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Effect of ovine luteinizing hormone (oLH) charge isoforms on VEGF and cAMP production☆

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ABSTRACT

Although an increase in VEGF expression and synthesis in association with LH has been established; it is unknown if all LH isoforms act similarly. This study evaluated the production of cAMP and VEGF among LH isoforms in two *in vitro* bioassays. The LH was obtained from hypophyses and the group of isoforms was isolated by chromatofocusing. cAMP production was assessed using the *in vitro* bioassay of HEK-293 cells and VEGF production was evaluated in granulosa cells. Immunological activity was measured with a homologous RIA. Immunoactivity and bioactivity for each isoform were compared against a standard, by estimating the IC_{50} and the EC_{50}. The basic isoforms were more immunoactive than the standard. The neutral and the moderately acidic had an immunological activity similar to the standard. The acidic isoform was the least immunoreactive. cAMP production at the EC_{50} dose was similar among the basic isoforms, the moderately acidic and the standard; for the neutral and the acidic, the EC_{50} dose was higher. It was observed that compared with the control, VEGF production at the lowest LH dose was no different in the standard and each isoform. In the intermediate dose, a positive response was caused in the standard and the neutral and basic isoforms. Although the acidic isoform showed a dose-dependent response, it was not significant relative to the control. In conclusion, the basic isoform generated the greatest cAMP and VEGF production, similar to the reference standard, and the acidic the smallest.

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1. Introduction

In ruminants, several waves of follicular growth occur during each estrous cycle. This process begins with the recruitment of a cohort or group of follicles within which selected follicles will continue to develop until becoming dominant. At that moment, their dependency on FSH decreases but increases for LH (reviewed by Webb and Campbell, 2007).

LH has been associated with the angiogenic process observed in the preexisting perifollicular vasculature of dominant follicle (Berisha et al., 2008; Chowdhury et al., 2010).

Follicular angiogenesis is partially regulated by growth factors, with the vascular endothelial growth factor (VEGF) being the main actor (reviewed by Kaczmarek et al., 2005). At follicular level, VEGF takes part in cell proliferation and survival, and it also induces changes in vascular endothelial permeability (Taylor et al., 2007). These changes have an impact on blood supply to the follicle, thus providing the necessary elements to ensure its final development and transition from dominant to preovulatory follicle (reviewed by Rocha-Araujo et al., 2011).

In ruminants, the predominant isoform is VEGF-A_{164}, whose expression and synthesis takes place mainly in granulosa cells of the dominant follicle (Chowdhury et al., 2010; Berisha et al., 2000; Greenaway et al., 2004). LH participation in VEGF production has been reported in various *in vitro* studies, where VEGF synthesis and expression by granulosa cells increases after treatment with hCG (Lee et al., 1997), human LH (Guimera et al., 2009) and bovine LH (Babitha et al., 2014); additionally, *in vivo* studies have demonstrated that the increase in VEGF in the follicular fluid of dominant follicles occurs after being treated with hCG (Hazzard et al., 1999). Particularly in sheep, immunohistochemistry and *in situ* hybridization studies on intraovarian structures have demonstrated that VEGF expression in follicles depends on the stage of the estrous cycle and follicular size (Chowdhury et al., 2010). In bovines with synchronized estruses, and hyperstimulated with FSH and treated with GnRH, it was observed that granulosa cells of preovulatory follicles showed a greater expression of the VEGF gene during the preovulatory LH surge (Berisha et al., 2008). Furthermore, marmoset monkeys treated with an antagonist to GnRH to avoid preovulatory LH secretion showed a reduction in the proliferation of endothelial cells, vascular density, and in VEGF expression in the granulosa cells of the preovulatory follicles (Taylor et al., 2004).

LH is a heterodimeric glycoprotein which, depending on the arrangement of its oligosaccharides, shows several isoforms that have physical–chemical, immunological and biological potency (Baenziger and Green, 1988; reviewed by Cooke et al., 1996; reviewed by Manzella et al., 1996; reviewed by Perera-Marin et al., 2007). In ruminants, it has been observed that the participation of different isoforms in circulating LH varies depending on the stage of the estrous cycle (Rojas-Maya et al., 2007; Arrieta et al., 2006; Perera-Marin et al., 2005).

Given the relationship between LH and VEGF, and the existence of different isoforms of the gonadotropin, this study was conducted to compare under standardized *in vitro* conditions the effect of different LH isoforms on VEGF production in granulosa cells obtained from dominant follicles in sheep.

2. Materials and methods

To carry out this work and prior to the development of the *in vitro* bioassay with granulosa cells from dominant ovarian follicles in ewes, it was necessary to obtain the LH isoforms in sufficient quantities. Ovine adenohypophyses were obtained and then subjected to chromatofocusing to separate and collect the isoforms. By means of the assay in HEK-293 cells transfected with cDNA for the rat LH receptor, the isoelectric point, immunological activity (homologous RIA for LH) and *in vitro* biological activity were determined for every isoform.

2.1. Collection of pituitary gland

Pituitary glands from healthy adult sheep were collected. Prior to slaughter, each animal was stunned with a captive bolt gun and its head removed. The pituitary glands were collected and deposited in phosphate buffer (50 mM, pH 7.2) for transport. The pituitary glands were cleaned of surrounding tissue, and the lobe anterior was stored at −80 °C until processing. Every LH extraction and purification step was performed at 4 °C.

2.2. Obtaining of glycoprotein extract from the lobe anterior

The glycoprotein extract (GPE) was extracted from ovine lobe anterior according to the method described for bovine GPE (Perera-Marin et al., 2004). First, 200 adenohypophyses with a wet weight of approximately 105 g were homogenized in 10% ammonium acetate solution, pH 7.0, containing 10.0 mM phenylmethylsulfonyl fluoride (PMSF) with a ratio of 10 ml of solution per gram of wet tissue.

Once the homogenate was obtained, it was mechanically stirred and 24 h later it was centrifuged at 12,000 × *g* for 45 min and the precipitate obtained after centrifugation (R0) was discarded and the supernatant received an ethanol volume corresponding to 40% of total volume. The ethanol suspension was stirred for 24 h and then centrifuged. The proteins precipitated (R1) after centrifugation was discarded and the supernatant received a volume of pure ethanol corresponding to 85% of total volume. This suspension remained undisturbed for 48 h, after which the supernatant was discarded and the precipitated proteins were collected by centrifugation. This group of proteins was named glycoprotein extract (GPE).

The GPE was re-suspended in deionized water, dialyzed (Spectra/Por # 4, cut-off 12–14 kDa) for 24 h with the water changed every 8 h, and, after that, lyophilized for later processing.

2.3. Glycoprotein extract purification

Of total GPE, only 285 mg of protein were purified in the CM-Sepharose cation exchanger in conditions similar to those described for bovine GPE (Perera-Marin et al.,