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Effects of Growth Hormone–Releasing Hormone-Related Peptide on Stem Cell Factor Expression in Cultured Rat Sertoli Cells

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The growth hormone–releasing hormone (GHRH) gene produces a precursor molecule that contains GHRH and a carboxyl-terminal peptide that we have named GHRH-related peptide (GHRH-RP). This peptide, like GHRH, stimulates the expression of stem cell factor (SCF), an important reproductive and hematopoietic cytokine, in vitro and in vivo. In the present study, using primary cultures of rat Sertoli cells, we compared the time course of action and the level of SCF stimulation seen following treatment with GHRH-RP and GHRH. Additionally, we investigated the activity of a truncated peptide, p75-92NH2, whose sequence is contained within GHRH-RP. All three of these peptides were shown to stimulate the steady-state levels of SCF mRNA to a comparable degree. However, the time course of action for GHRH-RP differed markedly from that of GHRH. GHRH-RP and p75-92NH2, similar to GHRH, induce SCF expression, at least in part, via the activation of the protein kinase A/cyclic adenosine monophosphate intracellular signaling pathway.

Key Words: Growth hormone-releasing hormone–related peptide; Sertoli cells; stem cell factor; protein kinase A.

Introduction

Growth hormone–releasing hormone (GHRH) is the primary hypophysiotropic hormone responsible for the synthesis and release of GH from pituitary somatotrophs. Rat preproGHRH is a 104 amino acid peptide that contains a signal peptide sequence (amino acids 1–29), the mature GHRH peptide (amino acids 30–74), and a carboxyl-terminal region (amino acids 75–104) *(1)*. Although the actions of GHRH have been well documented, the biological activity of this C-terminal moiety, which we have designated GHRH-related peptide (GHRH-RP), has only recently begun to be investigated.

Our laboratory has shown that GHRH-RP stimulates stem cell factor (SCF) gene expression in vitro in cultures of rat Sertoli cells *(2)*. SCF, also known as c-kit ligand or steel factor, is a pluripotent growth factor that plays a critical role in reproduction *(3)* and hematopoiesis *(4)*, in which it has effects on both primitive and mature progenitor and germ cells. More recently, we have shown that GHRH-RP regulates SCF expression in vivo as well. Using a GHRH-RP overexpressing transgenic mouse, we reported that constitutive expression of GHRH-RP in these animals induced overexpression of SCF that resulted in increased cell-cycling rates for myeloid and erythroid hematopoietic progenitor cells *(5)*. In comprehensive studies focused on proGHRH posttranslational processing, we determined that GHRH-RP likely undergoes further proteolytic modifications, following its formation, that result in the production of a smaller GHRH-RP-like peptide, designated p75-92NH2. In vitro, this peptide significantly increased cyclic adenosine monophosphate (cAMP) activity in cultured Sertoli cells, similar to the increases seen following treatment of cells with either GHRH-RP or GHRH *(6)*.

The goals of the present study were to characterize further the action of GHRH-RP on SCF gene expression in Sertoli cells. We examined the time course, the magnitude of SCF gene activation, and the intracellular signal transduction pathways activated by GHRH-RP. Based on the amino acid sequence and the presence of a possible enzymatic cleavage site located at amino acid residue 92 of the GHRH-RP peptide, a truncated peptide, p75-92NH2, was also synthesized and its actions were compared with those of GHRH-RP and GHRH in cultured Sertoli cells.

Results

The results of the time course experiments indicated that treatment of Sertoli cells with GHRH-RP increased SCF mRNA expression for up to 16 h (Fig. 1). By 24 h of treatment with GHRH-RP, SCF mRNA levels had returned to near baseline. These results were similar to those seen with

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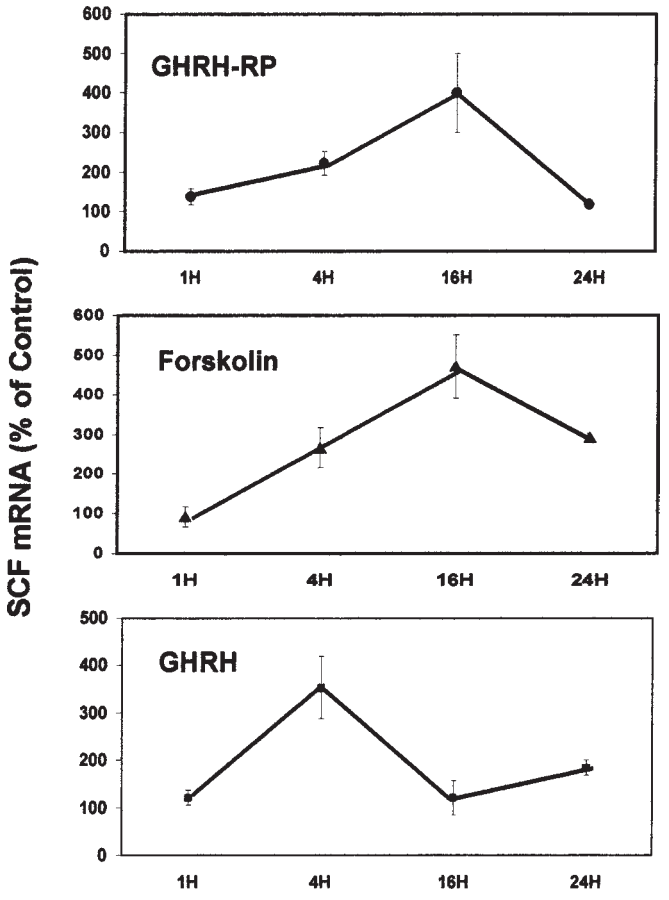

Fig. 1. Time course for the stimulation of SCF mRNA by GHRH-RP. Sertoli cells were cultured and treated for 1, 4, 16, and 24 h with GHRH-RP, GHRH (100 n*M* each), or forskolin (10 μ*M*, positive control), and Northern blotting was performed and the data quantified by densitometry. The results shown are the mean ± SEM of six replicates per treatment.

forskolin, which was used as a positive control. By contrast, GHRH appeared to induce a more rapid stimulation of SCF expression, with maximal SCF mRNA levels seen after 4 h of treatment.

Both GHRH-RP and GHRH significantly increased the steady-state levels of SCF mRNA in cultured Sertoli cells. Figure 2 shows a representative Northern blot of total RNA extracted from Sertoli cells treated with both peptides. GHRH-RP and GHRH increased SCF mRNA levels 2.6- and 3.5-fold, respectively, above those measured in the vehicle-treated control cells (Fig. 3). When cells were treated simultaneously with GHRH and GHRH-RP (RH + RP), no additive or synergistic effect was observed (Fig. 2). The truncated GHRH-RP peptide, p75-92NH2, was also shown to stimulate SCF mRNA. Treatment of Sertoli cells with this peptide increased SCF mRNA 2.7-fold over control values. Cells treated with PMA (12-O-tetradecanoyl-phorbol-13-acetate) (negative control) also failed to show any increase in SCF mRNA (data not shown).

To clarify whether the actions of GHRH-RP, p75-92NH2, or GHRH on the stimulation of SCF mRNA levels might be mediated by GH, Sertoli cells were treated with GH and RNA processed for Northern blot analysis. Figure 4 presents a representative Northern blot showing the effect of GH on SCF expression. Treatment with GH did not significantly increase SCF expression compared to the vehicle-treated cells (Fig. 4).

Transfection studies were used to determine which intracellular signaling pathways GHRH-RP and p75-92-NH2 activate. Sertoli cells were transfected with plasmid DNAs that monitor the stimulation of either protein kinase A (PKA) or Jun N-terminal kinase. Each of the DNAs used, pFA/cAMP-responsive element binding protein (CREB) and pFA/c-JUN, respectively, express a fusion protein composed of a pathway-specific transactivator fused to the yeast GAL-4 DNA-binding domain. If treatment of the cells results in the stimulation of either PKA or Jun N-terminal kinase, the transactivator region is phosphorylated, which induces the binding of the GAL-4 moiety to the pFR/luc reporter plasmid with subsequent luciferase expression.

The results of these transfection studies indicate that GHRH-RP and p75-92NH2 are both strong inducers of cAMP activity in Sertoli cells. GHRH-RP increased cAMP greater than threefold whereas p75-92NH2 induced an even greater stimulation of sixfold, identical to that seen with forskolin which was used as a positive control (Fig. 5). GHRH, previously shown to work through the PKA/cAMP pathway in pituitary somatotrophs for the stimulation of GH synthesis and release *(7)*, increased cAMP activity by 3.2-fold. None of the peptides examined had a significant effect on the activation of Jun N-terminal kinase (Fig. 6).

Discussion

We have previously reported that GHRH-RP stimulates SCF gene expression both in vitro and in vivo. In the present studies, we have further characterized this action and have shown that GHRH-RP as well as a truncated GHRH-RP peptide, p75-92NH2, and GHRH all significantly increase the steady-state levels of SCF mRNA. We also have shown that this increase in SCF is mediated through the stimulation of the PKA intracellular signaling pathway and that the time course of action for GHRH-RP and GHRH are significantly different. Finally, we have determined that the increase in SCF messenger RNA is not secondary to increased GH production, because GH had no direct effect on SCF expression in the Sertoli cell cultures.

The amino acid composition of the GHRH-RP and p75-92-NH2 peptides used for these experiments were determined based on the known sequence of the cDNA for preproGHRH in conjunction with studies that detailed the proteolytic processing of the GHRH precursor peptide. GHRH-RP is composed of the last 30 amino acids of the preproGHRH peptide and is generated subsequent to the endogenous formation of mature GHRH *(8,9)*. The sequence of p75-92-NH2 peptide, as previously mentioned, is composed of the

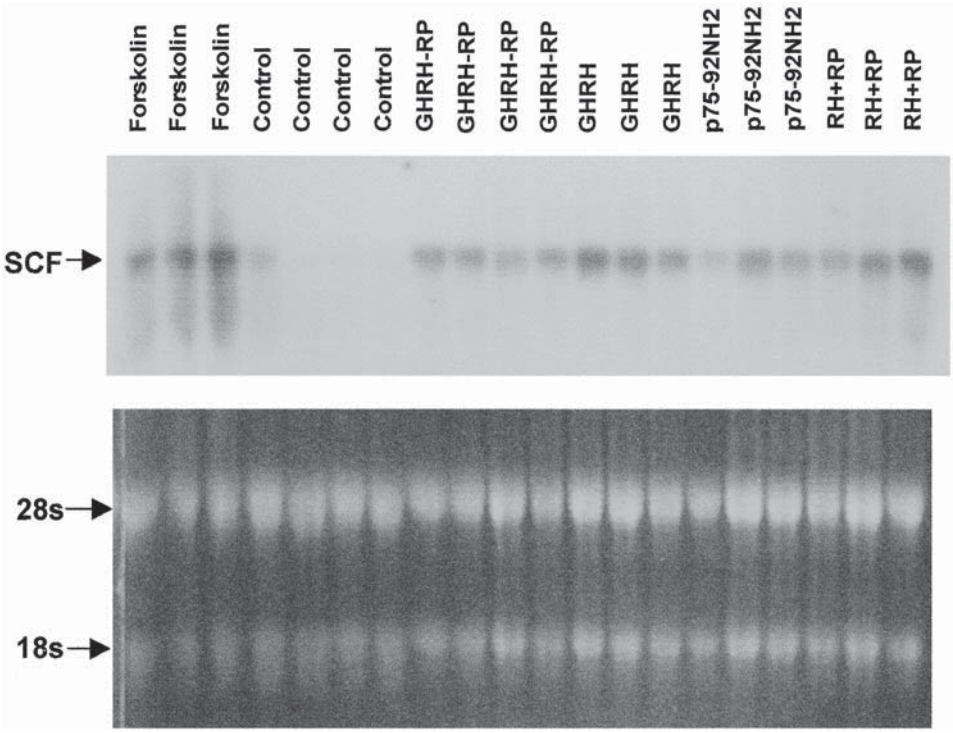

Fig. 2. Northern blot analysis showing SCF mRNA expression in cultured Sertoli cells treated for 6 hours with forskolin (10 μ*M*, positive control), vehicle (control), GHRH-RP, GHRH, p75-92NH2, and GHRH + GHRH-RP (RH + RP). All peptides were used at a final concentration of 100 n*M*).

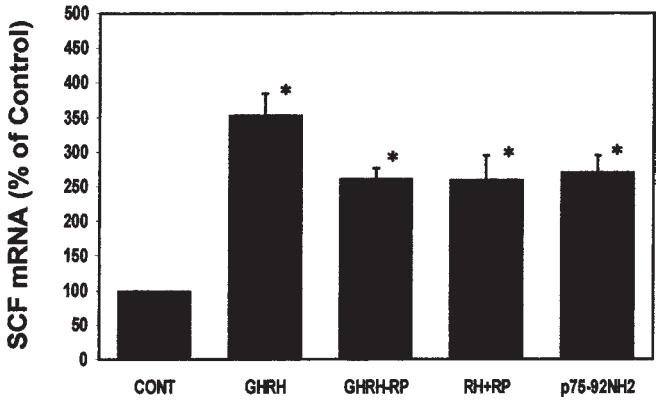

Fig. 3. Comparison of the effect of GHRH-RP, p75-92NH2, and GHRH on SCF mRNA levels in Sertoli cells (see Fig. 2 for details). Results shown are the mean ± SEM of two experiments each containing four to six replicates per treatment. $^{*}p < 0.05$. Cont (control). GHRH + GHRH-RP (RH + RP).

first 18 amino acid residues of GHRH-RP. This sequence was chosen based on the presence of the basic leucine residue at the 92 position of preproGHRH, which could serve as a likely proteolytic cleavage site *(10)* resulting in the generation of this truncated GHRH-RP peptide. Furthermore, in studies in where we described in detail the posttranslational processing of the GHRH precursor molecule in hypothalamic neurons, two peptide products, in addition to mature GHRH, were identified by pulse-chase analysis. Further investigation of

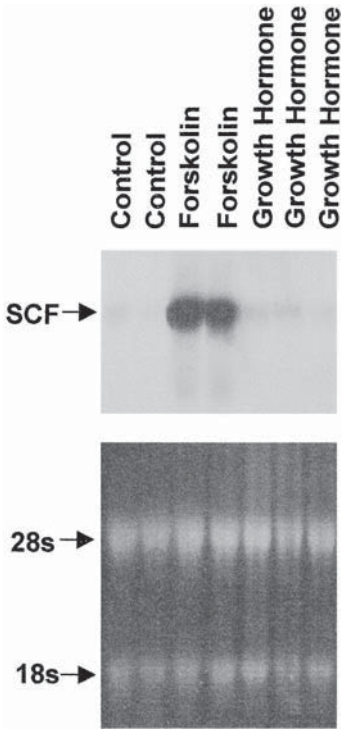

Fig. 4. Northern blot analysis showing the effect of GH on SCF expression in Sertoli cells treated for 4 h with vehicle (control; lanes 1 and 2), forskolin (10 μ*M*; lanes 3 and 4), or GH (100 n*M*; lanes 5–7). Results shown are from one of two separate Northern blot experiments performed, each showing identical results.

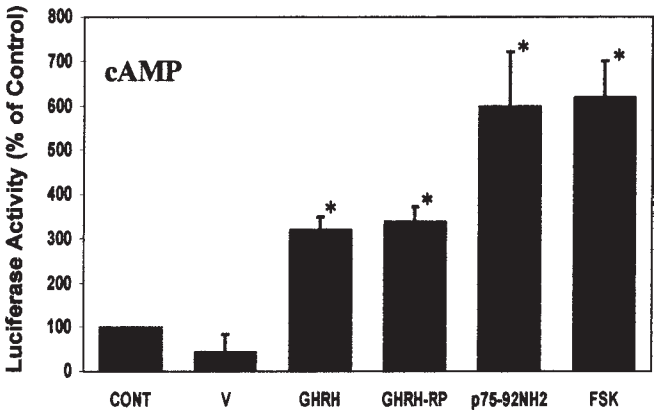

Fig. 5. Transient transfection studies showing the activation of PKA in cultured Sertoli cells treated for 7 h with vehicle (CONT), GHRH-RP, p75-92NH2, GHRH (100 n*M* each), or forskolin (FSK) (10 μ*M*). Sertoli cells transfected with pFRluc vector alone (V) served as control. Results shown are the mean ± SEM of three separate experiments with three transfections per treatment (p75-92a) $^{*}p < 0.05$.

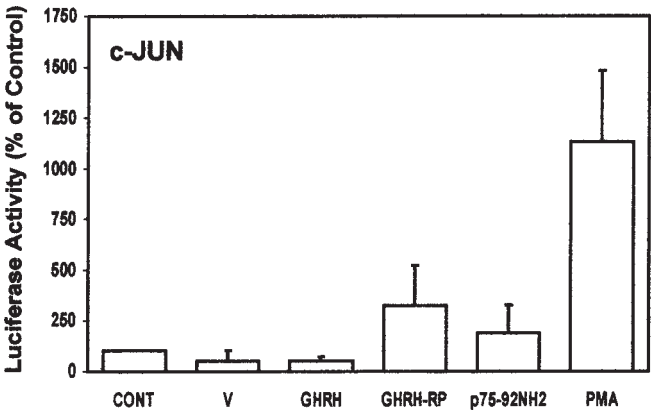

Fig. 6. Transfection studies showing the activation of the Jun N-terminal kinase follow treatment of Sertoli cells with vehicle (CONT), GHRH-RP, p75-92NH2, GHRH or PMA (see Fig. 5 for details). Sertoli cells transfected with pFRluc vector alone (V) served as control. Results shown are the mean ± SEM of two separate experiments with three transfections per treatment.

these peptides determined that the larger of these peptides was identical in size to synthetic GHRH-RP and was immunoprecipitated using specific GHRH-RP antiserum. The smaller peptide, approx 2 kDa, is comparable in size to the p75-92NH2 peptide used for the present studies *(6)*. However, the exact amino acid sequence of this endogenous smaller peptide is still unknown.

In the testis, Sertoli cells maintain intimate contact with developing germ cells and provide factors that directly regulate germ cell function. One such factor, SCF, has been shown to play an essential role in the survival, differentiation, and migration of gonadal germ cells *(11,12)* as well as in hematopoietic stem cells *(13)*. PreproGHRH is expressed in the gonads, germ cells, *(14–17)*, and hematopoietic tissue *(18–20)*. Thus, GHRH-RP and p75-92NH2 are also likely to be expressed in these tissues and could exert biological activity through a paracrine mechanism. Our findings that GHRH-RP and the p75-92-NH2 peptide both significantly increase SCF expression provides new information about the possible regulation of this important cytokine in the testis.

Testis-specific SCF gene expression has been shown to be under the control of pituitary follicle-stimulating hormone through elevation of intracellular cAMP levels *(21)* and by testosterone through its stimulatory effect on CREB expression *(22)*. Our finding that GHRH-RP and the p75-92NH2 peptide stimulate cAMP activity provides key information concerning the mechanism by which these peptides may also be regulators of SCF. Previously we were unable to demonstrate that GHRH-RP activates cAMP in Sertoli cells *(2)*. Discrepancies between the results reported here and those of the earlier study most likely reflect a markedly improved sensitivity in the methods employed in the current study.

In addition to the cAMP response element binding sequences within the SCF promoter region, several AP1 (Activator Protein-2) sites have also been identified *(23)*. Thus, we also explored the possibility that GHRH-RP or the p75-92NH2 peptide may act via the Jun N-terminal kinase pathway and its activation of the transcription factor c-jun. Results of these latter experiments indicate that this pathway is not likely to be involved in the GHRH, GHRH-RP, or p75-92NH2 stimulation of SCF gene expression in the testis. This, however, does not rule out that these peptides may activate additional intracellular signaling pathways in the testis as well as in other cell types. Further studies are necessary to identify fully the cell-specific signaling molecules that transduce the GHRH-RP and p75-92NH2 signals.

Materials And Methods

Peptides

The GHRH-RP peptide was synthesized based on the amino acid sequence of the C-terminal region (amino acids 75–104) of the preproGHRH molecule and has the following sequence: HLDRVWAEDKQMALESILQGFPRMKLSAEA. The second peptide, designated p75-92NH2, containing only the first 18 amino acids of GHRH-RP, has the amino acid sequence HLDRVWAEDKQMALESIL. The C-terminal leucine of p75-92NH2 was amidated. All peptides were synthesized by the American Peptide Company (Sunnyvale, CA).

Sertoli Cell Cultures

Sertoli cells were isolated from 20- to 22-d old male Sprague-Dawley rats using the method of Dorrington and Fritz *(24)* and as previously reported *(2)*. Briefly, whole testes were removed and decapsulated, and the seminiferous tubules were isolated by collagenase digestion (1 mg/mL in Ham's F12 media at 37°C for 10 min). The resulting seminiferous tubules were then minced, treated with trypsin (0.25% in Hank's bal-

anced salt solution [HBSS]) for 10 min at room temperature, washed, and redigested with collagenase for an additional 30–40 min to release the Sertoli cells. Sertoli cells were washed twice in HBSS and resuspended in serum-free Ham's F12 media supplemented with antibiotics and antimycotic mixture. All procedures using animals were approved by the Institutional Animal Care and Use Committee, Indiana University School of Medicine.

The Sertoli cells were plated either in 10-cm tissue culture dishes (4×10^6 cells/dish) for use in the Northern blot experiments or in 6-well tissue culture plates (4×10^5 cells/well) for use in the transfection studies. All cells were incubated in serum-free media for 4 d at 32°C and 5% CO_2. During this incubation period, contaminating germ or peritubular cells die off owing to the serum-free conditions in the culture media. This results in cultures that are almost exclusively composed of Sertoli cells. Fresh media was added on d 2 and 3 of incubation.

Northern Blot Analysis

On d 4 of incubation, cells were treated for various times with the following compounds: GHRH, GHRH-RP, p75-92NH2, GHRH + GHRH-RP (RH + RP), each at a final concentration of 100 n*M*. Forskolin (10 μ*M*) was used as a positive control. Total RNA was extracted using Tri-Reagent (MRI, Cincinnati, OH), separated by formaldehyde-agarose gel (1.2%) electrophoresis, transferred to a nylon membrane, and hybridized with a 600-bp, ^{32}P-labeled cDNA probe for SCF (kindly provided by Dr. David A. Williams, Indiana University) for 18 h at 42°C. Blots were washed and autoradiographed at –80°C for up to 4 d. Radiographs were quantified by phosphorimaging (Storm 860 scanner; Molecular Dynamics, Sunnyvale, CA, using ImageQuant software) and the results normalized to β-actin.

Transient Transfections

Following 4 d of incubation, the serum-free media was removed and replaced with OPTI-MEM media (Gibco, Rockville, MD). Sertoli cells were then cotransfected for 5 h at 32°C using Lipofectamine (4 μg/well) (Gibco), and the plasmid DNAs were provided in the PathDetect trans-reporting systems for evaluation of either PKA or Jun N-terminal kinase activation (Stratagene, La Jolla, CA). Briefly, cells (3.5×10^5/well) were cotransfected with 1 μg/well of the pFR/luc DNA and 50 ng/well of either the pFA/CREB or pFA/c-JUN plasmid DNAs, following the manufacturer's recommended protocol.

Following transfection, Sertoli cells were incubated for 18 h, and then the peptide treatments were added and the cells incubated for an additional 7 h. Next, cell lysates were prepared using the luciferase cell lysis reagent (Promega, Madison, WI) and luciferase activity was determined using the Promega Luciferase Assay system. Luminometry results were then normalized to total protein per well using the BCA Protein Assay Kit (Pierce, Rockford, IL) and graphed as relative light units.

Statisical Analysis

Results of the Northern analysis for the quantitation of GHRH-RP effect on SCF mRNA levels are reported as the mean ± SEM of two independent experiments each containing four to six replicates per treatment. Transfection experiments were repeated two or three times with three replicate transfections per treatment. Results of Northern blot and transfection studies were analyzed by student's *t*-test analysis.

Acknowledements

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