Reactive oxygen species are indispensable in ovulation

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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, H_2O_2 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). H_2O_2, 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. 1*A*). 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. 1 *B* 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. 2 *A* 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, H_2O_2 on its own effectively induced expansion of the cumulus in the preovulatory follicles (Fig. 2*A*); this effect was inhibited by BHA (Fig. 2*B*).

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.

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

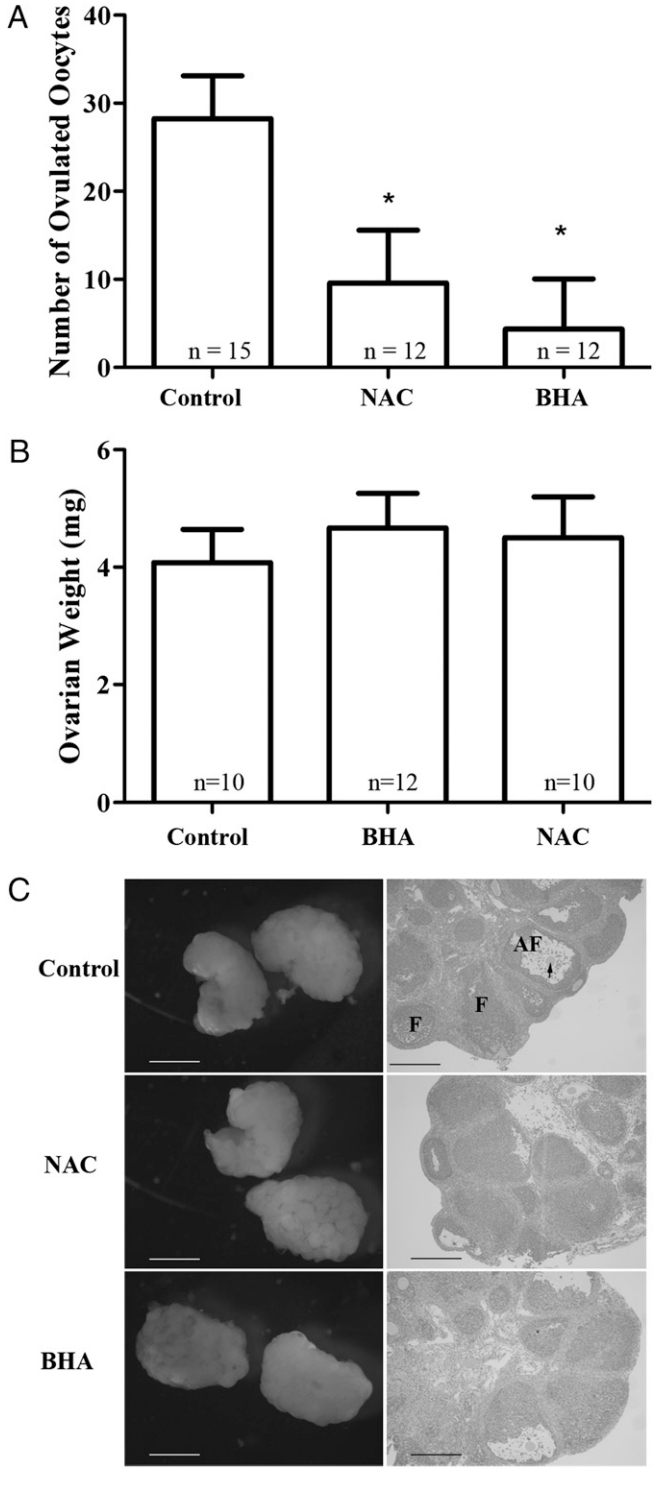

Fig. 1. Intrabursal administration of antioxidants significantly reduces ovulation rate. (*A*) Ovaries of eCG-primed, sexually immature mice were injected with two different antioxidant agents, BHA (250 μM) and NAC (100 mM), 3 h before hCG administration. Ovaries of control mice were injected with the 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 μm.)

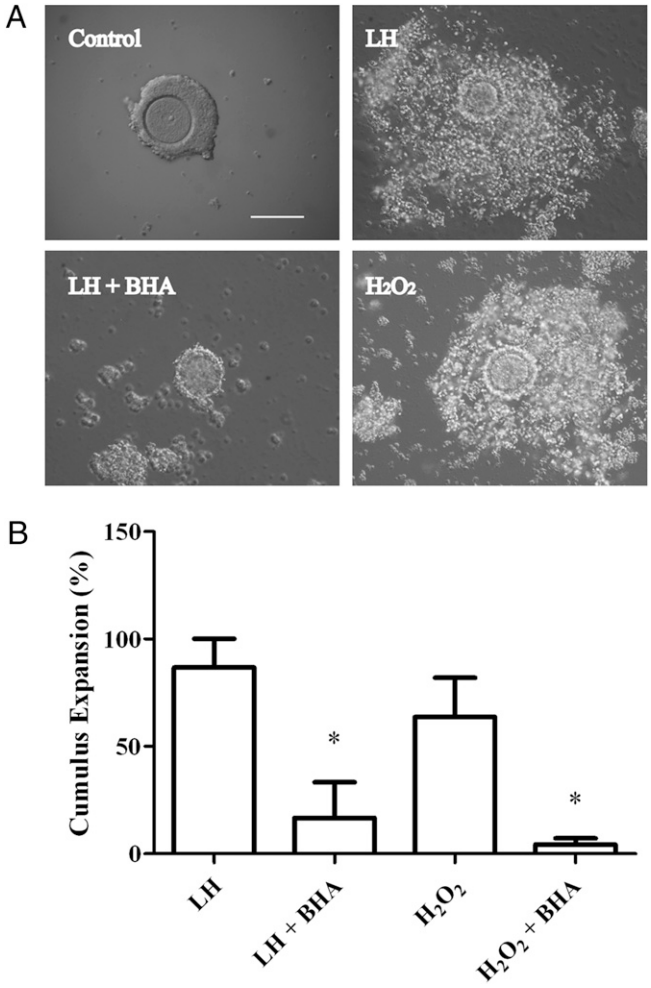

Fig. 2. Cumulus expansion depends on ROS formation. Large antral ovarian follicles were cultured with either LH (1 μg/mL) or H_2O_2 (20 μM) in the presence or absence of BHA (125 μM). (*A*) After 8 h, the COCs were released, and expansion was monitored by using differential interference contrast microscopy. (*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), Ptgs2 (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. 4*A*, 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, H_2O_2 on its own up-regulated the expression of the ovulatory genes mentioned above, mimicking the effect of LH (Fig. 4*B*). Specifically, Cebpb, Has2, and Tnfaip6 are inducible by H_2O_2 to the same extent as by LH, whereas Pgr and Ptgs2 reached lower levels compared with LH induction.

The possibility that LH and H_2O_2 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 H_2O_2-induced gene expression was also considerably, although not totally, inhibited by

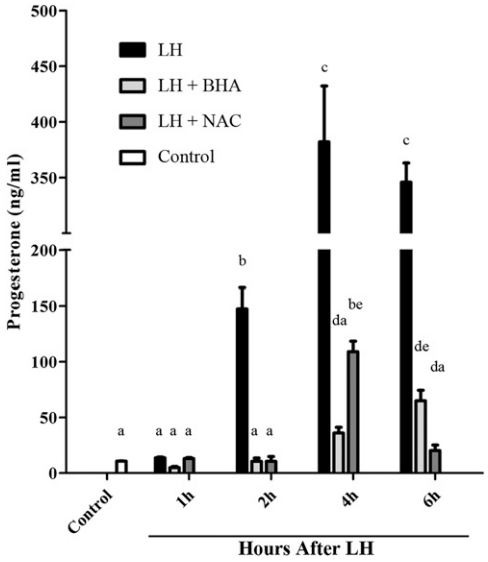

Fig. 3. ROS play a role in preovulatory progesterone production. Follicles were incubated in the presence of LH (1 μg/mL) with or without a preincubation with BHA (125 μM). Follicles incubated in LH-free medium for 6 h served as control. Progesterone concentrations were determined in the medium at the 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. 4*B*).

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. 5*A*), 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. 5*B*, 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.

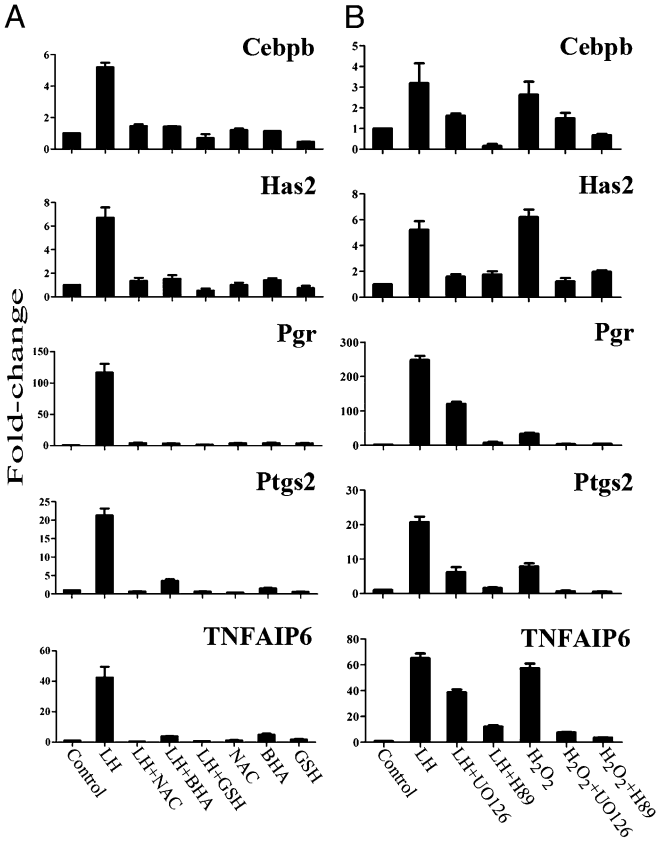

Fig. 4. ROS mediate LH-induced expression of preovulatory genes. (*A*) Groups of 35 follicles were incubated with LH (1 μg/mL) in the presence or the absence of one of the following antioxidant agents—NAC (10 mM), BHA (125 μM), and GSH (40 mM)—for 1 h before the addition of LH to the culture medium. 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 μM) or UO126 (10 μM) 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/

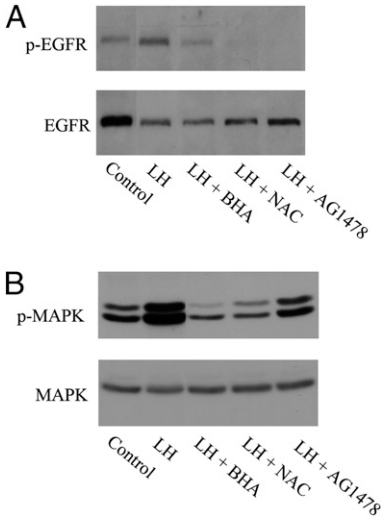

Fig. 5. LH-induced phosphorylation of both EGFR and p44/42 MAPK is reduced by ROS scavengers. (*A* and *B*) Follicles were preincubated for 1 h with either BHA (125 μM) or NAC (10 mM) or for 30 min with the EGFR tyrosine kinase inhibitor AG1478 (10 μm) before the addition of LH (1 μg/mL) for an additional 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 Tnfaip6 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 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).

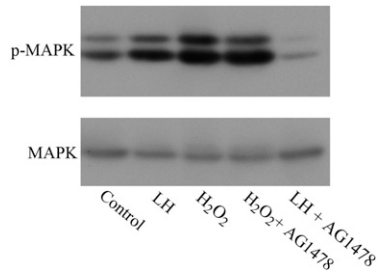

Fig. 6. H_2O_2 induces phosphorylation of p42/44 MAPK. The EGFR kinase inhibitor AG1478 (0.5 μM) was added to follicles incubated with either LH (1 μg/mL, control) or H_2O_2 (2 mM). The samples were subjected to Western blot analyses with anti–phospho-p42/44 MAPK antibodies followed by total MAPK antibodies.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

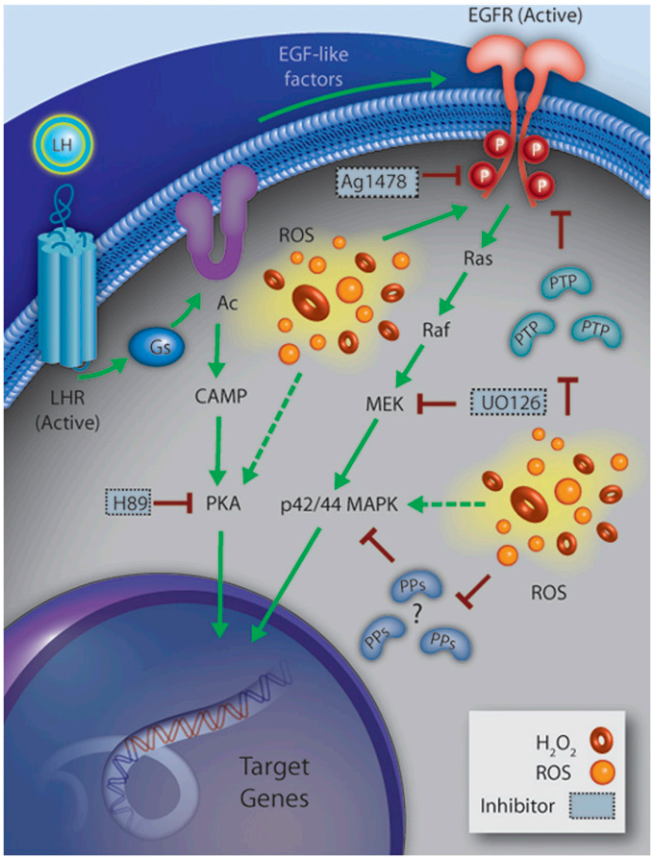

Fig. 7. The proposed mechanism for ROS involvement in ovulation. Interaction of LH with its receptor provokes an inflammatory-like response involving generation of ROS. ROS might be involved in activation of the EGFR by inhibition of either EGFR-specific phosphatases (PTPs) or MAPK phosphatases (PPs). 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 H_2O_2 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 H_2O_2 effect are up-regulated in the preovulatory ovary by LH. These mediators include cytokines such as TGF-β1, 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 O_2^- (56) as well as of superoxide dismutase (37). Collectively, these reports suggest that H_2O_2, 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 H_2O_2-induced expression of five ovulatory essential genes was significantly down-regulated by either a MEK inhibitor (UO126) or a PKA inhibitor (H89), implying that H_2O_2 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 μL) with or without BHA (250 μM; 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*.

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% O_2 plus 50% N_2. 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 H_2O_2 (20 μM; 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 H_2O_2. 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 μM), or GSH (40 mM) before the addition of LH or H_2O_2. 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 μg 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 μL) were carried out with 5 μL of mix (Fast SYBR Green Master Mix; Applied Biosystems), 2 μL of cDNA, and 2.5 pmol of each primer. β2-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 ± SE.

ACKNOWLEDGMENTS. We thank Professors Ari Elson and Alex Tsafriri 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.

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