LH Stimulation of Meiotic Resumption in Ovarian Follicles by Protein Kinase A and a PPP-Family Phosphatase

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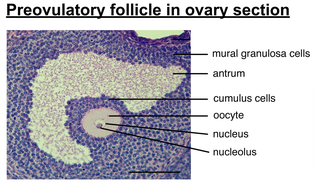
Abstract

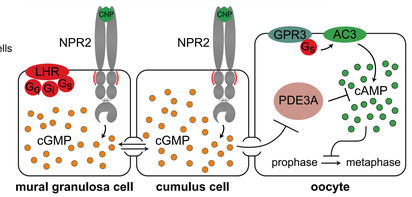
In mammalian preovulatory ovarian follicles, meiotic arrest of fully grown oocytes is maintaned by cyclic guanosine monophosphate (cGMP) that is produced by the membrane guanylyl cyclase natriuretic peptdide receptor 2 (NPR2) in the follicle's somatic cells and diffuses through gap junctions into the oocyte[1]. Phosphorylation of several juxtamembrane serines and threonines is essential for full NPR2 activity. The cyclic surge of luteinizing hormone (LH) acts on receptors in the outermost somatic cell layers to rapidly dephosphorylate and inactivate NPR2, lowering cGMP levels in the follicle and oocyte to trigger meiotic resumption[2,3]. Our goals are to determine whether protein kinase A (PKA) signaling mediates this process[4], and which LH-activiated PPP-family phosphatase dephosphorylates NPR2[2]. Application of the specific PKA inhibitor Rp-8-CPT cAMPS (Rp) to isolated mouse follicles prior to LH treatment (30 min) prevented NPR2 dephosphorylation, showing that the phosphatase activation is mediated by PKA. To identify PPP-family regulatory subunits that undergo rapid a LH-stimulated increase in phosphorylation, lysates of rat follicles treated with or without LH were enriched for phosphorylated peptides and analyzed by liquid chromatography-tandem mass spectrometry (LC-MS/MS) utilizing chemical labeling with tandem mass tags (TMT) for quantification. PPP1R12A and PPP2R5D emerged as two primary candidates. In PPP1R12A, peptides containing phosphorylated S507 residue had ~5-fold higher intensity in LH-treated follicles. PPP2R5D had four sites with elevated intensity with LH: S53 and S566 (10-fold and 4-fold, respectively), and S81/82 (~2-fold). Quantitative western blotting confirmed these results and showed that inhibition of PKA activity with Rp prevented the LH-induced phosphorylation changes. To determine whether phosphorylation of one or both proteins mediates NPR2 dephosphorylation after LH, we have made mice in which S507 of PPP1R12A or S53/S81/S82/S566 of PPP2R5D are replaced with alanines, and are testing their role in this process. Thus, we have identified two PPP-family regulatory subunits that are rapidly phosphorylated in response to LH-PKA signaling as candidates for effecting NPR2 dephosphorylation, an event required for timely oocyte meiotic resumption.

Graphical Background

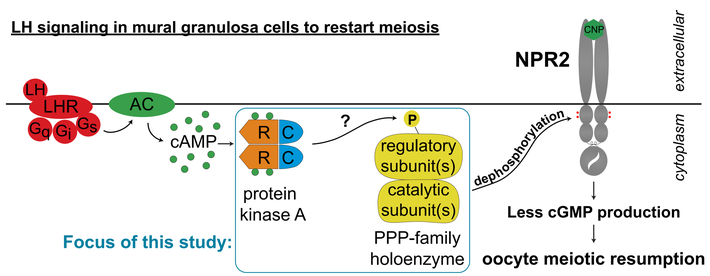
Preovulatory follicle in ovary section



How oocyte meiotic arrest is maintained



LH signaling in mural granulosa cells to restart meiosis



Questions and Approaches

1) **Is LH-induced NPR2 dephosphorylation mediated solely by PKA signaling?** **Test using a selective PKA inhibitor after validation.**

2) **Which PPP-family subunit(s) is responsible for effecting LH-induced NPR2 dephosphorylation? Use TMT-mass spec to identify candidates; test using genetically modified mice.**

Results

1) LH-induced NPR2 dephosphorylation is mediated solely by PKA signaling.

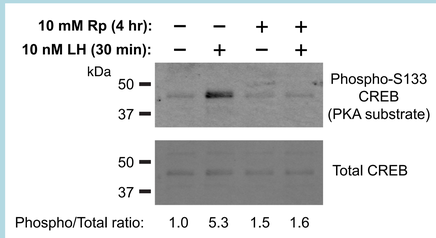


Figure 1: Complete inhibition of rapid, LH-induced PKA activity in intact, isolated mouse follicles using Rp-8-CPT-cAMPS.

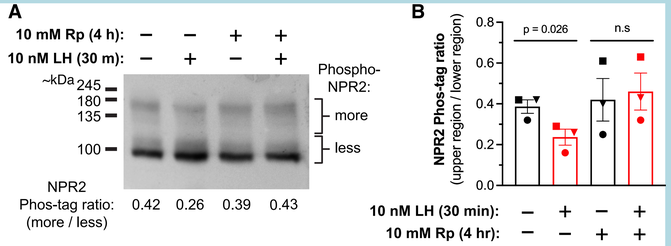


Figure 2: LH-induced NPR2 dephosphorylation is PKA-dependent. (A) HA-*Npr2* mouse follicles were run on a Phos-tag gel and probed with anti-HA. (B) Summary of 3 experiments showing mean ± SEM.

2a) Identification of candidate PPP-family subunit(s) that are phosphorylated in response to LH/PKA signaling.

Gene name	Amino acid	#	Phosphopeptide intensity (mass spec) (mean ± SEM) Control (PBS)	300 nM oLH	Fold change
PPP2R5D	**S**	**53**	16.20 ± 0.03	19.49 ± 0.27	**10.4**
PPP1R12A	**S**	**507**	16.42 ± 0.06	18.57 ± 0.59	**4.7**
PPP2R5D	**S**	**566**	19.94 ± 0.32	21.90 ± 0.35	**4.1**
PPP2R5A	S	38	16.45 ± 0.13	17.92 ± 0.07	2.9
PPP1R9A	S	185	14.20 ± 0.07	15.36 ± 0.14	2.4
PPP2R5C	S	497	19.03 ± 0.18	20.08 ± 0.31	2.2
PPP2R5D	**S**	**81***	22.39 ± 0.12	23.15 ± 0.15	**1.8**
PPP2R5D	**S**	**82***	22.12 ± 0.11	22.84 ± 0.17	**1.7**

Table 1: PPP-family phosphatase subunits that are significantly more phosphorylated in rat follicles after LH. *indicates marginally significant FDR q-values (0.041 and 0.046, respectively).

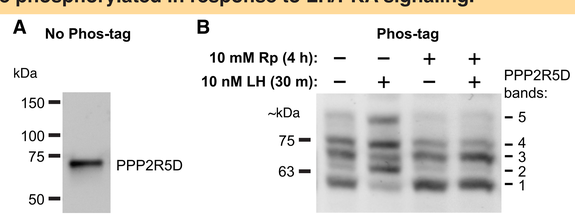


Figure 3: (A) PPP2R5D in isolated mouse follicles runs as a single band on a standard gel/blot. (B) Mouse follicle PPP2R5D is detected as 5 bands on a Phos-tag gel/blot. LH-induced PPP2R5D phosphorylation is PKA-dependent.

2b) Investigation of candidate phosphatase subunits using genetically modified mice.

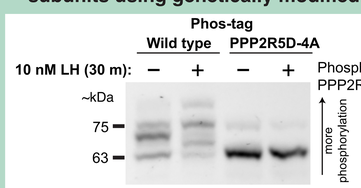


Figure 5: PPP2R5D-4A mutation prevents nearly all phosphorylation both before and after LH.

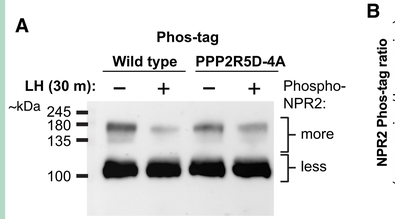


Figure 6. FIRST RESULT: PPP2R5D-4A mutation *may partially* inhibit LH-induced NPR2 dephosphorylation. (A) Phos-tag gel/blot. (B) Quantification of blot in (A).

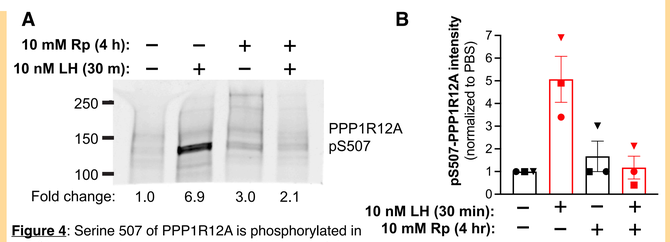
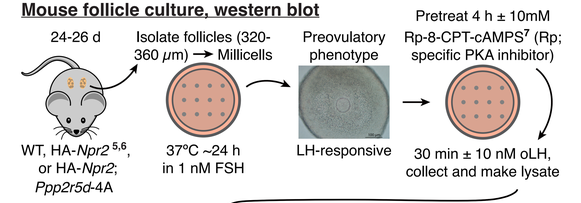


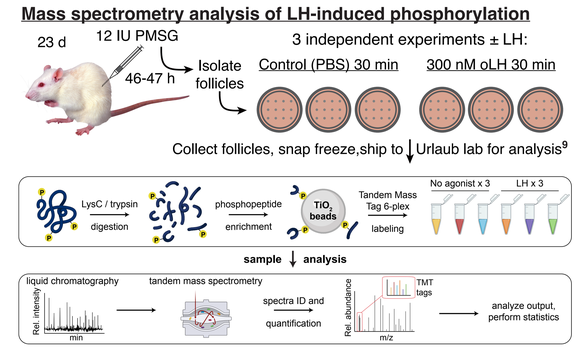
Figure 4: Serine 507 of PPP1R12A is phosphorylated in a PKA-dependent manner in response to LH in isolated mouse follicles. (A) Standard gel/blot probed with a phosphospecifc antibody. (B) Summary of 3 experiments.

Methods

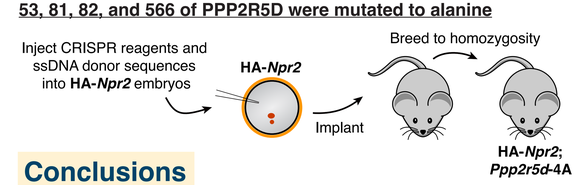
Mouse follicle culture, western blot



Mass spectrometry analysis of LH-induced phosphorylation



Generation of HA-*Npr2*;*Ppp2r5d*-4A mouse line, where serines 53, 81, 82, and 566 of PPP2R5D were mutated to alanine



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Acknowledgements

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Conclusions

- We report a method for specific and complete inhibition of PKA signaling in intact mouse ovarian follicles.
- LH-induced NPR2 dephosphorylation that leads to meiotic resumption is PKA-dependent.
- LH-PKA signaling phosphorylates PPP2R5D on multiple residues, as well as S507 of PPP1R12A.
- PPP2R5D-4A mutation does not prevent, but **may** partially inhibit, LH-induced NPR2 dephosphorylation.
- Effects of these mutations on NPR2 dephosphorylation and meiotic resumption are currently being tested.