NADPH Oxidase and Hydrogen Peroxide are Essential for Egg Activation

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NADPH Oxidase and Hydrogen Peroxide are Essential for Egg Activation

by

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Dedication:

To those who made UConn my second home.
Abstract

Following ovulation, *Drosophila* eggs undergo activation. Egg activation allows oocytes to become mature so that they can be fertilized and sustain embryonic development. Egg activation includes hardening of the vitelline membrane (eggshell), resumption of meiosis, and changes in protein and mRNA within the oocyte. These changes allow the oocyte to prepare for further development (Kaneuchi et al 2015). Egg chambers are surrounded by a layer of follicle cells. Little is known about the follicle cells’ contribution to egg activation, specifically eggshell hardening. Therefore, this study aims to determine whether certain follicle cell genes are important in eggshell hardening. Previously, NADPH oxidase (Nox) in follicle cells was found to be essential in ovulation (Li et al 2018). In the following study, it was found that knockdown of *Nox* in follicle cells causes an eggshell hardening defect. Knockdown of *Oamb* and *Gaq*, genes that activate Nox, also cause a defect. Nox generates superoxide, which is converted to *H₂O₂* by Sod3. Knockdown of extracellular *Sod3* in follicle cells caused an eggshell hardening defect, which suggests that follicle cell generated *H₂O₂* diffuses to the oocyte to participate in egg activation. This study will relate different ovulation pathways to pathways involved in eggshell hardening.
Introduction

Egg activation is a process that follows ovulation. It is suggested that reactive oxygen species play a role in regulating ovulation (Li et al 2018). Since egg activation and ovulation are closely related processes, it is predicted that the pathways that affect ovulation also influence egg activation. The purpose of this study is to analyze the effects of the gene Nox that codes for NADPH oxidase on egg activation. In addition to this, the effects of hydrogen peroxide on egg activation will be determined. It is predicted that NADPH oxidase and hydrogen peroxide are involved in egg activation, therefore, Nox knockdown in Drosophila will cause an egg activation defect and so will the knockdown of genes upstream of Nox. It is also predicted that hydrogen peroxide is a key signaling molecule involved in egg activation. Therefore, hydrogen peroxide will be able to rescue the phenotype when any of the genes that cause an activation defect are knocked down. The following will provide background on ovulation pathways, egg activation, and the role of reactive oxygen species in reproductive processes.

Ovulation

Ovulation is an essential reproductive process in both mammals and insects such as Drosophila. Ovulation is when an oocyte (egg cell) is released from the ovary and is ready to be fertilized by sperm (Deady & Sun 2015). Many signaling molecules influence ovulation including various hormones, growth factors, and prostaglandins. These molecules allow an oocyte to become fertilizable by breaking down the follicle wall of the oocyte, thereby signaling ovulation to occur (Li et al
In this paper, ovulation and follicle rupture will be used interchangeably. The follicle cells that surround the oocyte interact with the oocyte and allow for various processes to occur prior to ovulation, preparing the egg for activation. Several genes and associated pathways have been identified in previous studies in this lab to affect ovulation. The NADPH oxidase pathway is of interest in particular as it involves many reactive oxygen species that have been found to be essential for ovulation. This study aims to investigate follicle cell genes and reactive oxygen species involved in ovulation and to determine their role in the process of egg activation, which immediately follows ovulation.

_Egg Activation_

Egg activation is a process that follows ovulation and involves many processes that prepare the egg for fertilization. These processes include eggshell hardening, resumption of meiosis, and changes in protein and mRNA in the egg. All of these changes occur at the same time within the egg and they help to prepare the egg for eventual fertilization and embryogenesis (Kaneuchi et al 2015). This study mainly focuses on eggshell hardening. The vitelline membrane of the oocyte is referred to as the eggshell. It is the outer layer of the oocyte that is eventually penetrated by sperm for fertilization. When an egg is activated and the eggshell is hardened, it appears “shiny” like the egg chambers in Figure 1.
Figure 1. Activated Eggs are Mature and Impermeable to Bleach. Activation causes hardening of the vitelline membrane, which decreases permeability (Mahowald et al 1983). Oocytes that are not activated are indicated by arrows.

Figure 1 shows what activated eggs look like, in addition to what an egg with an activation defect looks like. Eggs with an activation defect are able to be dissolved by bleach in this experiment because the eggshell hardening does not occur, meaning that the eggshell is permeable to the bleach. Activation allows the eggshell to become impermeable to bleaching. Figure 2 below shows how eggs look at different points in meiosis. The different meiotic phases can be observed when the eggs are stained. Eggs that have been activated will resume meiosis in order to prepare for embryogenesis, meaning they will appear in the phases D-G.
Figure 2. Meiosis Resumes Following Activation. Prior to activation, eggs arrest at metaphase I in meiosis, then after activation, meiosis resumes. When the nuclei are stained, meiotic phase indicates activation state. A-C depict nuclei in prometaphase and metaphase. D-G show various phases in activated eggs that have resumed meiosis.

Calcium is an important signaling molecule and triggers activation. When ovulation occurs, calcium levels in the oocyte rise, and these calcium waves move across oocytes during egg activation. The trigger for activation is IP$_3$ mediated calcium release. In addition to chemical calcium release from ovulation, mechanical pressure also stimulates calcium release. When eggs enter the oviduct after ovulation, they are squeezed through the oviduct (in Drosophila). Calcium mechanosensitive channels take in extracellular calcium, which triggers activation (Kaneuchi et al 2015). In this experiment, mechanical stimulation is simulated with the addition of a hypotonic buffer, which causes the oocytes to swell. This swelling
simulates the squeezing of the eggs in the oviduct, which triggers calcium uptake and therefore activation.

**NOX and Reactive Oxygen Species**

NADPH oxidase (NOX) is able to convert oxygen to superoxide in *Drosophila* follicle cells. In a previous study, it was found that NOX is involved in the ovulation pathway and is essential for follicle rupture. It has also been shown that reactive oxygen species (ROS) are involved in ovulation (Li et al 2018) so it is suspected that ROS are also involved in the egg activation pathway. ROS are small molecules that can cause oxidative stress, but are often key signaling molecules in physiological processes. A few important ROS for this study include superoxide dismutase and hydrogen peroxide (H$_2$O$_2$). Superoxide dismutase (SOD) converts superoxide to H$_2$O$_2$ and it is involved in follicle rupture/ovulation. It is known that NOX knockdown causes an ovulation defect and that there is cross-talk between the follicle cells that surround the oocyte and the oocyte itself. Therefore, it is suspected that these ROS and NOX play a role in egg activation.
Materials and Methods

Drosophila Genetics

The described experiments utilized the model organism *Drosophila* with the following fly stocks: OreR: UAS-DCR2/+; 44E10-Gal4,UAS-RFP/OreR, Gaq-i1: UAS-DCR2/UAS-Gaqv50729; 44E10,UAS-RFP/+, Gaq-i2: UAS-DCR2/UAS-Gaqv105300; 44E10,UAS-RFP/+, Nox-i: UAS-DCR2/UAS-NOXV4914; 44E10,UAS-RFP/+, Oamb-i: UAS-DCR2/UAS-OAMBV2861; 44E10,UAS-RFP/+, Sod3-i: UAS-DCR2/UAS-SOD3v37793; 44E10,RG6/+, Sod3-Nox-i: 44E10/UAS-NOXV4914; UAS-SOD3/+, Sod1-Nox-i: 44E10/NOXV4914; UAS-SOD1/+. Flies were bred and raised on cornmeal molasses in a 25°C incubator. The “i” at the end of the noted genotypes indicates that RNA-i knockdown experiments were done to achieve these flies. These flies were kept in a 29°C incubator. Control flies in this experiment were Oregon-R type flies. These control flies were created by crossing Gal4 drivers with Oregon-R males.

Fly Preparation and Breeding

Fly stocks were prepared by members of the Sun lab and these pre-prepared stocks were used for this experiment. Virgin female flies were collected and kept in the vials with molasses agar for 2 days in 29°C, then transferred to a new vial and fed with wet yeast before each experiment. This preparation was done for each set of flies of the various genotypes listed in the *Drosophila Genetics* section.
Dissection and Egg Activation Assay

The following protocol was adapted from a 1983 study by Mahowald. New flies that had been fed with wet yeast for at least two days were dissected using a well plate and forceps. Flies were placed in Grace’s medium in one of the wells in the well plate for dissection. Ovaries were removed using forceps and transferred to culture media (Grace’s medium, 10% fetal bovine serum, 1X penicillin/streptomycin). To ensure follicle rupture, 20μM of octopamine was added to the culture media that contained the ovaries. Following this, the ovaries were squeezed using the forceps in order to obtain mature follicles. Once the ovaries had been broken apart, the ruptured follicles were isolated using a fluorescent microscope. Follicle cells were labeled with florescence and ruptured follicles were selected using a needle. Ruptured follicles are defined as those oocytes with more than 80% area lacking follicle-cell covering. Using a needle, between 20 and 30 egg chambers were isolated and transferred to a new well containing culture media using a micropipette. This was repeated twice more to have a total of three groups of egg chambers for each genotype. The wells were labeled with the number of egg chambers that they contained.

An egg activation buffer was prepared (3.3mM NaH₂PO₄, 16.6mM KH₂PO₄, 10mM NaCl, 50mM KCl, 5% 8000 polyethylene glycol, 2mM CaCl₂) and 1mL of the buffer was added to each of the wells containing egg chambers (Mahowald et al 1983). The eggs were incubated in the activation buffer for 30 minutes at room temperature.
Using a micropipette, culture media was removed from the egg chambers. A solution of 50% bleach was added to each of the wells containing the eggs. Eggs were incubated in the bleach solution for a total of 2 minutes. After this, the bleach was removed using a micropipette and the eggs were washed with deionized water to remove any remaining bleach solution. Under a dissection microscope, the remaining egg chambers were counted. Only full, hardened eggs that appeared to be ovular and shiny were counted – any partial or dull eggs were excluded from the count. The percentage of surviving eggs was calculated and the standard error was also calculated. A Student’s T test was performed for statistical analysis.

Hydrogen Peroxide Rescue

Dissection and isolation were done as in the method described in the previous section. Five wells of 20-30 egg chambers were prepared, the number of eggs in each well was recorded. Prior to activation with the activation buffer, different amounts of H_2O_2 were added to five wells (0μM, 1μM, 10μM, 100μM, 1000μM). The eggs were incubated in the H_2O_2 for 15 minutes. Following this, activation and bleaching were done in the method previously described and the remaining eggs were observed under a dissection microscope counted. A percentage of surviving eggs was calculated for each well and the standard error was calculated. A Student’s T test was performed for statistical analysis.

DAPI Staining

The following protocol was adapted from the 1997 study by Page and Orr-Weaver. The dissection process as described previously was performed in order to obtain 70-80 ruptured follicles from each genotype. For this experiment, Oregon-R
control flies were used. The egg chambers were treated with an isolation buffer (55mM NaOAc, 40mM KOAc, 110mM sucrose, 1.2mM MgCl₂, 1mM CaCl₂, 100mM Hepes). The eggs were allowed to incubate in this buffer for 15 minutes. Following this, eggs were activated using activation buffer for periods of 15, 30, and 45 minutes. Then, eggs were incubated in Zalokar's buffer (9mM MgCl₂, 10mM MgSO₄, 2.7mM glutamic acid, 33mM glycine, 2mM malic acid, 7mM CaCl₂, 2.9mM NaH₂PO₄, 0.22mM NaOAc) for periods of 15, 30, and 45 minutes. These two buffers were used over a total period of 60 minutes, meaning that when the incubation time for activation was 15 minutes, there was a 45 minute incubation time in the Zalokar’s buffer and so on. Following the use of the two buffers, the eggs were fixed using methanol and stained using DAPI. Egg chambers were transferred to Eppendorf tubes and labeled with the genotype of the flies. Heptane was added to the eggs and an equal volume of methanol was added to the eggs. The tubes were vigorously shaken for 2 minutes. The heptane layer was discarded and most of the methanol was removed from the eggs. The eggs were rinsed with 1mL of methanol three times. The eggs were left in a 4°C refrigerator overnight for fixing. The following day, the methanol was removed from the eggs. The eggs were rehydrated in solutions of methanol and PBS (900uL:100uL; 750:250; 500:500; 250:750). When each solution was added, the eggs were placed on a nutator for 5 minutes. Following five minutes, the solution was removed from the eggs and another was added. After washing with these solutions the eggs were rinsed in PBS for 1 minute on a nutator then with PBST (0.5% TxF100) for 30 minutes. Then, DAPI stain was added to a 1:100 dilution of 1xPBS. This was added to the eggs and the eggs were allowed to
stain on a nutator for 15 minutes. The stain was removed and the eggs were mounted on a slide.

Under a fluorescent microscope, the fixed and stained eggs were counted. The phase of meiosis of each egg was recorded and the eggs in each phase were quantified. A percentage of eggs in each phase was calculated and this is how the data was presented.
Results and Discussion

Based on the following experiments, it was concluded that NADPH oxidase and hydrogen peroxide are involved in egg activation. The following results describe the bleaching assay experiments, the reactive oxygen species rescue experiments, and the DAPI staining experiments that involved the determination of meiotic phases of egg chambers.

Nox is Essential for Egg Activation

Figure 3. Nox, Oamb, and Gαq are involved in Eggshell Hardening. Knockdown of Nox in follicle cells and knockdown of genes upstream of Nox showed a significant eggshell hardening defect following egg activation and bleaching.

Based on the results from the various bleaching assays, it was determined that Nox is involved in egg activation. Based on Figure 3A, there is a significant activation defect when Nox is knocked down in follicle cells. Compared to the
control that was at about 55% survival, Nox RNA-i knockdown had only 5% survival. Many of the Nox-i eggs were dissolved by the bleach, meaning that the eggshell had not been hardened. Therefore, it can be said that Nox knockdown causes an egg activation defect.

In order to solidify this point, other experiments that included knockdown of genes upstream of Nox including Oamb, and Gaq were performed. The results obtained from this experiment were similar to that of the Nox experiment. Since these genes are upstream of Nox, the idea is that if these genes are not functional, NADPH oxidase will not function, therefore there will be an egg activation defect. The results of the Oamb RNA-i knockdown indicated that there was an egg activation defect because compared to the control, the Oamb egg activation percentage was significantly lower. For both of the Gaq knockdown experiments in Figure 3B, the control was at about 88% and the Gaq activation percentages were both less than 30%. Based on statistical analysis, the Gaq activation was significantly different from the control. Since it was so much less, it indicated that there was an egg activation defect for both types of Gaq RNA-i knockdown eggs.

Based on the results from this set of experiments, it was determined that Nox, Oamb, and Gaq all play a role in egg activation, specifically in eggshell hardening, as the defects allowed the eggs to dissolve in bleach, indicating that the eggshell had not been hardened properly by the activation buffer.

Since the percent errors were relatively low for these experiments, it can be said that the data is valid. However, the control activation percentage for the Nox and Oamb knockdown experiments was relatively low at 55% where it should be at
about 90%. This means that the experiment should be repeated so that the control has a higher activation percentage. This is most likely a result of experimenter error. The Nox knockdown experiments were performed early on in the experimental process, when the experimenter was still learning the proper method of dissection. It is possible that the egg chambers were incubated for too long in the culture media while the experimenter dissected the three genotypes and the culture media became too old and so the eggs did not activate properly. In the future, Nox knockdown experiments should be performed and recorded after the experimenter can perform dissection with three genotypes in under an hour so that the culture media does not expire and the eggs remain viable.
Hydrogen Peroxide, Not Superoxide is Essential for Egg Activation

**Figure 4. H₂O₂, Not Superoxide is the Key Molecule in Eggshell Hardening.** When Sod3 is overexpressed, there is a partial rescue of the Nox phenotype (A). When follicle cell Sod3 is knocked out, there is a significant eggshell hardening defect (B). Since Sod3 generates H₂O₂, both A and B indicate that H₂O₂ is necessary for eggshell hardening to occur. C and D show attempts at rescuing the Nox phenotype with the
addition of H$_2$O$_2$. Neither rescue was successful, which is most likely due to the specificity of time and concentration necessary for H$_2$O$_2$ to affect eggshell hardening.

Based on the results of the rescue experiments, it can be concluded that hydrogen peroxide is a key signaling molecule in the process of egg activation. The two types of experiments that were performed confirmed that H$_2$O$_2$ and not superoxide plays an essential role in eggshell hardening.

In the experiment in Figure 4A, two variants of superoxide dismutase (SOD) expressing egg chambers were tested. When Sod3 is knocked down in follicle cells, superoxide builds up in the egg cell, meaning less hydrogen peroxide is produced. When Sod3 or Sod1 is knocked down and not functioning, there is an egg activation defect, indicated by Figure 4A-B. The significant defect that is caused by knockdown of Sod3 and Sod1 does not rescue the Nox phenotype, instead, an eggshell hardening defect is observed. These defects suggest that hydrogen peroxide is essential for egg activation rather than superoxide. Superoxide is converted into hydrogen peroxide by SOD, so if SOD does not function, hydrogen peroxide is not present. The experiments in Figure 4A-B show a significant defect when SOD is not functioning, meaning that it is necessary for hydrogen peroxide to be present for egg activation. Hydrogen peroxide is also able to diffuse through the follicle cell membrane and into the oocyte, while superoxide does not, meaning that follicle cell generated hydrogen peroxide that diffuses into the oocyte is likely responsible for egg activation (Li et al 2018).
To further test this, hydrogen peroxide was used in the experiments shown in Figure 4C-D in an attempt to rescue the NOX and SOD3 phenotypes in eggs with these genes knocked down. Figure 4C shows the results of the *Nox* knockdown experiment. These results suggest that there is a slight rescue of the NOX phenotype when the concentration of hydrogen peroxide is at about 1μM. In Figure 4D, it appears that there was a slight rescue of the SOD phenotype when the concentration of hydrogen peroxide added was 10μM. This means that the presence of hydrogen peroxide allows eggshell hardening to occur despite the fact that *Nox* or *Sod3* is knocked down, both of which have already been shown to cause egg activation defects. Given these results, it becomes evident that hydrogen peroxide is a key signaling molecule in egg activation. However, the error in these experiments was very high. These experiments should be repeated with different incubation times in the hydrogen peroxide and with different concentrations to assure that the rescue does indeed occur. It is possible that there are specific hydrogen peroxide concentrations necessary to rescue these phenotypes, but the exact physiological concentration necessary is unknown. Future experiments will confirm the exact time and concentrations that hydrogen peroxide is able to induce egg activation.
Meiotic Resumption Occurs During Egg Activation

Table 1. Percent of Nuclei in Different Phases of Meiosis for Different Genotypes

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Meiosis I (A-C)</th>
<th>Meiosis II (D)</th>
<th>Normal Products (E)</th>
<th>Recondensed (F)</th>
<th>Rosetta Structure (G)</th>
</tr>
</thead>
<tbody>
<tr>
<td>OreR</td>
<td>65.30(153)</td>
<td>6.976</td>
<td>9.770</td>
<td>7.053</td>
<td>10.90</td>
</tr>
<tr>
<td>NOXi-SOD3</td>
<td>59.10(38)</td>
<td>8.263</td>
<td>14.85</td>
<td>10.08</td>
<td>10.08</td>
</tr>
<tr>
<td>NOXi-SOD1</td>
<td>75.63(36)</td>
<td>5</td>
<td>5.625</td>
<td>2.5</td>
<td>11.25</td>
</tr>
<tr>
<td>NOX</td>
<td>73.81(10)</td>
<td>2.381</td>
<td>2.381</td>
<td>11.90</td>
<td>9.523</td>
</tr>
<tr>
<td>Unruptured</td>
<td>94.74(19)</td>
<td>0</td>
<td>0</td>
<td>5.263</td>
<td>0</td>
</tr>
</tbody>
</table>

The results of the meiosis experiments indicate that – regardless of genotype – most of the oocytes remain in meiosis I following activation. This is not the predicted result. According to previous studies, the majority of nuclei should be in stage G, the rosette structure (Page & Orr-Weaver 1997). Since many of the nuclei did not enter meiosis II, it suggests that the protocol was ineffective. The resumption of meiosis is supposed to occur as a result of activation, meaning that these eggs may not have undergone activation. In addition to this, 94% of the egg chambers are unruptured when viewed under the microscope, making it very difficult to visualize the nucleus and determine the meiotic phase. The unruptured eggs are a result of the lack of isolation. For this experiment, a large number of eggs was needed for staining, however, the ruptured eggs were not isolated. In order to correct this error, octopamine will needed to be added to the eggs prior to activation. The octopamine incubation time will need to be as long as possible in order to obtain as many ruptured eggs as possible. Then, the unruptured eggs will
need to be isolated and removed from the well before fixing and staining the eggs, as was done in previous experiments.

The large number of eggs in meiosis I suggests that there was a problem with the activation buffer, the Zolakar’s buffer, or both. To rectify this, different incubation times in both of these buffers should be tried. Although increments of 15, 30, and 45 minutes of incubation for both buffers were tried and it was found that there was little difference, there still could be a better incubation time for either of the buffers. If length of the buffer incubation was not an issue, then another suggestion is to prepare the eggs in an isolation buffer before activation at different time intervals. The isolation buffer described in the 1997 Page and Orr-Weaver study was used prior to activation. This buffer was used for this experiment, but only for 15 minutes. Increasing this time could affect the number of activated eggs, as the buffer is used to prepare the eggs for activation. In conclusion, meiosis resumption will have to be studied in further detail in other studies. However, this experiment did provide an understanding and visualization of the different types of phases present in oocyte nuclei during meiosis I and meiosis II.

Future Directions

To further understand the process of egg activation and eggshell hardening, other experiments can be performed in the future. Firstly, the hydrogen peroxide rescue experiments should be repeated to confirm rescue of Nox and Sod3 phenotypes. An Sod3 overexpression bleach assay should be done to confirm whether or not Sod3 is essential for egg activation. In addition to these, Mmp2 and EcR knockdown experiments could be performed in order to determine whether
other ovulation pathways independent of those that involve reactive oxygen species affect eggshell hardening. Also, to determine how inhibiting Nox affects egg activation, experiments with a Nox inhibitor (VAS2870 or DPI) can be performed. An experiment with the ROS scavenger BHA could help determine whether egg activation is inhibited by a lack of hydrogen peroxide.
Conclusion

From this study, it was determined that Nox is involved in egg activation and that hydrogen peroxide is an essential signaling molecule for egg activation. The pathway detailed in Figure 5 shows a visual representation of the conclusion of this experiment.

Figure 5. Nox is involved in egg activation and $H_2O_2$ is an important signaling molecule for egg activation.

The pathway in Figure 5 shows that OA binds to follicle cell OAMB, which activates Gaq. Gaq causes a calcium signal to activate NADPH oxidase, which causes NADPH to become NADP+, resulting the formation of superoxide from peroxide. Superoxide dismutase converts superoxide into hydrogen peroxide. This generated hydrogen peroxide diffuses through the oocyte membrane and allows egg activation
to occur. In conclusion, this study shows that NADPH and hydrogen peroxide are essential in the process of egg activation in *Drosophila.*
Reference List


