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AN EXPLANATION FOR THE DISPARATE EFFECTS OF SYNTHETIC PEPTIDES CORRESPONDING TO HUMAN FOLLICLE-STIMULATING HORMONE BETA-SUBUNIT RECEPTOR BINDING REGIONS (33-53) AND (81-95) AND THEIR SERINE ANALOGS ON STEROIDOGENESIS IN CULTURED RAT SERTOLI CELLS

Patricia Grasso[1], John W. Crabb[2] and Leo E. Reichert, Jr.[1]

[1]Department of Biochemistry and Molecular Biology
Albany Medical College
Albany, New York 12208

[2]W. Alton Jones Cell Science Center
Lake Placid, New York 12946

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SUMMARY: We have recently reported that synthetic peptide amides corresponding to regions of human FSH ß-subunit, hFSH-ß-(33-53) and hFSH-ß-(81-95), bind to receptor and stimulate estradiol biosynthesis by cultured rat Sertoli cells. Because of experimental difficulties caused by the presence of free sulfhydryl groups in these peptides, synthetic analogs were prepared in which all Cys residues were replaced with Ser. These analogs, [Ser-51]-hFSH-ß-(33-53) and [Ser-82,84,87,94]-hFSH-ß-(81-95), also bound to receptor but did not stimulate estradiol biosynthesis by cultured rat Sertoli cells. In order to explain this observation, we compared the effects of hFSH-ß-(33-53) and hFSH-ß-(81-95) and their Ser analogs on another recently recognized effect of FSH in Sertoli cells, namely its ability to promote influx of extracellular calcium. We and others have shown that estradiol biosynthesis by these cells is markedly decreased in the presence of high intracellular calcium. Cys-containing hFSH-ß-(33-53) and hFSH-ß-(81-95) did not increase influx of extracellular calcium over basal levels, whereas [Ser-51]-hFSH-ß-(33-53) and [Ser-82,84,87,94]-hFSH-ß-(81-95) induced 2.8- and 1.8-fold increases, respectively. Cellular cAMP and estradiol biosynthesis in reponse to each Ser-substituted peptide were not significantly different from basal levels. Thus, the explanation for the observed disparate effects of Cys and Ser analog peptides on estradiol biosynthesis may be related to the ability of the Ser peptides to stimulate calcium entry but not cAMP accumulation in cultured rat Sertoli cells.

The recently identified ability of FSH to bind calcium (1) and stimulate its entry into cultured rat Sertoli cells (2-4) suggests that FSH action in the testis may involve second messengers other than cAMP (5). Although the role of calcium in Sertoli cell function is not yet fully defined, its importance in FSH binding (6,7) and steroidogenesis (2,8) is becoming apparent. Using a synthetic peptide approach, we synthesized analogs of previously-identified receptor binding domains within the ß-

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subunit of hFSH (9,10) in which all Cys residues were replaced with Ser. We then tested the ability of the Ser analogs to stimulate uptake of extracelluar calcium (as $^{45}Ca^{2+}$), cAMP accumulation and androstenedione conversion to estradiol by cultured rat Sertoli cells. Our results indicate that, although sulfhydrl groups within these regions may not be essential for FSH binding to receptor (11), they do have the ability to modulate calcium influx and estradiol biosynthesis by cultured rat Sertoli cells.

METHODS: Peptide Synthesis. Synthetic peptide amides were synthesized as previously described by standard solid phase methodology (12) utilizing an Applied Biosystem model 430 automated synthesizer. Homogeneity of the peptides was demonstrated by analytical reverse-phase HPLC. Amino acid composition (13) and peptide sequence (14) were determined to verify the synthesis. The amino acid sequence of hFSH-ß-subunit was that provided by cloning data (15) and agreed with the sequence determined by microsequencing (16).

Sertoli Cell Culture and Isolation. Primary cultures of Sertoli cells were prepared from testes of 14- to 16-day old Sprague-Dawley rats as described in detail elsewhere (3).

Calcium Uptake by Cultured Rat Sertoli Cells. Sertoli cells were plated at a density of 1 x 10^6 viable cells/ml on glass coverslips (12-mm diameter) in 24-well tissue culture plates. Cultures were treated as indicated in the figure legends 72 h after plating. At the end of the treatment period, the medium was removed and the cultures were washed with 0.5 mM $LaCl_3$ to remove adhering $^{45}Ca^{2+}$ and to prevent efflux of accumulated $^{45}Ca^{2+}$ (17). The coverslips were transferred to 20-ml glass scintillation vials containing 10 ml Fluorosol (National Diagnostics, Manville, NJ) and incubated at 50 C overnight to solubilize the Sertoli cell monolayers. Radioactivity was counted in a Beckman LS 7500 counter with an efficiency of 100% for ^{45}Ca.

RESULTS: The amino acid sequences of synthetic peptide amides containing previously-identified receptor binding domains of the ß-subunit of hFSH (9,10) are shown in Fig. 1. Cys residues within these peptides (asterisks) were replaced with Ser. Calcium influx in response to [Ser-51]-hFSH-ß-(33-53) was 2.8-fold greater than basal levels, while [Ser-82,84,87,94]-hFSH-ß-(81-95) induced a 1.8-fold increase in calcium uptake (Fig. 2). Cys-containing peptide amides were unable to initiate a calcium response.

hFSH-β-(33-53) YTRDLVYKDPARPKIQKTCTF-amide

hFSH-β-(81-95) QCHCGKCDSDSTDCT-amide

Fig. 1. Amino acid sequences of hFSH-ß-(33-53) and hFSH-ß-(81-95) synthetic peptide amides. Cys residues are marked with asterisks (*) and indicate positions of Ser substitutions.

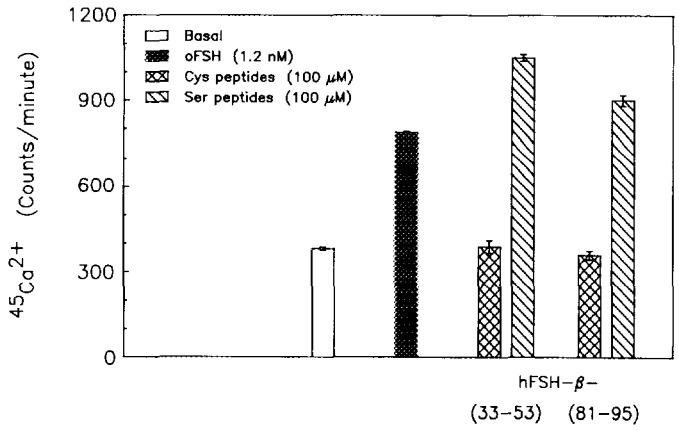

Fig. 2. Effects of Cys- and Ser-containing peptide amides corresponding to receptor binding domains of hFSH-ß-subunit on $^{45}Ca^{2+}$ uptake by cultured rat Sertoli cells. Sertoli cell monolayers were incubated in the absence or presence of 1.2 nM oFSH or 100 μM Cys- or Ser-containing peptide amides of hFSH-ß-(33-53) and hFSH-ß-(81-95) in DMEM/F12 labeled with 0.4 μCi $^{45}Ca^{2+}$ for 24 h at 37°C. Each bar and vertical line represents mean ± SD (n=4) $^{45}Ca^{2+}$ uptake during the 24-h culture period.

The effects of Ser substitution on estradiol biosynthesis and cAMP accumulation by cultured rat Sertoli cells are shown in Fig. 3. The dose-dependent agonist effect demonstrated for native sequence hFSH-ß-(33-53) (9) and hFSH-ß-(81-95) (10) on androstenedione conversion to estradiol could not be reproduced after Cys replacement. Ser-containing peptides, even at 100 uM concentration, were not able to increase estradiol biosynthesis (Fig. 3A) or elevate cellular cAMP (Fig. 3B) over basal levels.

DISCUSSION: Although we have previously shown that Cys residues present within receptor contact regions of the ß-subunit of hFSH are not required for binding of hormone to receptor (11), the influence of Cys on postbinding events reflecting transduction of the FSH signal are, for the most part, unknown. Our data suggest that Cys residues within these binding domains may be involved in regulation of FSH-stimulated calcium entry, cAMP accumulation and estradiol biosynthesis in Sertoli cells.

There is a substantial body of evidence indicating that the narrowly-regulated rise in cytosolic free calcium in Sertoli cells in response to FSH stimulation occurs via influx of extracellular calcium through voltage-sensitive and voltage-independent pathways (2-4, 18,19) which do not require elevation of cAMP (3). Using a synthetic peptide approach, we have recently shown that regions of the ß-subunit of hFSH, not identified as

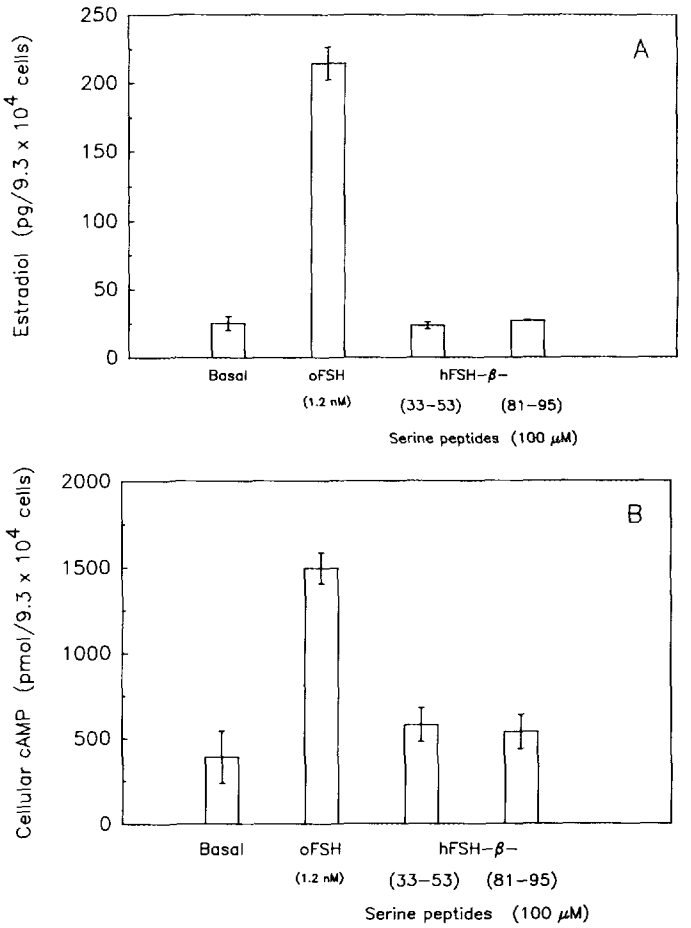

Fig. 3. **Effects of Ser-peptide amides corresponding to receptor binding domains of hFSH-ß-subunit on (A) estradiol biosynthesis and (B) cellular cAMP accumulation in cultured rat Sertoli cells.** Sertoli cell monolayers were incubated in the absence or presence of 1.2 nM oFSH or 100 μM Ser-substituted peptide amides of hFSH-ß-(33-53) or hFSH-ß-(81-95) in DMEM/F12 for (A) 24 h or (B) 1 h at 37° C. Estradiol content of spent media samples and cellular cAMP were measured by specific radioimmunoassays (3). Each bar and vertical line represents mean ± SD (n=4) estradiol biosynthesis during the 24-h culture period (A) and cellular cAMP accumulation during the 1-h culture period (B).

receptor contact regions, have the ability to induce entry of extracellular calcium into liposomes (4) and cultured rat Sertoli cells (19). Schiffer-Edmundson helical wheel projections suggested amphiphilic a-helical structures for these peptides (4), making penetration of the peptides into lipid bilayers and ionic permeability possible. Presumably, release of channel-forming peptides by lysosomal hydrolysis of FSH following receptor-mediated endocytosis of FSH-receptor complexes, contributes to the sustained phase of FSH-stimulated calcium influx observed in these cells (19).

Receptor-mediated calcium channel activity, as well as vesicular uptake of extracellular calcium which accompanies internalization of FSH-receptor complexes (19), would favor constitutive entry of high (millimolar) concentrations of calcium into the cytosol. Our data support a model which suggests that the presence of Cys residues within receptor binding domains of hFSH ß-subunit may, by way of the apparent negative influence of these residues on calcium influx, regulate calcium entry. In conjuction with calcium extrusion mechanisms (i.e., Ca^{2+}-ATPase) and mobilization to intracellular storage compartments (SER, mitochondria), it is conceivable that Cys residues within FSH receptor binding domains may contribute to maintaining optimal cytosolic calcium levels (nanomolar) for FSH action.

This notion is supported by (1) the observed ability of Ser-substituted peptides to promote uptake of extracellular calcium by cultured rat Sertoli cells and (2) the concomittant inhibitory effect of these peptides on cAMP accumulation and steroidogenesis. Ser substitution provides additional negative charges and potential calcium chelating residues within hydrophilic regions of these peptides, thereby increasing their calcium binding and transport capabilities. Apparently, these substitutions, alone or in conjuction with as yet undetermined conformational or other changes, result in the conversion of Cys-containing hFSH-ß-(33-53) and hFSH-ß-(81-95) into calcium-mobilizing analogs wich cannot induce estradiol biosynthesis. These observations suggest that the failure of Ser-substituted peptides to induce androstenedione conversion to estradiol may be related to their ability to enhance calcium uptake, since we (2) and others (8) have shown that high intracellular calcium inhibits estradiol biosynthesis.

The results of this study suggest that elevation of intracellular free calcium may exert autocrine negative control of testicular steroidogenesis, and that this effect may be modulated by the presence of Cys residues within receptor binding domains of the ß-subunit of FSH. Although the exact mechanism by which this regulation occurs is as yet undefined, our data suggest the possibility that the Ser analogs, presumably through their effects on calcium influx, may activate calcium-dependent phosphodiesterases known to be present in high concentrations in the immature rat Sertoli cell (5). Thus, no increase in cellular cAMP over basal levels was apparent. Assuming that changes in cAMP levels are coupled to changes in estradiol biosynthesis in response to FSH binding, then the observed failure of the Ser analogs to stimulate androstenedione conversion to estradiol may be due to their inability to induce accumulation of cAMP, an effect which may be mediated by elevations in cytosolic free calcium.

We report here that Ser analogs of receptor binding regions of hFSH ß-subunit promoted uptake of extracellular calcium by cultured rat Sertoli cells. These peptides, in contrast to their Cys-containing counterparts, were unable to elevate cellular cAMP or stimulate estradiol biosynthesis, suggesting that Cys residues in these regions are important for transducing the FSH signal. It is becoming increasingly apparent that Sertoli cell responsiveness to FSH stimulation may involve more than one second messenger. The effects of Ser analogs of hFSH ß-subunit receptor binding regions on calcium flux, cAMP accumulation and estradiol biosynthesis presented in this study provide additional evidence for the involvement of calcium in FSH signal transduction.

Multiple control of intracellular events in steroidogenic tissues, i.e., activation of adenylate cyclase at the plasma membrane and elevation of cytosolic calcium by proteolytic fragments of internalized FSH, may be related to the large size of FSH (and each of the other glycoprotein hormones) compared to other G-protein linked ligands, such as catecholamines. Proteolytic fragments of internalized FSH may also have other cytosolic and /or nuclear targets. The results of this and earlier studies (4,19) suggest that different domains of FSH ß-subunit may exert physiologic regulatory functions associated with estradiol biosynthesis by cultured rat Sertoli cells.

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