NADPH oxidase-generated reactive oxygen species in mature follicles are essential for Drosophila ovulation

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Ovarian reactive oxygen species (ROS) are believed to regulate ovulation in mammals, but the details of ROS production in follicles and the role of ROS in ovulation in other species remain unexplored. In Drosophila ovulation, matrix metalloproteinase 2 (MMP2) is required for follicle rupture by degradation of posterior follicle cells surrounding a mature oocyte. We recently demonstrated that MMP2 activation and follicle rupture are regulated by the neuronal hormone octopamine (OA) and the octopamine receptor in mushroom body (OAMB). In the current study, we investigated the role of the superoxide-generating enzyme NADPH oxidase (NOX) in Drosophila ovulation. We report that Nox is highly enriched in mature follicle cells and that Nox knockdown in these cells leads to a reduction in superoxide and to defective ovulation. Similar to MMP2 activation, NOX enzymatic activity is also controlled by the OA/OAMB-Ca2+ signaling pathway. In addition, we report that extracellular superoxide dismutase 3 (SOD3) is required to convert superoxide to hydrogen peroxide, which acts as the key signaling molecule for follicle rupture, independent of MMP2 activation. Given that Nox homologs are expressed in mammalian follicles, the NOX-dependent hydrogen peroxide signaling pathway that we describe could play a conserved role in regulating ovulation in other species.

NADPH oxidase | superoxide dismutase | hydrogen peroxide | ovulation | octopamine

Ovulation is a key step in animal reproduction and involves multiple endocrine, paracrine, and autocrine signaling molecules, such as progesterone, epidermal growth factors, and prostaglandins. These molecules ultimately activate proteinases that break down the ovarian follicle wall, releasing a fertilizable oocyte (1–3). Several lines of evidence indicate that reactive oxygen species (ROS) also play indispensable roles in mammalian ovulation (4–8). However, there is no genetic evidence to support an in vivo role of ROS in ovulation, and the enzymes responsible for ROS production during ovulation are still unknown.

ROS are oxygen-derived, chemically reactive small molecules and include superoxide anion (O2•−), hydrogen peroxide (H2O2), and hydroxyl radicals (OH•) (9). The physiological generation of ROS can occur as a byproduct of aerobic metabolism or as the primary function of the family of NADPH oxidases (NOXs). NOX enzymes transfer an electron across the cell membrane from NADPH in the cytosol to oxygen (O2) in the luminal or extracellular space. This movement of an electron generates O2•−, which can be rapidly converted into H2O2 by superoxide dismutases (SODs).

The mammalian NOX family comprises seven members (NOX1–5 and DUOX1–2), which have marked differences in tissue distribution and play a variety of physiological roles (10, 11). Members of this family are also expressed in mammalian ovaries. Nox4 and Nox5, for example, are expressed in human granulosa cells (12). NOX4 and its accessory proteins in human granulosa cells show age-dependent reductions in protein expression, which correlates with low fertility (13). Importantly, pharmacological inhibition of NOX enzymes blocks follicle-stimulating hormone-induced oocyte maturation in mouse cumulus–oocyte complex in vitro (14). Despite these observations, a role for NOX in mammalian ovulation has not been demonstrated.

Significance

Reactive oxygen species (ROS) cause oxidative stress and damage in many pathological conditions, but they can also function as signaling molecules in physiological processes. It is difficult, however, to decipher where ROS come from and which ROS are involved in these processes. In this article, we demonstrate that a NADPH oxidase (NOX) and an extracellular superoxide dismutase (SOD3) function in follicle cells of Drosophila egg chambers to produce hydrogen peroxide, which regulates follicle rupture and ovulation, a process essential for reproduction. NOX and SOD3 are expressed in human follicles and could potentially play similar roles in humans. Our work thus provides potential targets for treating ROS-related infertility or developing novel contraceptive approaches.

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absence of ovarian muscles and oviducts (23). This work casts doubt on the proposed involvement of ovarian muscles in follicle rupture/ovulation.

In this study, we investigated the role of Nox in Drosophila ovulation. To our surprise, we found that ovarian muscle Nox does not play a major role in ovulation but rather that Nox is enriched in mature follicle cells and is essential for follicle rupture/ovulation. OA/OAMB-Ca$^{2+}$ signaling activates NOX enzymatic activity to produce extracellular O$_2^{**}$, which is converted into H$_2$O$_2$ by an extracellular SOD3. Our results suggest that NOX-produced ROS in mature follicles play a conserved role in regulating follicle rupture/ovulation across species.

**Results**

**NOX Functions in Mature Follicle Cells for Drosophila Ovulation.** Previous work indicated that NOX functions in ovarian muscles to control muscle contraction and ovulation (19). However, a careful examination of the Gal4 drivers used previously (SI Appendix, Fig. S1) and our observation of almost-normal egg laying by females with Nox knockdown in muscles (SI Appendix, Table S1) indicated that ovarian muscle NOX does not likely play a major role in ovulation. Microarray and RNA-sequencing analysis (24, 25) showed that Nox is enriched in stage-13/14 egg chambers but not in activated oocytes (SI Appendix, Fig. S2A). RT-PCR analysis of isolated follicle cells and oocytes from mature follicles (SI Appendix, Fig. S2B) further supports that Nox is enriched in follicle cells.

To probe the function of follicular NOX in late oogenesis and ovulation, we knocked down Nox in mature follicle cells. We used two independent RNA interference (RNAi) lines driven by two well-characterized Gal4 drivers, 47A04-Gal4 and 44E10-Gal4 (21–23, 26). 44E10-Gal4 is specifically expressed in follicle cells of all stage-14 egg chambers, whereas 47A04-Gal4 is only expressed in follicle cells of late-stage-14 egg chambers (21). Both Nox-RNAi lines significantly reduced Nox mRNA levels in mature follicles, with Nox-RNAi1 showing a more potent reduction (Fig. 1A). Females expressing Nox-RNAi were subjected to an egg-laying assay and showed a significant reduction in their ability to lay eggs, indicating that Nox in mature follicle cells is required for efficient egg laying (Fig. 1B). This egg-laying defect in Nox-knockdown females is not likely to be due to an oogenesis problem, as ovaries from these females contained normal or even higher numbers of mature follicles (Fig. 1C).

Next, we examined whether Nox-knockdown females are defective in ovulation and/or oviposition (the process of laying down eggs). Females with Nox knockdown (particularly with Nox-RNAi1) took a much longer time to ovulate than control females, indicating an ovulation defect (Fig. 1D and SI Appendix, Table S2). Together, these data suggest that Nox in mature follicle cells is required for normal ovulation.

To determine whether Nox regulates follicle rupture, a process induced by follicular OA/OAMB signaling during ovulation (23), we cultured Nox-knockdown follicles ex vivo by OA stimulation. Consistent with previous results (21), control follicles isolated based on 47A04 and 44E10 expression showed 76% and 39% rupture, respectively, after a 3-h culture with OA (Fig. 1E). The difference in rupture rate is due to the fact that 47A04 is expressed only in fully matured follicles (21). By contrast, Nox-knockdown follicles showed a significant reduction in OA-induced follicle rupture (Fig. 1E and SI Appendix, Fig. S2 C–H), indicating that Nox is required for normal follicle rupture. Consistent with this conclusion, pretreatment of mature follicles with diphenyleneiodonium (DPI) or VAS2870, potent NOX enzymatic inhibitors (27), was sufficient to inhibit OA-induced follicle rupture in a dose-dependent manner (SI Appendix, Fig. S2 I and J). Furthermore, the addition of butylated hydroxyanisole (BHA), a broad-spectrum ROS scavenger, in the culture medium also inhibited OA-induced follicle rupture (SI Appendix, Fig. S2K). Together, these data suggest that NOX functions in mature follicle cells to promote OA-induced follicle rupture and ovulation.

**NOX Does Not Interfere with the OA/OAMB-Ca$^{2+}$–MMP2 Pathway.** OA/OAMB signaling in mature follicle cells leads to an intracellular Ca$^{2+}$ rise and MMP2 activation (23). To determine whether NOX functions upstream of the Ca$^{2+}$ rise in the OA/OAMB-Ca$^{2+}$–MMP2 pathway, we used ionomycin, a potent Ca$^{2+}$ ionophore, to stimulate follicle rupture directly. More than 90% of control follicles ruptured after a 3-h ionomycin stimulation, in contrast to 70% (in the case of 47A04) and 40–60% (in the case of 44E10) of Nox-knockdown follicles (Fig. 2A and SI Appendix, Fig. S3 A–F). This defect was more obvious when examined before the end of the 3-h culture (Fig. 2B). These data suggest that Nox regulates molecules downstream of Ca$^{2+}$ in the OA/OAMB-Ca$^{2+}$–MMP2 pathway, or alternatively that Nox regulates a different pathway for follicle rupture that is independent from MMP2.

To differentiate between these two hypotheses, we measured MMP2 expression and activation in Nox-knockdown follicles. MMP2 protein is properly expressed in posterior follicle cells of Nox-knockdown egg chambers (Fig. 2C and D). In situ zymography showed that Nox-knockdown follicles had slightly reduced MMP activation following OA stimulation (Fig. 2E and SI Appendix, Fig. S3 G–L), however, there were no differences in collagen IV [a target of MMP2 (21), encoded by Viking (Vkg)] between control and Nox-knockdown follicles (Fig. 2F and G). These data suggest that MMP2 is unlikely to be a major downstream effector of NOX in the follicle rupture process. Consistent with this, MMP2 mRNA and genes regulating MMP2 expression and activation, including Oamb and Hnt (21), were not down-regulated in Nox-knockdown follicles (SI Appendix, Fig. S3M).

ROS regulate steroid progesterone production during mammalian ovulation (7). In addition, parallel eddy steroid signaling is required for Drosophila ovulation (26). To determine whether NOX interferes with eddy steroid production in mature follicle cells, we attempted to rescue the rupture defect of Nox-knockdown follicles with 20-hydroxyecdysone (20E). As previously reported, the addition of
Fig. 2. NOX does not interfere with the OA/OAMB-Ca$^{2+}$–MMP2 pathway. (A) Quantification of follicle rupture after 3-h culture with 5 μM ionomycin. The numbers of follicles used in each genotype are 199, 134, 111, 446, 357, and 268. (B) Cumulative follicle rupture in 3 h in response to ionomycin stimulation. Mature follicles were isolated according to 47A04-Gal4 and three groups of each genotype (−90 follicles) were used. (C and D) Representative images show MMP2::GFP expression (green in C and D and white in Insets) in mature follicles of control (C) and Nox-i1 (D) driven by 44E10-Gal4. The mature follicle cells are marked by 44E10-Gal4 driving UAS-RFP (44E10-RFP; red in C and D). Only the posterior portions of the follicles are shown. DAPI (blue in C and D) is used to mark nuclei. (E) Quantification of posterior MMP activity in control and Nox-i mature follicles with 47A04-Gal4 or 44E10-Gal4 after 3-h culture with OA using in situ zymography. The numbers of mature follicles used in each genotype are 556, 539, 322, 478, 466, and 259. (F) Representative images show three categories of BM configurations (according to Vkg::GFP expression in green) in isolated mature follicles. (G) Quantification of BM configuration of isolated mature follicles from control or Nox-i females with 44E10-Gal4. (H) Quantification of follicle rupture after treatment with or without 20 nM 20E for 30 min followed by a 6-h OA culture. The numbers of mature follicles used in each genotype are 327, 355, 324, 367, 169, and 226. **p < 0.01, ***p < 0.001. BM, basement membrane; Iono, ionomycin; Nox-i, Nox-RNAi.

20E partially rescues the defect of shd-knockdown follicles (26), which lack the ability to convert E to 20E. By contrast, the addition of 20E had no effect on the ability of Nox-knockdown follicles to respond to OA-induced rupture (Fig. 2H). It is thus unlikely that NOX affects 20E production. In addition, receptors for ecdysoyter signaling were not affected in Nox-knockdown follicles (SI Appendix, Fig. S3A). Given that ecdysoyter signaling strongly interferes with OA-induced MMP2 activation, we believe that NOX does not interfere with ecdysoyter signaling. Together, these data suggest that NOX regulates an unidentified target/pathway for follicle rupture.

Fig. 3. OA activates NOX to produce superoxide extracellularly. (A–F) Representative images show DHE staining (white in A–F) in control (A–D) and Nox-i (E and F) follicles after 30-min culture without (A and B) or with (C–F) OA stimulation. The Insets show low-magnification images with 44E10-GFP expression (green, marking stage-14 follicles) and DHE staining (red). (G–N) ∼012 Luminescence-dependent O$_2^-•$ quantification in mature follicles stimulated with OA (G–I and K–N) or ionomycin (J) at the 5-min time point. Mature follicles with different genotypes were isolated according to 44E10-RFP expression. Mature follicles in I were pretreated with BAPTA-AM for 30 min before ∼012 detection. Mature follicles in N were supplemented with SOD extract from bovine erythrocytes in the culture medium. Iono, ionomycin; Nox-i, Nox-RNAi; RLU, relative luminescence unit.

OA Activates NOX in Mature Follicle Cells to Produce Superoxide. Although NOX does not interfere with the OA/OAMB-Ca$^{2+}$–MMP2 pathway, OA/OAMB signaling may still regulate the enzymatic activity of NOX, as its N-terminal region contains EF-hand domains for Ca$^{2+}$ binding. To test this hypothesis, we examined O$_2^-•$ production in follicle cells upon OA stimulation. The fluorescent signal of dihydroethidium (DHE), a specific O$_2^-•$ indicator (28, 29), was dramatically increased in stage-14 follicle cells throughout the entire egg chamber after OA stimulation, but not in stage-13 follicle cells (Fig. 3 A–D). This increase was blocked in Nox-knockdown follicle cells (Fig. 3 E and F). To quantify O$_2^-•$ production in mature follicles, we developed a luminescence assay based on the dye L-012, which has been used to detect O$_2^-•$ in ovaries previously (19). Consistent with DHE staining, OA induced a sharp increase in O$_2^-•$ production in control follicles, which peaked at ∼30–40 min (Fig. 3G). In contrast, the increase in O$_2^-•$ production was significantly dampened in Nox-knockdown follicles (Fig. 3G) or follicles treated with the NOX inhibitor DPI or the ROS scavenger BHA (SI Appendix, Fig. S4A). In addition, when we used entire ovaries to measure OA-induced O$_2^-•$ production, Nox knockdown in mature follicle cells almost completely blocked the OA-induced O$_2^-•$ production (SI Appendix, Fig. S4B). This finding indicates that OA-induced O$_2^-•$ production is mainly restricted to mature follicle cells and depends on NOX. Thus, these data suggest that OA activates NOX in mature follicle cells to generate O$_2^-•$. Not surprisingly, OA-induced
O$_2^-$ production required OAMB (Fig. 3H). In addition, chelating the intracellular Ca$^{2+}$ with 1,2-Bis(2-aminophenoxy)ethane-N,N,N',N'-tetraacetic acid tetrakis(acetoxymethyl ester) (BAPTA-AM) blocked OA-induced O$_2^-$ production (Fig. 3J), and ionomycin was sufficient to induce O$_2^-$ production in a NOX-dependent manner (Fig. 3J). These results suggest that follicular adrenergic signaling induces an intracellular Ca$^{2+}$ rise, which activates NOX enzymatic activity in all mature follicle cells, in addition to MMP2 enzymatic activity in posterior follicle cells, during *Drosophila* ovulation.

**NOX Functions to Produce Superoxide Extracellularly.** It is unknown where NOX is localized subcellularly in mature follicle cells, as a NOX antibody is not available. To probe where NOX is localized to produce O$_2^-$ for follicle rupture, we overexpressed three distinct Sods—cytoplasmic Sod1 (30), mitochondrial Sod2 (31), and extracellular Sod3 (32, 33)—in mature follicle cells to dismutate O$_2^-$ into H$_2$O$_2$. Superoxide can hardly diffuse through cell membranes; thus, subcellularly localized SOD is required to dismutate O$_2^-$.

Overexpression of Sod1 in mature follicle cells did not reduce the amount of O$_2^-$ generated by OA stimulation (Fig. 3K), nor did overexpression of Sod2 (Fig. 3L). In contrast, overexpression of Sod3 significantly reduced the amount of OA-induced O$_2^-$ in mature follicles (Fig. 3M). We also confirmed that ectopic SOD3 is indeed secreted into the extracellular space (SI Appendix, Fig. S5 A-C). Furthermore, the addition of SOD extract from bovine erythrocytes in the culture medium was sufficient to reduce OA-induced O$_2^-$ in a dose-dependent manner (Fig. 3N). These data not only confirm the specificity of the 0.012 for O$_2^-$ detection but also suggest that NOX produces extracellular O$_2^-$, which can be dismutated by extracellular SOD3 but not cytoplasmic SOD1 or mitochondrial SOD2.

**H$_2$O$_2$, but Not Superoxide, Is the Key Signaling Molecule for Follicle Rupture.** Despite the fact that NOX regulates follicle rupture by generating O$_2^-$, which can be quickly converted to H$_2$O$_2$ by SOD3, it is still unknown whether O$_2^-$ or its derivative H$_2$O$_2$ is the signaling molecule responsible for follicle rupture. We reasoned that if O$_2^-$ is the signaling molecule for follicle rupture, overexpression of Sod3 in WT or Nox-knockdown follicles, which reduces or further reduces the O$_2^-$ level (Fig. 3M), would lead to defective rupture or an enhanced rupture defect, respectively. By contrast, overexpression of Sod1 or Sod2, which did not affect the O$_2^-$ level, would have a minimal effect. To our surprise, mature follicles with Sod3 overexpression alone had normal or even better follicle rupture in response to OA stimulation, and Sod3 overexpression in the Nox-knockdown follicles fully rescued the defect of OA-induced follicle rupture (Fig. 4A). This result indicates that H$_2$O$_2$, but not O$_2^-$, is likely the signaling molecule for follicle rupture. Unfortunately, Sod3 overexpression only partially rescued the egg-laying defect of Nox-knockdown females (Fig. 4B). This could be due to an insufficient amount of O$_2^-$ converted to H$_2$O$_2$ to execute normal physiology or because O$_2^-$ plays other roles in the egg-laying process in addition to being converted to H$_2$O$_2$ for follicle rupture/ovulation.

Consistent with the idea that H$_2$O$_2$ is the key signaling molecule for follicle rupture, overexpression of Sod1, which could produce intracellular H$_2$O$_2$ to compensate for the loss of NOX/SOD3-generated extracellular H$_2$O$_2$, exerted a similar rescue effect as Sod3 (Fig. 4 C and D). In contrast, overexpression of Sod2 in mitochondria did not show any rescue effect (Fig. 4 E and F), indicating that subcellular production of H$_2$O$_2$ is essential for follicle rupture. Consistent with this, overexpression of Catalase (*Cat*), an enzyme converting H$_2$O$_2$ to H$_2$O and O$_2$ (34), in mature follicle cells led to a strong reduction in OA-induced follicle rupture and egg-laying number (Fig. 4 G and H), but did not affect O$_2^-$ production (Fig. 3K). Notably, Sod1 overexpression alone caused a severe defect in OA-induced follicle rupture and egg laying (Fig. 4 C and D), indicating that too much intracellular H$_2$O$_2$ may be toxic for follicle rupture. Not surprisingly, the addition of H$_2$O$_2$ in the culture medium did not rescue the rupture defect of Nox-knockdown follicles (SI Appendix, Fig. S5D). Taken together, we favor the idea that a spatiotemporal burst of H$_2$O$_2$ production in the extracellular environment of mature follicle cells is critical for OA-induced follicle rupture.

**SOD3 Is Required to Convert Superoxide to H$_2$O$_2$ for Follicle Rupture.** The above studies indicate that SOD3 likely functions outside the mature follicle cells to convert NOX-produced O$_2^-$ to H$_2$O$_2$ to regulate follicle rupture/ovulation. To test this hypothesis, we specifically knocked down Sod3 in mature follicle cells. Females with Sod3 knockdown laid <20 eggs/female per day, similar to Nox-knockdown females (Fig. 5A). In addition, Sod3-knockdown mature follicles were defective in OA-induced follicle rupture (Fig. 5B). Furthermore, the defective follicle rupture/ovulation in Sod3-knockdown females could be significantly rescued by overexpression of Sod3 (Fig. 5 A and B). These data suggest that follicular SOD3 is indeed required for follicle rupture/ovulation. As predicted, O$_2^-$ accumulated fivefold in Sod3-knockdown follicles in comparison with control follicles and this accumulation could be partially reduced by overexpression of Sod3 (Fig. 5C). These results further demonstrate that H$_2$O$_2$, not O$_2^-$, is responsible for regulating follicle rupture. However, it is unclear whether H$_2$O$_2$ acts extracellularly or diffuses through the cell membrane to reach its targets for follicle rupture. In conclusion, we identified an OA/OAMB-Ca$^{2+}$-NOX-SOD3 pathway that regulates H$_2$O$_2$ production and follicle rupture in all mature follicle
cells in addition to the previously identified OA/OAMB-Ca$^{2+}$-MMP2 pathway in posterior follicle cells (Fig. 5D).

Discussion

Ovarian ROS are indispensable for ovulation in mice (7). However, the site of production of ROS is unknown and it is unclear whether ROS play a conserved role in ovulation across species. In this study, we provide genetic evidence that follicular ROS are required for ovulation in Drosophila. We demonstrate that NOX, whose activity is regulated by follicular adrenergic signaling, regulates follicle rupture and ovulation by producing O$_2^-•$ in the extracellular space of mature follicle cells (Fig. 5D). In addition, our data suggest that an extracellular SOD3 converts this O$_2^-•$ into H$_2$O$_2$, which is the key signaling molecule responsible for regulating follicle rupture (Fig. 5D). H$_2$O$_2$ can partially mimic LH in regulating cumulus expansion and gene expression in mammalian follicles (7). It is thus plausible that H$_2$O$_2$ plays a conserved role in regulating follicle rupture/ovulation from insects to mammals.

Members of the NOX family are also expressed in mouse and human granulosa cells and are functional in producing ROS (12–14). Norepinephrine, the mammalian counterpart of OA, is highly enriched in human follicular fluid and causes ROS generation in human granulosa cells (35). It will be interesting to determine whether norepinephrine plays a similar role as OA in generating ROS through regulating NOX activity during follicle rupture/ovulation in mammals.

Why would Drosophila mature follicles use NOX to generate ROS during follicle rupture? ROS can be generated through the mitochondrial respiratory chain and membrane-bound NOX family enzymes, as well as by a host of intracellular enzymes, such as xanthine oxidase, cytochrome oxidases, cytochrome P450 enzymes, and lipoxygenases that produce ROS as part of their normal enzymatic function (36). As high-level cytoplasmic ROS are detrimental to cell function and viability, limiting O$_2^-•$/H$_2$O$_2$ production in the extracellular environment may be essential for cell viability and function. This is consistent with our finding that overexpression of Sod1, which presumably produces extra-cytoplasmic H$_2$O$_2$, led to a disruption in follicle rupture and egg laying (Fig. 4C and D). Interestingly, Nox-knockdown follicles overexpressing Sod1 had normal follicle rupture (Fig. 4C), likely due to compensation of NOX-generated H$_2$O$_2$ by intracellularly produced H$_2$O$_2$, whereas bathing Nox-knockdown follicles in H$_2$O$_2$ did not rescue the defect in OA-induced follicle rupture (SI Appendix, Fig. S5D). These findings suggest that local ROS production is essential for cellular physiology, while global ROS may be detrimental.

Interestingly, Sod3 knockdown alone was sufficient to cause follicle rupture defects in Drosophila (Fig. 5A and B), yet mice lacking SOD3 are healthy and fertile (37). It is possible that SOD1 can compensate for the loss of SOD3 in mouse follicles, as mice lacking SOD1 or both SOD1 and SOD3 are subfertile or infertile, respectively (38–40).

This study solved a conundrum in Drosophila ovulation. Previous work demonstrated that follicle rupture requires OA/OAMB induction of MMP2 activity in posterior follicle cells. However, OA/OAMB induces a rise in intracellular Ca$^{2+}$ in all mature follicle cells (22, 23). What is the role of OA/OAMB-Ca$^{2+}$ in nonposterior follicle cells? Our work demonstrated that OA/OAMB-Ca$^{2+}$ signaling activates NOX in all follicle cells to produce O$_2^-•$ and H$_2$O$_2$, which are important for follicle rupture (Fig. 3 A–F). NOX-generated ROS had a minimal effect on MMP2 activity, implying that these ROS regulate an independent pathway that is required for follicle rupture (Fig. 5D). Further studies should test whether region-specific Nox knockdown, such as only in nonposterior follicle cells, causes a follicle rupture defect.

The targets of H$_2$O$_2$ in regulating follicle rupture are still unknown. Biological redox reactions catalyzed by H$_2$O$_2$ typically affect protein function by promoting the oxidation of cysteine residues (41). The best-characterized examples of H$_2$O$_2$-mediated signal transduction include several protein tyrosine phosphatases in growth factor signaling pathways, such as platelet-derived growth factor, epidermal growth factor (EGF), insulin, and B cell receptor signaling (36, 41, 42). Oxidation of the cysteine residue in the active-site motif of these phosphatases reversibly inactivates phosphatase activity and promotes growth factor signaling. The timing of H$_2$O$_2$ production and follicle rupture makes it unlikely that H$_2$O$_2$ promotes follicle rupture in Drosophila follicle cells by regulating growth factor signaling. The peak production of O$_2^-•$ (and presumably of H$_2$O$_2$) is ~30–40 min after OA stimulation (Fig. 3), which coincides with the beginning of follicle rupture (23). There is not enough time to allow growth factor signaling-mediated transcription and translation to occur before rupture happens. Alternatively, H$_2$O$_2$ is also involved in the activation of the ADAM (a disintegrin and metalloprotease) family of metalloproteinases, possibly through direct oxidation of a cysteine residue that prevents the inhibition of catalytic domain by the prodomain of the enzyme (14, 43, 44). We favor the idea that NOX-generated H$_2$O$_2$ activates ADAM or other proteinases to regulate follicle rupture in addition to MMP2 activation. Microarray and RNA-sequencing analysis identified multiple proteinases that are up-regulated in Drosophila follicle cells during ovulation (24, 25), and at least six different proteinases have been suggested to be involved in mammalian ovulation (45). Recent bioinformatics and large-scale proteomic analyses have predicted >500 proteins containing redox-active cysteine residues (46, 47), some of which could serve as the downstream effectors of H$_2$O$_2$ for follicle rupture.

Materials and Methods

Details are described in SI Appendix, SI Materials and Methods. This includes information on Drosophila genetics, egg laying and ovulation time, ex vivo follicle rupture, in situ zymography, qRT-PCR, ROS detection, immunostaining, and microscopy.
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