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Luteinizing hormone reduces the activity of the NPR2 guanylyl cyclase in mouse ovarian follicles, contributing to the cyclic GMP decrease that promotes resumption of meiosis in oocytes

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ABSTRACT

In preovulatory ovarian follicles of mice, meiotic prophase arrest in the oocyte is maintained by cyclic GMP from the surrounding granulosa cells that diffuses into the oocyte through gap junctions. The cGMP is synthesized in the granulosa cells by the transmembrane guanylyl cyclase natriuretic peptide receptor 2 (NPR2) in response to the agonist C-type natriuretic peptide (CNP). In response to luteinizing hormone (LH), cGMP in the granulosa cells decreases, and as a consequence, oocyte cGMP decreases and meiosis resumes. Here we report that within 20 min, LH treatment results in decreased guanylyl cyclase activity of NPR2, as determined in the presence of a maximally activating concentration of CNP. This occurs by a process that does not reduce the amount of NPR2 protein. We also show that by a slower process, first detected at 2 h, LH decreases the amount of CNP available to bind to the receptor. Both of these LH actions contribute to decreasing cGMP in the follicle, thus signaling meiotic resumption in the oocyte.

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Introduction

Mammalian oocytes are maintained in meiotic prophase for prolonged periods. During prophase arrest, the oocyte is located in a follicle in which it is surrounded by granulosa cells (Fig. 1(A)). As the follicle grows to its full size (~400–500 μm in mice), the oocyte acquires the ability to resume meiosis, but due to inhibitory signals from the granulosa cells, the oocyte remains in prophase (Jaffe and Norris, 2010; Conti et al., 2012). Then during each reproductive cycle, luteinizing hormone (LH) from the pituitary acts on the granulosa cells of the fully grown follicle to cause the oocyte to mature into a fertilizable egg and be ovulated. This process begins with the transition from prophase to metaphase, marked by the breakdown of the nuclear envelope about 2 h after LH exposure. However, other events of the prophase-to-metaphase transition occur before nuclear envelope breakdown: microtubule organizing centers assemble (Schuh and Ellenberg, 2007), chromatin condenses (Racowsky and Baldwin, 1989), and cell cycle regulatory proteins undergo changes in activity and localization (Solc et al., 2010).

Recent studies of the mouse ovary have shown that a key inhibitory substance for maintaining prophase arrest is cGMP, which diffuses from the granulosa cells into the oocyte through gap junctions (Norris et al., 2009; Vaccari et al., 2009). In the oocyte, cGMP inhibits the cAMP phosphodiesterase PDE3A, and thus prevents the degradation of cAMP. Elevated cAMP activates protein kinase A, which acts through a complex of mechanisms to inhibit the activity of the CDK1-cyclin B kinase and thus to inhibit the prophase-to-metaphase transition (Solc et al., 2010; Conti et al., 2012). If cGMP in a follicle-enclosed oocyte is experimentally decreased, by injection of a cGMP-specific phosphodiesterase, cAMP is decreased, and as a consequence meiosis resumes (Norris et al., 2009).

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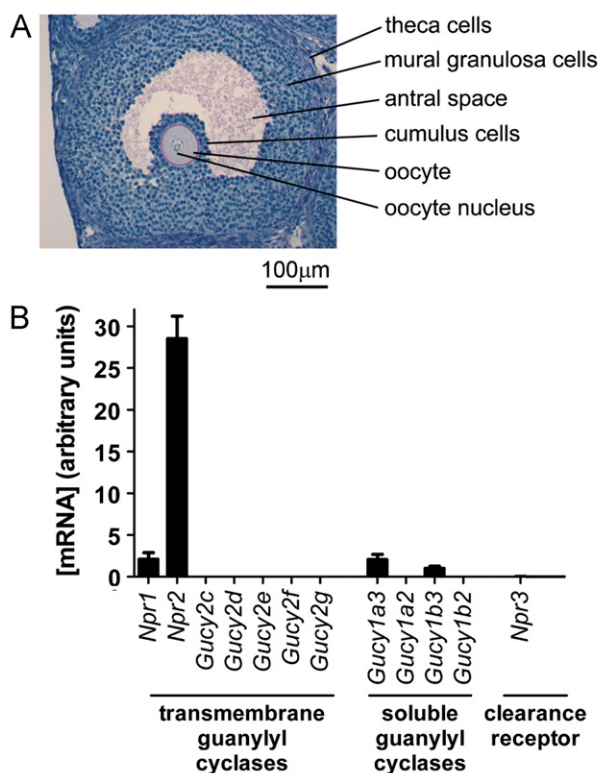

Fig. 1. In mural granulosa cells, *Npr2* mRNA is present at a higher concentration than mRNAs for other guanylyl cyclases. (**A**) Histological section of a mouse ovary, showing an antral follicle, and indicating the mural granulosa cells collected for analysis, as well as other cell types and structures in and around the follicle. (**B**) Relative concentrations of each guanylyl cyclase mRNA in isolated mural granulosa cells. Results for the natriuretic peptide clearance receptor, *Npr3*, are also shown. Where no bars are visible, concentrations of mRNAs were $<0.1\%$ of *Npr2*. The results show the mean ± s.e.m. for 3 RNA preparations.

The generation of the cGMP that maintains meiotic arrest requires the function in the granulosa cells of the transmembrane guanylyl cyclase natriuretic peptide receptor 2 (NPR2, also known as guanylyl cyclase-B) and its extracellular agonist C-type natriuretic peptide (CNP, also known as natriuretic peptide C, NPPC) (Zhang et al., 2010). In ovaries of mice carrying mutations in *Npr2* or *Nppc* genes, meiosis resumes precociously (Zhang et al., 2010). Although there is also evidence for expression of other guanylyl cyclases in granulosa cells (Sriraman et al., 2006) and some evidence that these may contribute to the maintenance of meiotic arrest (Törnell et al., 1990; Sela-Abramovich et al., 2008; Vaccari et al., 2009) and the response of the follicle to LH (Sriraman et al., 2006), CNP-dependent activation of NPR2 is fundamental for generating the inhibitory levels of cGMP.

CNP is synthesized by the outer (mural) granulosa cells, and binds to NPR2 throughout the follicle to stimulate cGMP production (Jankowski et al., 1997; Zhang et al., 2010). The connection of the cumulus cells to the mural granulosa cells is essential for maintaining meiotic arrest, since when this connection is broken, leaving the cumulus-oocyte complex free in the antral space, meiosis resumes (Racowsky and Baldwin, 1989). This supports the concept that although *Npr2* mRNA is most concentrated in the cumulus cells (Zhang et al., 2010), cGMP generated by NPR2 in the mural layers also provides a critical part of the inhibitory cGMP to the oocyte.

Despite this knowledge of how CNP, NPR2, and cGMP function to maintain meiotic arrest, less is known about how signaling by LH reverses the arrest. LH acts on a G-protein-linked receptor (LHCGR) (Rajagopalan-Gupta et al., 1998), which in rats and mice, is located in the mural granulosa cells, mostly within the outer several layers of cells, and is absent in the cumulus cells (Amsterdam et al., 1975; Eppig et al., 1997). In response to LH, the permeability of the gap junctions between the granulosa cells throughout the follicle is reduced, such that intercellular diffusion within the follicle of molecules of the size of cGMP is slowed (Sela-Abramovich et al., 2005; Norris et al., 2008). In parallel, cGMP levels in the follicle decrease (Hubbard, 1986; Norris et al., 2009; Vaccari et al., 2009), from a basal level of $\sim 3\,\mu M$, to $\sim 0.5\,\mu M$ at 20 min and $\sim 0.1\,\mu M$ at 1 h after applying LH (Norris et al., 2010). CNP levels also decrease (Jankowski et al., 1997; Kawamura et al., 2011), but the earliest of these measurements were made at 4 h after LH application, while the cGMP decrease occurs by 20 min, so their functional significance has not been certain. As cGMP in the follicle decreases, cGMP in the interconnected oocyte falls correspondingly, to a few percent of the basal level at 1 h. As a consequence, the inhibition of PDE3A is relieved, cAMP decreases, and meiosis resumes (Norris et al., 2009; Vaccari et al., 2009).

The decrease in cGMP in the follicle could be caused by a decrease in cGMP synthesis, an increase in cGMP degradation, and/or an increase in cGMP efflux. Here we report that one mechanism by which LH signaling reduces cGMP is by reducing the activity of the guanylyl cyclase NPR2.

Materials and methods

Mice and hormones

Ovaries were obtained from prepubertal B6SJLF1 mice (23–25 day old) from The Jackson Laboratory (Bar Harbor, ME); procedures were approved by the animal care committees of the University of Connecticut Health Center, China Agricultural University, and The Jackson Laboratory. For granulosa cell collection, cumulus-oocyte complex collection, CNP ELISA assays, and histological analysis, the mice were injected with 5 I.U. equine chorionic gonadotropin (eCG) 40–48 h before use, to stimulate follicle growth and LH receptor expression. Mice for antral follicle isolation were not injected with eCG; instead the follicles were exposed to 10 ng/ml follicle stimulating hormone (FSH) in vitro.

Ovine LH, human LH, ovine FSH, and eCG, purified from biological sources, were obtained from A.F. Parlow (National Hormone and Peptide Program, Torrance, CA). Human recombinant LH was obtained from EMD Serono Research Institute, Inc. (Rockland, MA). Human chorionic gonadotropin (hCG) was purchased from Sigma-Aldrich (St. Louis, MO). Ovine LH was used for studies of isolated follicles (10 μg/ml). Because of their slower rate of degradation (Mock and Niswender, 1983), human LH or hCG was used for injection into mice (10 μg or 5 I.U., respectively).

Measurement of relative amounts of guanylyl cyclase mRNAs in granulosa cells

Mural granulosa cells were collected by puncturing antral follicles of isolated ovaries with 30 gauge needles. RNA was extracted using TRIzol reagent (Invitrogen Corporation, Carlsbad, CA). DNAse I digestion was performed to remove residual genomic DNA, and mRNAs were reverse transcribed using random hexamers.

Quantitative TaqMan analysis was performed using the Applied Biosystems PRISM 7900 sequence detection system, to determine the relative concentration of each guanylyl cyclase mRNA in granulosa cells. Primer sequences are listed in Table S1 in the supplementary material. Differences in primer efficiency

were determined by measuring the cycle threshold (C_t) values for each primer pair using 30 ng of genomic DNA. Only small differences were detected, and these were corrected for by use of the following formula:

C_t (corrected)$=C_t$ (measured)$+C_t$ (mean of all probes, genomic DNA)$-C_t$ (genomic DNA)

Normalization was performed using the housekeeping gene *Rpl32* as a control. The resulting expression is given in arbitrary units.

2.3. Measurement of guanylyl cyclase activity in a crude membrane fraction of follicles

For each experiment, antral follicles from 4 mice were isolated and cultured for 24–30 h in the presence of FSH to stimulate follicle growth and LH receptor expression (Norris et al., 2008, 2010). The follicles were divided into 2 equal groups, and half were exposed to LH for the indicated time. The 40–50 follicles in each group were washed in PBS and lysed in phosphatase inhibitor buffer (Dickey et al., 2007) in a 100 μl glass homogenizer. To obtain a crude membrane fraction, the homogenate (200 μl volume) was centrifuged at 10,000 × *g* for 20 min; the pellet (~1 μl volume) was resuspended in 50 μl of phosphatase inhibitor buffer and sonicated briefly. Protein content was determined by solubilizing a 4 μl aliquot in 1% SDS and performing a BCA assay (Pierce, Thermo Fisher Scientific, Rockford, IL). The crude membrane fraction contained ~1 μg of protein per follicle. The samples were frozen in liquid N_2 and stored at −80 °C.

Guanylyl cyclase assays were conducted for each pair of follicle samples prepared as described above (one sample that had been treated with LH, one control sample without LH), using methods as previously described (Robinson and Potter, 2011). Assays were performed at 37 °C using 1–2 μg of follicle protein per assay tube, in the presence or absence of 1 μM CNP (or ANP), which are maximally activating concentrations for their respective receptors (Abbey-Hosch et al., 2005; Dickey et al., 2008). 0.5 mM isobutylmethylxanthine (IBMX) was included to inhibit cGMP phosphodiesterase activity. CNP (or ANP) dependent guanylyl cyclase activity refers to the activity measured in the presence of the natriuretic peptide minus the activity measured in the absence of the natriuretic peptide. Statistical significance of the data was tested using two-way repeated measures ANOVA with a Bonferroni post-test; control and LH-treated samples that had been prepared and assayed together were analyzed as pairs. The analysis was performed using Prism software (GraphPad Software, Inc., La Jolla, CA).

Measurement of cGMP in cumulus cells

Cumulus-oocyte complexes were isolated at various times after hCG injection, and cultured as previously described (Zhang et al., 2010), with or without 30 nM CNP for 1 h. Cumulus cells were then separated for measurement of cGMP using an ELISA method as previously described (Zhang et al., 2010). Statistical significance of the data was tested using one-way ANOVA with a Dunnett multiple comparisons post-test.

Measurement of CNP in ovaries

CNP in ovaries was assayed by an ELISA method (FEK-012-03, Phoenix Pharmaceuticals Inc., Burlingame, CA) with a primary antibody made against the 22 amino acid form of CNP. This antibody also recognizes the 53 amino acid form of CNP, and presumably the precursor forms, which include the same 22 amino acids at their C-termini (Wu et al., 2003; Potter et al., 2009).

Samples were prepared by a method modified from Jankowski et al. (1997). Two ovaries in 70 μl of 1.0 M acetic acid were heated at 95 °C for 10 min, then lysed with a probe sonicator. 350 μl of MeOH was added to solubilize lipids, and the tube was centrifuged at 30,000 × *g* at 4 °C for 15 min. The supernatant ("ovary extract") contained ~250 μg of protein. ~50% of the CNP was recovered in this extract (determined by adding a known amount of CNP to the ovaries before extraction). For each sample, 10, 5, and 2 μg of the extract protein were lyophilized and assayed, following the manufacturer's instructions. Data were analyzed using Prism software. Statistical significance of the data was tested using one-way ANOVA with a Dunnett multiple comparisons post-test. The concentration of CNP in the ovary, if CNP was uniformly distributed, was estimated based on a volume per ovary of ~4 μl (~4 mg wet weight).

Histological analysis of nuclear envelope breakdown kinetics

Serial sections of mouse ovaries were prepared as previously described (Mehlmann et al., 2004). Follicles with a diameter of ≥350 μm in at least one dimension, as measured in the section containing the nucleolus or chromosomes, were analyzed for the presence of an intact nucleus (see Fig. 1(A)).

Results and discussion

In mural granulosa cells, mRNA encoding NPR2 *is present at a higher concentration than mRNAs encoding other guanylyl cyclases*

Although NPR2 is known to be present in mural granulosa cells and functionally important for maintaining meiotic arrest, there is also evidence that NPR1 and soluble guanylyl cyclase subunits could contribute to the control of meiotic arrest and progression (see Introduction). Because previous studies did not determine the relative expression levels of mRNA for NPR2 and other guanylyl cyclases in mouse granulosa cells, and because not all of the guanylyl cyclases were investigated, we quantitatively compared the amounts of mRNA in mural granulosa cells for each of the mouse guanylyl cyclase genes (Fig. 1(B)).

The mouse genome contains 7 transmembrane and 4 soluble guanylyl cyclase genes (Potter, 2011a). We detected mRNA encoding two transmembrane guanylyl cyclases, NPR1 and NPR2, and two soluble guanylyl cyclase subunits, GUCY1A3 (soluble guanylyl cyclase alpha 1) and GUCY1B3 (soluble guanylyl cyclase beta 1). Among these, *Npr2* mRNA was expressed at a high level, ≥14 times higher than any of the other guanylyl cyclases. We also tested for mRNA encoding NPR3, which has sequence similarity to the extracellular domains of NPR1 and NPR2, but lacks the guanylyl cyclase domain and activity (Potter, 2011a). NPR3 is a clearance receptor for natriuretic peptides. Little or no *Npr3* mRNA was detected.

Although concentrations of mRNAs are not directly proportional to the amounts of the proteins they encode, these measurements further support the conclusion that NPR2 is the primary guanylyl cyclase that produces cGMP in the follicle.

LH signaling reduces NPR2 activity in the follicle

One way that LH activation of its receptors in the mural granulosa cells could decrease cGMP levels within the follicle is by reducing NPR2 activity. Two aspects of this question were considered: (1) whether LH signaling decreases NPR2 activity in the follicle as a whole, of which most of the volume is mural granulosa cells, and (2) whether LH signaling decreases NPR2 activity in the cumulus cells. A decrease in NPR2 activity in either

or both of these regions could contribute to the measured decrease in cGMP in the oocyte. This section describes our studies of a crude membrane fraction from whole follicles, and a subsequent section describes our studies of cumulus cells.

Guanylyl cyclase activity was measured using the particulate fraction obtained by centrifuging a homogenate of follicles. When this crude membrane fraction was incubated without CNP, guanylyl cyclase activity was too low to measure accurately, but addition of 1 μM CNP increased the activity to 0.21 ± 0.02 nmole cGMP/mg protein/minute (n=15 follicle preparations, see Fig. 2(A) and (B)). After a 20 min exposure of follicles to LH, CNP-dependent guanylyl cyclase activity fell to 50% of the activity measured in the membrane fraction from follicles without LH exposure, and remained depressed for 2 h after applying LH (Fig. 2 (A) and (B)).

The decrease in follicle cGMP that will result from a 50% decrease in NPR2 activity depends on the cGMP affinity of the phosphodiesterases present in the granulosa cells (Fig. S1). If the affinity is higher (lower K_m), the cGMP concentration will fall to a lower level. Much of the cGMP phosphodiesterase activity in the follicle is sensitive to sildenafil and tadalafil, indicating an important PDE5 component (Vaccari et al., 2009). Based on K_m values for PDE5, a 50% reduction in NPR2 activity could potentially account for the decrease in follicle cGMP from 3 μM before LH treatment to ~0.5 μM after 20 min (Norris et al., 2010) (Fig. S1).

Because a small amount of *Npr1* mRNA is also expressed in granulosa cells (Fig. 1(B)), we also evaluated the effect of LH on NPR1 activity, by measuring guanylyl cyclase activity in the presence of 1 μM atrial natriuretic peptide (ANP). Studies of human NPR1 and NPR2 have shown that 1 μM ANP activates NPR1, but has almost no effect on NPR2 (Dickey et al., 2008). In the crude membrane fraction from follicles, ANP-dependent guanylyl cyclase activity was 0.07 ± 0.03 nmole cGMP/mg protein/minute, or 33% of the CNP-dependent activity (n=4). However, the ANP-dependent activity was unchanged by LH (Fig. 2(C) and (D)). Some of the ANP-dependent guanylyl cyclase activity that we measured might be due to NPR1 expressed in membranes from theca cells and blood vessels that were not completely removed from the follicle by microdissection (Fig. 1(A)). The lack of effect of LH on ANP-dependent cGMP accumulation serves as a control to indicate that the LH-induced decrease in CNP-dependent cGMP accumulation is not due to an LH effect on phosphodiesterase activity that could have been present in the crude membrane fraction despite the presence of IBMX.

The LH-induced decrease in NPR2 activity in the follicle occurs without a corresponding decrease in NPR2 protein

Previous studies have shown that other biological factors that rapidly decrease NPR2 activity in cultured cells do so in a manner that is independent of NPR2 protein levels (Abbey and Potter, 2003; Abbey-Hosch et al., 2004). To test if LH decreased the amount of NPR2 protein in follicles, we first tried Western blotting, and immunoprecipitation followed by Western blotting. However, with the available antibodies, it was not possible to detect endogenous levels of the protein using these methods. So instead, we measured guanylyl cyclase activity in follicle membrane fractions after treatment with 1% Triton X-100 and 5 mM $MnCl_2$, a condition known to maximally activate NPR1 and NPR2 in the absence of natriuretic peptide and to be indicative of guanylyl cyclase protein levels (Potter and Hunter, 1999; Abbey and Potter, 2003). Guanylyl cyclase activity measured in the presence of Triton X-100 and $MnCl_2$ is independent of modification of the NPR2 protein by phosphorylation (Potter and Hunter, 1998).

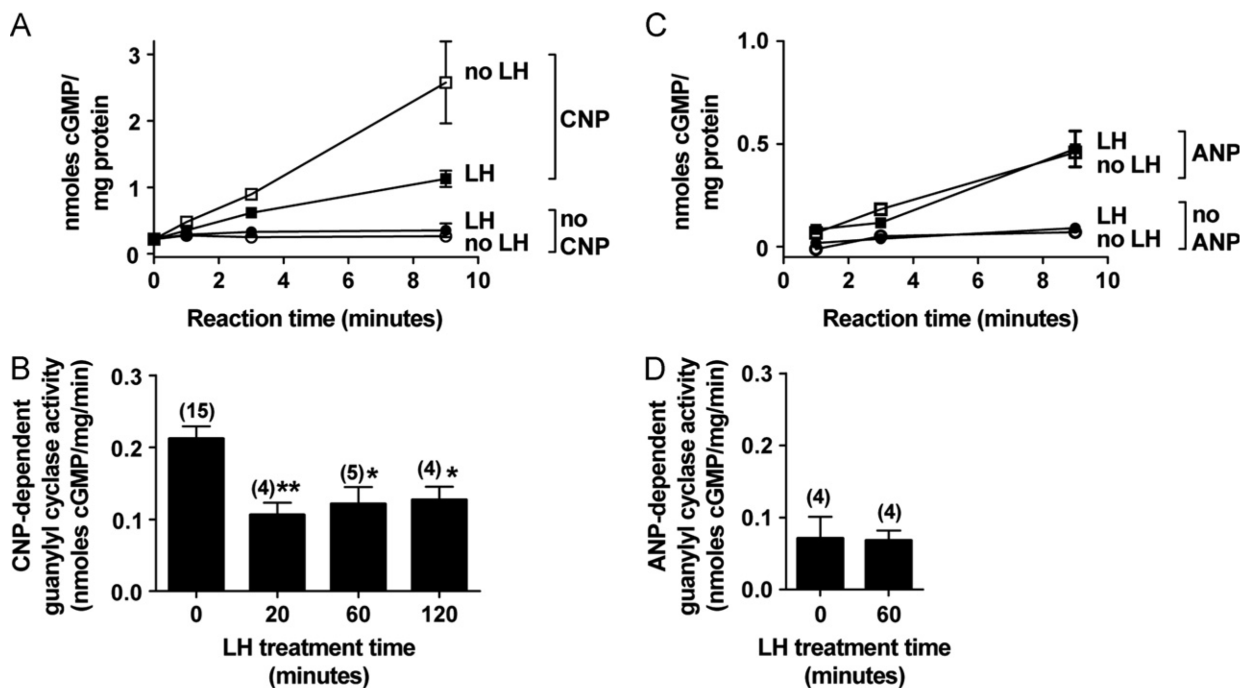

Fig. 2. LH signaling reduces NPR2 activity in the follicle. (A) Guanylyl cyclase activity of crude membrane fractions prepared from follicles treated with or without LH for 20 min was measured with or without 1 μM CNP. Values indicate the mean ± range of duplicate measurements for each condition, using one follicle preparation made after LH treatment, and another preparation made in parallel but without LH treatment. Where not visible, the error bars are contained within the symbol. (B) Combined data from 15 experiments like that in A, showing CNP-dependent guanylyl cyclase activity of crude membrane fractions from follicles treated with LH for the indicated times. CNP-dependent activity values were determined by subtracting the basal values measured in the absence of CNP. Activities are expressed as the mean ± s.e.m. for n follicle preparations. Activities for follicles treated with LH for 20, 60, or 120 min differed significantly from the activity for follicles without LH treatment (*$p < 0.05$; **, $p < 0.01$). (C) and (D) Guanylyl cyclase activity of crude membrane fractions prepared from follicles treated with or without LH for 60 min was measured with or without 1 μM ANP. Data are presented as described for A and B. LH did not decrease the ANP-dependent guanylyl cyclase activity.

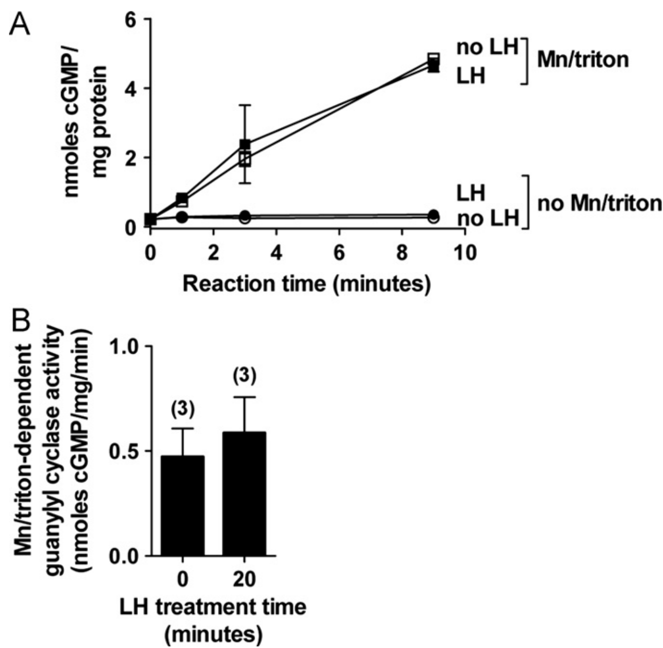

Fig. 3. The LH-induced decrease in NPR2 activity occurs without a corresponding decrease in NPR2 protein. (A) Guanylyl cyclase activity of crude membrane fractions prepared from follicles treated with or without LH for 20 min was measured with or without 1% Triton X-100 and 5 mM $MnCl_2$, to maximally activate guanylyl cyclase. Values indicate the mean ± range of duplicate measurements for each condition, using one follicle preparation made after LH treatment, and another preparation made in parallel but without LH treatment. (B) Combined data from 3 experiments like that in A, showing Mn/triton-dependent guanylyl cyclase activity of crude membrane fractions from follicles treated with or without LH for 20 min (mean ± s.e.m.). LH did not decrease the Mn/triton-dependent guanylyl cyclase activity.

Detergent-dependent guanylyl cyclase activity was the same in samples from follicles with or without LH treatment for 20 min (Fig. 3). Since NPR1 and NPR2 are the only detectable membrane guanylyl cyclases in granulosa cells, and since NPR1 is a relatively minor component, these detergent measurements indicate that at 20 min, LH does not decrease the amount of NPR2 protein. A possible cause of the rapid LH-induced decrease in NPR2 activity is dephosphorylation, which can result from elevation of intracellular Ca^{2+} and/or activation of protein kinase C (Abbey-Hosch et al., 2005; Potter, 2011b).

In the cumulus cells, CNP-dependent cGMP production decreases in response to LH receptor stimulation, but more slowly than in the mural granulosa cells

Because of the direct connection between the cumulus cells and the oocyte, and because of the higher level of *Npr2* mRNA in the cumulus cells compared with the mural cells (Zhang et al., 2010), it was of particular interest to investigate whether LH signals that are initiated in the mural granulosa cells regulate NPR2 activity in the cumulus cells. As described above, LH receptors are not present in the cumulus cells, so such regulation would have to involve signaling between different regions of the follicle.

Due to the small amount of protein that could be obtained, we could not analyze guanylyl cyclase activity in a cumulus cell membrane fraction as we did for the more abundant material from whole follicles. Instead, we isolated cumulus-oocyte complexes from ovaries at various times after injection of mice with human chorionic gonadotropin (hCG) to stimulate the LH receptor, incubated the complexes in the presence or absence of 30 nM CNP for an additional hour, then isolated the cumulus cells and measured their cGMP content. hCG is often used instead of LH, since both hormones act on the same receptor. 30 nM CNP was used because this is approximately the minimum concentration needed to prevent spontaneous meiotic resumption in isolated cumulus-oocyte complexes (Zhang et al., 2010). Under these experimental conditions, measurements of a change in cellular cGMP content in response to LH receptor stimulation could indicate a change in guanylyl cyclase activity, or a change in cGMP phosphodiesterase activity, or a change in cGMP efflux. However, by measuring the effect of LH receptor stimulation on cGMP content in the presence and absence of CNP, we were able to distinguish between these possibilities.

Without injection of the mice with hCG, addition of 30 nM CNP to cumulus-oocyte complexes elevated the cGMP content of the cumulus cells by 4.1 ± 0.9 times ($n=6$). When cumulus-oocyte complexes were isolated from mice at 1 h after hormone injection, and then incubated in the presence of CNP for an additional hour, the cGMP content of the cumulus cells was the same as that in cumulus cells from mice without hormone injection (Fig. 4). However, when the cumulus-oocyte complexes were isolated at 2 h after hormone injection, and incubated with CNP until 3 h, cGMP had decreased to 70% of values obtained without hormone treatment (Fig. 4). With isolation of the complexes at 3 h, followed by a CNP incubation and measurement at 4 h, cGMP had decreased to 42% (Fig. 4).

In the absence of CNP, the cGMP content of the cumulus cells was low, as expected for this in vitro condition in which NPR2 would not be activated. Under this condition, the cGMP content was not decreased by LH receptor stimulation (Fig. 4), indicating that LH receptor signaling does not increase cGMP phosphodiesterase activity in these cells, or cause an increase in cGMP efflux. Thus the LH receptor-induced decrease in cumulus cell cGMP seen in the presence of CNP can be attributed to a decrease in cGMP production. These findings indicate that LH signaling decreases NPR2 activity in the cumulus cells, but only after 2–3 h, versus 20 min in the mural cells. This delay is likely to be a consequence of the localization of the LH receptors in separate cells (the mural granulosa). As will be discussed below, this intercellular signaling is most likely mediated by the release of EGF-like growth factors from the mural granulosa cells.

The decrease in cGMP production in the cumulus cells could result from a decrease in the amount of NPR2 protein, or from a

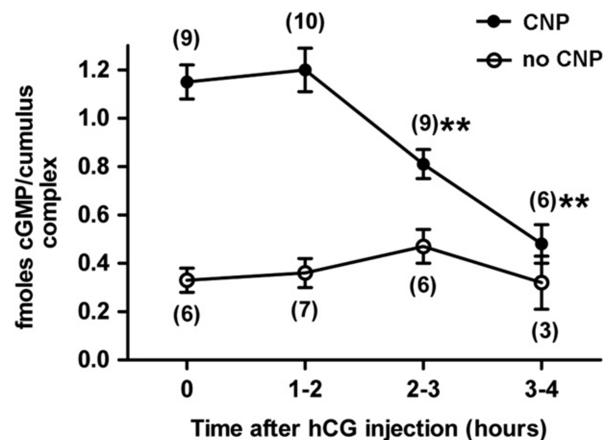

Fig. 4. In the cumulus cells, CNP-dependent cGMP production decreases in response to LH receptor stimulation, but more slowly than in the mural granulosa cells. Cumulus-oocyte complexes were collected at various times after hCG injection into mice and incubated for one hour with or without 30 nM CNP. The cumulus cells were separated and cGMP content was measured. Values indicate the mean ± s.e.m. for the number of experiments shown in parentheses. Values for 2–3 and 3–4 h post hCG were significantly different from the no hCG value (**$p < 0.01$).

modification of the NPR2 protein such as dephosphorylation. In support of the first possibility, the amount of *Npr2* mRNA in the cumulus cells decreases to ~50% of the basal level at 3 h after LH receptor stimulation (Zhang et al., 2011). However, it is unknown how rapidly NPR2 protein would decrease as a consequence. Protein turnover rates for NPR2 have not been investigated, but turnover of the closely related protein NPR1 in cultured cells is a slow process, with a half-time of ≥ 8 h (Flora and Potter, 2010).

The amount of CNP in the ovary decreases in response to LH, preceding nuclear envelope breakdown

Another factor that could contribute to the LH-induced reduction in cGMP levels in the follicle is a decrease in CNP. CNP decreases have been reported previously for rat, mouse, and human (Jankowski et al., 1997; Kawamura et al., 2011), but the earliest of these measurements were made at 4 h after LH receptor stimulation, so it was unclear if the CNP decrease occurred early enough to contribute to causing nuclear envelope breakdown and the events that precede it, vs later events leading to ovulation. To investigate the time course of the decrease in CNP, we injected mice with LH, and at various times afterwards, collected their ovaries for analysis of CNP content using an ELISA based on an antibody that should recognize all forms of CNP and its precursor NPPC.

Without LH injection, there were ~150 fmoles of CNP per mg of ovary extract protein (Fig. 5(A)), corresponding to an overall concentration of ~10 nM. However, the immunoreactive material detected by the ELISA contains both extracellular peptide and intracellular precursor protein, and only the peptide that has been secreted into the extracellular space can activate NPR2. Thus the concentration of peptide that could function to regulate NPR2 is unknown.

No change from the pre-LH level of CNP was seen at 1 h after injection of LH, but by 2 h, the amount of CNP had decreased to 37% (Fig. 5(A)). The decrease in CNP at 2 h corresponds to the time at which nuclear envelope breakdown in the oocyte is beginning, as determined from histological sections of ovaries of similarly treated mice (Fig. 5(B)). Thus, the CNP decrease occurs well after cGMP decreases in the follicle (detected at 20 min after LH, Norris et al., 2010), but early enough to potentially contribute to stimulating nuclear envelope breakdown. After nuclear envelope breakdown, CNP continued to decrease, reaching 15% at 4 h after LH, and 7% at 8 h (Fig. 5(A)).

A likely cause of the CNP decrease is that *Nppc* mRNA decreases to about half of the basal level by 2 h after LH receptor stimulation (Kawamura et al., 2011). Thus LH signaling might reduce *Nppc* mRNA synthesis or increase its degradation. Because the turnover of CNP is very rapid, with a half-life of about 3 min in plasma (Hunt et al., 1994), a decrease in *Nppc* mRNA could rapidly decrease the amount of CNP. Other possible factors that could contribute to the decrease in CNP are an increase in the natriuretic peptide clearance receptor NPR3, and an increase in the activity of proteases that degrade CNP (Potter, 2011c).

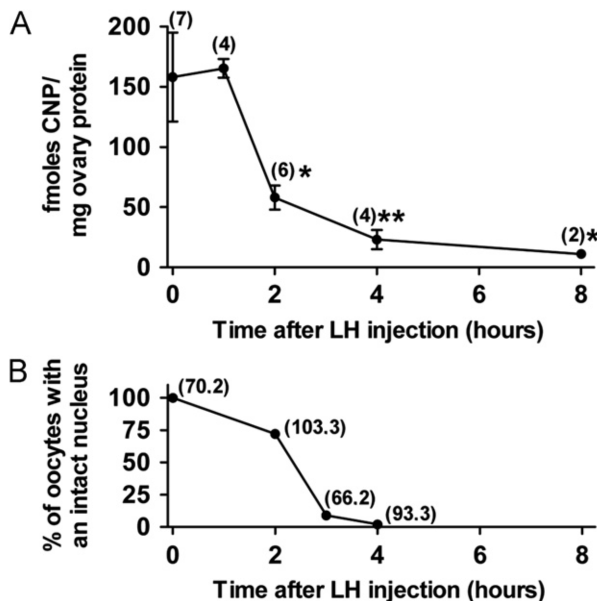

Fig. 5. The amount of CNP in the ovary decreases in response to LH, preceding nuclear envelope breakdown. (A) Time course of the decrease in the CNP content of ovary extracts, following LH injection into mice. Values indicate the mean ± s.e.m. for the number of mice shown in parentheses. Values for 2, 4, and 8 h LH treatments were significantly different from the no LH value (*$p < 0.05$; **$p < 0.01$). (B) Time course of nuclear envelope breakdown, following LH injection into mice. Values indicate the percentage of fully grown follicles (≥ 350 μm in diameter) in which the oocyte contained a prophase-arrested nucleus; the number of follicles and the number of mice counted are indicated.

The amount of CNP in the ovary increases as follicles develop to the preovulatory stage

We also examined the effect of equine chorionic gonadotropin (eCG, also called PMSG) on CNP levels. Unlike human chorionic gonadotropin (hCG), which binds to the LH receptor, eCG binds to the follicle stimulating hormone receptor, which stimulates antral follicle growth and LH receptor expression. eCG is often used experimentally to cause follicles to grow and to progress to the preovulatory stage; it was used for this purpose for the CNP measurements described above.

We found that eCG injection of the mice, 44 h before collecting the ovaries, increased their CNP content (Fig. 6). The increase in CNP in response to eCG is consistent with findings that mRNA encoding the CNP precursor (NPPC) increases in mouse ovaries in response to eCG (Kawamura et al., 2011), and that CNP and cGMP increase between the days of diestrus and proestrus, in rats and hamsters (Jankowski et al., 1997; Hubbard and Greenwald, 1982). Thus our findings add to the accumulating evidence that during follicle growth to the preovulatory stage, CNP and cGMP content of the ovary increases. At the preantral stage, cyclic nucleotide regulation is not needed to maintain meiotic arrest (Erickson and Sorensen, 1974). Then with follicle growth, as the oocyte accumulates more CDK1 and other factors that result in meiotic competence (Chesnel

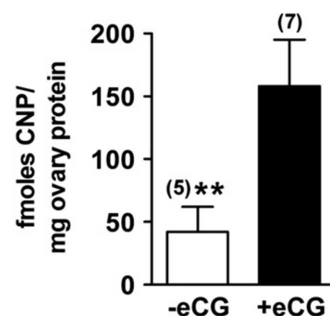

Fig. 6. The amount of CNP in the ovary increases in response to activation of follicle stimulating hormone receptors. The mice to be used for the CNP measurements shown in Fig. 5(A) were injected 44 h previously with eCG to stimulate follicle growth. In response to eCG, the amount of CNP per mg of ovary protein increased ~4 times. The +eCG data shown in Fig. 6 are the same as the no LH (0 h) data shown in Fig. 5(A), so the statistical significance of these data was tested together. Values for ovaries from mice with or without eCG treatment were significantly different.

and Eppig, 1995), CNP is synthesized in order to prevent premature meiotic progression.

Pathways by which LH signaling in the mural granulosa cells causes cGMP to decrease in the oocyte

The connections between LH-induced activation of G-proteins in the outer layers of the mural granulosa cells and the ensuing events in the follicle that lead to the decrease in cGMP and resumption of meiosis in the oocyte are only partially understood (Fig. 7) (Vaccari et al., 2009; Norris et al., 2010; Hsieh et al., 2011; Conti et al., 2012). These connections include not only the pathways leading to a decrease in granulosa cell guanylyl cyclase activity, but also pathways that reduce gap junction permeability through MAP kinase-dependent phosphorylation of connexin 43 (Sela-Abramovich et al., 2005; Norris et al., 2008), thus reducing diffusion of cGMP into the oocyte. Both the decrease in gap junction permeability and the decrease in granulosa cell guanylyl cyclase activity contribute to the decrease in oocyte cGMP.

At the level of the mural granulosa cells, LH receptor signaling activates G_s and adenylyl cyclase (Rajagopalan-Gupta et al., 1998), thus elevating cAMP. LH receptor signaling also activates G_i, G_q, and phospholipase Cβ (Rajagopalan-Gupta et al., 1998; Kühn and Gudermann, 1999), thus elevating calcium (via IP_3) in granulosa cells in culture (Davis et al., 1986; Flores et al., 1998). However, activation of protein kinase C by the diacylglycerol that is generated by phospholipase C has, to our knowledge, not been detected so far (Salvador et al., 2002). Further studies of these signaling events using intact follicles will be informative, because both calcium elevation and protein kinase C activation can lead to dephosphorylation and inactivation of NPR2 (Abbey-Hosch et al., 2005). Calcium elevation could also increase the activity of the PDE1 family of cGMP phosphodiesterases (Francis et al., 2011).

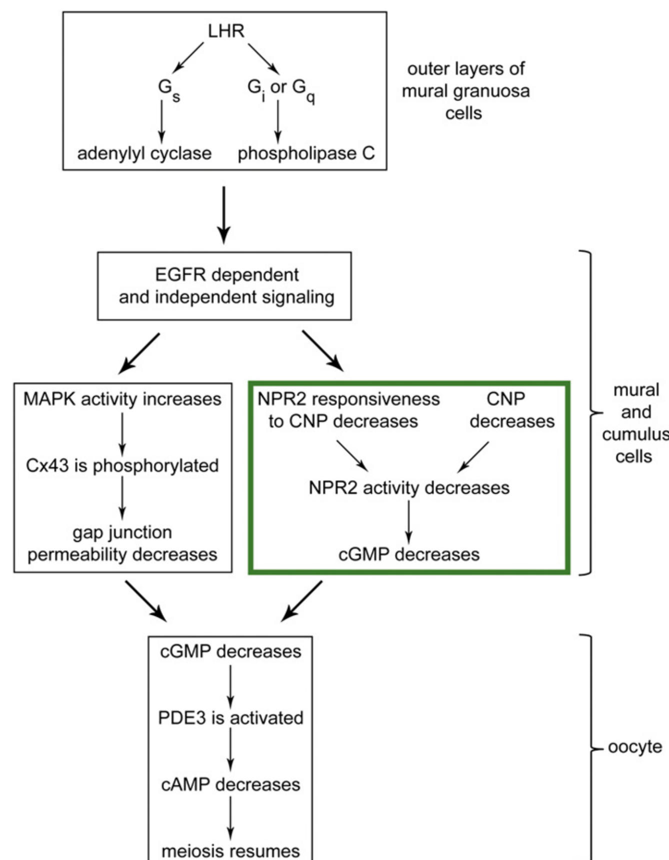

Fig. 7. Signaling pathways connecting LH binding to its receptors in the outer layers of the mural granulosa cells to resumption of meiosis in a mammalian oocyte. The green box indicates the findings of this study in the context of other aspects of the signaling network.

Since the mRNA encoding the precursor protein of CNP is expressed in the same cells as the LH receptor (Zhang et al., 2010), exposure to LH could result in a reduction in the amount of CNP by signaling within the same cells. Likewise, since the LH receptor is expressed in the outer several layers of the mural granulosa cells (Amsterdam et al., 1975), the LH-induced decrease in NPR2 activity in these cells could result from signaling within the same cell. However, the LH-induced decrease in NPR2 activity in the cumulus cells, or in the inner layers of the mural cell epithelium, both of which regions lack LH receptors (Amsterdam et al., 1975; Eppig et al., 1997), must involve signaling between cells.

Based on evidence that the cGMP decrease in the follicle is partially dependent on EGF receptor signaling (Vaccari et al., 2009; Norris et al., 2010; Hsieh et al., 2011; Conti et al., 2012), EGF-like growth factors released from the outer layers of mural granulosa cells in response to LH are mediators of the paracrine signals, and could also contribute to autocrine signaling in the outer layers of the mural granulosa cells. EGF receptor signaling is essential for LH-induced nuclear envelope breakdown (Park et al., 2004), and EGF receptor activation, as indicated by increased phosphorylation of the receptor protein, occurs as early as 30 min after LH treatment (Panigone et al., 2008). However, it remains unknown how LH receptor signaling triggers the synthesis and/or release of the EGF-like growth factors epiregulin and amphiregulin. RNA encoding precursors of these growth factors increases by 2 h after LH receptor stimulation (Park et al., 2004), but in addition, LH signaling might activate the proteases that release epiregulin and amphiregulin from pre-existing precursors (Blobel et al., 2009; Killock and Ivetic, 2010).

EGF receptor signaling is required for much of the increase in MAP kinase activity in response to LH (Panigone et al., 2008), and thus contributes to phosphorylation of connexin 43 and the resulting decrease in gap junction permeability (Norris et al., 2008; Conti et al., 2012). EGF receptor signaling also activates phospholipase Cγ (Chattopadhyay et al., 1999), and could thus elevate calcium and protein kinase C activity, amplifying the LH receptor signaling that may occur through through phospholipase Cβ. As discussed above, these signaling events could decrease NPR2 activity, and possibly increase PDE1 activity, thus lowering cGMP in the granulosa cells and oocyte.

Conclusions

By 20 min after applying LH to ovarian follicles, the guanylyl cyclase activity of NPR2 elicited by a saturating concentration of CNP is decreased by half. This correlates with a similarly rapid decrease in follicle cGMP. There is then a slower decrease in NPR2 responsiveness to CNP in the cumulus cells, first seen at 2–3 h. By 2 h, LH signaling also induces a decrease in the amount of CNP in the ovary. Together, these 3 factors that decrease guanylyl cyclase activity contribute to the decrease in cGMP in the follicle. Because the mural granulosa cells, cumulus cells, and oocyte are connected by gap junctions to form a syncitium with respect to cGMP, cGMP in the oocyte equilibrates with that in the surrounding somatic cell compartment, and the resulting decrease in oocyte cGMP promotes meiotic resumption.

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Appendix A. supplementary material

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.ydbio.2012.04.019.

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