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Expression and functional activity of PACAP and its receptors on cumulus cells: Effects on oocyte maturation

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Pituitary adenylate cyclase-activating polypeptide (PACAP) and its receptor PAC1-R (PACAP type 1 receptor) are transiently expressed in granulosa cells (GCs) of mouse preovulatory follicles and affect several parameters associated with the ovulatory process. We investigated the expression of PACAP and its receptors in cumulus cells (CCs) after the LH surge and their role on cumulus expansion/apoptosis and oocyte maturation. PACAP and PAC1-R expression increased in CCs isolated at different times after treatment with human chorionic gonadotropin (hCG). Moreover, PACAP was able to reverse the inhibition of oocyte meiotic maturation caused by hypoxantine in cumulus cell-oocyte complexes (COCs) and efficiently promoted male pronuclear formation after fertilisation. PACAP was also able to induce cumulus expansion and prevent CC apoptosis. Our results demonstrated the induction of PACAP and its receptors in CCs by LH and EGF, suggesting that PACAP may play a significant role in the complex interactions of gonadotropin and growth factors during ovulation and fertilisation.

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1. Introduction

Mammalian oocytes are arrested at the dictyate stage until shortly before ovulation. After the preovulatory LH surge, the cumulus expands, and the oocyte resumes meiotic maturation and becomes fertilisable (Baker, 1972). In addition to gonadotropins, a large number of growth factors modulate oocyte maturation. Most of these factors do not directly affect the oocyte but exert their activity via cumulus cells (CCs) (Apa et al., 1994, 1995; Canipari, 2000; Canipari et al., 2012). It is well known that mouse CCs express few or no LH-Rs and are unable to directly respond to LH. Among these factors, EGF-like factors, namely amphiregulin (AREG), epiregulin (EPI), and beta-cellulin (BTC), have been demonstrated to be involved in CC functions and to mediate LH effects on CCs (Park et al., 2004). Furthermore, granulosa cells (GCs) respond to LH by secreting these EGF-like factors, which in turn act on CCs to mediate cumulus expansion. Moreover, inhibitors of EGF-Rs dramatically suppress cumulus expansion. All of these factors may affect each other, and their subsequent regulatory interactions are quite complex.

Several studies have demonstrated that peptides, such as pituitary adenylate cyclase-activating polypeptide (PACAP), vasoactive intestinal polypeptide (VIP) and growth hormone-releasing factor (GRF), affect important ovarian functions (Canipari et al., 2012; Reglodi et al., 2012; Thomas et al., 2012). PACAP is expressed in a large variety of species ranging from zebrafish (Wang et al., 2003) to humans (Morelli et al., 2008; Park et al., 2010), and the presence of PACAP has also been demonstrated in human follicular fluids (FFs) (Brubel et al., 2011) where it has been inversely correlated to the number of oocytes retrieved after superovulation treatment (Koppan et al., 2012).

PACAP is a member of the secretin–glucagon–VIP family of peptides. In its mature form, it is expressed in two alternative and tissue-specific polypeptides, PACAP-27 and PACAP-38, which share the same 27 amino acids at the N-terminal and are generated from the proteolytic digestion of a single 127-amino acid precursor (Arimura, 1992a). PACAPs, VIP and their receptors have been found in many organs, tissues, and cell types, including the lung, testis, adrenal gland, and ovary (Arimura, 1992b; Gottschall et al., 1990;

Abbreviations: CCs, cumulus cells; COCs, cumulus cell-oocyte complexes; GCs, granulosa cells; GV, germinal vesicle; GVBD, germinal vesicle breakdown; PB, polar body; 2PN, 2 pronuclei; Met II, metaphase II; IVM, in vitro maturation; IVF, in vitro fertilization; FF, follicular fluid; AREG, amphiregulin; EPI, epiregulin; BTC, betacellulin; Hx, hypoxanthine.

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Sherwood et al., 2000). PACAP and VIP are recognised by three distinct G protein-coupled receptors: PACAP type 1 receptor (PAC1-R), which displays a lower affinity for VIP; and VIP type 1 and 2 receptors (VPAC1-R and VPAC2-R), which bind the two peptides with equivalent affinity (Lutz et al., 1993). In their active forms, all three receptors trigger the adenylate cyclase signalling pathway, whereas only the PAC1-R appears to be coupled to the phospholipase C pathway (Spengler et al., 1993).

In the mammalian ovary, PACAP promotes steroidogenesis, cAMP production, and plasminogen activator synthesis in rat granulosa cells (Apa et al., 2002; Heindel et al., 1996; Zhong and Kasson, 1994) and reduces the rate of apoptosis (Barberi et al., 2007; Lee et al., 1999; Morelli et al., 2008) in the preovulatory phase in rat, mouse and human GCs. Moreover, PACAP also accelerates the kinetics of meiotic maturation in cumulus-enclosed rat oocytes (Apa et al., 1997).

The increased expression in vivo of PAC1-R in GCs after human chorionic gonadotropin (hCG) administration may reflect an involvement of PACAP in the mediation of gonadotropin activity during the periovulatory period (Vaccari et al., 2006). In mice, the enhanced expression of the PACAP/PAC1-R system in preovulatory follicles after hCG stimulation and the anti-apoptotic effect of PACAP and VIP on GCs in vitro suggest that PACAP may be involved in a more extensive and general regulation of follicle development (Barberi et al., 2007; Morelli et al., 2008; Vaccari et al., 2006).

To further characterise the PACAP/receptor system in the mouse preovulatory follicle, this study aimed to verify the presence of this peptide and its receptor transcripts in mouse cumulus cells, as well as its effect on cumulus expansion and oocyte nuclear and cytoplasmic maturation.

2. Materials and methods

2.1. Animals

Immature CD1 mice (Charles River, Como, Italy) were housed under controlled temperature (25 °C) and light (12 h light/day) conditions with ad libitum access to food and water. Twenty-one-day-old immature mice were injected i.p. with 7 i.u. pregnant mare's serum gonadotropin (PMSG, Intervet, Milan, Italy) to enhance multiple follicular development. After 46–48 h, the PMSG-primed animals were either sacrificed by cervical dislocation or injected with 5 i.u. human chorionic gonadotrophin (hCG, Intervet). The animals were maintained in accordance with the Italian Department of Health Guide for Care and Use of Laboratory Animals. The experimental protocols were approved by the 'La Sapienza' University Committee for Animal Care and Use.

2.2. Isolation and culture of intact cumulus cell–oocyte complexes (COCs), CCs and mural granulosa cells

We performed in vivo studies in which PMSG-primed animals were injected with hCG, and the ovaries and oviducts were excised at different times after hCG injection (respectively, from 0 to 9 h and at 17 h after hCG injection). GCs and COCs were obtained as previously described (Barberi et al., 2007). Briefly, the largest follicles from each ovary were punctured with a 25-gauge needle and gently pressed to release the cells or by cutting and gently squeezing the oviducts. The complexes were removed with a fine-bore Pasteur pipette, and the remaining GCs were centrifuged at 250*g* for 5 min. CCs were dissociated from the oocytes by serial passages through micropipettes, as previously described (Canipari et al., 1995). CCs and GCs were resuspended in lysis buffer for RNA extraction (RNeasy Kit, Qiagen S.p.A, Milano, Italy).

We also performed in vitro studies using intact COCs or GCs that were isolated from PMSG-primed mice either before (t0) or 1 h after hCG injection and cultured in Dulbecco's Modified Eagle Medium (DMEM, Gibco) buffered with 25 mM HEPES and supplemented with 2 mM glutamine, antibiotics (100 mM penicillin, 100 μg/ml streptomycin), 100 mM MEM-non-essential amino acids and 0.23 mM pyruvate, 1 mg/ml BSA or 1% FCS. The GCs were cultured at a density of 1×10^6 per ml. Groups of 30 COCs were transferred into 30-μl drops of medium and covered with mineral oil (Sigma) to prevent evaporation. After culturing, the CCs were dissociated from the oocytes. The GCs and CCs were then either resuspended in lysis buffer for RNA extraction or fixed for the evaluation of apoptosis.

2.3. RNA extraction and RT

Total RNA from GCs and CCs (about 30 COCs/sample) was isolated using a silica gel-based membrane spin column (RNeasy Kit, Qiagen S.p.A.). The purity and integrity of the RNA were checked spectroscopically and using gel electrophoresis. Total RNA (1 μg) was reverse transcribed in a final volume of 20 μl using the M-MLV Reverse Transcriptase kit (Invitrogen, Milano, Italy) according to the manufacturer's instructions.

2.4. Multiplex PCR

To determine the presence of specific transcripts, a multiplex-PCR was performed. The reactions were performed using a Multiplex PCR Kit (Qiagen) according to the manufacturer's instruction; the housekeeping gene β-actin was used as an internal control. To increase the specificity and quality of PACAP, PACAP/VIP receptors, and AREG transcript products, touchdown PCR was performed. The initially high annealing temperature (T_a, 67 °C) was lowered by 1 °C per cycle to a 'touchdown' temperature of 59 °C. This 'touchdown' temperature remained the same for the remaining 22 cycles.

The selected primer sequences are shown in Table 1. Each primer pair was previously individually tested for specific amplification. For each primer set, the numbers of cycles for the PCR was chosen in the exponential phase of amplification. Primers for PAC1-R and FSH-R were selected in a region that allowed for the detection of all of the splice variants.

For each sample, 10 μl of PCR product was subjected to electrophoresis on a 2% (w/v) agarose gel and stained with ethidium bromide. The densitometric evaluation of the bands was performed using the AIDA software (Advanced Image Data Analyser 2.11 raytest GmbH, Straubenhardt, Germany). The relative mRNA levels were normalised against the expression of the housekeeping gene, β-actin. DNA contamination controls were performed using gene-specific primers on RNA in the absence of reverse transcriptase treatment.

2.5. Morphological analysis of cumulus cell apoptosis

To evaluate the in vitro effect of PACAP on CC apoptosis, COCs obtained from PMSG-treated mice before or 1 h after hCG stimulation (hCG-primed) were cultured for 24 h in DMEM supplemented with 1 mg/ml BSA in the presence of medium alone (Ctrl), 100 ng/ml FSH, and 450 ng/ml PACAP (Merck-Millipore, Darmstadt, Germany) or 10 ng/ml EGF. To evaluate the effect of PACAP on CC apoptosis in in vivo expanded cumuli, COCs, isolated from PMSG-treated mice 16 h after hCG stimulation, were cultured for an additional 8 h in DMEM alone (Ctrl) or in the presence of the same hormonal stimulation previously described. PACAP concentration was chosen according to data previously obtained on cumulus-enclosed rat oocyte meiotic maturation (Apa et al., 1997). After culture, the COCs were transferred into a 50-μl drop of PBS containing 1 mg/ml

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