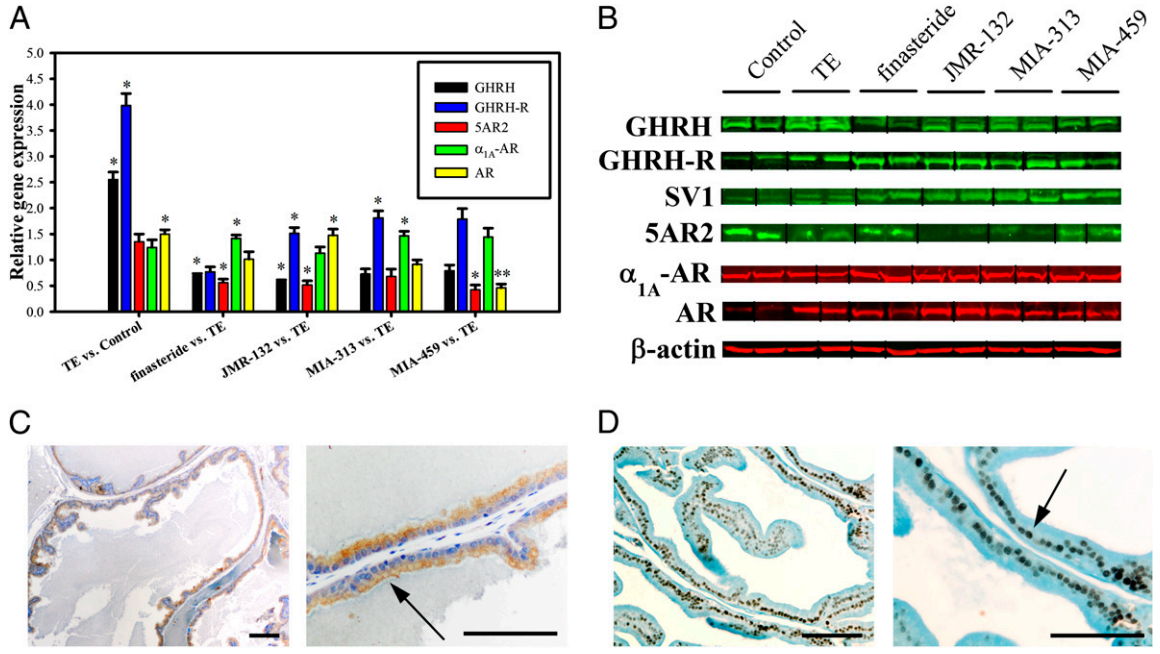

Fig. 1. (*A* and *B*) Effect of GHRH antagonists JMR-132, MIA-313, and MIA-459 on the expression of GHRH, GHRH-R, SV1, 5AR2, α_{1A}-AR, and AR. (*A*) Bar graph showing real-time RT-PCR analysis of GHRH, GHRH-R, 5AR2, α_{1A}-AR, and AR. Bars represent relative expression of individual genes in prostate samples ($n = 3$) between TE-treated and control groups or between TE-treated groups and groups treated with TE and finasteride, JMR-132, MIA-313, or MIA-459. Values >1.00 indicate up-regulation of individual genes; values <1.00 indicate down-regulation. Data are shown as means ± SEM. Asterisks indicate a significant difference (*$P < 0.05$ and **$P < 0.01$ by Student's *t* test). (*B*) Western blot analysis of GHRH, GHRH-R, SV1, 5AR2, α_{1A}-AR, and AR. Representative blots of three independent experiments are presented and include β-actin as an internal standard; corresponding signal intensity values are shown in Fig. S1*A*. Grouping of representative bands for each experimental group was digitally performed. (*C*) Expression of GHRH receptors in representative ventral prostate of control rats was confined to the cytoplasm and luminal membrane of prostatic acinar cells (arrow). Localization of GHRH receptors is shown in 10× (*Left*) and 40× (*Right*) magnification. (Scale bars: 50 μm.) (*D*) Androgen receptors in representative ventral prostate of control rats were localized in the in the nuclei of prostatic acinar cells (arrow), as shown by 20× (*Left*) and 40× (*Right*) magnification. (Scale bars: 50 μm.)

Table 1. Effect of GHRH antagonists JMR-132, MIA-313, and MIA-459 on morphological parameters

Treatment group	Body weight (g)		Relative prostate weight (mg/100-g rat)	Prostatic DNA content (ng DNA/mg tissue)	Relative testicle weight (mg/100-g rat)
	Day −28	Day 42	Day 42	Day 42	Day 42
Control	400.9 ± 9.9	551.6 ± 13.3	234.9 ± 16.7	224.8 ± 8.6	617.6 ± 22.2
TE	392.8 ± 6.8	482.6 ± 7.1*	365.4 ± 20.3†	266.8 ± 5.9	565.5 ± 25.7
TE/finasteride (0.1 mg·kg^{-1}·d^{-1})	396.0 ± 8.8	476.0 ± 12.7*	337.6 ± 26.0	214.1 ± 9.4‡	507.2 ± 30.6†
TE/JMR-132 (40 μg/d)	409.2 ± 10.2	517.4 ± 20.2	300.4 ± 18.1‡	216.1 ± 8.7‡	522.2 ± 28.5
TE/MIA-313 (20 μg/d)	403.7 ± 8.4	499.0 ± 14.2	303.4 ± 17.3‡	186.0 ± 8.3§	541.6 ± 10.4
TE/ MIA-459 (20 μg/d)	410.4 ± 8.8	510.1 ± 15.2	287.0 ± 16.3‡	214.6 ± 7.8‡	525.4 ± 19.1

Statistical analysis was performed by one-way ANOVA, followed by Bonferroni *t* test.
*$P < 0.05$ and †$P < 0.001$ compared with control; ‡$P < 0.05$ and §$P < 0.01$ compared with TE.

MIA-459, as well as finasteride, significantly lowered protein levels of 5AR2 ($P < 0.05$ for all) (Fig. 1*B*; protein signal intensity values are shown in Fig. S1). Although finasteride and JMR-132 significantly elevated the α_{1A}-AR protein levels ($P < 0.05$ for both) (Fig. 1*B* and Fig. S1), MIA-313 and MIA-459 caused a nonsignificant increase in α_{1A}-AR protein levels. Levels of prostatic AR protein were significantly elevated in TE-induced BPH ($P < 0.05$); only treatment with JMR-132 resulted in significant change in AR protein level (2.30 fold up-regulation; $P < 0.05$) (Fig. 1*B* and Fig. S1). AR was localized to the nuclei of prostatic acinar cells by immunohistochemical staining (Fig. 1*D*).

GHRH Antagonists Suppress Proinflammatory IL-1β, NF-κβ, and COX-2. Prostatic IL-1β protein was increased significantly after TE treatment compared with control ($P < 0.001$), whereas the GHRH antagonists JMR-132, MIA-313, and MIA-459 and finasteride significantly reduced IL-1β levels ($P < 0.001$ for all) (Fig. 2*B*; signal intensity values are shown in Fig. S1). Expression of NF-κβ/p65 (RelA) protein was significantly elevated after treatment with TE ($P < 0.01$). GHRH antagonists JMR-132, MIA-313, and MIA-459 and finasteride significantly decreased prostatic NF-κβ/p65 protein levels compared with TE-induced BPH ($P < 0.001$, $P < 0.01$, $P < 0.01$, and $P < 0.01$, respectively) (Fig. 2*B* and Fig. S1). Prostatic COX-2 protein was elevated after TE treatment, but not significantly. All three GHRH antagonists and finasteride significantly lowered prostatic COX-2 protein levels ($P < 0.05$ for all) (Fig. 2*B* and Fig. S1). There was a suppression of NF-κβ2 and RelA genes after treatment with all three GHRH antagonists and finasteride ($P < 0.01$for all) (Fig. 2*A*). The COX-2 gene was significantly down-regulated after MIA-313, MIA-459, or finasteride ($P < 0.05$, $P < 0.01$, and $P < 0.01$, respectively) (Fig. 2*A*).

GHRH Antagonists Inhibit Cell Division and Induce Apoptosis. Morphologic evaluation on H&E slides revealed that the size of average epithelial areas in the ventral prostate did not differ among the study groups. Mitoses were significantly fewer in all groups than in the TE-induced BPH controls. Apoptotic cell numbers were higher in the groups treated with GHRH antagonists MIA-313 and MIA-459 and finasteride, but the differences from TE-treated controls were not statistically significant (Table 2). Representative apoptotic cells among epithelial cells in ventral prostates of rats treated with MIA-459 are shown in Fig. 3*F*.

We found transcriptional up-regulation of B-cell lymphoma 2 (Bcl-2) and down-regulation of Bcl-2–associated X protein (Bax) after TE treatment ($P < 0.05$ for both) (Fig. 3*A*). The mRNA expression of Bax was elevated after treatment with all three GHRH antagonists or finasteride ($P < 0.05$ for all) (Fig. 3 *B–E*), but the mRNA expression of Bcl-2 was decreased after treatment with JMR-132, MIA-313, and MIA-459 ($P < 0.05$, $P < 0.05$, and $P < 0.01$, respectively) (Fig. 3 *C–E*). No significant change in prostatic proliferating cell nuclear antigen (PCNA) protein levels occurred after TE treatment. The GHRH antagonists JMR-132 and MIA-459 significantly reduced PCNA protein ($P < 0.05$ for all) (Fig. 3*G*).

GHRH Antagonists Cause Transcriptional Down-Regulation of Multiple Genes Involved in Growth, Inflammatory Response, and Signaling. Growth factors, inflammatory cytokines and receptors, and signal transduction factors were evaluated for control mice, mice with TE-induced BPH, and mice with TE-induced BPH treated with the GHRH antagonists JMR-132, MIA-313, and MIA-459 by real-time RT-PCR arrays for rat. We identified important functional molecules affected by treatment with GHRH antagonists and selected genes potentially related to prostate shrinkage. More than

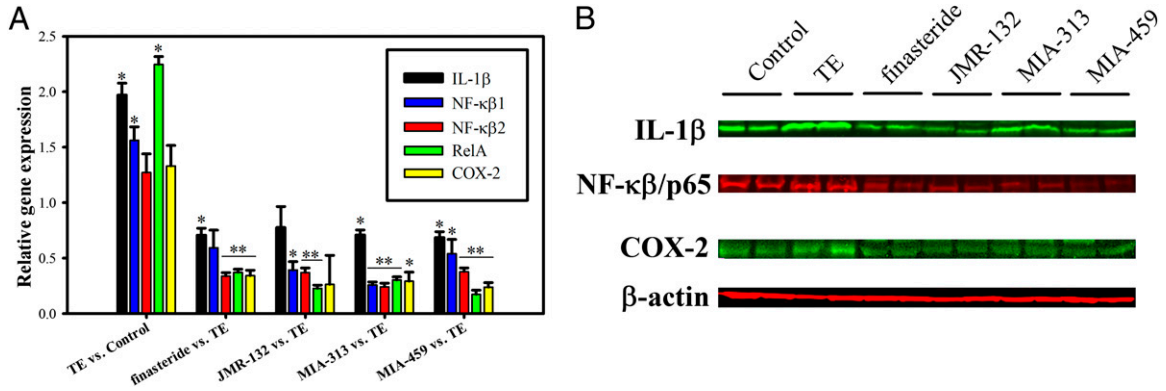

Fig. 2. GHRH antagonists suppress expression of IL-1β, NF-κβ, and COX-2 in rat prostates. (*A*) Bar graph showing real-time RT-PCR analysis of IL-1b, NF-κβ1, NF-κβ2, RelA, and COX-2. Bars represent relative expression of individual genes between prostate samples ($n = 3$) from TE-treated and control groups or between TE-treated groups and groups treated with TE and finasteride, JMR-132, MIA-313, or MIA-459. Values >1.00 indicate up-regulation of individual genes; values <1.00 indicate down-regulation. Data are shown as means ± SEM. Asterisks indicate a significant difference (*$P < 0$ 0.05 and **$P < 0.01$ by Student's *t* test). (*B*) Western blot analysis of IL-1β, NF-κβ/p65, and COX-2. Representative blots of three independent experiments are presented and include internal standard β-actin; corresponding signal intensity values are shown in Fig. S1*B*.

Table 2. Effect of GHRH antagonists on cell proliferation and apoptosis in rat prostatic epithelium

Group	Mean epithelial area in view fields (%)	Number of mitoses in one theoretical field composed entirely of epithelial cells	Number of apoptotic cells in one theoretical field composed entirely of epithelial cells
Control	14.3 ± 1.6	1.93 ± 1.32	3.40 ± 0.43
TE	17.1 ± 2.3	5.86 ± 1.81*	3.12 ± 0.69
TE/finasteride (0.1 mg·kg^{-1}·d^{-1})	15.9 ± 2.6	0.69 ± 0.35†	6.42 ± 2.30
TE/JMR-132 (40 μg/d)	15.0 ± 0.4	0.87 ± 0.43†	3.55 ± 0.16
TE/MIA-313 (20 μg/d)	14.7 ± 1.3	1.73 ± 0.47†	5.18 ± 1.10
TE/MIA-459 (20 μg/d)	16.0 ± 2.3	0.00 ± 0.00†	5.69 ± 1.47

The data were evaluated by one-way ANOVA, followed by the Student–Newman–Keuls method.
*$P < 0.05$ compared with control; $^{\dagger}P < 0.05$ compared with TE.

80 genes were significantly altered after treatment with TE and GHRH antagonists ($P < 0.05$) (Tables S1, S2, and S3).

Transcriptional levels of several growth factors including bone morphogenic proteins 1 (Bmp1), 2 (Bmp2), 3 (Bmp3), and 8a (Bmp8a), Egf, Fgf1, Fgf2, Fgf11, Fgf12, Fgf14, Igf2, Igf22, neurotrophin 4 (Ntf4), Pdgfa, secreted phosphoprotein 1 (Spp1), Tgf-α, Tgf-β1, Tgf-β2, Tgf-β3, and Vegf-α were significantly lowered by GHRH antagonists ($P < 0.05$) (Table S1).

Expression of inflammatory cytokines including IL-1α, IL-1β, IL-13, IL-15, IL-17β, Spp1, and Cd40lg were decreased by GHRH antagonists ($P < 0.05$) (Table S2). Among chemokines and their receptors, expression of chemokine (C-C motif) ligands 6 (Ccl 6), 7 (Ccl7), and 12 (Ccl12), chemokine (C-C motif) receptors 1 (Ccr1) and 6 (Ccr6), chemokine (C-X-C motif) receptor 3 (Cxcr3), and Gpr2 was significantly decreased by GHRH antagonists ($P < 0.05$) (Table S2).

From the PCR array for signal transduction (Table S3), we identified putative downstream pathways responsible for GHRH antagonist effects in this model. We found mRNAs for early growth response protein 1 (Egr1), Fos, Jun, and nerve growth factor inducible A (*NGFI-A*)-binding protein 2 (Nab2) mitogenic pathway genes significantly down-regulated after treatment with GHRH antagonists ($P < 0.05$). Expression of Hedgehog pathway target genes [Bmp2, Bmp4, Hedgehog interacting protein (Hhip), protein patched homolog 1 (Ptch1), Wingless-type family member 1 (Wnt1), and Wnt2] was significantly affected by GHRH antagonists ($P < 0.05$). Levels of PI3K/protein kinase B (AKT) pathway-related genes such as Bcl2, fibronectin 1 (Fn1), Jun, and matrix metalloproteinase 7 (Mmp7) decreased after treatment with GHRH antagonists ($P < 0.05$). Expression of genes involved in the phospholipase C pathway, such as Bcl-2, Egr1, Fos, intercellular adhesion molecule 1 (Icam1), Jun, Junb, and vascular cell adhesion molecule 1 (Vcam1) was significantly lowered by GHRH.

We used real-time RT-PCR (Table S4) to verify changes in the expression of selected proinflammatory and growth factor genes (Fig. S2). Levels of mRNA for IGF1, IGF2, TGF-α, TGF-β2, EGF, and FGF-2 were significantly elevated in TE-induced BPH by 1.36-, 1.64-, 1.89-,1.98-, 2.21-, and 1.83-fold, respectively ($P < 0.05$ for all) (Fig. S2*A*). Finasteride (0.1 mg/kg) significantly decreased the expression of mRNA for IGF2 and IL-6 by 1.97- and 2.21-fold, respectively ($P < 0.05$ for all) (Fig. S2*B*). Antagonist JMR-132 significantly lowered the expression of IGF2, TGF-β1, TGF-β2, EGF, VEGF-A, and IL-6 by 1.36-, 1.96-, 1.59-, 1.61-, 1.51-, and 1.65-fold, respectively ($P < 0.05$ for all) (Fig. S2*C*). Treatment with MIA-313 significantly decreased the expression of TGF-α, TGF-β2, EGF, FGF-7, VEGF-A, and IL-6 by 1.49-, 1.47-, 1.53-, 1.72-, 1.63-, and 1.52-fold, respectively ($P < 0.05$ for all) (Fig. S2*D*). The GHRH antagonist MIA-459 exerted the greatest down-regulation at the transcriptional level: 2.35-

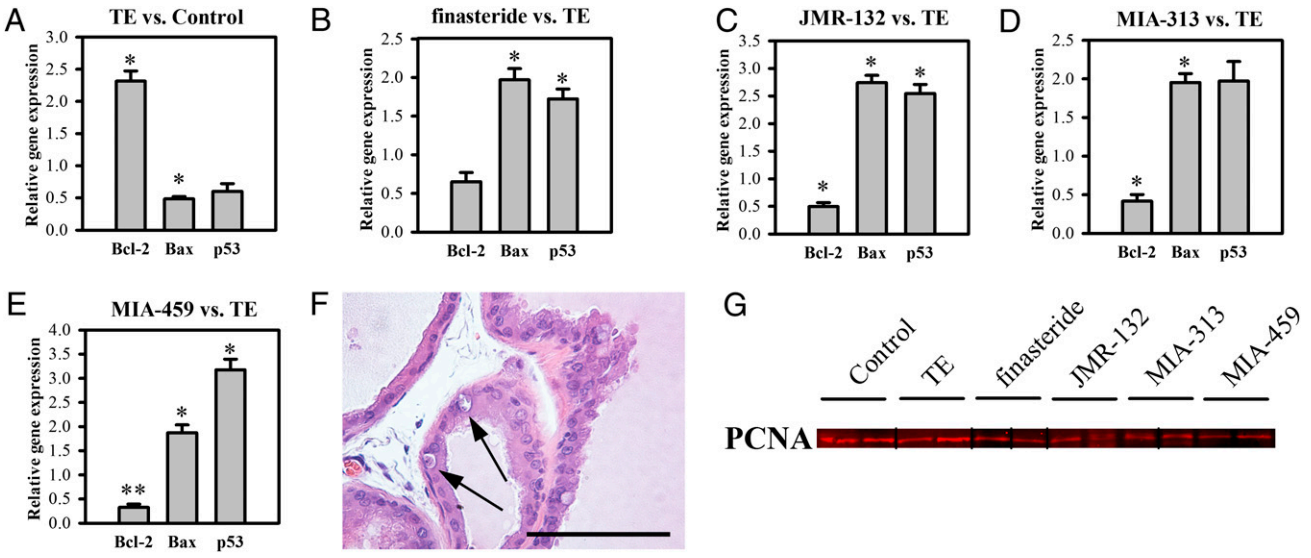

Fig. 3. GHRH antagonists induce apoptosis and inhibit proliferation of prostatic epithelial cells in rats. (*A–E*) Bar graphs show real-time RT-PCR analysis of Bcl-2, Bax, and p53. Bars represent relative expression of individual genes between prostate samples ($n = 3$) from TE-treated and control groups (*A*) or between TE-treated groups and groups treated with TE and finasteride (*B*), JMR-132 (*C*), MIA-313 (*D*), or MIA-459 (*E*). Values >1.00 indicate up-regulation of individual genes; values <1.00 indicate down-regulation. Data are shown as means ± SEM. Asterisks indicate a significant difference (*$P < 0.05$ by Student's *t* test). (*F*) Representative apoptotic cells (arrows) among prostatic acinar epithelial cells of an MIA-459 treated rat are shown in an H&E-stained slide. (Magnification: 40×.) (Scale bar: 50 μm.) (*G*) Western blot analysis of PCNA. Representative blots of three independent experiments are presented; corresponding signal intensity values are shown in Fig. S1*B*. Grouping of representative bands for each experimental group was digitally performed.

fold for IGF-2 ($P < 0.01$), 4.98-fold for TGF-α ($P < 0.01$), 1.63-fold for TGF-β1 ($P < 0.05$), 1.45-fold for TGF-β2 ($P < 0.05$), 2.75-fold for EGF ($P < 0.01$), 2.10-fold for FGF-2 ($P < 0.05$), and 1.87-fold for FGF-7 ($P < 0.05$) (Fig. S2*E*).

Effect of GHRH Antagonists on Serum GH, IGF1, DHT, and Prostate-Specific Antigen. TE markedly increased serum GH (104.8 ± 13.4 ng/mL [$P < 0.01$]) compared with control (49.4 ± 3.9 ng/mL). The GHRH antagonists and finasteride caused a decrease in serum GH levels, but the differences from TE-treated controls were statistically not significant (Table S5). Serum IGF1 was significantly elevated after TE treatment (4.9 ± 0.6 ng/mL [$P < 0.05$]), whereas the GHRH antagonists lowered serum IGF1 levels, although not significantly. In TE-induced BPH rats there was an ∼10-fold increase in serum DHT compared with control at day 42 (337.4 ± 53.5 pg/mL [$P < 0.001$]); however, no significant differences in serum DHT levels were observed after treatment with GHRH antagonists. A significant 60% reduction in DHT was observed after treatment with finasteride 0.1 mg/d ($P < 0.05$). Changes in serum prostate-specific antigen (PSA) levels were not significant. Values for serum markers are given in Table S5.

Discussion

The main finding of our study is that the GHRH antagonists JMR-132, MIA-313, and MIA-459 reduce prostate size in an experimental model of BPH. In addition to prostate shrinkage in rats, multiple factors related to growth and inflammation, which are crucial in the pathogenesis and progression of BPH (17), were markedly reduced by treatment with GHRH antagonists. The expression of GHRH, GHRH-R, and the GHRH-R splice variant SV1 in rat prostate was demonstrated by Western blot. The antibody used to detect GHRH receptors identifies both pituitary-type GHRH-R and its splice variant SV1 (24). Furthermore, the ligand competition assay detected specific high-affinity receptors for GHRH in rat prostate, and immunohistochemical analyses revealed that this expression of GHRH-R is confined to luminal epithelial cells of the rat prostate. Changes in serum GH, IGF1, DHT, and PSA were not significant after treatment with GHRH antagonists. Recently we showed that GHRH antagonists inhibit the proliferation of the human prostate epithelial BPH-1 cell line in vitro (25).These findings strongly suggest that prostate shrinkage is a result of direct inhibitory effects of GHRH antagonists exerted through prostatic GHRH receptors, not involving the GH/IGF1 axis. The demonstration of the coexpression of GHRH and its receptors in rat prostate supports the hypothesis that GHRH produced locally in the prostate could act in an autocrine/paracrine manner through an interaction with the GHRH receptors (6). The presence of this pathway, which is disrupted by GHRH antagonists, provides a mechanistic explanation for the antiproliferative effects of such antagonists in prostate cell growth in culture (25) and in nude mice xenograft models of prostate cancer (1, 10, 11). Our data also imply that GHRH could be involved in the pathogenesis of BPH.

In this study we used real-time PCR arrays to investigate the beneficial molecular mechanisms of GHRH antagonists in a BPH-model. The analyses showed that several growth factors were up-regulated in TE-induced BPH control rats and markedly down-regulated in such animals treated with GHRH antagonists. Growth factors are regulatory peptides that govern the response of cells to injury and mediate the highly coordinated processes of cell growth, differentiation, and apoptosis. Among them are many polypeptides that use autocrine or paracrine pathways to signal stromal and epithelial cells in the microenvironment (17). To verify PCR array results and more precisely determine changes in gene expression, we studied the expression of selected growth factor- and proinflammatory-encoding genes by real-time RT-PCR. We confirmed that GHRH antagonists suppress transcriptional expression of IGF-2, TGF-α, TGF-β1 and -β2, EGF, FGF-2, VEGF-A, and IL-1β. Several of these growth factors were reported to be involved in the pathogenesis of BPH: EGF and TGF-α (EGF family); FGF-2, FGF-7, and FGF-9 (FGF family); IGF-1 and IGF-2 (IGF family); TGF-β1 and TGF-β2 (TGF-β family), and VEGF (VEGF family) (17).

Our observation of the transcriptional activation of inflammatory cytokines (e.g., Ccl6, Ccl12, IL-1α, IL-1β, IL-13, IL-15, and IL-17β) in the prostate of rats with induced BPH is consistent with clinical findings (26) and with experimental findings in rats (27). We found that GHRH antagonists significantly lowered transcriptional expression of several cytokines including IL-1α, IL-1β, IL-13, IL-15, and IL-17β. These cytokines are part of an inflammatory network in BPH including several growth factors (15); they promote T-lymphocyte infiltration and the subsequent inflammation progression associated with BPH (28).

We showed that treatment with TE results in elevated levels of IL-1β, NF-κβ, and COX-2 protein in the rat prostate, whereas the GHRH antagonists JMR-132, MIA-313, and MIA-459 caused a pronounced comparative decrease in IL-1β, NF-κβ, and COX-2 protein levels. Vykhovanets et al. (29) recently demonstrated that IL-1β, an inflammatory cytokine, causes NF-κβ activation in the mouse prostate. The NF- κβ family proteins, such as NF-κβ/p65 (RelA), are inducible transcription factors that regulate the expression of hundreds of genes in immune response, angiogenesis, cell adhesion, proliferation, differentiation, and apoptosis. The activation of NF-κβ is one of the earliest events in chronic inflammation (30). COX-2, an inducible isoform of the cyclooxygenase enzyme, is an early-response gene up-regulated by specific stimuli such as mitogens, growth factors, and a variety of cytokines including IL-1 (31). Expression of COX-2 and COX-2–dependent prostanoid production induced by proinflammatory mediators is regulated predominantly by NF-κβ–dependent gene transcription (32), suggesting a causal relationship between the lowered NF-κβ/p65 levels, inhibition of COX-2 up-regulation, and decreased IL-1β production caused by GHRH antagonists. Overexpression of COX-2 in human BPH samples has been reported (33), and GHRH antagonists were shown to lower levels of COX-2 in experimental lung cancer (34) and prostate cancer (8). COX-2 also was shown to up-regulate antiapoptotic Bcl-2 with an associated decrease in apoptosis in prostate tissue (35).

All three GHRH antagonists were demonstrated to inhibit cell proliferation, elevate tumor suppressor p53, and lower PCNA levels in rat prostatic epithelium. We observed an increased expression of antiapoptotic Bcl-2 in TE-induced BPH prostates. This overexpression of Bcl-2 corresponds to observations of Alonso-Magdalena et al. (19) in human BPH samples, which suggest that BPH is not a proliferative disease but rather is an accumulation of cells resistant to death. Our work shows that treatment with GHRH antagonists causes significant translational up-regulation of proapoptotic Bax and suppression of antiapoptotic Bcl-2 in rat prostates. The number of apoptotic cells in prostatic epithelium after treatment with GHRH antagonists also was decreased, although this decrease was not statistically significant. These propapoptotic effects of GHRH antagonists might be caused by the significant suppression of prostatic COX-2 or by inhibition of both intrinsic and extrinsic pathways of p53-mediated apoptosis (36).

Analyzing transcriptional changes in signal transduction pathways with quantitative PCR arrays, we observed the involvement of the mitogenic, hedgehog, PI3/AKT, and phospholipase C pathways and their downstream effectors. These pathways may be responsible for transmitting beneficial effects of GHRH antagonists in experimental BPH. GHRH antagonists can strongly inhibit the proliferation rate of cancer cells through the inhibition of the MAPK pathway (37).

The therapeutic effects of GHRH antagonists were superior to those of finasteride in many aspects of our study, including prostatic shrinkage and suppression of growth factors and proinflammatory COX-2 as well as antiproliferative and proapoptotic effects. The adverse effects of finasteride may encourage the use of GHRH antagonists as an alternative medical therapy of BPH.

In summary, in this study we demonstrated that the GHRH antagonists JMR-132, MIA-313, and MIA-459 reduce prostate volume in an experimental BPH model. Our data suggest that this

reduction in prostate volume is caused by the direct inhibitory effects of GHRH antagonists exerted through prostatic GHRH receptors as well as by transcriptional suppression of enumerated growth factors and proinflammatory cytokines. We also showed strong inhibition of proinflammatory IL-1β, NF-κβ, and COX-2. The antiapoptotic effects of these GHRH antagonists also have been demonstrated. These findings suggest mechanisms of action of GHRH antagonists in BPH and also indicate a role for GHRH as a locally acting growth factor in BPH. It is possible that GHRH antagonists could be clinically useful for therapy of BPH, alone or in combination with other agents.

Materials and Methods

Drugs and Chemicals. Testosterone enanthate (TE) (Watson Pharmaceuticals), corn oil vehicle (Sigma-Aldrich), and finasteride (Sigma-Aldrich) were used. The GHRH antagonists JMR-132, MIA-313, and MIA-459 were synthesized as described (*SI Text*). For daily injection, finasteride and GHRH antagonists were dissolved in 0.1% DMSO in 10% aqueous propylene glycol solution.

Animals. Adult male Wistar rats (Charles River Laboratories) between 10 and 11 wk of age were housed in a climate-controlled environment with a 12-h light/dark cycle and were fed standard laboratory diet with water ad libitum. Body weights were determined weekly. All animals remained healthy throughout the experiment. Animal care was in accordance with institutional guidelines and complied with National Institutes of Health policy.

Study Design. After a 7-d acclimatization, rats were divided randomly into five experimental groups and one negative control group of 10 animals each. BPH was induced in experimental groups by daily s.c. injection into the right flank of long-acting TE (2 mg/d) dissolved in corn oil from Day −28 to Day 0 (induction phase). Negative control animals received s.c. injections of corn oil alone on the same schedule. The dosage and duration of testosterone treatment was based on the reports by Maggi et al. (20) and by Scolnik et al. (21). One group of rats was administered daily s.c. injections of the 5AR2 inhibitor finasteride, 0.1 mg/kg, in the left flank from days 1–42 after BPH induction. This dosage of finasteride is comparable to the human dosage (5 mg/d). The other three groups of animals were given daily s.c. injections of the GHRH antagonists JMR-132, MIA-313, or MIA-459 (40, 20, and 20 μg/d, respectively). The dosage of GHRH antagonists was based on prior experimental oncological use (38). Experimental groups consisted of (*i*) TE only, (*ii*) TE/finasteride (0.1 $mg \cdot kg^{-1} \cdot d^{-1}$), (*iii*) TE/JMR-132, (*iv*) TE/MIA-313, and (*v*) TE/MIA-459. TE-only positive control animals received 0.1% DMSO in 10% aqueous propylene glycol solution instead of finasteride or GHRH antagonists on the same schedule. Venous blood samples were collected before the experiment (day −28) and on the last day of the experiment (day 42). Serum was separated by centrifugation and stored at −80 °C. Rats were weighed and killed under anesthesia on the morning of day 42. Whole prostates were removed immediately, weighed, and snap frozen. Alternate prostate lobes were immersed in phosphate-buffered 10% formalin (pH 7.4) and embedded in paraffin for histological analysis.

Additional information is provided in *SI Text*.

ACKNOWLEDGMENTS. This study was supported by Departments of Pathology and Medicine of the Medical Research Service of the Veterans Affairs Department, by the Division of Hematology/Oncology of the Miller Medical School, University of Miami, and by the South Florida VA Foundation for Research and Education (all A.V.S.) and by the L. Austin Weeks Endowment for Urologic Research (N.L.B.).

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