Reactive oxygen species and Udx1 during early sea urchin development

Julian L. Wong, Gary M. Wessel*

Department of Molecular Biology, Cellular Biology, and Biochemistry, Box G-J4, Brown University, Providence, RI 02912, USA

Received for publication 7 June 2005, revised 8 July 2005, accepted 8 July 2005

Abstract

Sea urchin fertilization is marked by a massive conversion of molecular oxygen to hydrogen peroxide by a sea urchin dual oxidase, Udx1. This enzyme is essential for completing the physical block to polyspermy. Yet, its expression is maintained during development, as indicated by the presence of both Udx1 mRNA and Udx1 protein enriched at the surface of all non-mesenchymal blastomeres. When hydrogen peroxide synthesis by Udx1 is inhibited, either pharmacologically or by specific antibody injection, cleavage is delayed. Application of exogenous hydrogen peroxide, however, partially rescues a fraction of these defective embryos. We also report an unequal distribution of reactive oxygen species between sister blastomeres during early cleavage stages, suggesting a functional role for Udx1 in intracellular signaling.

Introduction

Fertilization in sea urchins is accompanied by a respiratory burst that lasts approximately 10 min (Foerder et al., 1978; Warburg, 1908). This consumption of oxygen ultimately provides the hydrogen peroxide (H2O2) necessary for the activity of ovoperoxidase, a tyrosine cross-linking enzyme derived from the egg cortical granules (Foerder and Shapiro, 1977; LaFleur et al., 1998). A calcium-dependent pH-sensitive dual oxidase, Udx1, is responsible for this H2O2 synthesis (Wong et al., 2004).

Unlike genes utilized exclusively during the physical block to polyspermy – such as the structural matrix proteins SFE1, SFE9, proteolaiasin, rendezvin, and the enzyme ovoperoxidase that are all expressed exclusively during oogenesis (Wessel et al., 2001; Wong and Wessel, 2004) – Udx1 transcript is present in eggs and 1-cell zygotes (Wong et al., 2004), suggesting that it may also play a role during development. One possible function is in pathogen defense, based on the analogy between the sea urchin oxidative burst and the anti-microbial respiratory burst of neutrophils (Heinecke and Shapiro, 1992; Shapiro, 1991). In such a case, attack on an embryo may trigger a respiratory response that protects it from the offending microbes (Klebanoff et al., 1979), whereas high cytoplasmic concentrations of reactive oxygen species of scavengers, such as glutathione and ovothiol, would protect the embryo (Fahey et al., 1976; Turner et al., 1988). Alternatively, Udx1 might have a role in cell signaling: hydrogen peroxide was recently identified as a transducer of autocrine and paracrine signaling (Kamata and Hirata, 1999; Neill et al., 2002) and an oxidizing inactivator of protein tyrosine phosphatases (Blanchetot et al., 2002a; Denu and Tanner, 1998; van der Wijk et al., 2003). This reactive oxygen species (ROSs) and its precursors or derivatives – such as singlet oxygen (1O2), superoxide (O2•−), and hydroxyl radical (•OH) – possess...
relatively short half-lives in vivo due to their extreme reactivity and potential toxicity to target proteins (Bergendi et al., 1999). Nevertheless, these characteristics have proven to be essential for the participation of ROSs in rapid signal transduction (Finkel, 2000; Kochevar, 2004; Neill et al., 2002), especially in cell cycle regulation (Arnold et al., 2001; Chen et al., 2004; Nasr-Esfahani et al., 1990) and in promoting proliferation (Burdon, 1995; Maulik and Das, 2002; Stone and Collins, 2002)—two critical events during embryogenesis that also appear to be regulated by transient increases in intracellular calcium concentrations (Browne et al., 1996; Whitaker and Larman, 2004). Nevertheless, these characteristics have proven to be essential for the participation of ROSs in rapid signal transduction (Finkel, 2000; Kochevar, 2004). (Bergendi et al., 1999; Burkitt and Wardman, 2001; Yazaki, 2001). Successful utilization of ROSs for signaling, however, requires tight regulation of both the production and the extinction of these reactive species to minimize damage to biomolecules (Davies, 1987; Davies and Delsignore, 1987; Davies et al., 1987) and to keep cytoplasmic redox status homeostatic rather than apoptotic (Bergendi et al., 1999; Burkitt and Wardman, 2001; Finkel, 2000; Kochevar, 2004).

Here, we find Udx1 present throughout early development and active upon calcium stimulation. Functional blocking studies indicate a role for this enzyme during cleavage, but the spatial and temporal resolution of its activity could not be determined since endogenous production of extracellular H$_2$O$_2$ during development was below the threshold of detection. We do, however, find significant elevation in intracellular ROSs following unequal division of the vegetal blastomeres at the 16-cell stage. Together, these data suggest a role for ROSs during sea urchin cleavage.

Materials and methods

Animals and embryos

*Lytechinus variegatus* were collected and handled in artificial seawater (ASW) as previously described (Wong and Wessel, 2004). Unless noted, embryos were cultured in ASW at 23°C.

RNA analysis

Total RNA samples from *L. variegatus* eggs and embryos were blotted and probed using full-length Udx1, as previously described (Bruskin et al., 1981; Wong et al., 2004). Single-tube RT-PCR reactions were run as described (Wong et al., 2004), using template derived from oocyte, eggs, and various embryonic stages.

Immunoblot and immunolocalization

Antiserum against separate domains of Udx1 was produced in New Zealand White rabbits, and immunoglobulins were affinity purified as described (Wong et al., 2004). Equal concentrations of affinity-purified antibodies against the three domains of Udx1 (Wong et al., 2004) were combined for use in each experiment, unless otherwise noted. For immunoblot analysis, total protein samples from eggs or embryos were subjected to SDS-PAGE on precast 4–20% polyacrylamide Tris–glycine gels (Life-Therapeutics Limited, Frenchs Forest, New South Wales, Australia) and stained, or transferred to nitrocellulose and probed as described (Wong and Wessel, 2004). Immunolocalization was accomplished on both paraffin-embedded tissue and whole mount cells and embryos as described (Wong et al., 2004; Wong and Wessel, 2004).

Inhibition and partial rescue of peroxidase-mediated developmental

Approximately 500 μl of loosely packed *L. variegatus* eggs or embryos was exposed to a final concentration of 0.0 μM (DMSO), 0.1 μM, or 1.0 μM diphenyleneiodonium (DPI; Sigma-Aldrich Corporation, St. Louis, MO, USA) for 30 min at various times during development, usually spanning 15 min before and after a cell cleavage. Various concentrations of exogenous H$_2$O$_2$ were provided over the same period of time, as necessary. Embryos were then washed two times with 100 volumes of ASW, resuspended in 25 ml ASW containing 100 μg/ml ampicillin, and transferred to a 100-mm Petri dish to continue developing. Samples of each culture were taken at key stages of development and fixed in a final concentration of 10% formalin in ASW. Sampled cohorts were scored based on morphology, with normal embryos categorized to the appropriate stage while abnormal embryos were generically scored as “abnormal” if they displayed any of the following: asymmetry or a disruption in blastomere quantity, irregular morphology, and/or collapse of the blastocoele. At least 300 individual embryos were scored per sampling, per treatment.

Quantifying effect of DPI on the rate of oxygen consumption

A 5% [v/v] solution of *L. variegatus* eggs or 30-min old zygotes in 23°C ASW was loaded into a Clarke electrode chamber under continuous mixing and then allowed to stabilize for 5 min. Traces of the basal rate of oxygen consumption were measured over 10 min, and then a 100 × solution of DPI or sodium azide in DMSO (data not shown) was added to the chamber of zygotes. Effects of these drugs were then recorded for 15 min longer. Experiments were conducted at least three times per drug concentration using eggs from 7 different sea urchins. Slopes of trend lines fitted by eye to the traces over 10-min windows before and after addition of chemicals or their control solvents were calculated for quantitative comparison. The difference in these slopes (Δ) were used as a proxy to assess the effects of each drug on oxygen.
consumption. Phenotypes of zygotes following treatments were recorded using Nomarski light microscopy by an Orca-ER CCD camera (Hamamatsu Corporation, Bridgewater, NJ, USA) on a Zeiss AxioPlan microscope (Carl Zeiss Incorporation, Thornwood, NY, USA), controlled by Metamorph® software (Universal Imaging Company, Downingtown, PA, USA).

**Injection and phenotyping 2-cell embryos**

In vivo inhibition studies tested the effects of affinity-purified anti-NADPH or preimmune IgGs and monovalent Fabs on cleavage. Fabs were isolated according to the ImmunoPure® preparation kit instructions (Pierce Biotechnology Incorporation, Rockford, IL, USA). As previously published (Wong et al., 2004), antibodies equilibrated to 50 mM KCl were resuspended with fluorophore-conjugated dextran (10,000 MW) to give a final concentration of 0.5 mg/ml whole IgGs or 0.25 μg/ml Fabs. Anti-NADPH injection solutions contained 0.2 mM Texas Red dextran (Molecular Probes, Eugene, OR, USA); preimmune injection solutions contained both 0.2 mM Texas Red dextran and 50 μM AlexaFluor488® dextran (Molecular Probes, Eugene, OR, USA).

One blastomere of an L. variegatus 2-cell embryo was injected with antibody solution as described previously (Wong et al., 2004). Embryos were injected within 20 min of completing first cleavage and then transferred to 40-mm glass-bottom culture dishes (World Precision Instruments, Incorporation, Sarasota, FL, USA) containing ASW. To maintain their position, mixed populations of injected and uninjected embryos were settled within wells punched from 1% agarose poured as a base in the culture dishes. Development in these cohorts was imaged with an Axiovert 200M microscope (Carl Zeiss Incorporation, Thornwood, NY, USA) fitted with an Orca-ER CCD camera (Hamamatsu Corporation, Bridgewater, NJ, USA) driven by AxioVision® Software (Carl Zeiss Incorporation, Thornwood, NY, USA). Populations were imaged every minute for 90 min; some were cultured longer and monitored periodically to assess later development.

Delays in cytokinesis were quantified by comparing the timing of injected blastomere division to its uninjected sister blastomere. Differences in these times are reported if observed over the first 90-min time lapse. Injected blastomeres that failed to cleave by the end of the 90-min period were scored as 90 min minus the time of uninjected control blastomere cleavage. Only embryos whose uninjected blastomeres continued to divide were scored. Mass of antibody injected per zygote was estimated by Texas Red fluorescence as described (Wong et al., 2004) and then normalized ratiometrically to auto-fluorescence of uninjected sister blastomere. Control injections of preimmune IgGs or boiled Fabs were identified by AlexaFluor488® fluorescence. Calibrations for the final antibody concentration in the egg were calculated from the ratio of injection volumes versus blastomere volume.

**Measurement of hydrogen peroxide production during development using an imaging photon detector**

Wells were punched from 1% agarose overlaying a 40-mm glass-bottom culture dishes as above, and then dishes were filled with a solution of 0.5 units per ml horseradish peroxidase (HRP; Sigma-Aldrich Corporation, St. Louis, MO, USA) and 100 μM luminol (diluted from a 10 mM stock made up in 100 mM K2CO3 [pH 11.5]) in ASW. L. variegatus eggs were settled into these wells, and 1 μl of diluted sperm (1:100 of dry stock) was added near the well to inseminate the population. Hydrogen peroxide (H2O2) production was imaged via photons emitted from luminol conjugation as described (Wong et al., 2004) for a total of 5.5 h after insemination at 23°C. Brightfield images were taken at 15-min intervals for the duration of the experiment. Recording and analysis of photon generation per zygote were made using IpdWin95 software (Sciencewares, East Falmouth, MA, USA). Traces were generated for circular regions centered over each embryo, calculating photon emission over running 50-s windows. The peak amplitude of the oxidative burst was designated as 100% photon emission per trace. Traces per embryo were then compared based on the timing and peak amplitude of the oxidative burst following fertilization.

**Measuring potential for calcium-dependent hydrogen peroxide synthesis during development**

Approximately 100 eggs or embryos in ASW were loaded into triplicate wells of a 96-well plate containing a 2× reaction buffer. The final 200 μl reaction volume contained 100 μg/ml A23187 (Sigma-Aldrich Corporation, St. Louis, MO, USA) or DMSO, 50 μM Amplex Red® (Molecular Probes, Eugene, OR, USA), and 0.5 units of HRP in ASW. Wells may have also contained 0.0 μM (DMSO), 0.1 μM, or 1.0 μM diphenyleneiodonium (DPI; Sigma-Aldrich Corporation, St. Louis, MO, USA), as needed. Loaded plates were transferred to an Ascent Fluoroskan® fluorimeter (Labsystems, Franklin, MA, USA) to measure kinetics of resorufin formation over a 20-min period as described (Wong et al., 2004). The average rate per reaction well was calculated from data points generated within the first 5 min. For each developmental time point, eggs were activated as controls; these reactions all exhibited greater than 95% of cells with fertilization envelopes. Data were corrected by subtracting the average rate of resorufin formation of triplicate unactivated reaction wells (DMSO) from the activated (A23187) reaction wells and then normalized to the average rate obtained from eggs activated at the same time. Where applicable, normalized drug-treated kinetics are reported as percentage of the untreated control (0 μM inhibitor).
Measuring intracellular reactive oxygen species

*L. variegatus* eggs or embryos were cultured in the presence of 10 μM 5-(and-6)-carboxy-2',7'-dichlorodihyderofluorescein diacetate (H$_2$DCFDA; Molecular Probes, Eugene, OR, USA) for 30 min during development, usually spanning 15 min before and after cleavage. Various concentrations of DPI or 100 μM H$_2$O$_2$ were also included, as needed, during the 30-min incubations. DCF fluorescence was imaged over the entire embryo using an Orca-ER CCD camera (Hamamatsu Corporation, Bridgewater, NJ, USA) on a Zeiss AxioPlan upright microscope fitted with a FITC dichroic prism (Carl Zeiss Incorporation, Thornwood, NY, USA) controlled by Metamorph® software (Universal Imaging Company, Downingtown, PA, USA). Reacted levels of DCF fluorescence were quantitated per embryo by measuring total fluorescence within the fertilization envelope. Data were normalized to total DCF fluorescence in eggs treated under the same conditions as the specified stage of embryos.

For more specific localization of reactive oxygen species during unequal cleavage, embryos were either: (1) pretreated with various concentrations of DPI for 5 min just after fourth cleavage into 16-cell stage or (2) treated with 0.002% SDS at 4-cell stage for 20 min followed by washing in ASW and then resumption of culturing to the 16-cell stage (Tanaka, 1976). Embryos were then exposed to 10 μM H$_2$DCFDA with 1 μg/ml Hoechst (Molecular Probes, Eugene, OR, USA) for 20 min, as above. Experiments conducted without Hoechst yielded no difference in DCF fluorescence patterns (data not shown). Fluorescence was imaged in 5-μm z-stacks that consisted of 30 total optical sections through an animal–vegetal plane, using a TCS SP2 AOBS® confocal microscope (Leica Microsystems). Average levels of reacted DCF were quantitated in individual cells at each optical section using Metamorph® software (Universal Imaging Company, Downingtown, PA, USA) and then averaged over the stack for each cell. For comparison of blastomere content, micromere fluorescence was normalized to fluorescence of its sister macromere.

Statistics

All pair-wise comparisons were made using the two-tailed Student *t* test.

Results

Characterization of gene expression during development

We previously identified the diphenyleneiodonium-sensitive NADPH-dependent dual oxidase, Udx1, that is active at the cell surface of sea urchin eggs immediately after fertilization (Wong et al., 2004). Its NADPH-oxidase activity – referred to here as “oxidase activity” – is responsible for generating the hydrogen peroxide (H$_2$O$_2$) necessary for cross-linking the fertilization envelope (Foerder et al., 1978; Wong et al., 2004). Here, we initially characterize Udx1 expression during early development. Patterns of Udx1 expression are similar in *L. variegatus* and *Strongylocentrotus purpuratus* (data not shown); we focus here on *L. variegatus* because of its ease of use and optical clarity.

RNA gel blot analysis failed to detect any dual oxidase transcripts in *L. variegatus* eggs or embryos (Fig. 1A) (Wong et al., 2004). Multiplex reverse-transcriptase PCR amplification, however, detected *Udx1* transcript in all stages of oogenesis and early development (Fig. 1B), whereas the cortical-granule-specific transcript SFE9 is absent following oogenesis, as expected (Wessel, 1995).

In agreement with the RNA analysis, we find that full-length Udx1 protein is detectable in all early stages of development tested and can be seen in both *L. variegatus* (Fig. 1C, arrow) and *S. purpuratus* embryos (data not shown). The protein is present in cytoplasmic vesicles of the egg, is enriched in the apical plasma membrane of all cells of early embryos, and is present in epithelial cells following gastrulation (Fig. 1; data not shown). The surface localization of Udx1 is consistent with its transmembrane organization (Wong et al., 2004). Of particular interest is the accumulation of lower molecular weight bands in stages of development beyond 2-cell (Fig. 1C), which may reflect a high turnover rate of the Udx1 cohort required for the oxidative burst following fertilization (Wong et al., 2004). A

Embryonic exposure to diphenyleneiodonium blocks cell division

We used the covalent modification of diphenyleneiodonium (DPI) near heme groups (Doussiere et al., 1999) as a tool to test whether or not Udx1 plays a role during development. DPI is an irreversible chemical inhibitor that, through a free radical mechanism involving superoxide (O$_2^-$), covalently adds to groups adjacent to the heme domain of its target reductase (Doussiere et al., 1999), although some additional conjugation may occur at nearby flavin domains if the inhibitor is in excess (O’Donnell et al., 1993; Wang et al., 1993). This modification results in steric interference and quenching of the heme group, affecting a broad spectrum of NADPH-dependent oxidases found in endothelium, synoviocytes, chondrocytes, neuroepithelial cells (reviewed in Babiör, 1999), and sea urchin zygotes (Wong et al., 2004). Inhibition of a cytochrome *b$_{558}$* respiratory burst has effectively been blocked by phenolic analogs of apocynin, such as acetosyringone (Karlsson et al., 2000; Leusen et al., 1996; Van den Worm et al., 2001), but these reagents do not have any effect on the sea urchin dual oxidase (Wong et al., 2004). This leaves DPI as the primary – albeit non-specific (Riganti et al., 2004) – pan oxygen reductase inhibitor described to date. In the absence of additional cytochrome *b$_{558}$* or NADPH-oxidase homo-
logs in the sea urchin genome (Wong et al., 2004; data not shown), we initially used DPI to probe for a role of Udx1 during development.

Under continuous exposure to DPI at the IC50 concentration of 1 μM of the oxidative burst at fertilization in *L. variegatus* (Wong et al., 2004), all embryos were arrested in their treated stage, whereas 0.1 μM DPI slightly delayed cleavage compared to controls (data not shown). This suggested that Udx1 is functional during cell cycle progression. We then reduced the exposure time to a 30-min window overlapping the completion of cytokinesis. For example, 1-cell zygotes were exposed to DPI 15 min before first cleavage until about 15 min after. Then, the inhibitor was washed out, and the treated embryos were allowed to continue developing in artificial seawater. We exposed various embryonic stages – from eggs being fertilized (Fig. 2A) to the 60-cell stage (data not shown) – to DPI for 30 min and scored their phenotypes based on their morphology at specific time points. Per 30-min exposure, 0.1 μM DPI delayed cleavage or generated slightly more abnormal embryos than controls (0 μM), while 1 μM DPI completely arrested cell cycle progression at the treated stage (Figs. 2A, B; data not shown). After several hours, however, populations exposed to 1 μM DPI recovered such

---

**Fig. 1.** Udx1 is present through early development. (A) RNA gel blot containing total RNA (10 μg each) from ovary, egg, and embryo, probed for the sea urchin dual oxidase. Ethidium bromide staining of the 28S rRNA is shown as a loading control. (B) Multiplex reverse-transcriptase polymerase chain reaction from whole cell lysates from oocytes, eggs, and embryos. Primers for the dual oxidase, SFE9, and 18S rRNA were used simultaneously in the amplification reaction. Image shows ethidium bromide staining. No lysate blanks and no-reverse-transcriptase (−RT) control reactions showed no amplification products (data not shown). (C) Immunoblot of early cleavage and pre-larval stages. Fifty micrograms of total protein was loaded per stage, as indicated by the Coomassie-stained loading control. Blots probed with preimmune antisera were blank (data not shown). Arrow indicates the mass of the full Udx1 protein (185 kDa); smaller molecular weight bands are indicative of endogenous proteolysis and may represent turnover of the enzyme in later stages. (D–I) Immunofluorescence images of tissue sections (D, F, G) and whole mount (E, H, I) *L. variegatus* eggs and embryos. All samples probed with preimmune (D, E) or an equal mix of antibodies against the dual oxidase (F, G, H, I). Egg (egg), 1-cell zygote (zyg), 2-cell (2c), 4-cell (4c), 16-cell (16c), blastula (bl), gastrula (ga), and pluteus (pl) are indicated. Micromeres in 16-cell stage are indicated (arrowheads). Scale bar equals 50 μm.
that a fraction of the embryos regained a normal morphology for their age; the remaining embryos were alive, but developed abnormally. The most complete recovery was observed from embryos exposed during fertilization (Fig. 2A), whereas those receiving prolonged treatment up to the 60-cell stage did not achieve normal larval morphology in the same time as the controls (Fig. 2B; data not shown).

At high concentrations, DPI can detrimentally affect more than just flavoenzymes—specifically the productivity of the pentose phosphate pathway (Riganti et al., 2004) and mitochondrial activity (Gatley and Sherratt, 1976a; Gatley and Sherratt, 1976b; Li and Trush, 1998). In light of these observations, we measured the effects of DPI on oxygen consumption as a proxy to assess whether or not the...
abnormal phenotypes we observed were caused by perturbation of either the embryonic metabolic or respiratory pathways. One micromolar DPI does not affect zygotes, whereas 10 μM DPI (Fig. 2C) and sodium azide (data not shown) significantly inhibit oxygen consumption at the same stage of development. Thus, we hypothesize that the effects of low DPI concentrations on cleavage may be linked to the functional inhibition of Udx1.

Specific inhibition of Udx1 causes a delay in cytokinesis

We tested whether inhibiting Udx1 activity would specifically alter development by injecting functional blocking antibodies against the dual oxidase’s NADPH binding domain (Wong et al., 2004). One blastomere of a 2-cell embryo was injected within 20 min of completing first cleavage and then monitored for developmental defects compared to its sister blastomere. Delays in cleavage to the 4-cell stage were scored as the difference in time required to complete cytokinesis relative to the uninjected sister blastomere. Across a 5-fold titration in whole IgG concentration or in purified Fabs, we see a significant delay in second cleavage from anti-Udx1-injected blastomeres when compared to preimmune IgG (Figs. 3A, B; P = 0.04) or boiled Fabs-injected controls (Figs. 3C, D; P = 0.008). For example, regardless of which form of antibodies was used, 70% of all the control-injected blastomeres showed a cleavage delay of up to 25 min, whereas a comparable population of anti-Udx1-injected blastomeres required up to 75 min to divide—a difference of at least 50 min or at least a full cleavage cycle in L. variegatus (Figs. 3A, C). Specific antibody injection does not, however, abolish the blastomere’s ability to continue development. Prolonged culturing of select injected populations shows recovery of cytokinesis, albeit at a rate often slower than uninjected sister blastomeres (Figs. 3E, F). Hence, blastomeres exposed to functional blocking anti-Udx1 antibodies exhibit a recoverable phenotypic delay in cleavage similar to temporary exposure to 1 μM DPI (Fig. 2). We interpret the embryos’ recovery to mean that the antibodies were turning over in the cell and transitioning from inhibitory to limiting in effect. In addition, the participation of other ROS sources, such as mitochondria, could have accelerated the recovery, but the normal timing of cytokinesis appears to require Udx1 activity.

Exogenous hydrogen peroxide rescues DPI block of cleavage

Based on the hypothesis that ROSs function in cell signaling and cytokinesis (see Discussion), we asked whether or not exogenous H₂O₂, the primary product of Udx1, could rescue the DPI-induced delay in cleavage. Since DPI irreversibly inhibits Udx1 (Doussiére et al., 1999), this allowed us to assess the putative role of H₂O₂ alone on cleavage. Fifteen-minute old and 2-cell zygotes were exposed simultaneously to DPI and H₂O₂ for a 30-min window spanning cleavage and then allowed to continue developing in normal seawater. The clearest continuation of cleavage stages associated with 1 μM DPI could be seen with the two highest concentrations of H₂O₂ tested, 10 and 100 μM (Fig. 4). While these combinations did not completely rescue development, the exogenous H₂O₂ allowed 40% more of the DPI-treated embryos to progress through the cell cycle in a timeframe comparable to solvent-treated controls. This incomplete rescue may be a result of our inability to expose embryos to local bursts of H₂O₂ synthesis, as would be expected in vivo. Another contribution to the low recovery percentage might also result from the efficient ROS-neutralizing mechanisms available to the embryo (Fahey et al., 1976; Turner et al., 1988) that could prematurely extinguish the secondary message before it can initiate the proper signaling mechanisms.

Production of hydrogen peroxide during development

We used an imaging photon detection (IPD) system to determine the timing of H₂O₂ production during development, a proxy for Udx1 activity (Wong et al., 2004). While the IPD system can detect as few as 500 molecules of H₂O₂ (6 photons) per second per embryo (Creton and Jaffe, 2001), the background chemiluminescence under our conditions in the absence of embryos is 3000 molecules of H₂O₂ (37 photons) per second per equal embryonic area or 3–4% of the peak oxidative burst (Fig. 4A). Unfortunately, we were not able to measure significant H₂O₂ production from embryos following the initial oxidative burst—an observation that parallels the comparatively higher concentration of Udx1 transcript in oocytes versus embryos (Figs. 1A, B). The absence of clear extracellular signal over background
during cleavage, however, does not exclude the possibility that essential short-lived bursts of localized activity are present during development.

We then asked whether or not embryonic Udx1 (Fig. 1) is functional. As shown previously in eggs (Wong et al., 2004), artificially increasing concentrations of intracellular calcium with ionophore can activate or increase the \( \text{H}_2\text{O}_2 \) generating machinery such that high concentrations of \( \text{H}_2\text{O}_2 \) may be detected extracellularly. When embryos were exposed to ionophore, the rate of \( \text{H}_2\text{O}_2 \) production far exceeded the rate of synthesis in activated eggs (Fig. 5B). All embryonic stages during cleavage have the potential to generate \( \text{H}_2\text{O}_2 \) (Fig. 1C), and its production was abolished when embryos were exposed to 1 \( \mu \text{M} \) DPI, suggesting that Udx1 – as opposed to mitochondria (Fig. 2C) (Burdon, 1995; Li and Trush, 1998; Nohl et al., 2005) – is responsible for synthesizing most of this calcium-dependent \( \text{H}_2\text{O}_2 \) (Fig. 5C). Given the physiologically low endogenous production of \( \text{H}_2\text{O}_2 \) by Udx1 following fertilization versus egg activation (Wong et al., 2004), the level of activity observed in these experiments is likely in vast excess of what is used during embryogenesis. This conclusion is consistent with the 20-fold less anti-Udx1 (calculations not shown) necessary to elicit a cleavage phenotype compared to blocking the oxidative burst at fertilization (Fig. 3) (Wong et al., 2004). Three mechanisms probably act to repress \( \text{H}_2\text{O}_2 \) below detectable levels: first, ionophore treatments flood the cell with about 10 mM free calcium, whereas cytoplasmic levels are over 30,000-fold lower (between 250–350 nM) in cleavage stage embryos (Miller et al., 1994; Yazaki et al., 2004). Secondly, intracellular factors such as protein kinase C can carefully regulate oxidase activity in vivo (Heinecke and Shapiro, 1992; Wong et al., 2004) during development (Yang et al., 2004). Finally, intracellular concentrations of ROS scavengers such as glutathione and ovothiol would quench \( \text{H}_2\text{O}_2 \) before it could be detected extracellularly (Fahey et al., 1976; Turner et al., 1988).

**Accumulation of intracellular reactive oxygen species during embryogenesis**

Our inability to detect significant levels of extracellular \( \text{H}_2\text{O}_2 \) during development (Fig. 5) does not preclude an accumulation of cytoplasmic \( \text{H}_2\text{O}_2 \), a form that we cannot measure using the IPD. We tested this by measuring intracellular ROS levels with \( \text{H}_2\text{DCFDA} \), a membrane-permeable indicator that becomes fluorescently active upon peroxidase-dependent oxidation by ROSs, especially \( \text{H}_2\text{O}_2 \) (LeBel et al., 1992; Rota et al., 1999). Cytoplasmic glutathione peroxidases likely catalyze the oxidation of the intracellular esterified \( \text{H}_2\text{DCF} \) (Bergendi et al., 1999), although other sea urchin molecules such as the glutathione-peroxidase-like activity of ovothiol (Turner et al., 1988) or other intracellular peroxidases (M.L. Leguia, personal communication) may also participate. Exposure of cleaving embryos to \( \text{H}_2\text{DCFDA} \) results in an intracellular ROS profile quite similar to the levels of \( \text{H}_2\text{O}_2 \) synthesis observed extracellularly, peaking over early embryos but leveling off during later developmental stages (Fig. 6A). We hypothesized that the higher concentrations of ROS in 1- and 2-cell zygotes are a consequence of the accumulation of \( \text{H}_2\text{O}_2 \) from the oxidative burst during the first 15 min of post-fertilization. This was supported by the depletion of DCF fluorescence to levels equal to later stages of embryos when the oxidative burst is inhibited by 1 \( \mu \text{M} \) DPI, whereas exposure of 15-min old zygotes to DPI does not affect intracellular ROS levels (Fig. 6C). Interestingly, exposure to DPI during the 1- to 2-cell and 2- to 4-cell cleavages also retards the rate that ROSs are quenched (data not shown).

The correlation between Udx1 activity and DCF fluorescence following the oxidative burst suggests that any accumulation of ROSs might reveal where and when the calcium-dependent Udx1 is active during development. We noticed a slightly higher concentration of fluorescent DCF in micromeres compared to their sister macromeres or mesomes at the 16-cell stage (Fig. 6A, arrowhead), suggesting that their 30 nM elevation in intracellular calcium (270–320 nM in macromeres and mesomes; 300–350 nM in micromeres) (Yazaki, 2001; Yazaki et al., 2004) may activate Udx1. When measuring DCF fluorescence by confocal microscopy, we observed an accumulation of ROS around the nuclei or DNA and in micromeres at both the 16-cell (Fig. 7A) and 32-cell embryos (data not shown). Brief exposure of second cleavage stage embryos with 0.002% SDS results in the loss of unequal cell division at the fourth cleavage (Tanaka, 1976) with a concomitant loss of unequal calcium distribution (Yazaki, 2001; Yazaki et al., 2004). In our hands, embryos exposed in such a
manner to SDS showed a similar loss of DCF fluorescence among vegetal sister blastomeres (Fig. 7B), suggesting that the accumulation of ROSs in micromeres is attributed to a calcium-dependent mechanism. Micromeres exhibit calcium oscillations (Yazaki et al., 2004) that could potentiate Udx1 activity. We could not detect a decreased ratio of intracellular ROS in embryos exposed to DPI (Fig. 7C), however, suggesting that DPI-sensitive oxidase activity does not significantly contribute to the overall accumulation of intracellular ROSs. Instead, vegetal enrichment in mitochondria (Coffman et al., 2004) – whose activity is not disrupted by low concentrations of DPI (Fig. 2C) – may be responsible for the enrichment of DCF fluorescence we observe in 16-cell stage micromeres, a phenomenon similar
to the polarized hyperactivity of mitochondria at the posterior pole of *Drosophila* embryos (Ding et al., 1994).

**Discussion**

Reactive oxygen species (ROSs) such as singlet oxygen (‘O$_2$), superoxide (O$_2^-$), and hydrogen peroxide (H$_2$O$_2$) are intimately involved with cell homeostasis and signaling (Bae et al., 2004; Bergendi et al., 1999; Finkel, 2000; Kochevar, 2004; Liu et al., 2002; Neill et al., 2002). The extended half-life of H$_2$O$_2$ compared to the other candidate ROSs supports the participation of this molecule in complex signaling events (Bergendi et al., 1999). For example, H$_2$O$_2$ facilitates the transition into the DNA synthesis phase (S-phase) of the cell cycle (Chen et al., 2004; Stone and Collins, 2002), an event coincident with a local rise in intracellular calcium concentrations (Jaffe et al., 2001; Stricker, 1999; Whitaker and Larman, 2001; Yazaki et al., 2004). The parallel progress of these two signaling cascades...
suggests a common link: H$_2$O$_2$ may feed back to calcium release mechanisms, specifically by sensitizing the endoplasmic reticular inositol 1,4,5-trisphosphate receptor to its ligand, IP$_3$ (Hu et al., 2000). Thus, cytoplasmic H$_2$O$_2$ could mediate calcium at levels necessary to complete the transition through S- and G2-phases.
Hydrogen peroxide could also regulate any number of established signaling pathways, including activation of the mitogen-activated protein kinase (MAPK; Chen et al., 2004; Kamata and Hirata, 1999; Liu et al., 2002; Neill et al., 2002), stimulation of the ribosomal protein S6 kinase 1 (S6K1) through a Rac1-dependent pathway (Bae et al., 2004), or inactivation of protein tyrosine phosphatases (PTP; Denu and Tanner, 1998; van der Wijk et al., 2003). Such brief pulses are consistent with the transient nuclear localization of ROSs in blastomeres from various stages of development. In contrast, constitutively high levels of ROSs could delay proliferation via PTP activity. This family of phosphatases can stabilize homo- and heterodimeric membrane-bound enzymes (Blanchetot et al., 2002b; van der Wijk et al., 2003) or oxidize cytoplasmic enzymes to an inactive state (Blanchetot et al., 2002a; Finkel, 2000), thus affecting ligand–receptor signaling during cell cycle progression. Such PTP cascades are thought to be active during sea urchin development (Hiriyanna et al., 1995; Wessel et al., 1995; Wright and Schatten, 1995) and could prove to be effective during the delay in micromere division.
observed after the fifth cleavage of the blastomeres. During this event, the corresponding elevation in intracellular calcium (Yazaki et al., 2004) and high intracellular H₂O₂ (or other ROSs) may maintain a repressed state in all PTPs, resulting in cell cycle arrest. Only when both calcium and H₂O₂ levels are reduced does division proceed. At such a point, tyrosine phosphatase activity may be recovered through the reducing activity of both ovothiol (Turner et al., 1988) and glutathione (Fahey et al., 1976), albeit at rates 10- to 100-fold slower than the initial inactivation (Blanchetot et al., 2002a; Denu and Tanner, 1998, 2002).

One source of such intracellular ROSs is the family of heme-dependent animal reductases. These enzymes function in many aspects of adult homeostasis, including roles in immunity (Geiszt et al., 2003; Pullar et al., 2000), hormone synthesis (Morand et al., 2003), mechanical protection (Edens et al., 2001), and cellular proliferation (Arnold et al., 2001; Burdon, 1995; Chen et al., 2004). NADPH-dependent dual oxidases directly synthesize hydrogen peroxide (H₂O₂) from molecular oxygen, generating the least toxic, most enduring ROS (Bergendi et al., 1999). This neutral molecule can then serve as a substrate for extracellular animal peroxidases (Edens et al., 2001; Foerder et al., 1978; Geiszt et al., 2003; Morand et al., 2003; Wong et al., 2004) or as an intracellular signaling molecule (Bergendi et al., 1999; Hu et al., 2000; Kamata and Hirata, 1999). Since the source of H₂O₂ is often the same cell that synthesizes the peroxidases, it becomes evident that H₂O₂ may function as both an enzymatic substrate and an autocrine factor. Consider the thyroid dual oxidase p138Tox/Duox2: thyroid stimulating hormone (TSH) triggers the iodination of thyroglobulin, which requires H₂O₂ and thyroid peroxidase activity (Dunn and Dunn, 2001), as well as thyrocyte proliferation (Kraiem et al., 1990; Nitsch and Wollman, 1980). Excess H₂O₂ synthesis by mammalian p138Tox/Duox2 could enhance any proliferative signals initiated in some thyrocytes by TSH, with the ultimate effect of tissue expansion. Thus, p138Tox/Duox2 plays diverse, yet complimentary, roles in the thyroid. In a similar vein, we observe that a DPI-sensitive sea urchin oxidase is responsible for both the maturation of the fertilization envelope (Wong et al., 2004) and embryonic cleavage.

![Image](http://example.com/image.png)

**Fig. 8.** Possible roles of Udx1 during embryogenesis. Upper trace (dashed line) represents chronology of embryogenesis. Stages when intracellular calcium levels rise are indicated in gray, with corresponding hypothetical traces of calcium concentration. Predicted uses of H₂O₂, whether extracellular or intracellular, are indicated for each active cell. The enrichment of H₂O₂ in micromeres is not Udx1-dependent, although this enzyme may still participate in its production (see text). Inset represents a generic blastomere detailing regulation of Udx1 by intracellular pH, protein kinase C (PKC), and calcium. Dashed arrow indicates Udx1 sensitivity to pH and PKC activity (Wong et al., 2004). Some essential paths that may be regulated by H₂O₂ are shown, including the calcium (Ca⁺⁺) channel, phosphatidylinositol-3-phosphate receptor (IP3R) and mitogen-activated protein kinase (MAPK), ribosomal protein S6 kinase 1 (S6K1), and/or protein tyrosine phosphatase (PTP) cascades. Furthermore, H₂O₂ may be used to generate dityrosine cross-linking of fertilization envelope proteins (R – Y = Y – R) at fertilization by ovoperoxidase or as an anti-microbial defense mechanism during wound healing or infection.
We previously identified Udx1 as the H$_2$O$_2$ source of the oxidative burst at fertilization, an essential event necessary for fertilization envelope cross-linking by ovoperoxidase (Wong et al., 2004). Here, our evidence shows that Udx1 participates in modulating the cell cycle. The abundance of Udx1 in all embryonic stages, and its huge potential for calcium-dependent activation, is also consistent with a role in defense: any pathogenic invasion that causes intracellular calcium levels to rise could stimulate H$_2$O$_2$ synthesis to be used in an anti-microbial response (Foerder et al., 1978; Klebanoff et al., 1979). For example, the intracellular calcium concentrations required for cell/blastomere wound healing (McNeil and Steinhardt, 2003; McNeil et al., 2003; Terasaki et al., 1997) would trigger a burst of Udx1-dependent H$_2$O$_2$ production to ward off infective microbes in the vicinity of the injury. Under non-pathogenic conditions, however, Udx1 may also play a role in regulating the progression of cytokinesis (Fig. 8). Brief exposure to diphenyleinedione, the chemical that inhibits Udx1 activity at fertilization (Wong et al., 2004), affects cleavage in a manner similar to injection of function-blocking anti-Udx1 IgGs or Fabs. But the failure to properly organize daughter cells in embryos rescued by exogenous H$_2$O$_2$ implies that a more complex signaling network is necessary. The cleavage delay we observed is reminiscent of embryonic exposure to the general calcium influx inhibitor SKF 96365 (hydrochloride (1-β-[3-(4-methoxyphenyl)propoxy]-4-methoxy-phenethyl-1H-imidazole) (Yazaki et al., 2004). A key link between the two signaling cascades is the dependence on calcium, both as an activator of the dual oxidase Udx1 and as a signaling molecule during cell division (Berridge et al., 1998; Burdon, 1995; Ducibella et al., 2002; Whitaker and Larman, 2001). Thus, the embryo may calibrate the phases of cleavage using calcium transients to activate appropriate signaling cascades and examining reversible inactivation of protein tyrosine phosphatases by hydrogen peroxide: evidence for a sulfenic acid intermediate and implications for the role of reactive oxygen species in apoptosis. Biochem. Biophys. Res. Commun. 282, 329–333.

**Acknowledgments**

We would like to thank Drs. Sam Beale and Luiza Nogaj for the instruction and use of the Clarke electrode and Dr. Robbert Créton for use of his IPD microscope and microinjection set-up. We are also grateful to Dr. David Rand for his helpful statistics sessions and to members of the PRIMO laboratory for their constructive discussions. This work was funded by grants from the NIH and the NSF.

**References**


Van den Worm, E., Beukelman, C.J., Van den Berg, A.J., Kroes, B.H.,


