Regulation of the Ovarian Connexin43 by Luteinizing Hormone

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Summary

It has been recently shown that LH-induced reinitiation of meiosis in mouse oocytes is mediated by mitogen activated protein kinase (MAPK), however, the mechanisms involved in this action remained unknown. We hypothesized that activation of MAPK terminates gap junctional communication in the ovarian follicle leading in turn to oocyte maturation. Accordingly, we demonstrated that LH down regulates the translation of the ovarian gap junction protein, connexin43 (Cx43) and that this response is mediated by MAPK. However, LH-induced arrest of Cx43 synthesis was observed after, but not prior to reinitiation of meiosis. On the other hand, uncoupling of the ovarian cells as well as Cx43 phosphorylation, that is also MAPK-dependent, took place immediately after exposure to LH. We conclude that MAPK mediates LH-induced oocyte maturation by reducing the permeability of gap junctions within the ovarian follicle possibly through phosphorylation of Cx43.

Introduction

The two compartments of the ovarian follicle, the oocyte and the

somatic cumulus/granulosa cells are functionally coordinated due to the presence of a network of cell-to-cell communication generated by gap junctions (Dekel, 1998). Gap junctions are composed of proteins from the connexin gene family, the most abundant of which in the ovary is connexin43 (Cx43, reviewed by Grazul-Bilska et al., 1997). Cx43 is a multiphosphorylated protein that becomes phosphorylated in response to LH (Granot and Dekel, 1994). Sequence analysis of Cx43 shows that this protein can serve as a substrate for different kinases such as the cAMP-dependent protein kinase A (PKA), protein kinase C (PKC), glycogen synthase, kinase 3 (GSK3), and mitogene activated kinase (MAPK, reviewed by Lampe and Lau, 2000).

A major role of junctional communication in the ovarian follicle is to supply nutrients from the somatic cells, which support oocyte growth (Eppig, 1979; Brower and Schultz, 1982). In addition, gap junctions mediate the transfer of cAMP from the granulosa/cumulus cells to the oocyte (reviewed by Dekel, 1998; Webb et al., 2002). cAMP serves as the regulatory signal that maintains the fully-grown oocyte in meiotic arrest (Dekel and Beers, 1978; Dekel and Beers, 1980). Reinitiation of meiosis, also known as oocyte maturation, that occurs in response to the preovulatory surge of LH is subsequent to interruption of cell-to-cell communication within the ovarian follicle (Gilula et al., 1978; Larsen et al., 1981; Sherizly et al., 1988). Breakdown of communication stops the supply of cAMP from the somatic cells to the oocyte resulting in a decrease in intraoocyte concentration of this cyclic nucleotide (reviewed by Dekel, 1988). Recently it has been demonstrated that MAPK mediates LH-induced maturation of mouse follicle-enclosed oocytes (Su et al., 2003). However, the mechanisms involved in this action remained largely unknown. We undertook the task of examining the hypothesis that activation of MAPK terminates gap junctional communication in the ovarian follicle, further exploring the mechanism of action of this kinase.

Materials and Methods

Sexually immature 23 days old female Wistar rats injected with 8 IU of pregnant mare's serum gonadotropin (PMSG) were employed for these studies. The rats underwent euthanasia by cervical dislocation 48h later. The experiments were conducted in accordance with the Guide for the Care and Use of Laboratory Animals (National Research Council, National Academy of Science, Bethesda, MD).

Isolated intact follicles were prepared and cultured as described previously (Granot and Dekel, 1994). At the end of incubation the follicles were incised and the oocytes were recovered and monitored microscopically for their meiotic status (Sherizly et al., 1988). The somatic follicle cells were further processed for MAPK phosphorylation assay (Kalma et al., 2004). Expression of Cx43 was examined by Western blot analysis

(Granot and Dekel 1994; Kalma et al., 2004). Pulse chase experiments and analysis of cx43 translation were performed as described previously (Kalma et al., 2004). For immunohystochemistry, paraffin embedded ovarian follicle sections were incubated with anti Cx43 antibodies followed by incubation with an Alexa-conjugated second antibody.

Metabolic coupling assay in the cumulus-oocyte complexes was performed as described previously (Sherizly et al., 1988). Simultaneous, double whole cell patch recordings were performed as described previously (Devor and Yarom, 2002) on cultured granulose cells in order to assess electrical coupling. Cultured granulosa cells were also employed for the evaluation of dye transfer by the scrape-loading procedure (Moyer and Ehrlich, 2003).

Results

It has been well established that the somatic cells of the ovarian follicle express predominantly Cx43 (Beyer et al., 1989; Risek et al., 1990). However, information regarding connexin expression by the oocyte is fairly limited and somewhat controversial. RT-PCR analysis successfully demonstrated Cx43 mRNA expression in mouse oocytes (Valdimarsson et al., 1993) and immunostaining localized the Cx43 protein on the oolema of cattle oocytes (Sutovsky et al., 1993). On the other hand, immunofluo-

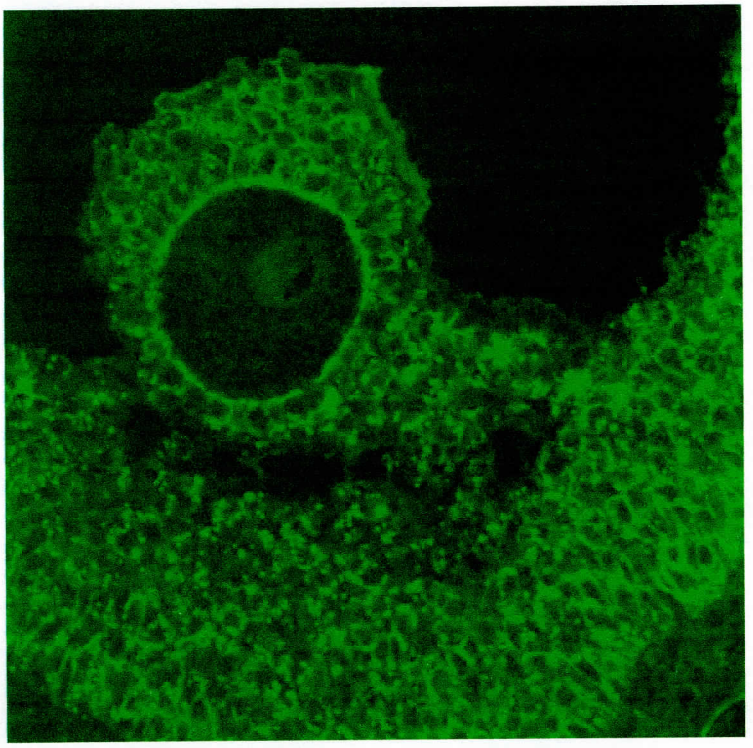

Fig1. Immunofluorescent staining of rat ovarian follicle section using specific anti Cx43 antibodies.

rescent analysis of mice ovarian sections suggested that the presence of Cx43 is restricted to the somatic follicular cells (Simon et al., 1997). In agreement with the last report, immunofluorescent staining of rat ovarian section, performed by us recently, indeed showed the high abundance of Cx43 in the granulose/cumulus cells but also exhibited an extensive staining at the interphase between the somatic cells and the oocyte (Fig. 1). The presence of Cx43 on the oolema suggested herein by immunocytochemistry, gains strong support from our previous study that used RT-PCR and Western blot analysis to demonstrate the expression of this connexin in rat oocytes (Granot et al., 2002). Complementary analysis of isolated rat oocytes by confocal microscopy as well as electron immunogold staining of ovarian sections supported these findings (Granot et al., 2002).

The expression of Cx43 mRNA and protein in the somatic cells of rat ovarian follicles were demonstrated by Northern and Western blot analysis, respectively (Granot and Dekel 1994; 1997). In addition to the developmental pattern of expression of this gene and its corresponding protein, these studies revealed that the amount of Cx43 protein is down-regulated by LH. The decreased abundance of a protein could possibly represent its enhanced degradation and/or some negative regulation elicited at its translation or at its gene transcription. Our recent studies revealed that LH does not affect the rate of Cx43 degradation but rather inhibits its synthesis (Kalma et al., 2004). The effect of LH on translatability of Cx43 was examined in intact follicles pulse-labeled with [^{35}S] methionine in the presence or absence of LH. These experiments revealed that LH remarkably reduced the incorporation of the radiolabeled amino acid into Cx43. This effect was mediated by MAPK (Kalma et al., 2004) and could not be demonstrated before 3h of exposure to the gonadotropin (Fig. 2).

Previous studies suggested that the mechanism by which LH induces oocyte maturation consists of interruption of cell-to-cell communication within the ovarian follicle, leading to a drop in intraoocyte concentration of cAMP (reviewed by Dekel, 1988). Reinitiation of meiosis in follicle

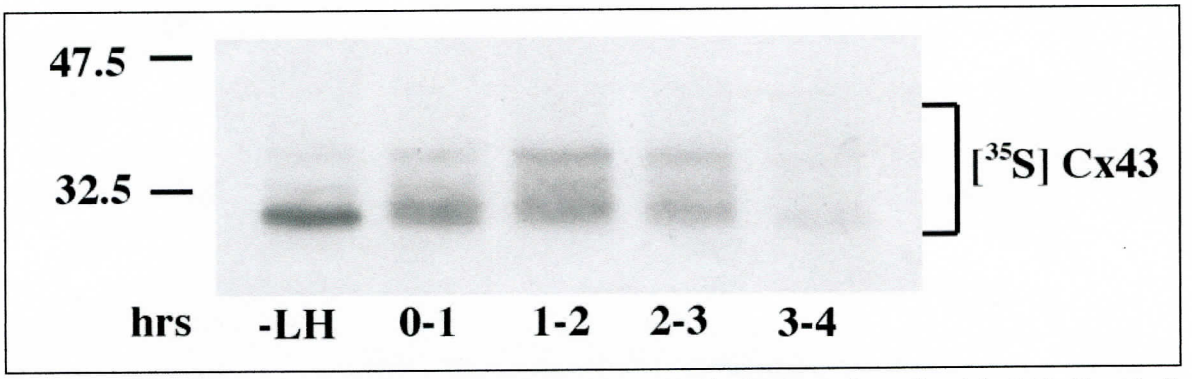

Fig. 2. The effect of LH on Cx43 translation. Follicles incubated with LH (1μg/ml) and labeled at the indicated times with [^{35}S]methionine (400 mCi/ml) were extracted, Cx43 was immunoprecipitated, and analysis by SDS-PAGE and autoradiography was conducted. Reproduced with permission from Kalma et al., (2004).

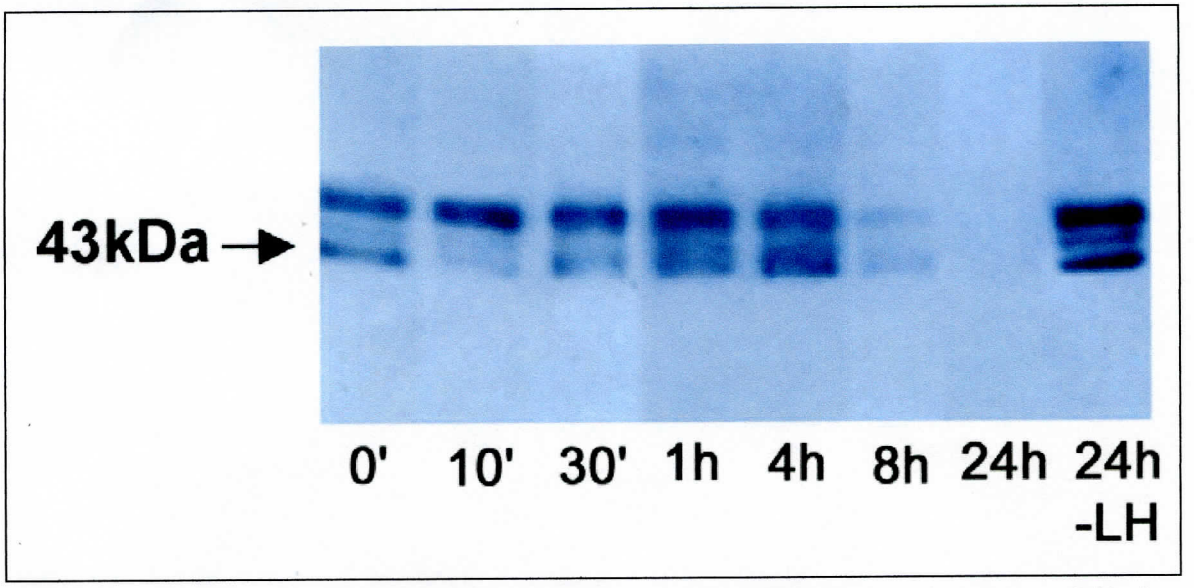

Fig. 3. Time course of LH-induced phosphorylation of Cx43 in rat ovarian follicles. Follicles were incubated with or without LH (1μg/ml) for the indicated times. At the end of incubations, cellular membranes were extracted and subjected to Western blot analysis using anti Cx43 antibodies. Reproduced with permission from Granot and Dekel (1994).

enclosed rat oocytes occurs at 2-3h after LH administration (Lazar et al., 2002). On the other hand, LH-induced inhibition of Cx43 translation takes place after 3h of exposure to the gonadotropin with about 5 additional hours required for a substantial reduction in the amount of the protein (Fig. 3, Granot and Dekel, 1994). These temporal relationships would not support the hypothesis that LH-down-regulation of Cx43 mediates the effect of this hormone on meiosis resumption. However, since incubation of isolated ovarian follicles with LH stimulated an immediate hyperphosphorylation of the Cx43 (Fig. 3, Granot and Dekel, 1994), we further suggested that conformational changes in the protein that are subsequent to its posttranslational modification may decrease the permeability of the gap junctions.

In agreement with this idea, an early decrease in metabolic coupling in response to LH was demonstrated by us in a previous study (Sherizly et al., 1988). A recent study in our laboratory employed two additional complementary methods to confirm these findings. Evaluation of electrical coupling by patch clamp analysis revealed that LH reduced the flow of current between two adjacent granulosa cells and that this effect was observed within seconds after administration of this gonadotropin. Similarly, assessment of dye transfer performed by the scrape loading technique also demonstrated an immediate closure of gap junctions within a granulosa cell monolayer manifested by inhibition of spread of Lucifer Yellow from the wounded cells to their immediate neighbors. These studies were extended to include additional evaluation of the possible in-

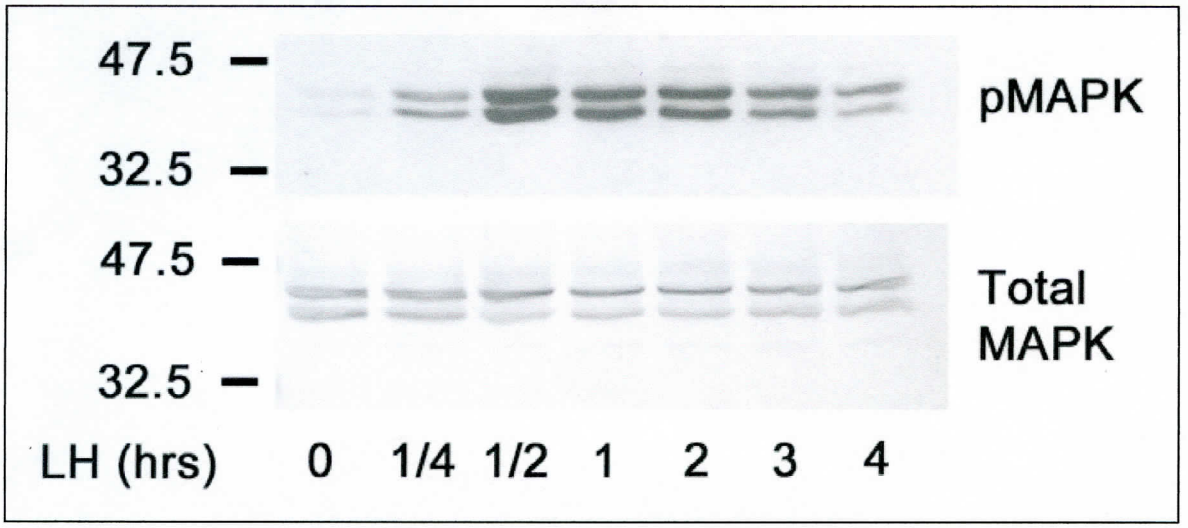

Fig. 4. Effect of LH on MAPK activation. Follicles incubated with LH (1μg/ml) for the indicated times were extracted and subjected to SDS-PAGE and Western blot analysis using antiphosphorylated and antitotal MAPK antibodies. Reproduced with permission from Kalma et al (2004).

volvement of MAPK-catalyzed Cx43 phosphorylation in the gating mechanism operated by LH on gap junctions in the ovarian follicle. This hypothesis takes into account recent demonstrations of LH-induced MAPK activation (Cameron et al., 1996) as well as the presence of MAPK consensus sequences on the Cx43 molecule (Warn-Cramer et al., 1996). In order to test our hypothesis we initially confirmed that LH activates MAPK in the somatic cells of the ovarian follicle further demonstrating that this response is observed soon after exposure to the hormone (Fig. 4).

We further employed UO126, which is a specific inhibitor of the MAPK signaling pathway. Inhibition of LH-induced MAPK activation resulted in reversal of down-regulation of cell-to-cell communication evaluated by the three complementary methods mentioned above: electrical coupling, dye transfer and metabolic coupling. Moreover, inhibition of LH-stimulated MAPK activation suppressed the effect of this hormone on oocyte maturation. In addition, LH-induced Cx43 phosphorylation was also influenced by the inhibitor of the MAPK signaling pathway.

Conclusions

Previous studies in our laboratory suggested that LH-induced breakdown of gap junctional communication within the ovarian follicle is an essential prelude for oocyte maturation. In this chapter we present more recent results that provide evidence that this response to LH is mediated by activation of MAPK in the cumulus/granulose cell compartment. These findings are compatible with the following sequence of events: binding of LH to the somatic cells of the ovarian follicle triggers an immediate activation of MAPK. The active MAPK phosphorylates Cx43 introducing

conformational changes in this protein that result in turn in closure of the gap junction channels. This early response to LH is followed by later suppression of Cx43 synthesis that is also dependent on MAPK activation. The immediate gating of the gap junction channels stops the supply of cAMP and triggers the resumption of meiosis. The later decrease in Cx43 availability ensures irreversibility of this process.

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