

Gonadotropin-Releasing Hormone Induces Apoptosis of Prostate Cancer Cells: Role of c-Jun NH₂-Terminal Kinase, Protein Kinase B, and Extracellular Signal-Regulated Kinase Pathways

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ABSTRACT

A standard therapy used today for prostate cancer is androgen ablation by gonadotropin-releasing hormone analogs (GnRH-a). Although most patients respond to androgen ablation as an initial systemic therapy, nearly all cases will develop androgen resistance, the management of which is still a major challenge. Here, we report that GnRH-a can directly induce apoptosis of the androgen-independent prostate cancer-derived DU145 and PC3 cell lines. Using specific inhibitors, we found that the apoptotic effect of GnRH-a is mediated by c-Jun NH₂-terminal kinase (JNK) and inhibited by the phosphatidylinositol 3'-kinase (PI3K)-protein kinase B (PKB) pathway. Indeed, in DU145 cells, GnRH-a activates the JNK cascade in a c-Src- and MLK3-dependent manner but does not involve protein kinase C and epidermal growth factor receptor. Concomitantly, GnRH-a reduces the activity of the PI3K-PKB pathway, which results in the dephosphorylation of PKB mainly in the nucleus. The reduction of PKB activity releases PKB-induced inhibition of MLK3 and thus further stimulates JNK activity and accelerates the apoptotic effect of GnRH-a. Interestingly, extracellular signal-regulated kinase is also activated by GnRH-a, and this occurs via a pathway that involves matrix metalloproteinases and epidermal growth factor receptor, but its activation does not affect JNK activation and the GnRH-a-induced apoptosis. Our results support a potential use of GnRH-a for the treatment of advanced prostate cancer and suggest that the outcome of this treatment can be amplified by using PI3K-PKB inhibitors.

INTRODUCTION

Carcinoma of the prostate is the most commonly diagnosed malignancy in older men and the second leading cause of cancer death in the Western world (1). Androgen ablation therapy is the standard approach for treatment of carcinoma of the prostate in the early, androgen-responsive stages of the tumor, and this ablation is mainly achieved by the administration of gonadotropin releasing hormone (GnRH) analogs (GnRH-a; Ref. 2). Thus, chronic stimulation by GnRH-a leads to pituitary desensitization, arrest of gonadotropin secretion, and suppression of androgen production in the gonads. The lack of androgen, which is required for the function and maintenance of the prostate cancer cells, inhibits their growth and decreases tumor volumes. However, at later stages of the disease, nearly all tumors develop androgen resistance and become hormone-refractory prostate cancer, the management of which is still a major challenge.

GnRH is a hypothalamic decapeptide that normally serves as a key regulator of the mammalian reproductive system. In the pituitary, GnRH acts via a specific G-protein coupled receptor (GnRH receptor) and triggers the synthesis of the common α - and β -chains of the

gonadotropins, luteinizing hormone, and follicle-stimulating hormone (2). Luteinizing hormone and follicle-stimulating hormone control the function of the gonads, induce steroidogenesis, and influence the growth and function of a variety of steroid-dependent tissues including the prostate gland. The GnRH receptor was first identified exclusively in pituitary gonadotrophs (3, 4). However, the expression of GnRH receptor was later reported also in several extra-pituitary tissues, including breast, ovary, testis, placenta, and endometrial cells (5). Moreover, GnRH receptor expression has been demonstrated in several tumors and tumor-derived cell lines in which the addition of GnRH-a resulted in the inhibition of cell growth (5). Interestingly, GnRH was found to exert a direct antiproliferative effect also on carcinoma of the prostate cells (6–9). However, the mechanism underlying this effect has not yet been elucidated.

GnRH receptor-mediated signaling pathways have been extensively studied in past years. Binding of GnRH to GnRH receptor in pituitary gonadotrophs is mediated primarily via G α_q , which activates PLC β and leads to the generation of the second messengers diacylglycerol and inositol 1,4,5-triphosphate, which in turn activate protein kinase C (PKC) isoforms and Ca²⁺ mobilization (10). Previous studies from our laboratory have shown that in pituitary-derived α T3–1 cells, GnRH activates all four mitogen-activated protein kinase (MAPK) cascades to various extents by a PKC- and tyrosine-kinase-dependent mechanism (5, 11). The activated MAPKs then participate in the regulation of expression of several genes such as the common α -subunit gene, the β -subunit of luteinizing hormone, and the GnRH receptor (5). We and others have shown that several distinct pathways are involved in the activation of MAPKs by GnRH (5, 11). GnRH-induced extracellular signal-regulated kinase (ERK) activation in α T3–1 cells is mainly dependent on PKC and supported by a pathway involving c-Src, dynamin, and Ras (12). On the other hand, the activation of c-Jun NH₂-terminal kinase (JNK) by GnRH in these cells involves PKC, c-Src, and CDC42/Rac1 (13). In GnRH receptor-expressing COS7 cells, used as a prototype for the extra-pituitary action of GnRH, the GnRH receptor transmits its signals to ERK mainly via G α_i , epidermal growth factor (EGF) receptor (EGFR), and c-Src, whereas the signals to JNK are transmitted by sequential activation of c-Src and phosphatidylinositol 3'-kinase (PI3K; Ref. 14). Thus, in different cell lines and under varying conditions, GnRH can use distinct mechanisms for the activation of MAPKs.

The mechanisms by which intracellular pathways regulate allular processes have attracted much attention recently. Activated MAPKs phosphorylate a large number of targets, which eventually initiate and regulate various cellular processes including proliferation, differentiation, and development (11). Although the ERK cascade is usually mitogenic, the JNK cascade mediates mainly stress response and apoptosis. However, the mechanism by which JNK mediates its apoptotic effects is not yet fully understood. Another important pathway is PI3K-protein kinase B (PKB), which has emerged as critical for cell survival in many systems including prostate cancer cells. Increased activity of PKB has been shown in the androgen-dependent LNCaP cell line (15, 16) as well as in advanced stages of carcinoma of the prostate (17).

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The present study was aimed to examine the direct effect of GnRH-a on a prostate cancer model and to study the signaling pathways involved in its antiproliferative function. Our results show that GnRH-a initiates a significant apoptotic response in DU145 and PC3 androgen-independent prostate cancer cells. Examining the signaling pathways that are involved in the apoptotic process, we found that GnRH-a activates both JNK and ERK MAPKs but reduces the activity of PKB in the nucleus of DU145 cells. Inhibition of JNK resulted in a marked reduction in the apoptotic effect of GnRH-a, whereas inhibition of PI3K-PKB increased the effect. On the other hand, inhibition of ERK did not affect GnRH-induced apoptosis. We then studied the mechanism of regulation by GnRH-a of these pathways and found that the activation of JNK was dependent on the concomitant decrease in PKB activity. This effect is mediated, at least in part, by inhibition of the upstream activator of JNK, MLK3. Our results support a potential use of a direct administration of GnRH-a for the treatment of hormone-refractory prostate cancer and suggest that the outcome of this treatment can be amplified by inhibition of the PI3K-PKB pathway.

MATERIALS AND METHODS

Buffers. Buffer A consisted of 50 mM β -glycerophosphate (pH 7.3), 1.5 mM EGTA, 1 mM EDTA, 1 mM DTT, and, 0.1 mM sodium orthovanadate. Homogenization buffer (buffer H) consisted of buffer A and 1 mM benzamide, 10 μ g/ml aprotinin, 10 μ g/ml leupeptin, and 2 μ g/ml pepstatin-A. Radioimmunoprecipitation assay (RIPA) buffer consisted of 137 mM NaCl, 20 mM Tris (pH 7.4), 10% (v/v) glycerol, 1% (v/v) Triton X-100, 0.5% (w/v) deoxycholate, 0.1% (w/v) SDS, 2 mM EDTA, 1 mM phenylmethylsulphonyl fluoride, 1 mM sodium orthovanadate, and 20 μ M leupeptin. HNTG buffer consisted of 20 mM HEPES (pH 7.5), 0.15 M NaCl, 0.1% Triton X-100, and 10% glycerol. PBS was from Life Technologies (Grand Island, NY).

Stimulants, Inhibitors, Antibodies, and Miscellaneous Reagents. [D-Trp]⁶ GnRH, a stable GnRH analog (GnRH-a), epidermal growth factor (EGF), genistein (PTK inhibitor), LY294002, and protein A-Sepharose were obtained from Sigma Chemicals Co. (St. Louis, MO). GF109203X, PD098059, SB203580, AG1478, PP2, and wortmannin were purchased from Calbiochem (San Diego, CA). GM-6001 was from Biomol Research Laboratories (Plymouth Meeting, PA). Mouse monoclonal antiactive MAPK antibodies (DP-ERK and DP-JNK), rabbit polyclonal anti-phospho-Ser-473 PKB, anti-ERK, anti-JNK, anti-PKB antibodies, and phospho-PKB peptide (461–477) were from Sigma, Israel (Rehovot, Israel). Protein G-agarose, polyclonal anti-c-Src, anti-EGFR, anti-heparin-binding EGF (C-18), and monoclonal anti-phospho-Tyr (PY-99) were from Santa Cruz Biotechnology (Santa Cruz, CA). Polyclonal anti-phospho-c-Src (Tyr-416) was purchased from New England Biolabs (Beverly, MA). Polyclonal anti-phospho-MLK3 (Thr-277/Ser-281), anti-phospho-ASK1 (Ser-83), anti-phospho-(Ser/Thr) PKB substrate, anti-MLK3, and anti-ASK1 were from Cell Signaling Technology, United States. Polyclonal mouse anti-ErbB1 antibody (mAb565) was a gift from Dr. Y. Yarden (Weizmann Institute of Science, Rehovot, Israel). Anti-GnRH receptor was a gift from Dr. F. Kohen (Weizmann Institute of Science, Rehovot, Israel). This antibody was generated by first immunizing rats with GnRH-13-BSA conjugate, followed by the use of the generated anti-GnRH antibody as an antigen for the production of polyclonal anti-idiotypic antibodies that were further purified on a protein-A column (18).

Cell Culture, Stimulation, and Harvesting of DU145 Cells. Human prostate carcinoma DU145 and PC3 cells were obtained from the American Type Culture Collection (Rockville, MD) and grown in RPMI 1640 supplemented with 10% (v/v) heat-inactivated FCS (Life Technologies, Grand Island, NY), 1% glutamine, and an antibiotic mixture added to a final concentration of 100 units/ml penicillin and 100 μ g/ml streptomycin. Cells were maintained in a humidified atmosphere containing 5% CO₂ at 37°C. For experiments, DU145 cells were serum-starved in medium containing 0.1% heat-inactivated FCS, 16 h before stimulation. After treatment, the cells were washed twice with ice-cold PBS and once with buffer A. For determination of MAPKs, the cells were subsequently harvested in ice-cold buffer H and sonicated (50 W, 2 \times 7 s), cell lysates were centrifuged (20,000 \times g, 15 min, 4°C), and the

supernatant was assayed for protein content using the Coomassie protein assay (Pierce, Rockford, IL) and subjected to SDS-PAGE analysis. For the determination of PKB, c-Src, EGFR, and GnRH receptor, the cells were lysed with RIPA buffer (10 min, 4°C), centrifuged (20,000 \times g, 15 min), and analyzed by SDS-PAGE and Western blotting.

Solid Phase Assay for c-Jun NH₂-Terminal Kinase Activity. Serum-starved (0.1% FCS, 16 h) DU145 cells were stimulated and then washed (twice with PBS and once with buffer A) and harvested in buffer H, sonicated (50 W, 2 \times 7 s), and centrifuged (20,000 \times g, 15 min). Protein content in cell extracts was determined as above, and JNK activity was detected as described by Hibi *et al.* (19). In brief, aliquots (150 μ g of protein) of the cell extracts were incubated with glutathione *S*-transferase-c-Jun (1–91) for 1 h at 4°C to allow JNK binding to the substrate. After extensive washing with HNTG buffer, JNK activity was measured by phosphorylation of glutathione *S*-transferase-c-Jun (1–91) in the presence of 20 mM MgCl₂, 20 μ M [γ -³²P]ATP (specific activity, ~3,000 cpm/pmol; Amersham Biosciences) for 20 min at 30°C. The reactions were terminated by the addition of SDS gel sample buffer, and the samples were subjected to 12% SDS-PAGE followed by autoradiography (Kodak X-100 film).

Determination of Protein Kinase B Activity. After stimulation, DU145 cells were washed (twice with PBS and once with buffer A) and harvested in RIPA buffer. After centrifugation (20,000 \times g, 15 min), aliquots of the resulting supernatant (300 μ g protein) were immunoprecipitated by anti-PKB COOH-terminal antibody (Sigma) using protein A-Sepharose (20 μ l). PKB activity was determined by the phosphorylation of 2 mg/ml histone H2B (Boehringer Mannheim) in kinase buffer [75 mM β -glycerophosphate (pH 7.3), 30 mM MgCl₂, 1.5 mM DTT, 0.15 mM sodium orthovanadate, 6 μ M PKI peptide, 3.75 mM EGTA, 0.3 mM ATP, 30 μ M calmidazolium, 2.5 mg/ml BSA, and 10 μ Ci [γ -³²P]ATP (3,000 cpm/pmol)] for 20 min at 30°C. Phosphorylation of histone H2B was analyzed by 15% SDS-PAGE followed by autoradiography.

Determination of Apoptosis. To analyze apoptosis, subconfluent DU145 cells were plated on glass coverslips in 60-mm-well plates under the standard culture conditions as described above. Twenty-four h after the initial seeding, cells were serum-starved (0.1% FCS) and treated with the examined stimulants. After 24, 48, 72, and 96 h, the cells were fixated with a freshly prepared paraformaldehyde solution [4% in PBS (pH 7.4)] for 1 h at 15–25°C, washed with PBS and then incubated with 0.1% Triton X-100 in 0.1% sodium citrate (2 min, 4°C), washed again with PBS, and incubated with terminal deoxynucleotidyltransferase-mediated nick end labeling (TUNEL) reaction mixture containing fluorescein-dUTP and terminal deoxynucleotidyl transferase (Roche Molecular Biochemicals) for 30 min at 37°C. Preparations were analyzed by fluorescence microscopy. The incidence of apoptosis in each preparation was determined by counting 200 cells and calculating the percentage of apoptotic cells.

Immunofluorescent Staining. DU145 cells were plated on glass coverslips as described above. After growing for an additional day, the cells were serum-starved (0.1% FCS, 16 h) and then treated with the examined stimulants. Fixation (3% paraformaldehyde solution), permeabilization (0.2% Triton X-100, 4 min), and staining were as described previously (20). The cells were sequentially incubated (45 min each) with anti-phospho-Ser-473 PKB antibodies (1:200) and rhodamine-conjugated goat-antirabbit antibodies (1:200; The Jackson Laboratory) together with 4'-diamino-2-phenylindole (Sigma). The cells were visualized by fluorescence microscopy.

RESULTS

Gonadotropin-Releasing Hormone Analog Induces Apoptosis in DU145 Cells. Previous studies have shown that GnRH receptor is expressed in both androgen-dependent (LNCaP) and androgen-independent (DU145) cells and that GnRH-a exerts a direct inhibitory action on the proliferation of human prostatic cancer cell lines (7, 21). We used anti-idiotypic antibodies to GnRH receptor and found that DU145 and PC3 cells express a *M_r* 55,000 GnRH receptor (Fig. 1A) similar to that of the pituitary receptor (data not shown). To examine whether the antiproliferative effect of GnRH-a involves apoptosis, serum-starved DU145 cells were treated with GnRH-a (10⁻⁷M) for several days (24, 48, and 72 h), and the number of apoptotic cells was

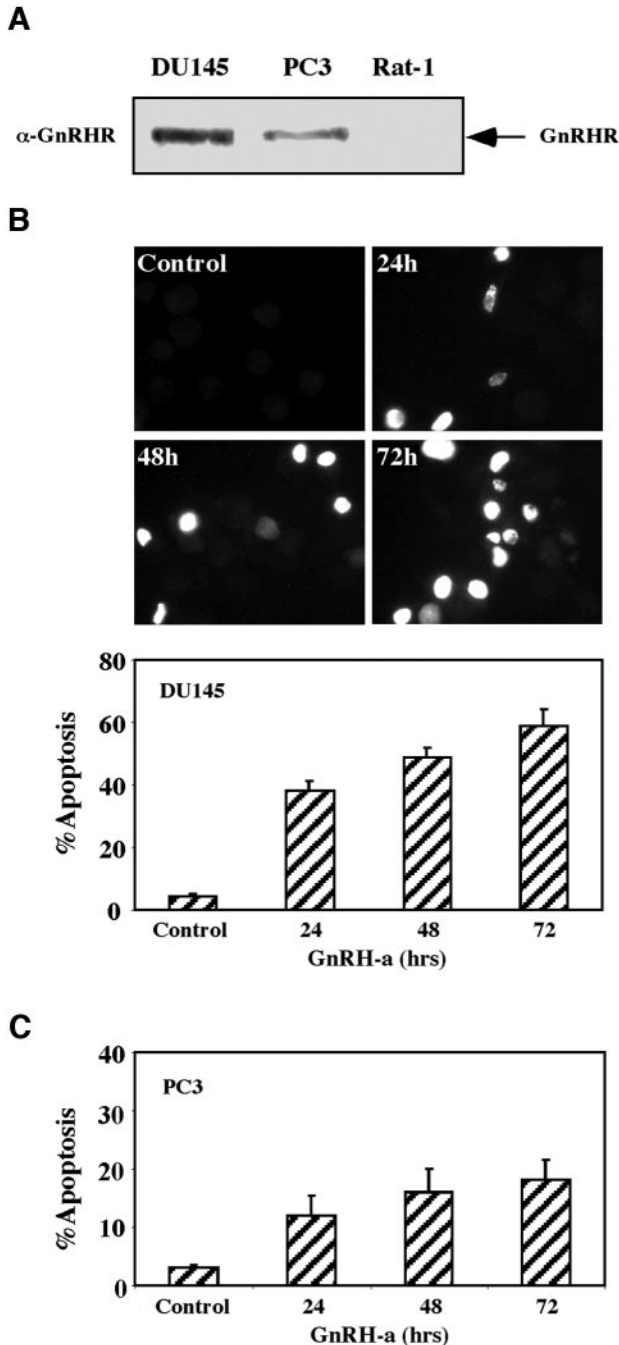


Fig. 1. GnRH-a induces apoptosis of DU145 cells. **A**, expression of GnRH receptor (*GnRHR*) in DU145 and PC3 cell lines. Cells were lysed with RIPA buffer and separated by SDS-PAGE. An extract from Rat1 cell was used as a negative control. GnRH receptor was detected by Western blotting with anti-GnRH receptor antibody (α -*GnRHR*). **B**, time course of apoptosis induced by GnRH-a in DU145 cells. Serum-starved DU145 cells (16 h, 0.1% FCS) were either treated with GnRH-a (10^{-7} M) for increasing periods of time (24, 48, and 72 h) or left untreated (*Control*). GnRH-induced cell death was detected by TUNEL. The results in the *bar graph* represent the percentage of apoptosis and are the mean \pm SE of three experiments. **C**, GnRH-a-induced apoptosis in PC3 cells. Serum-starved PC3 cells (0.1% FCS) were treated as above, and apoptosis was detected by terminal deoxynucleotidyltransferase-mediated nick end labeling. The results in the *bar graph* represent the percentage of apoptosis and are the mean \pm SE of two experiments.

examined by TUNEL (Fig. 1B). Significant time-dependent apoptosis was detected after 24 ($38\% \pm 3.3$), 48 ($49\% \pm 2.9$), and 72 h ($59\% \pm 5.3$) of treatment (Fig. 1B). In addition, we found that the effect of GnRH-a on cell death induction was dose dependent, because 24 h after treatment, 10^{-6} M GnRH-a induced $\sim 45\%$ apoptosis; 10^{-7} M GnRH-a, $\sim 40\%$; and 10^{-8} M, $\sim 15\%$ (data not shown). The ability

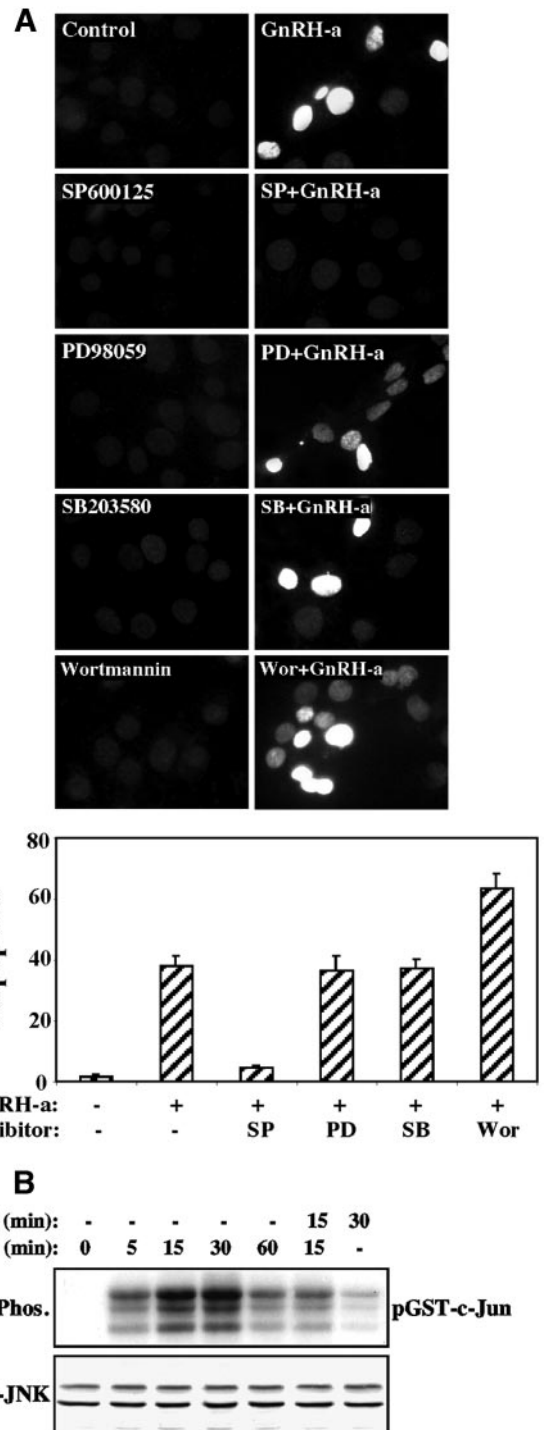


Fig. 2. GnRH-a induces apoptosis via JNK and PKB, but not ERK or p38MAPK cascades. **A**, serum-starved DU145 cells were either treated with GnRH-a (10^{-7} M, 24 h) or left untreated (*Control*) in the presence or absence of $5 \mu\text{M}$ SP600125 (*SP*), $25 \mu\text{M}$ PD098059 (*PD*), $20 \mu\text{M}$ SB203580 (*SB*), or 25 nM wortmannin (*Wor*). Cell death was detected by TUNEL. The results in the *bar graph* are the mean percentage of apoptosis \pm SE of three experiments. **B**, inhibition of JNK activation by SP600125. Serum-starved DU145 cells were either pretreated with $5 \mu\text{M}$ (15, 30 min) of the JNK inhibitor SP600125 (*SP*) or left untreated. GnRH-a (10^{-7} M; 15 min) was added to the pretreated and to the untreated cells. The activity of JNK toward glutathione *S*-transferase-c-Jun (1-91) was determined by *in vitro* kinase assay. The phosphorylation was detected by autoradiography (Jun Phos.), and the total amount of JNK was detected with anti-JNK antibody (α -*G-JNK*). The results are representative of three experiments.

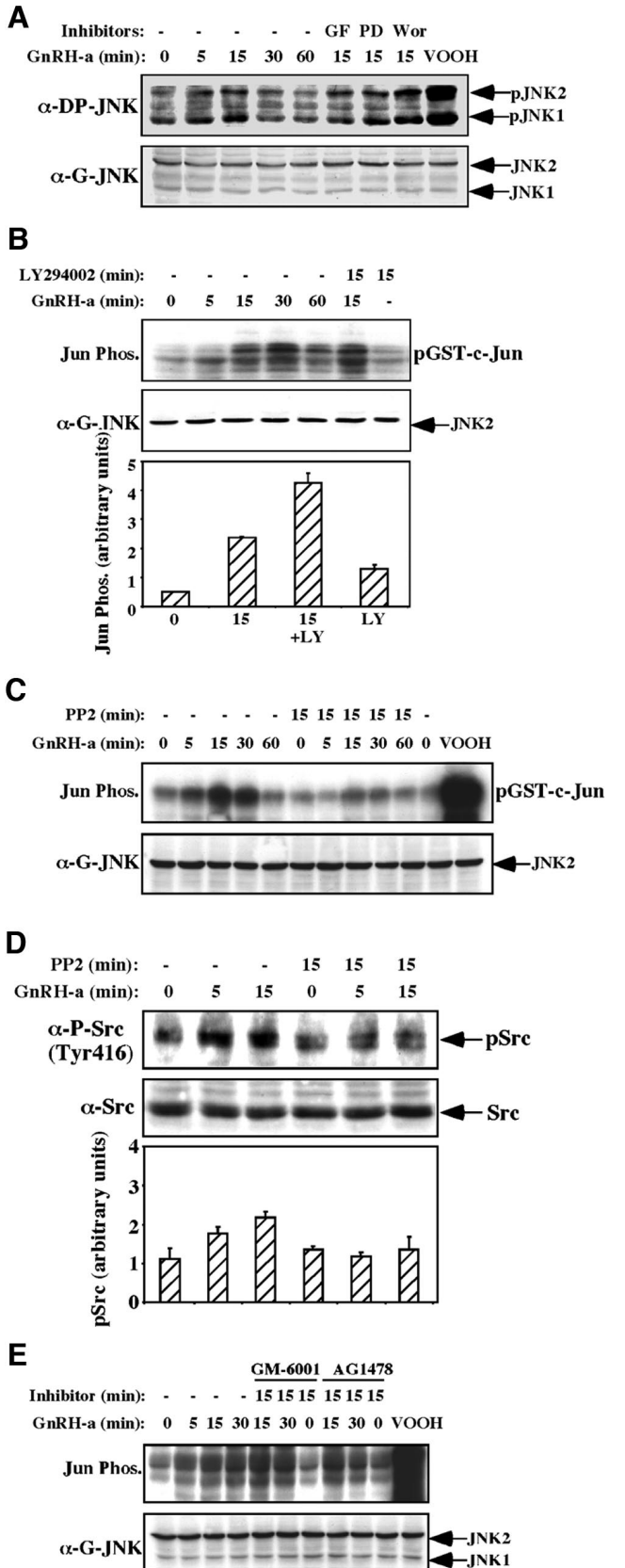


Fig. 3. JNK activation by GnRH-a in DU145 cells. **A**, DU145 cells were serum-starved (0.1% FCS, 16 h), and cells were pretreated (15 min) with 3 μM GF109203X (GF), 25 μM PD098059 (PD), and 25 nM wortmannin (Wor) or left untreated. H_2O_2 (200 μM) and 100 μM vanadate (VOOH) was used as a positive control (20 min). The cells were then stimulated with GnRH-a (10^{-7} M) for the indicated times. Phospho-JNK was detected with anti-DP-JNK antibody ($\alpha\text{-DP-JNK}$). The amount of JNK was detected with the anti-JNK antibody ($\alpha\text{-G-JNK}$). The results are representative of three experiments. **B**, JNK

activation by GnRH-a is increased by LY294002. Serum-starved DU145 cells were either pretreated with LY294002 (20 μM ; 15 min) or left untreated and then stimulated with 10^{-7} M GnRH-a. The activity of JNK toward glutathione S-transferase-c-Jun (1-91) was determined by *in vitro* kinase assay. The phosphorylation was detected by autoradiography, and the total amount of JNK was detected with $\alpha\text{-G-JNK}$. The results are representative of two experiments. **C**, JNK activation is inhibited by PP2. Serum-starved DU145 cells were pretreated with 5 μM PP2 (15 min) or left untreated and stimulated with GnRH-a (10^{-7} M). VOOH was used as positive control. JNK activity was detected as above. The results are representative of two experiments. **D**, effect of PP2 on GnRH-a-induced c-Src phosphorylation. DU145 cells were serum-starved and were either pretreated with 5 μM PP2 (15 min) or left untreated. The cells were then stimulated with GnRH-a (10^{-7} M) for the indicated times. Phosphorylation of c-Src on Tyr-416 was detected with anti-phospho-c-Src antibody ($\alpha\text{-P-Src}$). The total amount of c-Src was detected with anti-Src antibody ($\alpha\text{-Src}$). The results are representative of three experiments. **E**, GnRH-a-induced JNK activation is not dependent on EGFR and metalloproteinase activity. DU145 cells were serum-starved, then either pretreated with GM-6001 (200 nM, 15 min) or AG1478 (5 μM , 15 min) or left untreated, and then stimulated with 10^{-7} M GnRH-a for the indicated times. JNK activity was determined by *in vitro* kinase assay as in **B**. The results are representative of two experiments.

of GnRH-a to induce apoptosis was examined also by counting $4'6\text{-diamino-2-phenylindole}$ -stained cells with fragmented DNA and by monitoring the appearance of the active form of caspase 3. The results obtained by both of these methods correlated with the TUNEL results (data not shown), confirming the massive nature of GnRH-a-induced apoptosis in DU145 cells. Apoptosis was demonstrated also in the androgen-independent prostate cancer PC3 cell line treated with 10^{-7} M GnRH-a (Fig. 1C). This apoptotic effect was smaller than that shown for DU145 cells, and this may be due to a lower number of GnRH receptors expressed in PC3 cells.

Effect of c-Jun NH₂-Terminal Kinase, Extracellular Signal-Regulated Kinase, p38 Mitogen-Activated Protein Kinase, and Phosphatidylinositol 3'-Kinase Inhibitors on Gonadotropin-Releasing Hormone Analog-Induced Apoptosis. To elucidate the signaling pathways that mediate the GnRH-a-induced apoptosis in DU145 cells, the effect of various pharmacological inhibitors was examined. Specific inhibition of JNK (SP600125) abolished the apoptotic effect of GnRH-a (Fig. 2A). The efficacy of SP600125 was confirmed by its ability to block JNK activity upon GnRH-a treatment (Fig. 2B). On the other hand, inhibition of MEK1/ERK by PD98059 and of p38MAPK by SB203580 did not affect GnRH-a-induced apoptosis (Fig. 2A). In contrast, the PI3K inhibitor wortmannin increased the apoptotic effect of GnRH-a after 24 h of treatment (Fig. 2). These results suggest a role for JNK but not ERK or p38MAPK in the apoptotic effect of GnRH-a. The fact that the GnRH-a-induced apoptosis is enhanced by inhibition of PI3K indicates that this pathway confers a negative regulation on the apoptotic effect of GnRH-a.

c-Jun NH₂-Terminal Kinase Activation by Gonadotropin-Releasing Hormone Analog in DU145 Cells. We then analyzed the mechanism of activation of JNK in DU145 cells. For this purpose, serum-starved DU145 cells were stimulated with 10^{-7} M GnRH-a, harvested, and subjected to Western blot analysis or activity assay. Using anti-phospho-JNK antibody, the two bands of the M_r 46,000 JNK1 and the M_r 54,000 JNK2 were detected. Addition of GnRH-a to the cells resulted in increased phosphorylation of JNKs, predominantly JNK2 (Fig. 3A), which peaked 15–30 min after treatment and declined thereafter. Pretreatment of the GnRH-a-stimulated DU145 cells with GF109203X or PD098059 had no inhibitory effect on JNK activation by GnRH-a, whereas pretreatment with wortmannin significantly increased the phosphorylation of JNKs upon GnRH-a stimulation. These results suggest that PI3K but not the MEK/ERK or PKC pathways have a role in the inactivation of the JNK cascade.

To further assess the mechanism of JNK activation by GnRH-a, we examined its activation by a direct *in vitro* kinase assay toward c-Jun and analyzed the effect of signaling inhibitors on JNK activity. Indeed, this assay confirmed the activation of JNK obtained with the anti-phospho-JNK antibody (Fig. 3B). In addition, we found that

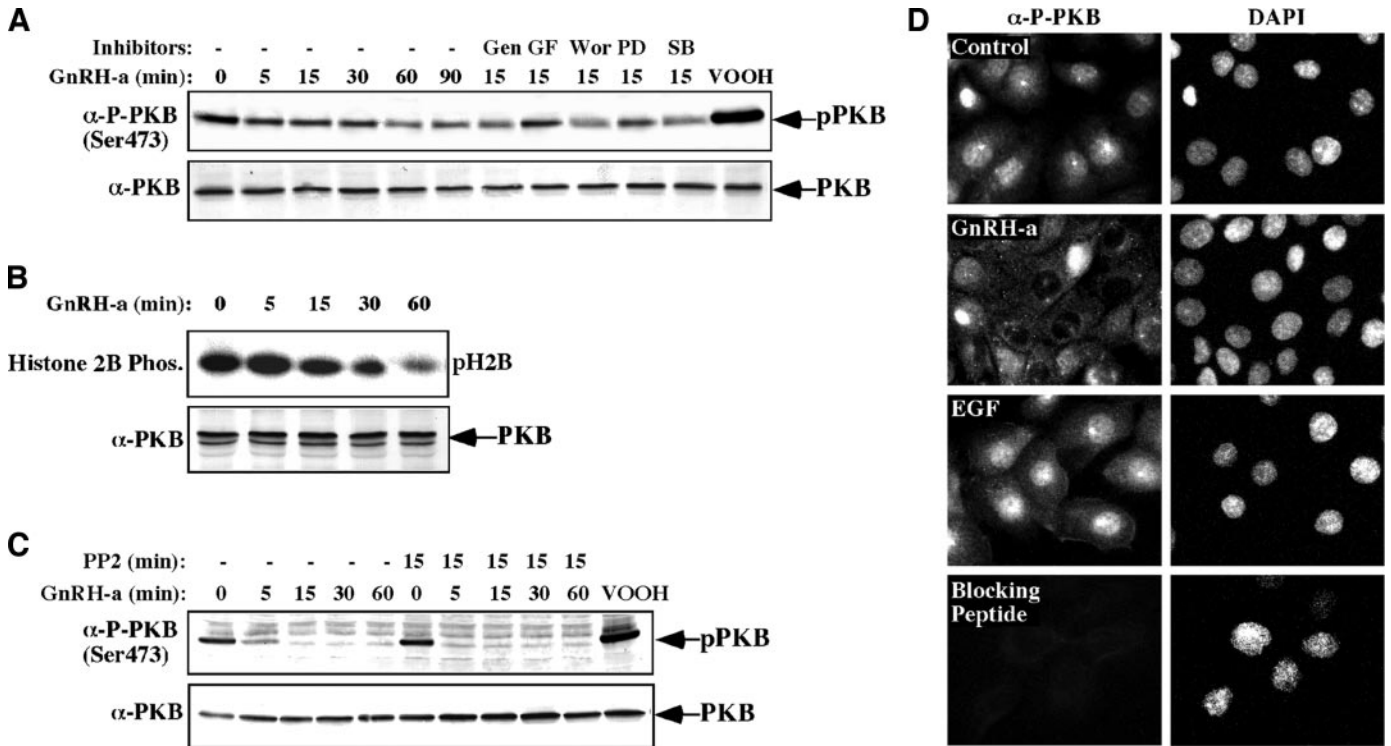


Fig. 4. Effect of GnRH-a treatment on PKB phosphorylation, activity, and localization. *A*, serum-starved DU145 cells were pretreated with 200 μ M genistein (*Gen*), 3 μ M GF109203X (*GF*), 25 nM wortmannin (*Wor*), 25 μ M PD098059 (*PD*), and 20 μ M SB203580 (*SB*) or left untreated. GnRH-a (10^{-7} M, 15 min) was added to the pretreated and to the untreated cells. H_2O_2 (200 μ M) and 100 μ M vanadate (*VOOH*) was used as positive control (20 min). Cell extracts prepared in RIPA buffer were subjected to Western blotting using either anti-phospho-PKB antibody [α -*P-PKB* (*Ser473*)] or anti-PKB antibody (α -*PKB*). The results are representative of three experiments. *B*, serum-starved DU145 cells were treated with 10^{-7} M GnRH-a, immunoprecipitated with anti-PKB antibody, and subjected to *in vitro* kinase assay using Histone H2B (*P-H2B*) as a substrate. The phosphorylation was detected by autoradiography, and total amount of PKB was detected with α -PKB. The results are representative of three experiments. *C*, DU145 cells were serum-starved and either pretreated with 5 μ M PP2 or left untreated. GnRH-a (10^{-7} M) was added to the pretreated and the untreated cells for the indicated times. VOOH was used as positive control. PKB was detected as in *A*. The results are representative of three experiments. *D*, subcellular localization of PKB upon GnRH-a treatment. Serum-starved DU145 cells were treated with 10^{-7} M GnRH-a (10 min) or 50 ng/ml EGF (5 min) or left untreated (*Control*). Cells were stained with anti-phospho-PKB antibody [α -*P-PKB* (*Ser473*)]. Cell nuclei were stained with 4',6-diamino-2-phenylindole. For competition studies, PKB nuclear staining was blocked in the presence of 100 μ g/ml anti-phospho-specific PKB peptide (461–477) incubated with the anti-phospho-PKB antibody for 30 min before staining. Cells were visualized by fluorescence microscopy. The results are representative of three experiments.

another PI3K-specific inhibitor, LY294002, significantly increased JNK activation (Fig. 3*B*), confirming that the PI3K-PKB pathway is involved in the regulation of JNK activity in DU145 cells.

Previous studies from our laboratory have shown that c-Src plays a role in the transmission of signals by the GnRH receptor to MAPKs in COS7 cells expressing GnRH receptor and as a supportive pathway in pituitary-derived α T3–1 cells (12). Addition of the c-Src inhibitor PP2 reduced the GnRH-a-induced activity of JNK (Fig. 3*C*) in the serum-starved DU145 cells. Moreover, anti-phospho-Src antibodies (Tyr-416) revealed that GnRH-a induces phosphorylation of c-Src, and pretreatment of cells with PP2 inhibited this phosphorylation (Fig. 3*D*). Our results suggest that GnRH-a activates JNK in a c-Src-dependent manner. We also examined the possible role of EGFR in the activation of JNK by GnRH-a (22). EGFR and metalloproteinase inhibitors AG1478 and GM-6001, respectively, had no effect on JNK activation by GnRH-a (Fig. 3*E*), indicating that they are not part of the GnRH-a-JNK pathway.

Gonadotropin-Releasing Hormone Analog Induces Dephosphorylation of Protein Kinase B in DU145 Cells. Because the PI3K-PKB pathway regulates the GnRH-a-induced JNK cascade and was shown to play a role in carcinoma of the prostate survival (15, 17), we examined the GnRH-a effect on this pathway. PKB activation was determined using anti-phospho-Ser-473 or phospho-Thr-308 PKB antibodies. We found that GnRH-a reduced the basal phosphorylation of Ser-473 (Fig. 4*A*), whereas the phosphorylation of Thr-308 was not significantly affected (data not shown). We then used signaling inhibitors and found that the PTK inhibitor genistein, as well as wort-

mannin and SB203580, further inhibited GnRH-a-induced PKB dephosphorylation (Fig. 4*A*). However, GF109203X, PD098059 (Fig. 4*A*), and AG1478 (not shown) had no effect on Ser-473 phosphorylation. PKB dephosphorylation was correlated with a decrease in PKB activity as determined by *in vitro* kinase assay using H2B as a substrate (Fig. 4*B*). To further study the involvement of c-Src in PKB regulation, the effect of PP2 was examined. Pretreatment of DU145 cells with PP2 further inhibited PKB phosphorylation 5 min after GnRH-a stimulation but had no detectable effect at other time points examined (Fig. 4*C*). Thus, c-Src and EGFR do not mediate the inactivation of PKB, and the upstream mechanism of PKB inhibition by GnRH-a is not yet clear.

Gonadotropin-Releasing Hormone Analog Reduces the Phosphorylation of Nuclear Protein Kinase B. We then examined whether the effect of GnRH-a on the relatively high basal PKB activity detected in DU145 cells is reflected in its intracellular localization. Immunofluorescent staining of DU145 cells with the anti-phospho-PKB (Ser-473) antibody revealed that in untreated cells, PKB is phosphorylated and localized mainly in the nucleus (Fig. 4*D*). After GnRH-a treatment, PKB phosphorylation in the nucleus is dramatically reduced in most cells, although some phosphorylation is still detected in the cytosol. Treatment of DU145 cells with EGF further increased the nuclear staining of active PKB and induced some membrane localization, and this correlated with induction of PKB phosphorylation as detected by a Western blot (data not shown). Incubation of the antigenic peptide (100 μ g/ml) with the anti-phos-

pho-PKB (Ser-473) antibody blocked PKB staining, confirming the specificity of the detection by the antibodies.

Role of Phosphatidylinositol 3'-Kinase-Protein Kinase B in the Regulation of c-Jun NH₂-Terminal Kinase on Gonadotropin-Releasing Hormone Analog Stimulation. A possible mechanism by which PI3K-PKB might be involved in the regulation of apoptosis upon GnRH-a treatment is a direct inhibition of JNK activity. One possible way by which PKB may inhibit JNK is by phosphorylating one of the kinases in the MAP3K level of the JNK cascade. Recent studies have shown that phosphorylation of MLK3 (23) and ASK1 (24) by PKB causes their inhibition and as a result inhibits the activity of the JNK cascade. We assessed the effect of GnRH-a on the activation of MLK3 and ASK1 by anti-phospho-specific antibodies. Thus, the use of anti-phospho-MLK3 (Thr-277/Ser-281) antibody revealed a significant activation of MLK3 by GnRH-a that peaked at 15 min after stimulation and was correlated with the time course of GnRH-a-induced JNK activation. Pretreatment of DU145 cells with the PI3K-PKB inhibitor LY294002 increased the GnRH-a-induced phosphorylation of MLK3 (Fig. 5B). To examine whether the activation of MLK3 by GnRH-a involves inhibition of MLK3 by PKB, MLK3 was immunoprecipitated and then probed with an anti-phospho-(Ser/Thr) PKB substrate antibody, which recognizes phosphorylated PKB substrates. Thus, treatment of DU145 cells with GnRH-a significantly reduced the phosphorylation of MLK3 on the PKB phosphorylation site (Fig. 5C), and a similar effect was observed by inhibition of PKB activity with wortmannin (data not shown). We then examined the phosphorylation of ASK1 upon GnRH-a stimulation and found that DU145 cells express relatively low levels of ASK1, and treatment of cells with GnRH-a increased the inhibitory phosphorylation of ASK1 (Fig. 5D), as detected by anti-phospho-Ser-83 antibody (25). Therefore, unlike MLK3, ASK1 is probably inhibited and not activated by GnRH-a and is not the MAP3K that mediates JNK activation upon GnRH-a treatment. Therefore, it is likely that MLK3 does play a role in the PKB-regulated JNK activity. However, due to the expression of PKB phosphorylation sites in other MAP3Ks, it is possible that additional MAP3Ks are regulated by the PI3K-PKB pathway and participate in the GnRH-a-JNK pathway in DU145 cells.

Extracellular Signal-Regulated Kinase Activation by Gonadotropin-Releasing Hormone Analog in DU145 Cells. Our results showed that unlike JNK and PI3K-PKB, ERK and p38MAPK are not involved in the apoptotic response of GnRH-a in DU145 cells. Nonetheless, we examined whether these cascades can be activated by GnRH-a in DU145 cells. Surprisingly, we found that ERK1/2 (but not p38MAPK nor ERK5; data not shown) were significantly activated and that their phosphorylation peaked 15 min after GnRH-a stimulation, declining thereafter (Fig. 6A). Pretreatment of cells with the PKC inhibitor GF109203X had only a small inhibitory effect on GnRH-a-induced phosphorylation of ERKs probably due to inhibition of basal ERK activity. However, pretreatment with the MEK1 inhibitor PD098059 and the EGFR inhibitor AG1478, completely prevented the phosphorylation of ERKs by GnRH-a. These results suggest a role for EGFR but not for PKC in the pathway that leads to ERK activation by GnRH-a. It is also possible that also other ErbB family members heterodimerize with the EGFR and participate in the GnRH-a to ERK signaling in the DU145 cells.

A proposed mechanism for G protein-coupled receptor-induced activation of EGFR is through the activation of membrane metalloproteinases, which induce the release of membrane-bound EGF-like ligands, such as heparin-binding EGF, and activate the EGFR (26). We evaluated the involvement of heparin-binding EGF in GnRH receptor-mediated signaling in DU145 cells by examining the shedding of heparin-binding EGF upon GnRH-a stimulation (Fig. 6B).

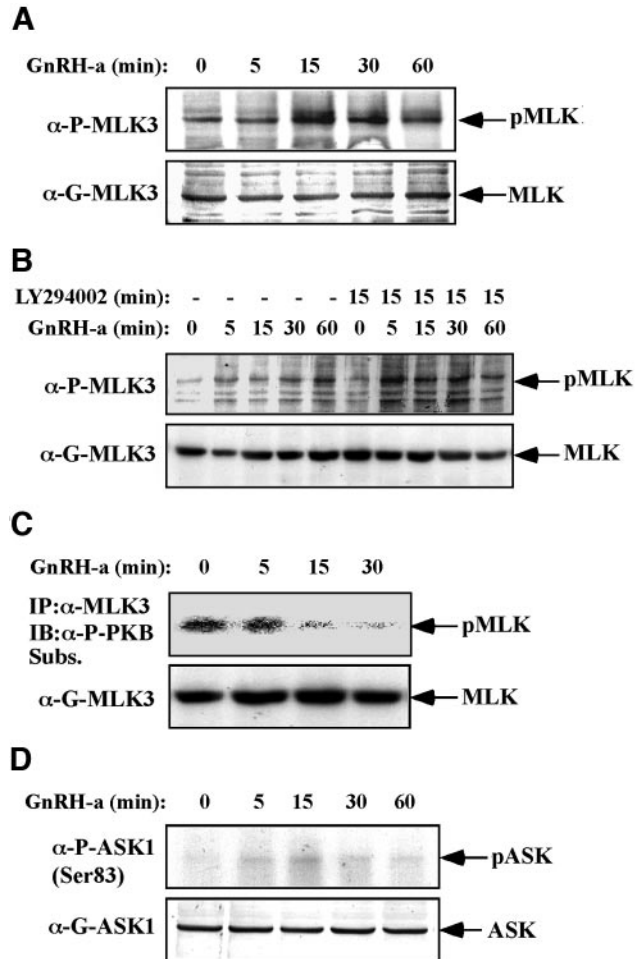


Fig. 5. Effect of GnRH-a on activation of MLK3 and ASK1. A, induction of MLK3 phosphorylation by GnRH-a. DU145 cells were serum-starved and either treated with GnRH-a (10^{-7} M) for the indicated times or left untreated. Phosphorylation of MLK3 was detected by Western blotting using anti-phospho-MLK3 antibody (Thr-277/Ser-281; α -P-MLK3). The total amount of MLK3 was detected with the anti-MLK3 antibody (α -G-MLK3). The results are representative of two experiments. B, GnRH-a-induced MLK3 phosphorylation is elevated by LY294002. Serum-starved DU145 cells were either pretreated with LY294002 (20 μ M; 15 min) or left untreated. GnRH-a (10^{-7} M) was added to the pretreated and untreated cells for the indicated times. Phosphorylation of MLK3 was detected as above. The results are representative of two experiments. C, phosphorylation of MLK3 by PKB is reduced by GnRH-a. Serum-starved DU145 cells were either treated with GnRH-a (10^{-7} M) for the indicated times or left untreated. DU145 cells were then subjected to immunoprecipitation with anti-MLK3 antibody, and its phosphorylation was detected by Western blotting using anti-phospho-(Ser/Thr) PKB substrate antibody (α -P-PKB Subs.). The total amount of MLK3 was detected with anti-MLK3 antibody (α -G-MLK3). The results are representative of two experiments. D, the inhibitory PKB phosphorylation of ASK1 is induced by GnRH-a. Phosphorylation of ASK1 was detected by Western blotting using anti-phospho-ASK1 (Ser-83; α -P-ASK1). The total amount of ASK1 was detected with the anti-ASK1 antibody (α -G-ASK1). The results are representative of two experiments.

Indeed, we found that heparin-binding EGF is rapidly secreted into the medium after 5 and 15 min of GnRH-a treatment, which correlates with the GnRH-a-induced ERK response. In addition, pretreatment with the selective metalloproteinase inhibitor GM-6001 (27) prevented GnRH-a-induced phosphorylation of ERKs (Fig. 6B). Similarly, we found that EGFR was rapidly phosphorylated upon GnRH-a stimulation (Fig. 6D), and pretreatment with GM-6001 blocked EGFR transactivation by GnRH-a. Thus, our results suggest that activation of ERKs by GnRH-a involves transactivation of EGFR, which is mediated through activation of metalloproteinases and shedding of heparin-binding EGF.

Because c-Src and EGFR often maintain bidirectional interactions and c-Src has been implicated in GPCR-mediated EGFR transactiva-

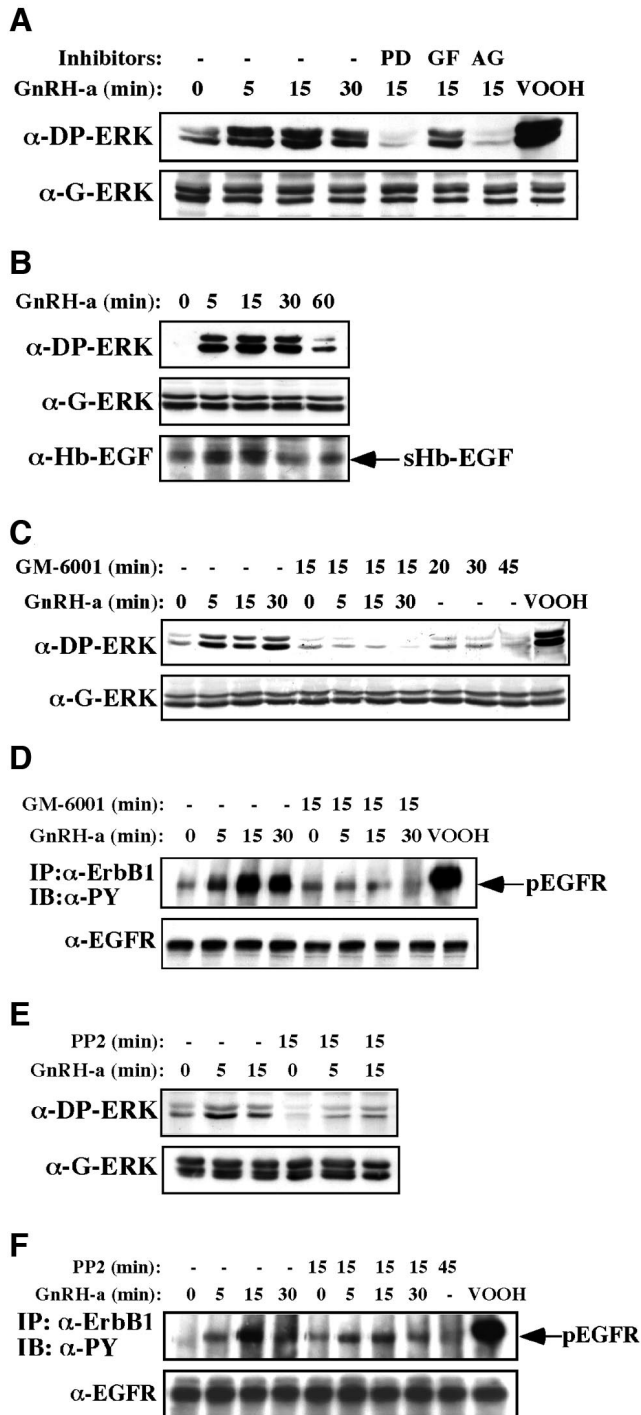


Fig. 6. GnRH-induced phosphorylation of ERKs in DU145 cells. **A**, ERK phosphorylation is induced by GnRH-a. DU145 cells were serum-starved and pretreated with 25 μM PD098059 (PD), 3 μM GF109203X (GF), or 5 μM AG1478 (AG) or left untreated. GnRH-a (10^{-7} M, 15 min) was added to the pretreated and the untreated cells. H_2O_2 (200 μM) and 100 μM vanadate (VOOH) was used as positive control (20 min). Phosphorylation of ERK was detected with anti-DP-ERK antibody (α -DP-ERK). The total amount of ERKs was detected with the anti-ERK antibody (α -G-ERK). The results are representative of three experiments. **B**, shedding of heparin-binding EGF upon GnRH-a treatment. Serum-starved DU145 cells were stimulated with 10^{-7} M GnRH-a for the indicated times. The medium was then collected and incubated with heparin-agarose beads to precipitate the secreted heparin-binding EGF ectodomain (sHb-EGF). The amount of secreted heparin-binding EGF was detected by Western blotting using goat anti-heparin-binding EGF antibody (α -Hb-EGF). The results are representative of three experiments. **C**, inhibition of GnRH-a-induced ERK phosphorylation by GM-6001. Serum-starved DU145 cells were either pretreated with GM-6001 (200 nM) for the indicated times or left untreated. The cells were then stimulated with GnRH-a (10^{-7} M). ERK was detected as in **A**. The results are representative of two experiments. **D**, inhibition of GnRH-a-induced EGFR phosphorylation by GM-6001. Serum-starved DU145 cells were pretreated with GM-6001

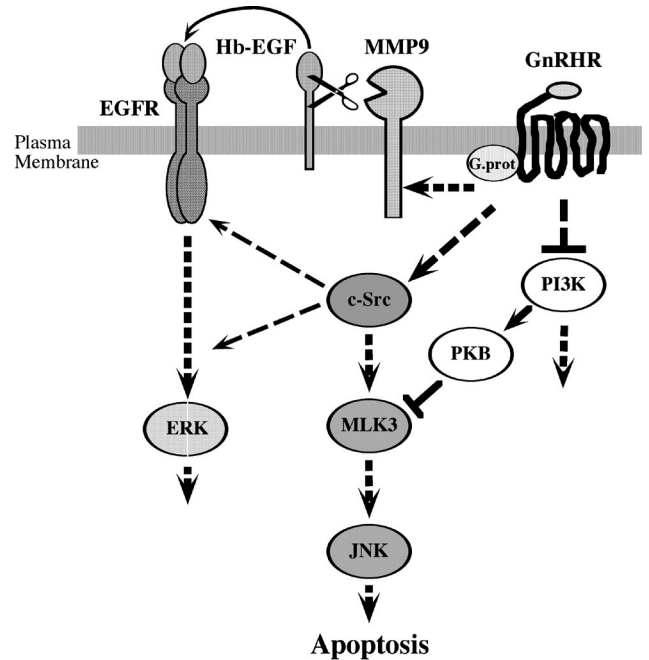


Fig. 7. Schematic representation of GnRH signaling toward the MAPK cascades and PKB in DU145 cells. Broken lines indicate an indirect activation.

tion (28, 29), we studied the interplay between EGFR and c-Src in the GnRH receptor to ERK pathway. Addition of the c-Src inhibitor PP2 significantly reduced the basal activity of ERK but only partially inhibited the GnRH-a-induced phosphorylation of ERKs (Fig. 6F). We also found that GnRH-a-induced EGFR phosphorylation was inhibited by PP2. These results indicate that c-Src participates in the regulation of EGFR as suggested in other systems (30, 31), possibly by preventing its down-regulation (32). On the other hand, the EGFR inhibitor AG1478 had no effect on c-Src phosphorylation upon GnRH-a stimulation (data not shown). The fact that AG1478 had no effect on the c-Src, JNK, and PKB phosphorylation suggests that GnRH-a stimulates ERKs via EGFR and JNK via c-Src, although c-Src may participate in the transactivation of EGFR by GnRH (Fig. 7).

DISCUSSION

GnRH-a is successfully used to treat androgen-sensitive carcinoma of the prostate, operating mainly via a reduction of testosterone production in the patients (2). However, there is increasing evidence showing that the effects of GnRH-a on carcinoma of the prostate are mediated not only through suppression of gonadal steroids, but also through a direct effect on cell growth (33–36). This effect may indicate that besides its effect on androgen-sensitive carcinoma of the prostate, GnRH-a may be efficient also in the treatment of hormone-refractory prostate cancer. In the present study, we evaluated the antiproliferative effect of GnRH-a on the hormone-refractory prostate

(200 nM; 15 min) and then stimulated with 10^{-7} M GnRH-a for 5, 15, and 30 min. EGFR was immunoprecipitated, and its phosphorylation was detected by Western blotting using anti-PY antibody (α -PY). The total amount of EGFR was detected with anti-EGFR antibody (α -EGFR). The results are representative of three experiments. **F**, inhibition of GnRH-a-induced ERK and EGFR phosphorylation by PP2. DU145 cells were serum-starved and either pretreated with 5 μM PP2 as indicated or left untreated. The cells were then stimulated with GnRH-a (10^{-7} M). Phosphorylation of ERK was detected with anti-DP-ERK antibody (α -DP-ERK), and the total amount of ERKs was detected with the anti-ERK antibody (α -G-ERK). EGFR was immunoprecipitated and detected as above. The results are representative of three experiments.

cancer cell lines DU145 and PC3. Our results indicate that prostate cancer-derived cells express GnRH receptors and that GnRH-a induces apoptosis of these cells. The expression of GnRH receptor in the DU145 and PC3 cells was confirmed by GnRH-a-binding assays (data not shown). Moreover, these surface GnRH receptors are functional as they induce apoptosis of the two prostate cancer cell lines.

It should be noted that the concentrations of GnRH-a used today for androgen-ablation are much lower than the concentrations found to induce apoptosis in the hormone-refractory prostate cancer-derived cell lines (2–3 orders of magnitude difference). Therefore, it is unlikely that the low concentrations of GnRH-a used for androgen-ablation are able to induce apoptosis of the hormone-sensitive carcinomas of the prostate at this stage of the disease. Moreover, due to the efficiency of the androgen-ablation treatment, it is probably not necessary to raise these concentrations for hormone-sensitive carcinomas of the prostate. However, high GnRH-a concentrations should be useful for hormone-refractory prostate cancers, and we show here that this treatment can benefit from the addition of low concentrations of PI3K-PKB inhibitors (Fig. 2). Taken together, we suggest that high concentrations of GnRH-a alone can be used for the treatment of the hormone-refractory prostate cancer, and the outcome of this treatment can be amplified by using PI3K-PKB inhibitors.

The DU145 and PC3 used here belong to a subgroup of carcinomas of the prostate that lost their hormone sensitivity due to their lack of expression of a functional androgen receptor (37). A larger group of hormone-refractory prostate cancers includes cells that do express androgen receptor but are not sensitive to androgen due to a variety of other reasons. Here, we concentrated on the effects of GnRH-a independently of androgen receptor, because it was shown that GnRH-a may interact with PSA and androgen receptor and therefore change the signaling and fate of these cells (38). Therefore, our results are directly applicable to hormone-refractory prostate cancers that do not express androgen receptor, as occurs in some cases of bone metastasis. However, we found that GnRH-a causes growth inhibition also in androgen-receptor-containing hormone-refractory prostate cancer, although the mechanism of this inhibition is probably distinct from the one in DU145 cells. Therefore, we do believe that GnRH-a might serve as a useful treatment in other hormone-refractory prostate cancers independent of the cause of androgen insensitivity of the disease.

We analyzed in detail the signaling cascades that are involved in the GnRH-a-induced apoptosis in DU145 (Fig. 7). We found that GnRH-a induced the activation of JNK and ERK, did not affect the activation of the other MAPKs p38MAPK and ERK5, and induced inhibition of the activity of PKB in the nucleus of DU145 cells. Interestingly, although the ERK cascade may participate in few apoptotic processes (39, 40), in most cell types and conditions, its activity is distinct from these processes and usually leads to proliferation (41). Indeed, our results indicate that inhibition of the ERK cascade does not influence the apoptotic activity of GnRH-a, and therefore it is unlikely that the elevated ERK activity plays a significant role in the survival of the DU145 cells. On the other hand, the role of the JNK cascade in apoptosis is well documented (42, 43), although its mechanism is not fully elucidated as yet. We show here that the activation of ERK and JNK is mediated by completely different mechanisms, because GnRH-a activation of JNK is mediated by c-Src but not at all via EGFR that activates ERK. This finding demonstrates that the two pathways, which lead to distinct physiological outcomes are separated already at the upstream levels and do not demonstrate much of a cross-talk between themselves.

PI3K-PKB signaling serves as a survival pathway in many systems (44–46). Our results indicate that inhibition of this survival pathway is important for the induction of apoptosis by GnRH-a. It was shown that inhibition of PI3K-PKB by 12-*O*-tetradecanoylphorbol-13-

acetate in the androgen-dependent LNCaP cells is correlated with their enhanced apoptosis (16). However, in these cells, the apoptosis was mediated by PKC and p38MAPK, which is a distinct mechanism from the one involved in the GnRH-a-induced apoptosis here. Moreover, it should be noted that in pituitary cells, activation of the JNK cascade by GnRH-a does not lead to apoptosis, but rather induces the expression of GnRH-a-dependent gene products (47, 48). Thus, in different cell types, GnRH-a exerts its function by activating a different set of intracellular signaling cascades.

The mechanism responsible for PKB down-regulation upon GnRH treatment is not yet clear. Due to the lack of functionally active PTEN, it is likely that the dephosphorylation of the Ser-473 is mediated by another PKB phosphatase. Indeed, in LNCaP cells, PKB dephosphorylation seems to involve a PP2A phosphatase, as suggested by the inhibitory effect of okadaic acid on phorbol 12-myristate 13-acetate-induced PKB dephosphorylation (16). In agreement with these results, we found that pretreatment of DU145 cells with okadaic acid prevented PKB dephosphorylation upon GnRH-a stimulation (data not shown). However, whether GnRH-a can activate the dephosphorylation of PKB remains to be determined.

Our results suggest that the JNK and PI3K-PKB signaling pathways are dependently regulated upon GnRH-a stimulation. PKB can regulate the JNK cascade by multiple mechanisms. Our results suggest that the elevated JNK activity upon GnRH treatment is mediated by the reduced phosphorylation of the inhibitory site on MLK3 by PKB, whereas ASK1 is probably not involved in this process. However, it is possible that other mechanisms are involved as well. Thus, it was shown that PKB phosphorylates and negatively regulates MKK4 (49). Furthermore, PKB interacts with scaffolds proteins for the JNK pathway such as JIP1 and POSH and prevents formation of active signaling complexes (50). The involvement of these processes still needs to be elucidated. However, independent of the mechanisms that underlie the interplay between the PKB and JNK cascades, it seems that in DU145 cells the high basal activity of PKB maintains the low activity of JNK, and inhibition of PKB upon GnRH-a treatment facilitates the c-Src-dependent activation of the JNK cascade.

In summary, we report here that GnRH-a induces apoptosis of DU145 cells and that this requires the activation and/or inhibition of multiple pathways. The JNK cascade is activated downstream of c-Src but not downstream of the EGFR and seems to be a major mediator of the GnRH-a signaling. On the other hand, the activity of PKB is reduced upon GnRH-a treatment, and this may exert a stronger activation of the JNK cascade due to reduced inhibitory phosphorylation of MLK3 that operates upstream of JNK. Interestingly, additional inhibition of the PKB cascade by pharmacological inhibition facilitates the apoptotic effect of GnRH-a. Although ERKs are activated by GnRH-a in an EGFR-dependent manner, ERKs do not seem to be involved in the GnRH-a-induced apoptosis. Our results support a potential use of GnRH-a for the treatment of hormone-refractory prostate cancer and suggest that the outcome of this treatment can be amplified by using PI3K-PKB inhibitors.

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