

Reactive oxygen species are indispensable in ovulation

Ketty Shkolnik^{a,1}, Ari Tadmor^{a,1}, Shifra Ben-Dor^b, Nava Nevo^a, Dalia Galiani^a, and Nava Dekel^{a,2}

Departments of ^aBiological Regulation and ^bBiological Services, Weizmann Institute of Science, Rehovot 76100, Israel

Edited by R. Michael Roberts, University of Missouri, Columbia, MO, and approved December 15, 2010 (received for review November 29, 2010)

Ovulation is stimulated by the preovulatory surge of the pituitary luteinizing hormone (LH). Because the ovulatory response is commonly identified with inflammation, we explored the involvement of reactive oxygen species (ROS) in this process. Our experiments show that administration of broad-range scavengers of oxidative species into the ovarian bursa of mice, hormonally induced to ovulate, significantly reduced the rate of ovulation. LH-induced cumulus mucification/expansion, a necessary requirement for ovulation, was prevented by antioxidants both in vivo and in an ex vivo system of isolated intact ovarian follicles. Along this line, H₂O₂ fully mimicked the effect of LH, bringing about an extensive mucification/expansion of the follicle-enclosed cumulus-oocyte complexes. Impaired progesterone production was observed in isolated follicles incubated with LH in the presence of the antioxidant agents. Furthermore, LH-stimulated up-regulation of genes, the expression of which is crucial for ovulation, was substantially attenuated upon ROS ablation. This system was also used for demonstrating the role of ROS in phosphorylation and activation of the EGF receptor as well as its downstream effector, p42/44 MAPK. Together, our results provide evidence that ovarian production of ROS is an essential preovulatory signaling event, most probably transiently triggered by LH.

ovary | cumulus expansion

Ovulation is an essential prelude for successful reproduction. The ovulatory process is initiated by the midcycle surge of the pituitary luteinizing hormone (LH) that induces substantial biochemical, molecular, and cellular changes, culminating in the release of a mature ovum surrounded by the cumulus cells (1). Before ovulation, the cumulus cells produce an extracellular, hyaluronan-rich matrix that brings about cumulus mucification and its expansion. This response to LH that is essential for ovulation (2) is subsequent to the expression of a set of specific genes (3) such as prostaglandin synthase 2 (Ptgs2) (4), hyaluronan synthase 2 (Has2) (5), tumor necrosis-stimulated gene 6 (Tnfaip6) (6), and CCAAT/enhancer-binding protein β (Cebpb), a downstream target of p42/44 MAPK (Erk1/2) (7). Also essential for ovulation is LH-induced progesterone receptor (Pgr) expression as well as progesterone production (8).

Numerous genes related to inflammation are induced in preovulatory follicles by the LH surge (9–12). Moreover, ovulation is suppressed by agents that inhibit acute inflammatory reactions (13). The analogy of ovulation with an acute inflammation (9, 14) may suggest a role for reactive oxygen species (ROS) along this process. ROS originate from inflammatory cells, such as macrophages and neutrophils, which are massively recruited after the LH surge to the ovary (15), and their depletion impairs ovulation (16, 17). Cyclooxygenase 2 (Ptgs2), the expression of which is a hallmark for LH-induced ovulation, is associated with inflammation and generates ROS (18). ROS are also byproducts of monooxygenase reactions mediated by the P450 systems in steroidogenic cells (19). H₂O₂, which is constantly produced in cells, has been implicated in several ovulation-related events such as steroid hormone production (20, 21) and EGF receptor (EGFR) activation (22). Overall, because ROS are massively generated during the inflammatory process (23),

we hypothesize that they may be involved in the signaling cascade leading to ovulation.

Results

Antioxidants Reduce Ovulation Rate in Equine Chorionic Gonadotropin (eCG)/Human Chorionic Gonadotropin (hCG)-Treated Mice in Vivo. The possible involvement of ROS in ovulation was examined by injecting either butylated hydroxyanisole (BHA) or *N*-acetyl cysteine (NAC), two broad-range scavengers of ROS, into the ovarian bursa of eCG-primed female mice before induction of ovulation by hCG. The number of ovulated oocytes recovered from the oviductal ampulae of mice injected with the vehicle was 28.5 ± 4.85 , whereas administration of BHA and NAC significantly reduced the ovulation rate (Fig. 1A). Morphological and histological analyses of the injected ovaries as well as their weight showed no major abnormalities, excluding a possible global toxic effect elicited by these agents (Fig. 1B and C).

ROS Are Indispensable for Cumulus Expansion. To examine the effect of ROS elimination on LH-induced expansion of the cumulus, we incubated large antral ovarian follicles in the presence of LH either with or without BHA. The cumulus-oocyte complexes (COCs) recovered from the cultured follicles were analyzed by differential interference contrast microscopy. As expected, LH treatment induced expansion and mucification of the cumulus mass, a response that was vastly inhibited by the ROS scavenger BHA (Fig. 2A and B). These findings were strongly supported by the injection of BHA into the ovarian bursa, an in vivo treatment that significantly inhibited COC expansion (Fig. S1). Interestingly, H₂O₂ on its own effectively induced expansion of the cumulus in the preovulatory follicles (Fig. 2A); this effect was inhibited by BHA (Fig. 2B).

LH-Induced Progesterone Production by Preovulatory Ovarian Follicles Is Mediated by ROS. The mediatory role of ROS in the ovulatory response was further examined by the evaluation of LH-induced progesterone production in large antral ovarian follicles cultured with or without antioxidants. As anticipated, in the presence of LH, progesterone production was stimulated in a time-dependent manner, reaching a maximal level at 4 h, with only a low baseline level detected in follicles incubated for 6 h in LH-free medium (control) (Fig. 3). However, LH added to follicles previously exposed to antioxidants almost completely failed to induce progesterone secretion (Fig. 3).

Author contributions: K.S., A.T., S.B.-D., and N.D. designed research; K.S., A.T., N.N., D.G., and N.D. performed research; K.S., A.T., S.B.-D., D.G., and N.D. analyzed data; and K.S., A.T., S.B.-D., and N.D. wrote the paper.

The authors declare no conflict of interest.

This article is a PNAS Direct Submission.

Freely available online through the PNAS open access option.

¹K.S. and A.T. contributed equally to this work.

²To whom correspondence should be addressed. E-mail: nava.dekel@weizmann.ac.il.

This article contains supporting information online at www.pnas.org/lookup/suppl/doi:10.1073/pnas.1017213108/-DCSupplemental.

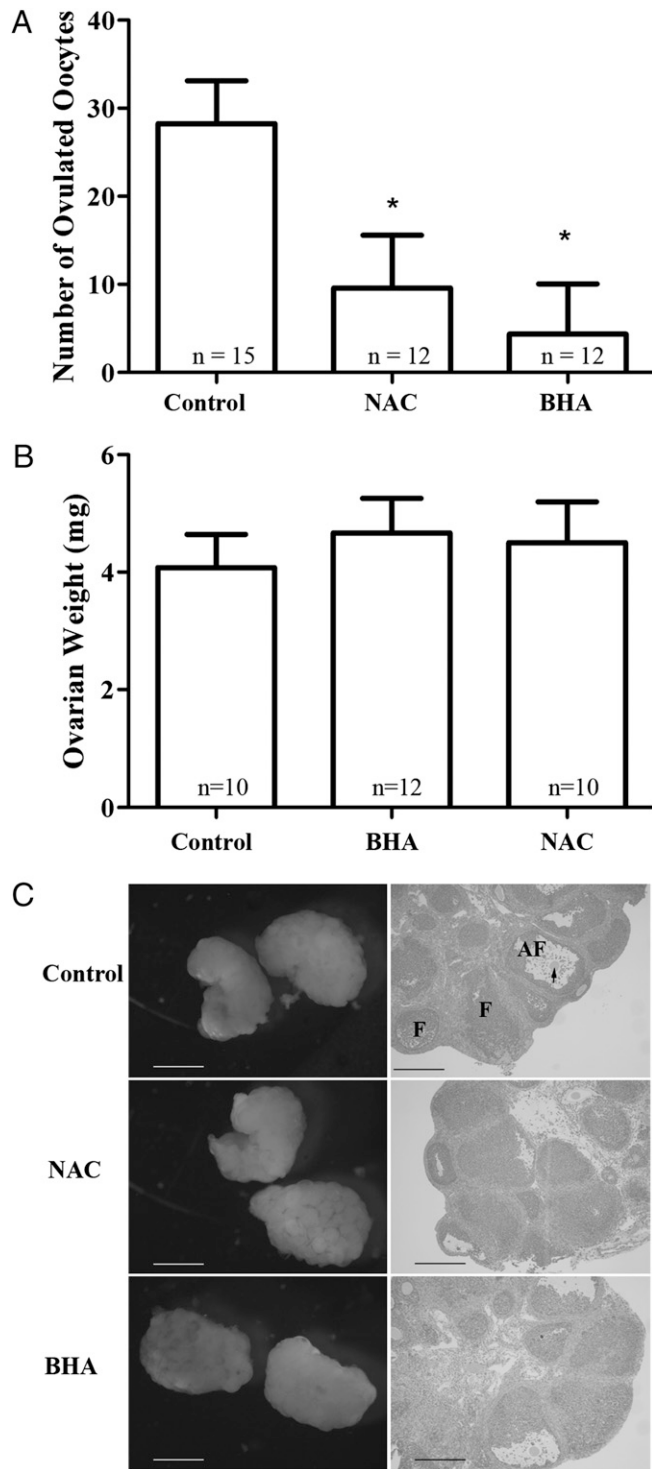


Fig. 1. Intrabursal administration of antioxidants significantly reduces ovulation rate. (A) Ovaries of eCG-primed, sexually immature mice were injected with two different antioxidant agents, BHA (250 μ M) and NAC (100 mM), 3 h before hCG administration. Ovaries of control mice were injected with the vehicle. Ovulated oocytes were flushed from the oviductal ampulae and counted 22 h later. Means having asterisks are significantly different from the control ($P < 0.05$). (B and C) The ovaries were then collected, weighted (B), examined for abnormalities (C Left), and further processed for histological analysis. Arrow points to an oocyte (C Right). n, Number of animals in each experimental sample; F, follicle; AF, antral follicle. (Scale bars: 100 μ m.)

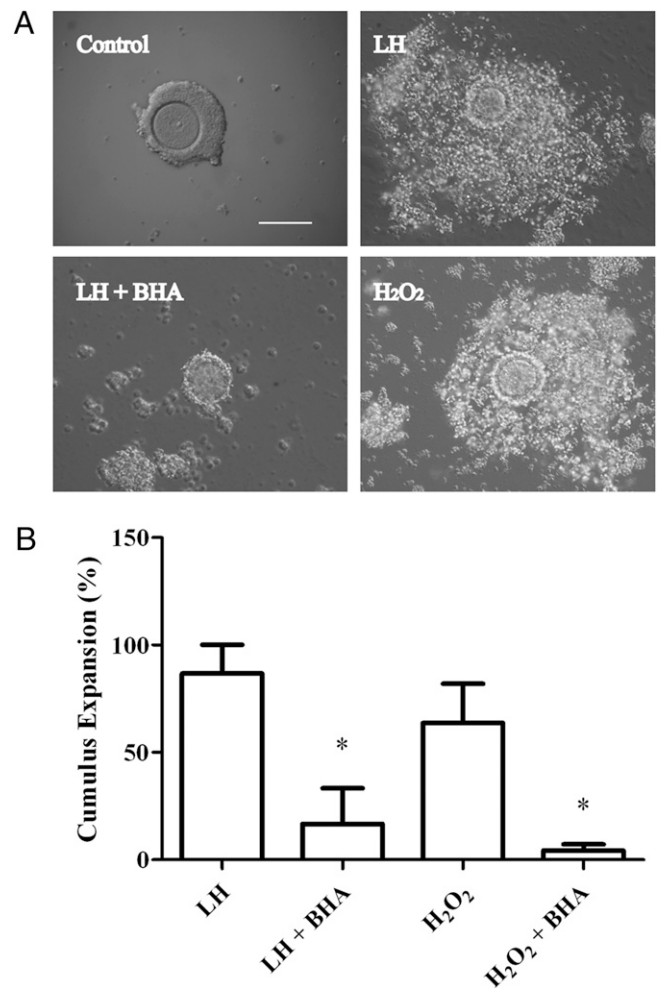


Fig. 2. Cumulus expansion depends on ROS formation. Large antral ovarian follicles were cultured with either LH (1 μ g/mL) or H₂O₂ (20 μ M) in the presence or absence of BHA (125 μ M). (A) After 8 h, the COCs were released, and expansion was monitored by using differential interference contrast microscopy. (B) The fraction of expanded cumuli under each treatment was calculated. A total of 12–18 follicles/COCs from each group were analyzed ($n = 3$).

ROS Are Essential Mediators of LH-Induced Expression of Preovulatory Genes. Large antral follicles were cultured with LH in either the presence or the absence of antioxidants, and the expression level of the following genes was evaluated: Cebpb (7), Ptg2 (24), Pgr (8), Has2 (25) and Tnfaip6 (26). Having confirmed that the mRNA expression of these genes increased from undetectable levels to reach a maximum at 4 h after the LH stimulus, the analysis of the effect of ROS ablation on the transcription level was performed at this time point. As demonstrated in Fig. 4A, the variable levels of up-regulated expression of five genes detected after LH stimulation was substantially reduced after the addition of each of the three antioxidants BHA, NAC, and glutathione (GSH). Interestingly, H₂O₂ on its own up-regulated the expression of the ovulatory genes mentioned above, mimicking the effect of LH (Fig. 4B). Specifically, Cebpb, Has2, and Tnfaip6 are inducible by H₂O₂ to the same extent as by LH, whereas Pgr and Ptg2 reached lower levels compared with LH induction.

The possibility that LH and H₂O₂ share, fully or in part, the same signaling pathways was further investigated. As expected, the effect of LH was significantly inhibited by a specific inhibitor of PKA (H89) as well as by an inhibitor of MEK1/2, the upstream regulator of p42/44 MAPK (UO126). The H₂O₂-induced gene expression was also considerably, although not totally, inhibited by

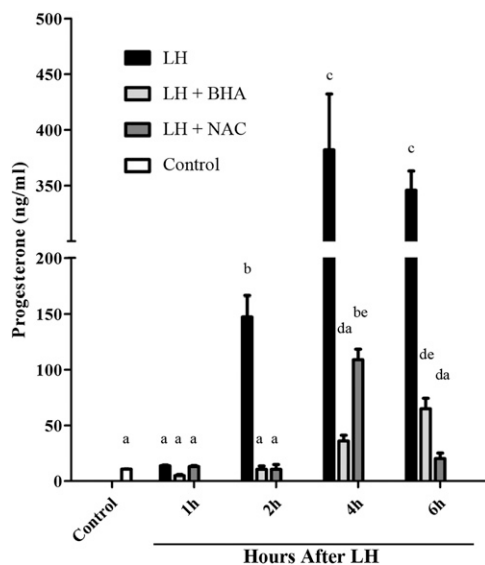


Fig. 3. ROS play a role in preovulatory progesterone production. Follicles were incubated in the presence of LH (1 μ g/mL) with or without a preincubation with BHA (125 μ M). Follicles incubated in LH-free medium for 6 h served as control. Progesterone concentrations were determined in the medium at the indicated time points. Data are representative of three independent experiments. Columns with no common superscripts are significantly different ($P < 0.05$).

these inhibitors, implying the existence of additional pathways that participate in the H_2O_2 -induced signaling cascade (Fig. 4B).

ROS Are Mediators of LH-Induced EGFR and p42/44 MAPK Activation. It has been previously demonstrated that LH induces EGFR phosphorylation in mouse ovaries *in vivo* (27) and that disruption of the EGFR signaling pathway impairs LH-induced ovulation (28). To address the role of ROS in the LH-induced EGFR-mediated ovulation, preovulatory follicles were cultured in the presence of LH with or without antioxidants, and Western blot analysis was performed to evaluate EGFR phosphorylation levels. The antibody used for detection of the phosphorylated EGFR recognizes the Y1068 residue, a major autophosphorylation site that binds Grb2 and that is involved in MAPK activation (29). We found that LH-induced phosphorylation of EGFR was substantially reduced after preincubation with either of the antioxidants, BHA or NAC (Fig. 5A), and was comparable to that obtained upon the addition of the known EGFR tyrosine kinase inhibitor AG1478. Next, we examined the effect of antioxidants on the EGFR downstream target, p42/44 MAPK. As seen in Fig. 5B, MAPK phosphorylation was significantly reduced after preincubation with the antioxidants. To further confirm the mediatory role of ROS in the LH-induced p42/44 MAPK phosphorylation, preovulatory ovarian follicles were incubated in the presence of H_2O_2 . As demonstrated in Fig. 6, the phosphorylation of p42/44 MAPK is induced by H_2O_2 in a LH-independent manner. Surprisingly, AG1478 did not affect the H_2O_2 -induced phosphorylation of p42/44 MAPK.

Discussion

In the present work, *in vivo* studies in combination with *ex vivo* and molecular analyses were used to provide the evidence that reactive oxidants present in the preovulatory ovarian follicles are essential for the ovulatory response. Our data demonstrate that ablation of the ovarian oxygen species hinders ovulation as well as a whole repertoire of essential preovulatory responses. We also show that H_2O_2 retains, at least partially, the ability to induce the abovementioned events in an LH-independent manner.

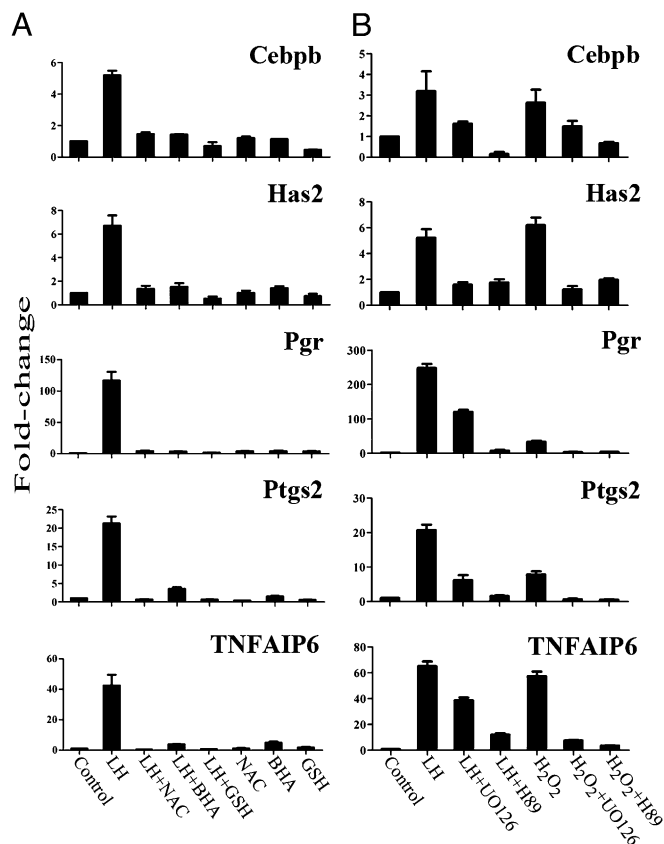


Fig. 4. ROS mediate LH-induced expression of preovulatory genes. (A) Groups of 35 follicles were incubated with LH (1 μ g/mL) in the presence or the absence of one of the following antioxidant agents—NAC (10 mM), BHA (125 μ M), and GSH (40 mM)—for 1 h before the addition of LH to the culture medium. After 4 h, the follicles were collected and homogenized, and mRNA was extracted for determination of gene expression by using quantitative real-time PCR. (B) Follicles were incubated in the presence of H_2O_2 or LH with or without either H89 (25 μ M) or UO126 (10 μ M) for 1 h. Gene expression profiles were determined by real-time PCR ($n \geq 3$).

ROS such as superoxide anion radical (O_2^-), hydrogen peroxide (H_2O_2), and hydroxyl radical (OH^-) that are produced in measurable quantities by every aerobic system were considered toxic to living cells (30, 31). However, recent reports in the field of free-radical biology describes oxyradicals as mediators of functionally significant events in a variety of biological systems (32–34). Of particular interest are previous works that have demonstrated ROS ability to activate p42/44 MAPK and EGFR, both of which are major components of the LH-induced signaling cascade in the ovary (28, 35). Moreover, a recently published paper has demonstrated the necessity of NADPH oxidase–derived ROS for the EGF/MAPK signaling pathway in uterine leiomyomas (36).

In fact, some involvement of oxyradicals in female reproduction (37), including ovulation, has been suggested previously (38). Furthermore, it was recently demonstrated that exposure to LH elevates ROS levels in ovarian follicles (39), and the suppression of ROS by superoxide dismutase in *in vitro*–perfused rabbit ovary preparations hinders ovulation (40). In this work we show that *in vivo* administration of broad-range scavengers of oxidative species such as BHA and NAC into the ovarian bursa of eCG/hCG-treated mice significantly reduces the rate of ovulation. These mice also failed to exhibit cumulus mucification/expansion, which is a prerequisite for ovulation (2). This observation gains further support by *ex vivo* experiments performed on isolated intact ovarian follicles. In this system, LH-induced cumulus mucification/

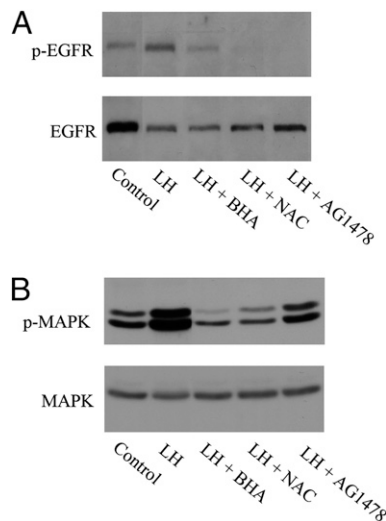


Fig. 5. LH-induced phosphorylation of both EGFR and p44/42 MAPK is reduced by ROS scavengers. (A and B) Follicles were preincubated for 1 h with either BHA (125 μ M) or NAC (10 mM) or for 30 min with the EGFR tyrosine kinase inhibitor AG1478 (10 μ M) before the addition of LH (1 μ g/mL) for an additional 30 min of incubation. Total protein was extracted and subjected to Western blot analysis. Representative experiment is shown ($n = 4$).

expansion was lost by exposure to antioxidants. Interestingly, both cumulus mucification as well as its expansion were generated by H_2O_2 on its own, whereas BHA totally prevented the LH-induced mucification.

The inhibitory effect of ROS scavengers on LH-induced ovulatory responses in the ovarian follicle was also manifested at the molecular level. The LH-stimulated up-regulation of ovulatory essential genes was substantially attenuated upon the addition of the antioxidants. Because *Ptgs2*, *Has2*, and *Tnfrsf6* are all involved in COC mucification/expansion, down-regulation of their expression by ROS scavengers may explain the impaired response of the cumulus cells discussed previously. Progesterone production, another parameter, which is tightly associated with ovulation, was also substantially reduced in isolated follicles exposed to LH in the presence of antioxidants. As progesterone synthesis and secretion take place immediately after LH receptor activation, the participation of ROS in the ovulatory process is probably an early event.

To achieve further insights regarding ROS localization along the LH-induced signaling cascade, we explored the molecular mechanism that leads to ovulation. Activation of LH-induced signaling pathways (41) generally occurs through LH-induced

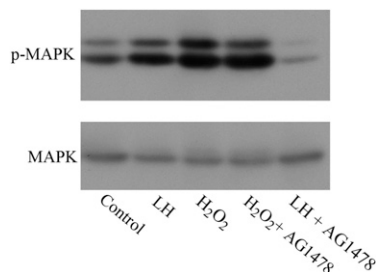


Fig. 6. H_2O_2 induces phosphorylation of p42/44 MAPK. The EGFR kinase inhibitor AG1478 (0.5 μ M) was added to follicles incubated with either LH (1 μ g/mL, control) or H_2O_2 (2 mM). The samples were subjected to Western blot analyses with anti-phospho-p42/44 MAPK antibodies followed by total MAPK antibodies. Representative experiment is shown ($n = 4$).

EGFR transactivation, which is essential for ovulation (28). Moreover, consistent with results in previous publications, LH-induced EGFR signaling-regulated MAPK activation in granulosa and cumulus cells mediates cumulus expansion and oocyte maturation (42–44). By ablating ROS using broad-range scavengers, we showed the role of ROS in phosphorylation and activation of the EGFR as well as its downstream effector, p42/44 MAPK. We also demonstrated that H_2O_2 can induce p42/44 MAPK phosphorylation in a LH-independent manner. Moreover, p42/44 MAPK activation by H_2O_2 is not affected by AG1478, an EGFR kinase inhibitor, suggesting that H_2O_2 molecules signal either downstream to the initial transactivation of the EGFR-mediated signaling cascade or through other, EGFR-independent pathways. Because the level of protein phosphorylation in a cell reflects the balance between opposing actions of protein kinases and phosphatases, inhibition of phosphatases apparently shifts the equilibrium toward phosphorylation of the p42/44 MAPK. H_2O_2 , a small and diffusible molecule that can be synthesized and destroyed rapidly in response to external stimuli, has been implicated as an intracellular messenger that modulates the extent of protein phosphorylation by inhibiting either serine/threonine or tyrosine phosphatases (32, 45, 46). Therefore, we hypothesize that H_2O_2 may stimulate phosphorylation of p42/44 MAPK either by inhibition of phosphatases or through other H_2O_2 -regulated pathways, as depicted in our proposed model (Fig. 7).

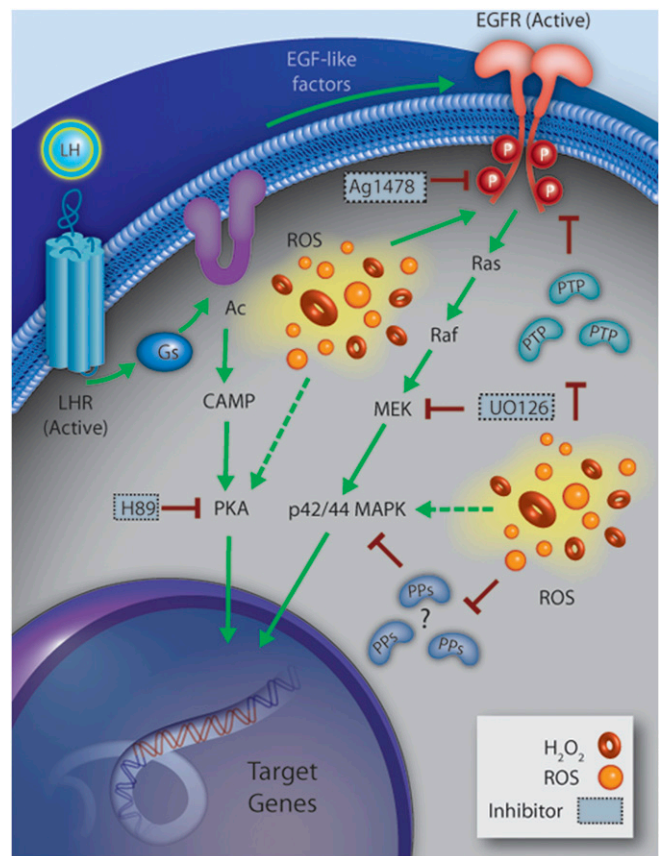


Fig. 7. The proposed mechanism for ROS involvement in ovulation. Interaction of LH with its receptor provokes an inflammatory-like response involving generation of ROS. ROS might be involved in activation of the EGFR by inhibition of either EGFR-specific phosphatases (PTPs) or MAPK phosphatases (PPs). The activated EGFR initiates a subsequent signaling cascade, which brings about p42/44 MAPK phosphorylation. Other signaling pathways take part in the activation of p42/44 MAPK as well. ROS may also be involved in activation of the PKA signaling and the MEK signaling cascade.

It was reported that the EGFR–ligand interaction causes the generation of hydrogen peroxide, followed by the direct activation of the EGFR by H₂O₂ in cells and in a cell-free system (47). The same scenario in the preovulatory ovary could be taken into account as well. The source of hydrogen peroxide can be intracellular, in association with receptor stimulation, or extracellular, produced by inflammatory cells, such as macrophages and neutrophils. Mediators of an inflammatory response that elicit an H₂O₂ effect are up-regulated in the preovulatory ovary by LH. These mediators include cytokines such as TGF- β 1, TNF- α (48–50), and IL-1 (51, 52), as well as endothelin (53, 54), platelet-activating factor (55), and others. There is also evidence for LH-inducible NADPH-dependent production of O₂⁻ (56) as well as of superoxide dismutase (37). Collectively, these reports suggest that H₂O₂, the production of which is likely to be a local event restricted to the microdomains adjacent to the site of stimulation, is an important signaling factor in various biological systems.

The predominant mediator of the LH-induced preovulatory cascade is the PKA signaling pathway (57). Whether p42/44 MAPK is downstream to the PKA cascade or activated in a PKA-independent manner is subject to dispute (58, 59). The H₂O₂-induced expression of five ovulatory essential genes was significantly down-regulated by either a MEK inhibitor (UO126) or a PKA inhibitor (H89), implying that H₂O₂ may possibly be a mediator of the LH function, upstream to PKA and p42/44 MAPK.

To summarize, this study suggests that ovarian production of ROS is an indispensable preovulatory signaling event, most probably transiently triggered by LH. In addition to their contribution to our basic understanding of the ovulatory process, our findings may have some exciting clinical implications. On the one hand, they point toward a possible detrimental effect of antioxidants on reproduction that should be carefully considered. On the other hand, they may potentially be translated into a practical, unique, nonhormonal approach for effective contraception.

Materials and Methods

Animals. Sexually immature 24-d-old female C57BL/6 mice were purchased from Harlan Laboratories and handled at the Animal Breeding Center of the Weizmann Institute of Science. The mice were injected with eCG (5 IU; Chronogest Intervest). Intrabursal injection was performed according to published procedures (60). Specifically, PBS (100 μ L) with or without BHA (250 μ M; Sigma-Aldrich) or NAC (100 mM; Sigma) was injected into both ovaries followed by an i.p. injection of hCG (5 IU; Organon) 3 h later. Twenty-two hours later, the mice were killed, the oviducts were excised and flushed, and the oocytes were counted. All experiments were conducted in accordance with the National Research Council *Guide for the Care and Use of Laboratory Animals*.

- Russell DL, Robker RL (2007) Molecular mechanisms of ovulation: Co-ordination through the cumulus complex. *Hum Reprod Update* 13:289–312.
- Chen L, Russell PT, Larsen WJ (1993) Functional significance of cumulus expansion in the mouse: Roles for the preovulatory synthesis of hyaluronic acid within the cumulus mass. *Mol Reprod Dev* 34:87–93.
- Richards JS (2007) Genetics of ovulation. *Semin Reprod Med* 25:235–242.
- Sirois J, Richards JS (1992) Purification and characterization of a novel, distinct isoform of prostaglandin endoperoxide synthase induced by human chorionic gonadotropin in granulosa cells of rat preovulatory follicles. *J Biol Chem* 267:6382–6388.
- Ashkenazi H, et al. (2005) Epidermal growth factor family members: Endogenous mediators of the ovulatory response. *Endocrinology* 146:77–84.
- Yoshioka S, et al. (2000) Expression of tumor necrosis factor-stimulated gene-6 in the rat ovary in response to an ovulatory dose of gonadotropin. *Endocrinology* 141:4114–4119.
- Fan HY, et al. (2009) MAPK3/1 (ERK1/2) in ovarian granulosa cells are essential for female fertility. *Science* 324:938–941.
- Lydon JP, et al. (1995) Mice lacking progesterone receptor exhibit pleiotropic reproductive abnormalities. *Genes Dev* 9:2266–2278.
- Richards JS, Russell DL, Ochsner S, Espey LL (2002) Ovulation: New dimensions and new regulators of the inflammatory-like response. *Annu Rev Physiol* 64:69–92.
- Liu Z, et al. (2009) Interleukin-6: An autocrine regulator of the mouse cumulus cell-oocyte complex expansion process. *Endocrinology* 150:3360–3368.
- Gérard N, Caillaud M, Martoriati A, Goudet G, Lalmanach AC (2004) The interleukin-1 system and female reproduction. *J Endocrinol* 180:203–212.
- Dinchuk JE, et al. (1995) Renal abnormalities and an altered inflammatory response in mice lacking cyclooxygenase II. *Nature* 378:406–409.

Culture of Follicles. Intact large antral ovarian follicles were recovered and placed in Leibovitz's L-15 tissue culture medium containing 5% FBS in 25-mL flasks gassed with 50% O₂ plus 50% N₂. For real-time PCR analysis, incubations were carried out at 37 °C in an oscillating water bath with or without ovine LH (0-LH-26 from National Hormone and Peptide Program, Harbor-UCLA Medical Center, Torrance, CA) or H₂O₂ (20 μ M; J. T. Baker) in the presence or absence of one of either H89 (Sigma) or UO126 (Calbiochem) added to the medium 1 h before the addition of LH or H₂O₂. For the experiments with the ROS scavengers, the samples were preincubated for 1.5 h with one of the following antioxidants: NAC (10 mM), BHA (125 μ M), or GSH (40 mM) before the addition of LH or H₂O₂. COCs were recovered from the cultured follicles and microscopically examined for cumulus expansion as described previously (61). Progesterone concentrations were determined in the cultured medium according to a published procedure (62).

Protein Extraction and Western Blot Analysis. Total protein was extracted from cultured follicles (50 per sample), and 70 μ g of protein was loaded onto an 8% SDS/PAGE for the detection of EGFR and p42/44 MAPK. The procedures of protein extraction and Western blot analysis of EGFR and p42/44 MAPK were performed as previously described by us (63).

Quantitative Real-Time PCR. RNA extraction and cDNA preparation were carried out according to the procedures previously described by us (63).

Primers were designed with Primer Express software (Applied Biosystems) and analyzed with the BLAT program for their specificity. The PCR primer pairs are described in Table S1. Relative quantification of the mRNA was performed by using the StepOne system v2.1 (Applied Biosystems). Quantitative real-time PCRs (10 μ L) were carried out with 5 μ L of mix (Fast SYBR Green Master Mix; Applied Biosystems), 2 μ L of cDNA, and 2.5 pmol of each primer. β 2-Microglobulin was used as internal control for normalization. The amplification process was monitored through the fluorescence of SYBR Green.

Statistical Analyses. Continuous data were evaluated for normality of distribution and for equality of variances by using the Shapiro–Wilk test and the Bartlett's test, respectively. Accordingly, comparisons between groups were performed with either ANOVA or Kruskal–Wallis nonparametric ANOVA; ANOVA test was used to analyze normally distributed data that had equal variances between groups, whereas Kruskal–Wallis nonparametric ANOVA was used to analyze data that were not normally distributed and/or had unequal variance between groups. Differences were considered significant at $P < 0.05$. All data are presented as means \pm SE.

ACKNOWLEDGMENTS. We thank Professors Ari Elson and Alex Tsafirri for helpful insight in this project and Ms. Rotem Kopel for protein extractions. This study was supported by the Dwek Fund for Biomedical Research. N.D. is the incumbent of the Philip M. Klutznick Professorial Chair in Developmental Biology.

- Espey LL, Stein VI, Dumitrescu J (1982) Survey of antiinflammatory agents and related drugs as inhibitors of ovulation in the rabbit. *Fertil Steril* 38:238–247.
- Espey LL (1980) Ovulation as an inflammatory reaction—a hypothesis. *Biol Reprod* 22:73–106.
- Brännström M, Mayrhofer G, Robertson SA (1993) Localization of leukocyte subsets in the rat ovary during the periovarial period. *Biol Reprod* 48:277–286.
- Van der Hoek KH, et al. (2000) Intrabursal injection of clodronate liposomes causes macrophage depletion and inhibits ovulation in the mouse ovary. *Biol Reprod* 62:1059–1066.
- Brännström M, Bonello N, Norman RJ, Robertson SA (1995) Reduction of ovulation rate in the rat by administration of a neutrophil-depleting monoclonal antibody. *J Reprod Immunol* 29:265–270.
- Baud L, Ardailou R (1986) Reactive oxygen species: Production and role in the kidney. *Am J Physiol* 251:F765–F776.
- Hanukoglu I (2006) Antioxidant protective mechanisms against reactive oxygen species (ROS) generated by mitochondrial P450 systems in steroidogenic cells. *Drug Metab Rev* 38:171–196.
- Agrawal P, Laloraya MM (1977) Induction of peroxidase in corpora lutea of rat ovary by lutropin. *Biochem J* 166:205–208.
- Nakamura Y, Ohtaki S (1990) Extracellular ATP-induced production of hydrogen peroxide in porcine thyroid cells. *J Endocrinol* 126:283–287.
- Reynolds AR, Tischer C, Verweir PJ, Rocks O, Bastiaens PI (2003) EGFR activation coupled to inhibition of tyrosine phosphatases causes lateral signal propagation. *Nat Cell Biol* 5:447–453.
- Johnson KJ, Ward PA, Kunkel RG, Wilson BS (1986) Mediation of IgA induced lung injury in the rat. Role of macrophages and reactive oxygen products. *Lab Invest* 54:499–506.

24. Lim H, et al. (1997) Multiple female reproductive failures in cyclooxygenase 2-deficient mice. *Cell* 91:197–208.
25. Chen L, et al. (1990) Hyaluronic acid synthesis and gap junction endocytosis are necessary for normal expansion of the cumulus mass. *Mol Reprod Dev* 26:236–247.
26. Fülöp C, et al. (2003) Impaired cumulus mucification and female sterility in tumor necrosis factor-induced protein-6 deficient mice. *Development* 130:2253–2261.
27. Park JY, et al. (2004) EGF-like growth factors as mediators of LH action in the ovulatory follicle. *Science* 303:682–684.
28. Hsieh M, et al. (2007) Luteinizing hormone-dependent activation of the epidermal growth factor network is essential for ovulation. *Mol Cell Biol* 27:1914–1924.
29. Rojas M, Yao S, Lin YZ (1996) Controlling epidermal growth factor (EGF)-stimulated Ras activation in intact cells by a cell-permeable peptide mimicking phosphorylated EGF receptor. *J Biol Chem* 271:27456–27461.
30. Nagy IZ (2001) On the true role of oxygen free radicals in the living state, aging, and degenerative disorders. *Ann N Y Acad Sci* 928:187–199.
31. Agarwal A, Gupta S, Sharma RK (2005) Role of oxidative stress in female reproduction. *Reprod Biol Endocrinol* 3:28.
32. Rhee SG, Bae YS, Lee SR, Kwon J (2000) Hydrogen peroxide: A key messenger that modulates protein phosphorylation through cysteine oxidation. *Sci STKE* 2000:pe1.
33. Papaiahgari S, Zhang Q, Kleeberger SR, Cho HY, Reddy SP (2006) Hyperoxia stimulates an Nrf2-ARE transcriptional response via ROS-EGFR-PI3K-Akt/ERK MAP kinase signaling in pulmonary epithelial cells. *Antioxid Redox Signal* 8:43–52.
34. Kim Y, et al. (2008) CD44-epidermal growth factor receptor interaction mediates hyaluronic acid-promoted cell motility by activating protein kinase C signaling involving Akt, Rac1, Phox, reactive oxygen species, focal adhesion kinase, and MMP-2. *J Biol Chem* 283:22513–22528.
35. DeYulia GJ, Jr., Cárcamo JM (2005) EGF receptor-ligand interaction generates extracellular hydrogen peroxide that inhibits EGFR-associated protein tyrosine phosphatases. *Biochem Biophys Res Commun* 334:38–42.
36. Mesquita FS, et al. (2010) Reactive oxygen species mediate mitogenic growth factor signaling pathways in human leiomyoma smooth muscle cells. *Biol Reprod* 82:341–351.
37. Laloraya M, Pradeep KG, Laloraya MM (1988) Changes in the levels of superoxide anion radical and superoxide dismutase during the estrous cycle of *Rattus norvegicus* and induction of superoxide dismutase in rat ovary by lutropin. *Biochem Biophys Res Commun* 157:146–153.
38. Fujii J, Iuchi Y, Okada F (2005) Fundamental roles of reactive oxygen species and protective mechanisms in the female reproductive system. *Reprod Biol Endocrinol* 3:43.
39. Yacobi K, Tsafirri A, Gross A (2007) Luteinizing hormone-induced caspase activation in rat preovulatory follicles is coupled to mitochondrial steroidogenesis. *Endocrinology* 148:1717–1726.
40. Miyazaki T, et al. (1991) Effect of inhibition of oxygen free radical on ovulation and progesterone production by the in-vitro perfused rabbit ovary. *J Reprod Fertil* 91:207–212.
41. Richards JS (2001) New signaling pathways for hormones and cyclic adenosine 3',5'-monophosphate action in endocrine cells. *Mol Endocrinol* 15:209–218.
42. Daub H, Weiss FU, Wallasch C, Ullrich A (1996) Role of transactivation of the EGF receptor in signalling by G-protein-coupled receptors. *Nature* 379:557–560.
43. Sela-Abramovich S, Chorev E, Galiani D, Dekel N (2005) Mitogen-activated protein kinase mediates luteinizing hormone-induced breakdown of communication and oocyte maturation in rat ovarian follicles. *Endocrinology* 146:1236–1244.
44. Su YQ, Wigglesworth K, Pendola FL, O'Brien MJ, Eppig JJ (2002) Mitogen-activated protein kinase activity in cumulus cells is essential for gonadotropin-induced oocyte meiotic resumption and cumulus expansion in the mouse. *Endocrinology* 143:2221–2232.
45. Fetrow JS, Siew N, Skolnick J (1999) Structure-based functional motif identifies a potential disulfide oxidoreductase active site in the serine/threonine protein phosphatase-1 subfamily. *FASEB J* 13:1866–1874.
46. den Hertog J, Ostman A, Böhmer FD (2008) Protein tyrosine phosphatases: Regulatory mechanisms. *FEB J* 275:831–847.
47. DeYulia GJ, Jr., Cárcamo JM, Bórquez-Ojeda O, Shelton CC, Golde DW (2005) Hydrogen peroxide generated extracellularly by receptor-ligand interaction facilitates cell signaling. *Proc Natl Acad Sci USA* 102:5044–5049.
48. Jasper M, Norman RJ (1995) Immunoactive interleukin-1 β and tumour necrosis factor- α in thecal, stromal and granulosa cell cultures from normal and polycystic ovaries. *Hum Reprod* 10:1352–1354.
49. Ohba M, Shibamura M, Kuroki T, Nose K (1994) Production of hydrogen peroxide by transforming growth factor- β 1 and its involvement in induction of egr-1 in mouse osteoblastic cells. *J Cell Biol* 126:1079–1088.
50. Lo YY, Cruz TF (1995) Involvement of reactive oxygen species in cytokine and growth factor induction of c-fos expression in chondrocytes. *J Biol Chem* 270:11727–11730.
51. Sattler M, et al. (1999) Hematopoietic growth factors signal through the formation of reactive oxygen species. *Blood* 93:2928–2935.
52. Simón C, et al. (1994) Interleukin-1 receptor antagonist suppresses human chorionic gonadotropin-induced ovulation in the rat. *Biol Reprod* 51:662–667.
53. Galle J, Lehmann-Bodem C, Hübner U, Heinloth A, Wanner C (2000) CyA and OxLDL cause endothelial dysfunction in isolated arteries through endothelin-mediated stimulation of O(2)(-) formation. *Nephrol Dial Transplant* 15:339–346.
54. Ko C, et al. (2006) Endothelin-2 in ovarian follicle rupture. *Endocrinology* 147:1770–1779.
55. Abisogun AO, Braquet P, Tsafirri A (1989) The involvement of platelet activating factor in ovulation. *Science* 243:381–383.
56. Jain S, Saxena D, Kumar GP, Laloraya M (2000) NADPH dependent superoxide generation in the ovary and uterus of mice during estrous cycle and early pregnancy. *Life Sci* 66:1139–1146.
57. Richards JS, et al. (2002) Novel signaling pathways that control ovarian follicular development, ovulation, and luteinization. *Recent Prog Horm Res* 57:195–220.
58. Kalma Y, Granot I, Galiani D, Barash A, Dekel N (2004) Luteinizing hormone-induced connexin 43 down-regulation: Inhibition of translation. *Endocrinology* 145:1617–1624.
59. Salvador LM, et al. (2002) Acute signaling by the LH receptor is independent of protein kinase C activation. *Endocrinology* 143:2986–2994.
60. Vaknin KM, Lazar S, Popliker M, Tsafirri A (2001) Role of meiosis-activating sterols in rat oocyte maturation: Effects of specific inhibitors and changes in the expression of lanosterol 14 α -demethylase during the preovulatory period. *Biol Reprod* 64:299–309.
61. Dekel N, Hillensjö T, Kraicer PF (1979) Maturation effects of gonadotropins on the cumulus-oocyte complex of the rat. *Biol Reprod* 20:191–197.
62. Girsh E, Dekel N (2002) Involvement of endothelin-1 and its receptors in PGF 2α -induced luteolysis in the rat. *Mol Reprod Dev* 63:71–78.
63. Reizel Y, Elbaz J, Dekel N (2010) Sustained activity of the EGF receptor is an absolute requisite for LH-induced oocyte maturation and cumulus expansion. *Mol Endocrinol* 24:402–411.