THE PROTEIN PHOSPHATASE INHIBITOR OKADAIC ACID STIMULATES OOCYTE MATURATION AND INHIBITS EGG ACTIVATION IN ZEBRAFISH.

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ABSTRACT

In vitro treatment of fully grown immature zebrafish oocytes with dihydroxyprogesterone (DHP) resulted in germinal vesicle migration (GVM) and breakdown (GVD), ooplasmic clearing, yolk proteolysis, osmoregulation, and blastodisc formation. The DHP-matured oocytes were activated upon transfer to double distilled water as evidenced by the formation of a perivitelline space. Experiments were conducted to study the effect of okadaic acid (OA), a specific inhibitor of protein phosphatase PP-2A, on zebrafish oocyte maturation, egg activation and early embryonic development. Microarray data indicated the presence of PP-2A regulatory subunit mRNA in oocytes and anti-PP-2A regulatory subunit antibodies labeled a ~65KDa band upon immunoblotting. Immature oocytes treated with OA (1 µg/ml) alone, showed GVM and GVD, clearing, yolk proteolysis, osmoregulation and blastodisc formation. The time course for maturation with OA was delayed relative to DHP-induced maturation. In addition, OA-matured oocytes exhibited an intermediate level of yolk proteolysis compared to DHP. OA-matured oocytes had increased cortical microtubules upon labeling with anti-tubulin (DM1A) antibody, suggesting an altered oocyte cortex. In addition, the micropylar cell that labeled strongly with DM1A could not be detected after OA treatment. OA and DHP were synergistic in promoting oocyte maturation. However, upon transfer to double distilled water, the OA-matured oocytes failed to form a perivitelline space indicating their inability to activate. DHP treatment did not rescue the OA-treated oocytes and they remained inactivatible. OA also inhibited the water-induced activation of DHP-matured oocytes. OA had no demonstrable effect on zebrafish early embryonic development at the dosages tested. Our data suggest the presence of PP-2A in the zebrafish oocyte and its role in oocyte maturation and egg activation.

INTRODUCTION

Oocytes of Zebrafish, *Brachydanio rerio* are arrested in prophase-1 of meiosis as they develop and grow in the ovarian follicles. Prior to ovulation, the oocytes enter into a final developmental stage of oocyte maturation when meiosis is reinitiated in response to a steroid hormone. Oocyte maturation involves a G2 to M phase transition in the cell cycle. During in vitro maturation with 17 alpha, 20 beta-dihydroxy-4- pregnen-3-one (DHP), the opaque oocytes become translucent, the centrally located germinal vesicle (GV, nucleus of the oocyte) migrates to the periphery and breaks down, a blastodisc forms at the animal pole, yolk proteins undergo limited proteolysis and the oocytes attain the capacity to osmoregulate. Protein phosphorylation and dephosphorylation are involved in the meiotic maturation of the oocytes. Although the emphasis of previous studies has been on protein phosphorylation and kinases, less attention has been paid to the role of protein phosphatases (PP). Okadaic acid is a polyether monocarboxylic acid synthesized by several types of dinoflagellates. It is a very specific inhibitor of protein phosphatases 1 and 2A. Okadaic acid (OA) has been found to be a very useful agent to study the role of protein phosphatases in *Xenopus*, starfish, mouse, and pig oocytes. OA induces hormone independent maturation in *Xenopus*, starfish, mouse, pig oocytes; correlated with germinal vesicle breakdown (GVD) is the appearance of maturation promoting factor activity. The aim of this study was to investigate the effect of OA on zebrafish oocyte maturation.

The maturation of oocytes is completed at metaphase 2 stage, when the first polar body is extruded and the chromosomes are arranged at metaphase plate. Meiosis is arrested at this stage. Further progression of meiosis and its completion depends on the activation stimulus. Activation of zebrafish eggs is not extensively studied. Parthenogenetic activation in zebrafish can be induced using double distilled water. In the present study we devised an in vitro assay for studying egg activation. We report here that in vitro DHP matured eggs of zebrafish are activatable only during a short window of time. Okadaic acid matured oocytes are inactivatable. Finally, OA can inhibit the activation of DHP matured oocytes.

Table 1. Microarray results for Phosphatase 2A In Zebrafish using Affymetrix zebrafish array. Results presented as relative signal intensity.

Egg	Ovary	Ratio Egg/Ovary	Description
954	1444	0.7	Moderate similarity to human serine threonine phosphatase 2A, 65 kDa regulatory subunit A, beta isoform
360	1140	0.3	Protein Phosphatase 2A, regulatory B subunit, B56
1082	895	1.2	Protein Phosphatase 2A, regulatory B subunit, B56
395	697	0.6	weak similarity to phosphoprotein phosphatase 2A regulatory chain, 74K-human
312	373	0.8	*Danio rerio*, similar to protein phosphatase 2, regulatory subunit B (B56) epsilon isoform mRna
178	57	3.2	Moderate similarity to human serine threonine protein phosphatase 2A, 55 kDa regulatory subunit B, alpha isoform

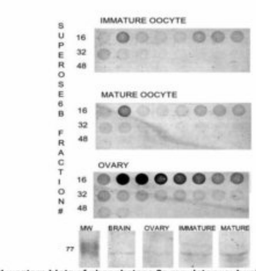

Figure 1. Dot and western blots of phosphatase 2a regulatory subunit from Superose 6B column fractions of immature oocytes, mature oocytes, and ovary.
(Upper 3 panels) Samples of 200 immature oocytes or 200 mature oocytes were homogenized in 400 µl of 0.15M NaCl 50mM Tris buffer. The samples were then centrifuged at 1000xg to spin down yolk. Samples of 400 µl of each supernatant were run on a Superose 6B column (1.5 x 30 cm) and 1ml fractions were collected. MW standards were eluted as follows: 2mDa fraction 18, 669 kDa fraction 25-26, 443 kDa fraction 28-29 and 68 kDa fraction 31-32. Aliquots (100 µl) were spotted using a Milli-D dot blot system from every other fraction starting from the 16th fraction up to 50th fraction. The nitrocellulose membrane was blocked, probed with rabbit anti-serine/threonine PP 2A/B (Exalpha P165P), then reacted with gold-labelled goat anti-rabbit IgG (GAR) and visualized with IntenSE II (GE/Amersham). Pooled zebrafish ovary weighing 135 mg from gravid females was homogenized in 270 µl of 0.15 mM NaCl 50mM Tris buffer. The sample was then centrifuged at 1000xg to spin down yolk. A supernatant sample of 270 µl was run on a Superose 6B column and fractions collected as above. Dot blotting was performed as described above.
(Lower panel) Western blot for phosphatase 2a in the ovary, immature, and mature oocytes. Samples of 30 immature oocytes, 30 mature oocytes, and 95 mg of ovary of zebrafish were homogenized in 60, 60, 190 µl of 0.15M NaCl 50mM Tris buffe,r respectively. The samples were then centrifuged at 16000xg for 5 minutes. Samples were mixed with loading buffer and 40 µl of the supernatant from each sample was run on a 1mm thick SDS-PAGE 10% gel. Western blot was performed on the resulting nitrocellulose membrane as described above for probing dot blots. MW = prestained molecular weight standards (kDa). A sample of adult zebrafish brain was run as a positive control.

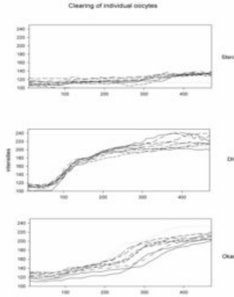

Figure 2. Computer-Aided Meiotic Maturation Assay (CAMMA) (Lessman et al, 2006 in press). Comparison of CAMMA assay of individual oocytes treated with DHP (1 µg/ml) and Okadaic acid (1 µg/ml) for 8 hours. Oocytes treated with DHP clear at a faster rate with out an initial lag period. Oocytes treated with Okadaic acid clear after an initial lag period. CAMMA uses an 8-bit grayscale with 0 = black (opaque) and 255 = white (clear).

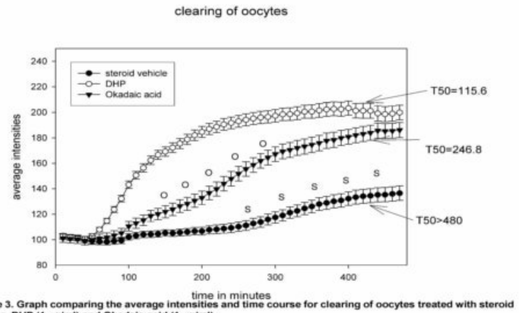

Figure 3. Graph comparing the average intensities and time course for clearing of oocytes treated with steroid vehicle, DHP (1 µg/ml) and Okadaic acid (1 µg/ml).
Data presented as mean +/- SEM and n = 40. Oocytes incubated in respective treatments were followed by CAMMA for 8 hours. The images from CAMMA were stacked and analyzed using Image J. Okadaic acid takes a longer time to cause clearing of the oocytes than DHP, ie minutes to produce a 50% clearing (T50) were significantly more for OA versus DHP. O superscript indicates significant difference between DHP versus okadaic acid treatments at the same incubation time (ANOVA). S superscript indicates significant difference between steroid vehicle versus okadaic acid treatments at the same incubation time (ANOVA).

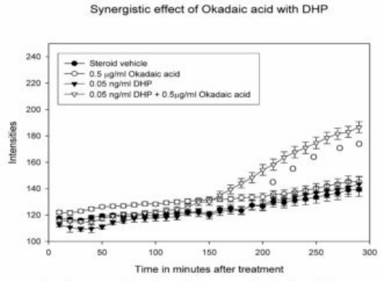

Figure 4. Graph comparing the average intensity and time course for clearing of the oocytes treated with steroid vehicle, very low doses of DHP (0.05 ng/ml), okadaic acid (0.5 µg/ml), and very low dose of DHP (0.05 ng/ml) + okadaic acid (0.5 µg/ml).
Data presented as mean +/- SEM intensity with n = 40. Oocytes treated with steroid vehicle, very low dose of DHP, or okadaic acid alone did not clear. Oocytes treated with 0.05 ng/ml of DHP and 0.5 µg/ml of Okadaic acid cleared indicating that they are synergistic in action. O superscript indicates the combined treatment is significantly different from other groups at the same time of incubation using the Students T-test ($p < 0.05$).

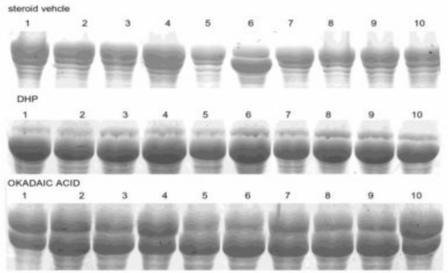

Figure 5. Individual oocyte protein profiles on SDS-PAGE after treatment with okadaic acid, DHP or steroid vehicle.
Zebrafish oocytes were treated with steroid vehicle, DHP (1µg/ml), Okadaic acid (1µg/ml) for 8 hours. Each oocyte was then homogenized in 10 µl of urea solubilization buffer. 10 µl of each individual oocyte sample was then loaded into separate lanes of a SDS-PAGE 7% 0.5mm thick gel. Gels were stained with Coomassie blue. The higher molecular weight (110 kDa) yolk band predominates in oocytes treated with steroid vehicle. The lower molecular weight (90 kDa) band predominates in oocytes treated with DHP suggesting yolk processing during maturation. Both the higher and the lower molecular weight bands are found in approximately equal proportions in the oocytes treated with okadaic acid suggesting the yolk processing is intermediate to that for DHP.

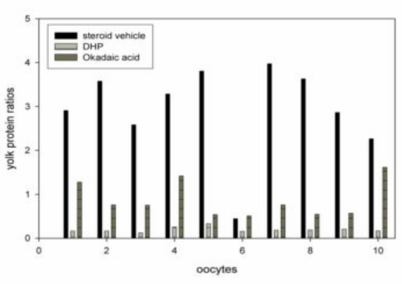

Figure 6. Density ratio of large/small MW bands (i.e. 110 and 90 kDa yolk bands, respectively) as determined by ImageJ from Fig. 5 gels above.
Graph comparing the ratio of yolk proteins of individual oocytes treated with steroid vehicle, DHP (1 µg/ml), and okadaic acid (1 µg/ml) for 8 hours. The yolk protein ratios were obtained by dividing the total signal from the higher molecular weight band (110 kDa) band with total signal from the lower molecular weight band (90 kDa). Oocytes treated with steroid vehicle have high yolk protein ratios i.e. >>1. Oocytes treated with DHP have low yolk protein ratios i.e. <<1. Oocytes treated with okadaic acid have intermediate yolk protein ratios.

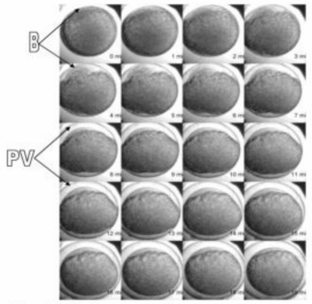

Figure 7. Activation assay montage.
Oocytes were matured with DHP (1 µg/ml) for 6 hours, the cleared oocytes were transferred to a 10ml Petri dish with double distilled water. 20 minutes after incubation in double distilled water, the oocytes were scored for the presence or absence of a perivitelline space (PV). Another feature of activation is the enlargement of the blastodisc (B).

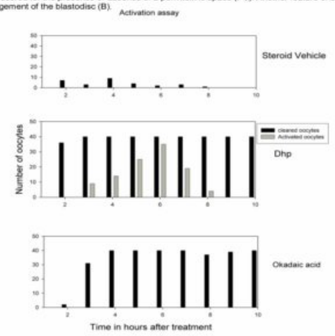

Figure 8. Activation assay after okadaic acid, DHP or steroid vehicle treatment.
Pooled oocytes dissected from gravid ovaries were divided into 30 groups and incubated with DHP (1 µg/ml), okadaic acid (1 µg/ml), or steroid vehicle for 1, 2, 3, 4, 5, 6, 7, 8, 9, and10 hours. After every hour starting from the first hour, the clearing oocytes from the respective group were transferred to a 10 ml Petri dish with double distilled water. After 20 minutes incubation in distilled water, the oocytes were scored for the presence or absence of a perivitelline space. The data were graphed with x-axis representing the time in hours after incubation and y-axis representing the number of oocytes. The black bars represent the number of clearing oocytes after treatment with DHP and the grey bars represent the number of clearing oocytes that formed a perivitelline space after 20 minute treatment with double distilled water. Oocytes started to clear after 1 hour incubation with DHP. The number of clearing oocytes that formed a perivitelline space increased from 1 hour after treatment with DHP, peaked at 6 hours after treatment, then the number decreased. By contrast, oocytes started to clear after 3 hour incubation with okadaic acid, but none of the clearing oocytes formed a perivitelline space after treatment with double distilled water. A few steroid vehicle treated oocytes showed spontaneous maturation, but these too failed to form a perivitelline space.

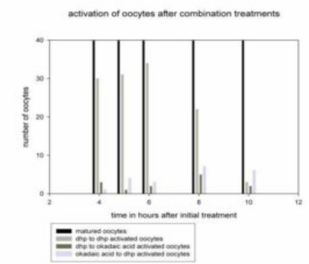

Figure 9. Effects of pretreatment with either DHP or okadaic acid on subsequent activation.
Pooled oocytes were divided into three groups. The first and second group of oocytes were pretreated with DHP (1 µg/ml). The third group of oocytes was pretreated with okadaic acid (1 µg/ml). After 4 hours of pretreatment, oocytes were washed several times in Cortland's solution. The first group of oocytes was incubated in DHP (1 µg/ml) again. The second group of oocytes was incubated in okadaic acid (1 µg/ml). The third group of oocytes was incubated in DHP (1 µg/ml). Oocytes from each group were divided into subgroups based on the duration of incubation with respective pretreatments as 4, 5, 6, 8, 10 hours. After every hour starting from the fourth hour, one subgroup of clearing oocytes were transferred to a 10 ml Petri dish with double distilled water. After a 20 minute treatment with distilled water, the oocytes were scored for the presence or absence of a perivitelline space. The data were graphed with x-axis representing the time in hours after initial pretreatment and y-axis representing the number of oocytes. The dark bar represents the number of clearing oocytes in each group. The various grayscale bars represent the number of clearing oocytes that formed a perivitelline space after 20 minute treatment with double distilled water in the respective groups. The number of clearing oocytes that formed a perivitelline space were less in the second and third groups with treatments (DHP to okadaic acid or okadaic acid to DHP) than in the first group with treatment (DHP to DHP).

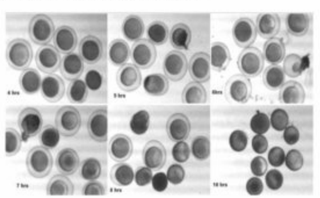

Figure 10. Montage showing the formation of perivitelline space in the oocytes pretreated with DHP then retreated with DHP and subjected to an activating stimulus (double distilled water) after varying durations of time (representative image data from Fig. 9 experiment).

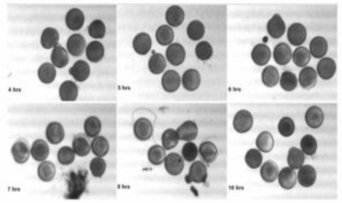

Figure 11. Montage showing the absence of perivitelline space in oocytes pretreated initially with DHP for four hours and transferred to okadaic acid. This suggests that okadaic acid can inhibit the activatability of DHP matured oocytes. Representative image data from Fig. 9 experiment.

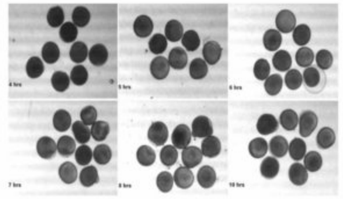

Figure 12. Montage showing the absence of perivitelline space in the oocytes pretreated initially with okadaic acid for four hours and transferred to DHP. This suggests that DHP cannot rescue the inactivatability of okadaic acid matured oocytes. Representative image data from Fig. 9 experiment

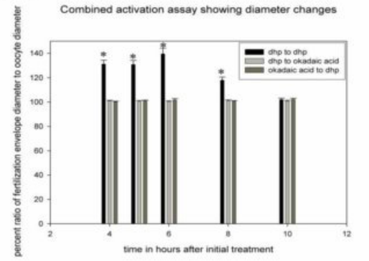

Figure 13. Quantitative analysis of perivitelline space formation after combinations of DHP/okadaic acid treatments.
The data were graphed with x-axis representing the time in hours after initial treatment and y-axis representing the ratio of fertilization envelope diameter to oocyte diameter x 100, i.e. which represents the amount of perivitelline space. The data are presented as the mean +/- SEM, n = 40. The amount of perivitelline space formed in the first group (DHP to DHP) is more than the second (DHP to okadaic acid) and third groups (okadaic acid to DHP) except for the 10 hr DHP to DHP group that fails to activate.* indicates that the diameters are significantly different.

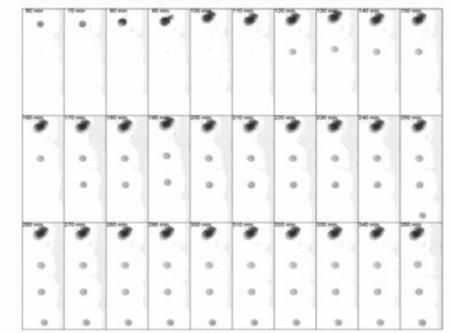

Figure 14. Montage showing the ability of maturing oocytes to osmoregulate.
Oocytes were divided into four groups and incubated with DHP (1 µg/ml) in 100% Cortland's solution for 1, 2, 3, and 4 hours respectively. After incubation, the maturing individual oocytes were transferred to wells containing double distilled water (osmotic shock). The first group of oocytes cytolysed in response to osmotic shock. Starting from the second group, as the oocytes matured they attained the capacity to osmoregulate and they survived the osmotic shock. A single representative oocyte from each group is shown in this time-lapse sequence.

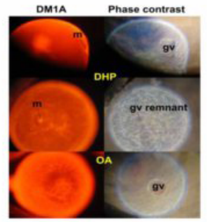

Figure 15. Alpha tubulin staining in DHP and okadaic acid maturing oocytes.
Oocytes were divided into two groups and treated with DHP (1 µg/ml) or okadaic acid (1 µg/ml). After 8 hours of incubation, oocytes were fixed in 4% paraformaldehyde overnight. Fixed oocytes were washed with PBS and incubated in mouse DM1A primary antibody. Oocytes were then incubated in Goat anti-mouse IgG-rhodamine 1:200 secondary antibody. Oocytes were cleared and examined with an epifluorescent microscope. Oocytes treated with DHP have a micropylar cell (m) in the follicular cell layer. Oocytes treated with okadaic acid (OA) have no micropylar cell in the follicular layer and there is increased signal in the cortical area suggesting clumping of tubulin in the oocyte cortex.

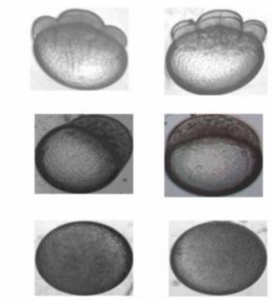

Figure 16. Absence of okadaic acid effect on early embryonic development in dechorionated zebrafish embryos.
Early stage embryos were dechorionated using dissecting forceps. The dechorionated embryos were divided into two groups. The first group was treated with steroid vehicle. The second group was treated with okadaic acid (1 µg/ml). The embryonic development was followed by automated scanner for 48 hours. No differences were found in the embryonic development between the two groups.

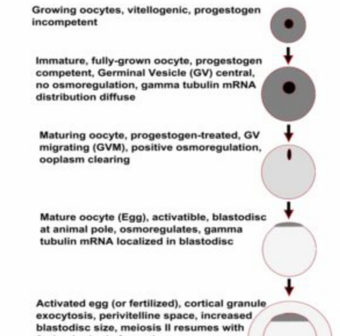

Figure 17. Cartoon summarizing some of the macro and molecular changes during oocyte maturation and egg activation in zebrafish.

CONCLUSIONS

1) **Okadaic acid causes clearing of oocytes, germinal vesicle migration and breakdown, blastodisc formation, yolk protein changes, and osmoregulation.**
2) **Okadaic acid-induced maturation is synergistic with DHP.**
3) **Okadaic acid matured oocytes have no micropylar cell and have a clumping of tubulin in the periphery.**
4) **Okadaic acid matured oocytes are inactivatable and cannot be rescued by treatment with DHP.**
5) **The activatability of DHP matured oocytes can be inhibited by Okadaic acid.**
6) **Okadaic acid (l µg/ml) has no demonstrable effect on zebrafish embryonic development .**

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