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Modulation of the steroidogenic related activity according to the design of single-chain bovine FSH analogs ☆

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ABSTRACT

Single-chain (SC) gonadotropins have been genetically engineered to increase the repertoire of analogs for potential use in humans and domestic animals. The major aim of the current study was to examine the steroidogenic related activity of SC FSH analogs carrying structural differences. To address this issue, we designed and expressed three SC bovine FSH analogs in CHO cells: (i) FSHβα in which the tethered subunit domains are linked in tandem; (ii) FSHβCTPα that contains the carboxy terminal peptide (CTP) of the human choriogonadotropin (hCG) β subunit as a spacer, and (iii) FSHβboCTPα in which the linker is derived from a CTP-like sequence (boCTP) decoded from the bovine LHβ DNA. The data suggested that the secretion efficiency of these variants from the transfected cells was unaffected by the presence or absence of the CTP linker, N-glycans were attached to the analogs and the hCGβ-CTP domain in the FSHβCTPα variant was O-glycosylated. In a rat immortalized granulosa cell bioassay the potency of the three variants towards progesterone secretion varied. In immature mice, the analogs increased the ovary weight and induced *StAR*, *Cyp11a* (P450scc), *Cyp17* (P450c17) and *Cyp19* (P450aromatase) transcripts. However, the dose dependence and amplitude of these transcript levels differed in response to FSHβα, FSHβboCTPα and FSHβCTPα. Collectively, these data suggest that the design of the FSH analog can modulate the bioactivity *in vitro* and *in vivo*. A systematic analysis of receptor activation with ligands carrying structural differences may identify new regulatory factor/s involved in the pleiotropic FSH activity.

1. Introduction

The gonadotropins luteinizing hormone (LH) and follicle stimulating hormone (FSH) are non-covalent heterodimers composed of the common α and the specific β subunit that confer receptor specificity (Pierce and Parsons, 1981). These hormones are members of the glycoprotein hormone family and are key regulators of steroidogenesis in the gonads. Despite the combination of structural and biochemical analyses now available, the relationship between the structure of the gonadotropins and the activity on multiple factors involved in the process of steroid hormone production is not completely understood. This issue is intriguing because the fine-tuning of the steroidogenic milieu in the ovary is crucial for the homeostasis and achieving this is a major challenge in assisted reproduction protocols.

It is well known that the endogenous gonadotropins and those administered in assisted reproduction protocols exist as a mixed glycoforms (Birken et al., 2001, 1996; Burgon et al., 1996; Chappel, 1995; D'Antonio et al., 1999; Keel et al., 1994; Keutmann et al., 1979; Pantel et al., 1998; Sairam and Li, 1975; Shome and Parlow, 1973; Stanton et al., 1996; Stockell Hartree and Shownkeen, 1991; Toll et al., 2006; Ulloa-Aguirre et al., 1999). In addition, the gonadotropins, especially LH and hCG, are converted in the body to variants with truncations in their protein backbone, further increasing the heterogeneity of the hormone population circulating in the body (Birken et al., 2001, 1996; Burgon et al., 1996; Chappel, 1995; D'Antonio et al., 1999; Keel et al., 1994; Keutmann et al., 1979; Pantel et al., 1998; Sairam and Li, 1975; Shome and Parlow, 1973; Stanton et al., 1996; Stockell Hartree and Shownkeen, 1991; Toll et al., 2006; Ulloa-Aguirre et al., 1999). Microheterogeneity of the amino and carboxy regions of the FSHβ subunits has also been reported (Fujiki et al., 1978; Saxena and Rathnam, 1976; Shome et al., 1988). Therefore, examining whether structural changes in the peptide backbone and carbohydrates of the ligand have consequences on the steroidogenic activity is relevant to further understand the

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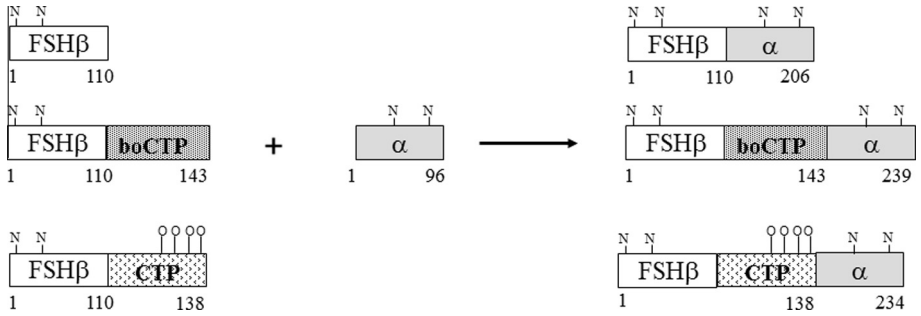

Scheme 1. The design of the SC FSH analogs. The bovine α (shaded box) and FSHβ subunit (open box) were genetically fused in tandem. The CTP domain of the hCGβ subunit (dotted box) and the CTP-like sequence cashed in the bovine LHβ gene (grey box) were used as linkers. The numbers on the top of the diagrams refer to amino acids, continuously numbered in the SC variants. The presence of N-linked (N) and O-linked (O) glycans are indicated.

physiology and pharmacology of the gonadotropins. In the current study we exploited the single-chain[1] (SC) gonadotropin approach to design bovine FSH analogs with structural changes and study their intracellular behavior and bioactivity *in vitro* and *in vivo*.

As a model to study structure–function analyses and for the purpose of analog design, bioactive SC gonadotropins from several species were previously genetically engineered, by linking the subunit genes in tandem (Aizen et al., 2007; Ben-Menahem, 2004; Dirnberger et al., 2001; Fidler et al., 2003; Garcia-Campayo and Boime, 2001; Kato et al., 1998; Narayan et al., 1995; Sato et al., 1997; Sugahara et al., 1995, 1996b). The generation of variants in the presence or absence of a linker sequence to space the tethered subunit domains, as well as switching their relative position, enabled to generate SC gonadotropin analogs with differences in the conformation (Argos, 1990; Ben-Menahem et al., 2001, 1997; Garcia-Campayo and Boime, 2001; Grinberg et al., 2008; Sugahara et al., 1995, 1996b). Interestingly, gonadotropin analogs, mostly SC variants but also mutated heterodimers and nonpeptide mimetics, with a conformation that is different from that of the wild-type heterodimer, bound to the cognate receptor and stimulated cAMP formation, which is a key signaling mediator for steroidogenesis (Ben-Menahem et al., 1997; Fralish et al., 2003; Garcia-Campayo and Boime, 2001; Grossmann et al., 1997; Heikoop et al., 1997; Jackson et al., 1999; Maclean et al., 2004; Moyle et al., 1987; Perlman et al., 2003; Sato et al., 1997; Sugahara et al., 1995; van Straten et al., 2002; Weenen et al., 2004; Xing et al., 2001). These and additional studies indicated that not all the interactions between the α and β subunits as seen in the crystal structure of the heterodimer are mandatory for receptor binding and activation (Bhowmick et al., 1996; Fan and Hendrickson, 2005; Fox et al., 2001; Lapthorn et al., 1994). This is consistence with the notion that permissiveness exists in ligand–receptor interactions, at least as related to receptor binding and the generation of cAMP (Ben-Menahem et al., 1997; Fralish et al., 2003; Hiro'oka et al., 2000; Jackson et al., 1999; Moyle et al., 1987; Sato et al., 1997). However, the outcome of receptor activation with ligands that differ in their structure on steroidogenesis is less clear, and is highly relevant to fertility because of the heterogenous nature of the gonadotropins and the importance of the steroidogenic milieu for reproduction. In a previous study with SC LH analogs with a different configuration we observed different effects on the production of two major steroids in bovine theca cells (Grinberg et al., 2008). That previous study with SC bovine LH variants in the arrangement of NH_2-α-CTP-LHβ-COOH (denoted as αCTPLHβ analog) and NH_2-LHβ-CTP-α-COOH (LHβCTPα variant) showed that these analogs elicited a different androstenedione/progesterone ratio in theca cells (Grinberg et al., 2008).

This information prompted us to study the steroidogenic activity of bovine FSH analogs with structural differences. For this purpose, we designed SC FSH analogs, in the absence or presence of a linker sequence to space the tethered subunit domains, and generated analogs with differences in the backbone and glycosylation of the linker (Scheme 1). As linkers we used the carboxy terminal peptide of the human CGβ subunit (denoted as CTP; (1)) and the cryptic CTP-like sequence we previously decoded from the bovine LHβ DNA (denoted as boCTP; (Nakav et al., 2006, 2005). The "linkerless" NH_2-FSHβ-α-COOH (FSHβα), and the spaced FSHβboCTPα (both had N-linked glycans) and FSHβCTPα (carrying N- and O-glycans) analogs were expressed in CHO cells. Several parameters were evaluated, including secretion efficiency from the transfected cells, activity *in vitro* and in mice.

2. Material and methods

2.1. Materials

Modifying and restriction enzymes were purchased from MBI Fermentas (Vilnius, Lithuania). Oligonucleotides used for PCR amplification and sequencing were purchased from Danyel Biotech (Rehovot, Israel) and Sigma (Rehovot, Israel). Polyethylenimine (PEI) for transfection and the N-glycosylation inhibitor tunicamycin were from Sigma (St. Louis, USA), and forskolin (an adenylyl cyclase activator) was from Biomol (USA). [^{35}S]-cysteine plus methionine mixture (Pro-mix) was purchased from Amersham Biosciences (UK). Media and reagents for tissue culture were from Biological Industries (Beit Haemek, Israel), except F-12 Ham's (Invitrogen Life Sciences, USA). Antisera against ovine FSH (AFPC5288113 Rb), and the pituitary bovine heterodimeric FSH (AFP5332B) were from the Pituitary Hormone Program of the NIH (Torrance, CA, USA) and provided by Dr. Albert Parlow. Pregnant mare serum gonadotropin (PMSG) and human chorionic gonadotropin (hCG) were purchased from Sigma. The anti ovine (o) FSH-α antibody (F64) was a generous gift from Dr. Stanley Lun (Wallaceville Animal Research Center, Upper Hutt, New Zealand) (Fidler et al., 2003). The monoclonal antibody (mAb) CTP104 raised against the O-glycosylated CTP of the hCGβ subunit was kindly provided by Dr. Steven Birken (formerly at Columbia university, New York City, NY, USA) (Birken et al., 2003; Krichevsky et al., 1994). Progesterone antibody was a gift from Dr. Fortune Kohen (Weizmann Institute of Science, Rehovot, Israel). For immunoprecipitation, Normal Rabbit Serum (NRS) and Pansorbin were purchased from Calbiochem and Sigma, respectively.

2.2. Methods

2.2.1. Mutagenesis and vector construction

The cDNAs of the bovine FSHβ and α subunits were used as templates for the engineering of subunit and SC variants, using

[1] *Abbreviation:* SC, single-chain.