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Angiogenic Activity of Swine Granulosa Cells: Effects of Hypoxia and the Role of VEGF

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Abbreviations: AOC, porcine aortic endothelial cells; HIF-1α, hypoxia inducible factor-1α; MC, microcarrier; VEGF, vascular endothelial growth factor

INTRODUCTION

Angiogenesis is a crucial event during follicular development driven by a variety of factors among which vascular endothelial growth factor (VEGF) likely plays a major role. Hypoxia is a powerful stimulus to VEGF production by means of hypoxia inducible factor-1α (HIF-1α), a factor that is rapidly degraded by cellular peroxisomes under normoxic conditions, and is instead stabilized in hypoxia. The granulosa layer, despite being avascular, appears to be involved in the control of follicular angiogenesis: as a matter of fact, granulosa cells have been identified as the main component involved in VEGF production both *in vivo* (Mattioli *et al.*, 2001) and *in vitro* (Grasselli *et al.*, 2002). Previous studies have shown that follicular growth is accompanied by a reduction in the pO_2 in follicular fluid and by an increase in VEGF production by granulosa cells (Basini *et al.*, 2004). The aim of the current study was to investigate the modulatory effects of hypoxia on swine granulosa cell angiogenic activity and the role of VEGF in the control of follicular angiogenesis. To this end, after incubation of granulosa cells under hypoxic conditions, we studied the effects of media conditioned by them on porcine aortic endothelial cell (AOC) growth in a three-dimensional fibrin gel matrix (Grasselli *et al.*, 2003). In order to verify the possible role played by VEGF produced by granulosa cells under these conditions, we evaluated AOC growth in the presence or absence of a VEGF inhibitor, the VEGF-TrapR1R2 (Wulff *et al.*, 2002).

MATERIALS AND METHODS

After collection of swine ovaries at a local abattoir, 10^6 granulosa cells from follicles (>5 mm) were seeded in 24-well plates and cultured under standard conditions for 24 h. After a subsequent 18-h incubation under normoxic (19% O_2), partial (5% O_2) or total

(1% O_2) hypoxic conditions, their culture media were collected. An *in vitro* angiogenesis assay was performed by pipetting a suspension of microcarrier (MC) coated by AOC into a fibrinogen solution (1 mg/ml) before the addition of thrombin (250 µl) to catalyse gel formation. Gels were treated with granulosa cell conditioned media for 192 h in the presence or absence of VEGF-TrapR1R2 (150 ng/ml). Media were changed at 48 h intervals and pictures of AOC were taken in order to evaluate their proliferation using Scion Image Beta software (Scion Corporation, MA, USA, http://rsb.info.nih.gov/nih-image/). Data are expressed as means ± SEM of five independent experiments, and statistical analysis was performed by means of multifactorial ANOVA (Statgraphics package, STSC Inc., Rockville, MD, USA). When significant differences were found, means were compared by Scheffe' F-test; p-values <0.05 were considered to be statistically significant.

RESULTS

A significant ($p < 0.01$) and constant increase in AOC proliferation in controls was observed during the 192 h of culture: the area covered by endothelial cells was enhanced by 25% on each evaluation. AOC incubated with media from granulosa cells subjected to normoxic conditions showed a growth rate similar to controls (AOC incubated with non-conditioned media) at each time of evaluation. Media under partial hypoxic conditions did not modify AOC growth during the first 48 h, while they significantly ($p < 0.01$) stimulated AOC proliferation during subsequent incubations. Media from granulosa cells in total hypoxia significantly ($p < 0.01$) enhanced AOC growth as compared to controls at each evaluation time. The addition of TrapR1R2 significantly ($p < 0.01$) reduced AOC growth induced by conditioned media.

CONCLUSIONS

Our results suggest that hypoxia represents an important regulatory factor of the follicular angiogenic process, probably by enhancing the VEGF-dependent angiogenic activity of granulosa cells. On this basis and according to our previous results (Basini *et al*., 2004), we can assume that a strong relationship between the pO_2 decrease in the follicular fluid, VEGF levels and the development of a proper thecal vascular sheath within the growing follicle. Although VEGF production by granulosa cells has been widely documented, our data show that under normoxic conditions these cells are devoid of any significant angiogenic activity. It is likely that the onset of an adequate vascular network in the developing follicle relies on the onset of an hypoxic environment, while under conditions of normal oxygen availability VEGF production by granulosa cells may not be sufficiently stimulated. The results obtained in our *in vitro* angiogenic model by the use of VEGF-TrapR1R2, a strong VEGF inhibitor (Wulff *et al*., 2002), strengthen the hypothesis that the enhanced granulosa cell angiogenic activity under hypoxic conditions depends primarily on increased VEGF levels. As a whole, these data confirm that the granulosa layer, despite being avascular, plays a pivotal role in the development of a thecal vascular sheath in the growing follicle through VEGF-dependent mechanisms.

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