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Ovothiol Replaces Glutathione Peroxidase as a Hydrogen Peroxide Scavenger in Sea Urchin Eggs

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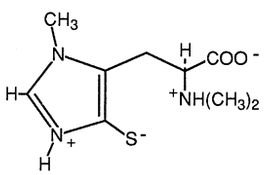
Despite its potential toxicity, H_2O_2 is used as an extracellular oxidant by *Strongylocentrotus purpuratus* eggs to cross-link their fertilization envelopes. These eggs contain 5 m*M* 1-methyl-N^{α},N^{α}-dimethyl-4-mercaptohistidine (ovothiol C), which reacts with H_2O_2. In consuming H_2O_2 and being reduced by glutathione, ovothiol acts as a glutathione peroxidase and replaces the function of the enzyme in eggs. The ovothiol system is more effective than egg catalase in destroying H_2O_2 at concentrations produced during fertilization and constitutes a principal mechanism for preventing oxidative damage at fertilization.

THE USEFUL BUT POTENTIALLY DESTRUCTIVE nature of oxygen in biological systems is especially well illustrated in sea urchin fertilization. Within minutes of gamete membrane fusion, sea urchin eggs form a protective fertilization envelope that is cross-linked by dityrosyl residues (*1–3*). Dityrosine formation, catalyzed by a secreted ovoperoxidase (*4*), requires H_2O_2 as an extracellular oxidant; H_2O_2 is produced in a respiratory burst that lasts during early embryonic activation (*5*). Thus the embryo creates a potential hazard by using a powerful oxidant like H_2O_2 at the beginning of its development. Sea urchin eggs and embryos contain one of the family of 4-mercaptohistidines (*6–9*), biological aromatic thiols with novel redox properties (*7, 10–13*). Ovothiols were discovered as cofactors for a cyanide-resistant reduced pyridine nucleotide [NAD(P)H] oxidase of ovoperoxidase (*11*), although this does not appear to be their physiological role (*7*). We show that the 5 m*M* ovothiol C (OSH) present in sea urchin eggs and embryos may function in the control of H_2O_2 toxicity by providing a nonenzymatic glutathione peroxidase (GPx)–like activity to protect the early embryo.



Ovothiol C

OSH is rapidly oxidized to ovothiol disulfide (OSSO) by H_2O_2, suggesting that OSH may control H_2O_2 toxicity at fertilization. Since glutathione is present in the reduced state (GSH) in eggs (*14*) and since the midpoint redox potential of ovothiol is 84 mV positive with respect to glutathione (*12*), ovothiol will be present as OSH in vivo. Oxidized ovothiol and oxidized glutathione (GSSG) are formed in the presence of H_2O_2 (*6, 12*)

$$2\ OSH + H_2O_2 \rightarrow OSSO + 2\ H_2O \quad (1)$$

$$2\ GSH + H_2O_2 \rightarrow GSSG + 2\ H_2O \quad (2)$$

The rate of formation of each oxidized species was first-order in H_2O_2 and thiol, yielding second-order kinetic constants (20 m*M* tris-Hepes, *p*H 7.2, 30°C) of 2.0 $s^{-1}\ M^{-1}$ (reaction 1) or 0.43 $s^{-1}\ M^{-1}$ (reaction 2). Whereas an increase in *p*H from 7.2 to 7.6 doubled the rate of oxidation of GSH by H_2O_2, the same *p*H change increased the rate of reaction of OSH with H_2O_2 by less than 10%; this is at least in part due to the difference in thiolate pK_a for GSH (8.6) (*15*) and OSH (2.3) (*10*). The rates of reaction of OSH and GSH with H_2O_2 were not dependent on the presence of divalent

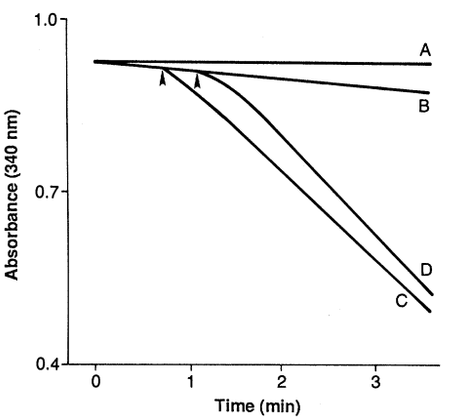


Fig. 1. Glutathione peroxidase activity of ovothiol. GPx was measured by the disappearance of NADPH (*26*) (Table 1). Reactions were initiated with H_2O_2; OSH or glutathione peroxidase (Sigma) were added later, as shown. Line A, background consumption of NADPH; line B, control, with 0.2 m*M* GSH present at time 0; line C, addition of 1 m*M* OSH at arrowhead; and line D, addition of 1 unit of bovine erythrocyte GPx (Sigma) at arrowhead.

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metal ions, in that pretreating buffers with Chelex 100 and the chelator diethylenetriamino pentaacetic acid (DTPA) (1 m*M*) had no effect.

If reaction 1 were used to trap H_2O_2, OSH could be regenerated according to

$$OSSO + 2\ GSH \rightarrow 2\ OSH + GSSG \quad (3)$$

The uncatalyzed reaction rate (determined by coupling reaction 3 to GSSG reduction by 1 unit of glutathione reductase in a mixture of 20 m*M* tris-HCl, 20 m*M* Hepes, 1 m*M* NaN_3, 0.32 m*M* NADPH, and 30 to 200 μ*M* OSSO, at *p*H 7.2, 30°C) increased with the concentrations of both OSSO and GSH, in each case somewhat less than predicted for a second-order reaction. At 0.02 m*M* OSSO and 1 m*M* GSH, reaction 3 proceeded at an order of magnitude more rapidly than the rate of OSSO production in reaction 1. We do not know whether reaction 3 is catalyzed by a thiol transferase in vivo.

Table 1. The GPx activity of sea urchin egg homogenates was examined in the presence of NaN_3 to inhibit catalase. Each assay represents 50 μl of whole, centrifuged, or boiled homogenates equivalent to 2.8×10^4 eggs (~1 mg of egg protein). *Strongylocentrotus purpuratus* were obtained from the Strait of Juan de Fuca and spawned as previously described (*11*). Assays were slightly modified from Tappel (*25*), in 1 ml of 20 m*M* tris, 20 m*M* Hepes, 1 m*M* NaN_3, 0.2 m*M* GSH, 0.16 m*M* NADPH, 1 unit of yeast glutathione reductase (Sigma), and 1.3 m*M* H_2O_2 at *p*H 7.2, 30°C. Egg homogenates were prepared by adjusting an egg suspension (280,000 eggs per milliliter) to *p*H 5.5 to remove the jelly layer, then washed with Mg^{2+}- and Ca^{2+}- free artifical seawater consisting of 500 m*M* NaCl, 10 m*M* KCl, 2.5 m*M* $NaHCO_3$, 20 m*M* tris, 20 m*M* Hepes, *p*H 7.2, treated with Chelex resin prior to use. Eggs were then homogenized (5.6×10^5 eggs per milliliter) in Chelex-treated tris, 20 m*M* Hepes, *p*H 7.2 (whole homogenate), and centrifuged at 15,000*g* for 5 min (homogenate supernatant) or boiled for 10 min and then centrifuged (boiled supernatant). Rates were measured spectrophotometrically at 340 nm, and the background NADPH consumption without homogenate, representing nonenzymatic oxidation of GSH by H_2O_2, was subtracted. Glutathione was determined as total GSH plus GSSG (*25*) on tissue homogenates after addition of 100% trichloroacetic acid to 5% final concentration, followed by centifugation. Up to 50 μl of the supernatant could be added to the assay mixture without significantly affecting the final *p*H.

Assay	NADPH consumed (nmol/min)
Egg homogenate	
Whole	3.81 ± 0.65*
Supernatant	3.58 ± 0.18*
Supernatant, boiled	3.64 ± 0.38*
Erythrocyte lysates†	
Supernatant	18.1
Supernatant, boiled	<0.5
Se-GPx†	
Selenoenzyme	142
Selenoenzyme, boiled	<0.5

*Mean ± SD of three determinations. †Assayed under slightly different conditions at *p*H 7.6, with 1.0 m*M* GSH, and 0.2 m*M* H_2O_2. Data for erythrocyte lysates prepared by osmotic lysis are expressed per milligram of protein. Purified GPx represents 0.2 manufacturer's units (Calbiochem).

Significant GPx-like activity was measured in egg preparations (Table 1). However, unlike the GPx activity of human erythrocyte lysates or the purified bovine selenoprotein, the activity in sea urchin eggs was heat stable. As with the nonenzymic reaction of OSH and H_2O_2, the GPx activity detected in egg homogenates did not require metal ions; eggs were washed with Chelex-treated buffers prior to homogenization, and addition of 1 m*M* DTPA, EGTA, or EDTA to the assay mixture did not affect the rate of NADPH consumption. The GPx activity of egg supernates increased linearly with H_2O_2 concentration from 0.2 to 5 m*M* with no evidence of saturation.

The rate of H_2O_2 consumption by homogenates (Table 1) was approximately that expected from known egg concentrations of OSH and GSH (~5 and ~2 m*M*). Thus the heat-stable GPx activity of sea urchin egg homogenates can be almost quantitatively accounted for by the nonenzymic reaction of GSH and OSH with H_2O_2, that is, by coupling reactions 1 and 3

$$2\ OSH + H_2O_2 \rightarrow OSSO + 2\ H_2O \quad (1)$$

$$OSSO + 2\ GSH \rightarrow 2\ OSH + GSSG \quad (3)$$

to give reaction 4

$$2\ GSH + H_2O_2 \rightarrow GSSG + 2\ H_2O \quad (4)$$

This GPx-like activity of purified ovothiol is illustrated in Fig. 1. GSH is regenerated from GSSG by glutathione reductase, the activity of which in egg homogenates was 51.5 μmol of NADPH consumed per minute per milligram of protein, nearly 20-fold as great as the specific activity of human erythrocyte lysates in a parallel assay.

The above data indicate that the sea urchin egg can consume H_2O_2 by using a system that replaces GPx with the amino acid OSH. Eggs also contain catalase as another system to metabolize H_2O_2. We were able to measure the relative contribution of the thiol-dependent and catalase-dependent systems for consumption of exogenously added H_2O_2, because catalase produces O_2. When H_2O_2 was added to eggs (Fig. 2) in the absence of inhibitors, most of the added H_2O_2 was consumed by a mechanism other than catalase (one that did not evolve O_2). In unfertilized eggs (Fig. 2A), this pathway accounted for metabolism of 73% of the H_2O_2, whereas the catalase-dependent system accounted for 27%. In fertilized eggs (Fig. 2B), the catalase-dependent system was responsible for 28% of H_2O_2 metabolism, whereas the system that did not evolve O_2 accounted for 72%. In the presence of the catalase inhibitor NaN_3, O_2

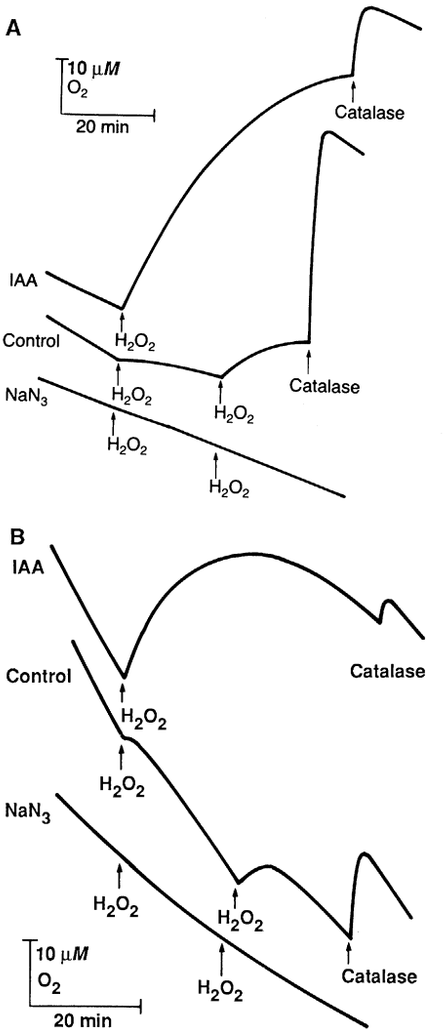

Fig. 2. Metabolism of exogenous H_2O_2 by sea urchin eggs. The production of O_2 from exogenous H_2O_2 by sea urchin eggs was measured in a Clark O_2 electrode (*11*) (Y.S.I., Yellow Springs, Ohio) in 5 ml of Mg^{2+}- and Ca^{2+}-free artificial seawater containing 2 m*M* DTPA, at *p*H 7.2 and 10°C, with 7×10^4 eggs per milliliter. Unfertilized eggs were washed twice in this artificial seawater prior to recording (**A**); fertilized eggs (**B**) were studied 60 min after insemination, at which time the respiratory burst had ended (*5*), but the steady-state O_2 consumption was still elevated. The electrode was calibrated by the addition of catalase to a known concentration of H_2O_2 and by measuring O_2 production. Where noted by arrows, 0.67 μmol (133 μ*M*) of H_2O_2 or 2 μg of bovine catalase (Sigma) were added. The evolution of O_2 upon the addition of exogenous catalase allowed estimation of the H_2O_2 that remained at the end of the incubation. This value was used to calculate total egg H_2O_2 consumption, and the relative contribution of egg catalase. The evolution of oxygen was measured in the absence of inhibitors (middle curve), in the presence of 2 m*M* iodoacetamide (IAA; top curve), and in the presence of 5 m*M* NaN_3 (bottom curve).

evolution was eliminated. In the presence of iodoacetamide (IAA), which alkylates OSH and GSH in vivo (7), essentially all of the H_2O_2 was metabolized by catalase and O_2 was produced with the expected 1:2 stoichiometry to H_2O_2.

A second means of evaluating the relative contributions of the catalase- and thiol-dependent systems was to measure the clearance of H_2O_2 from seawater by an embryo suspension. A scopoletin assay (5) was used to measure the residual H_2O_2 after removal of the embryos by centrifugation. Fertilized sea urchin eggs (70,000 embryos per milliliter) incubated in 133 μM H_2O_2 (under the conditions of Fig. 2) consumed H_2O_2 in an apparent first-order reaction with a time constant $t_{1/2}$ of 15 min. After preincubation with 5 mM NaN_3 alone, $t_{1/2}$ = 18 min, and after preincubation with 2 mM IAA alone, $t_{1/2}$ = 16 min. However, when eggs were treated with both NaN_3 and IAA, there was no destruction of H_2O_2; $t_{1/2}$ was >4 hours, identical to that found in seawater alone. These data indicate that the catalase- and thiol-dependent systems accounted for all of the H_2O_2 consumption by sea urchin embryos, and that inhibition of either system alone does not significantly change the rate-limiting step in whole eggs (which is likely to be H_2O_2 entry).

The consumption of oxygen by sea urchin eggs at fertilization, discovered 80 years ago (16), is the archetype of metabolic activation. The egg uses the strong oxidant H_2O_2, thereby produced in order to modify its extracellular matrix; it is protected from oxidative stress by the regenerable trap, OSH. The activations of the pentose phosphate pathway (17) and NAD kinase (18) after fertilization are probably required, at least in part, to produce NADPH as substrate for glutathione reductase to allow reaction 4 to continue.

High concentrations of an OSH trap may be preferable to the catalytic GPx system found in mammalian cells, in which H_2O_2 bursts of comparable magnitude are rarely used physiologically. The ovothiol system may also protect marine invertebrate eggs and embryos from the significant concentrations of H_2O_2 generated photochemically in the sea (19, 20). Ovothiols are not restricted to marine invertebrates, for eggs from the rainbow trout *Salmo gairdneri* contain ovothiols A and B (1.7 and 0.34 nmol per milligram of protein, respectively), and similar concentrations exist in Coho salmon eggs (21).

Biological aromatic thiols like OSH have chemical properties distinct from aliphatic thiols like glutathione, including their distinct thiolate pK_a, heightened nucleophilicity (10), and ability to reduce oxygen-centered radicals (13). Because reactive oxygen intermediates have been implicated in many types of cellular dysfunction (22–24) and OSH is not toxic at high intracellular concentrations (at least in these embryonic cells), OSH could have useful applications.

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Induction of Manganous Superoxide Dismutase by Tumor Necrosis Factor: Possible Protective Mechanism

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Manganous superoxide dismutase (MnSOD) scavenges potentially toxic superoxide radicals produced in the mitochondria. Tumor necrosis factor–α (TNF-α) was found to induce the messenger RNA for MnSOD, but not the mRNAs for other antioxidant or mitochondrial enzymes tested. The increase in MnSOD mRNA occurred rapidly and was blocked by actinomycin D, but not by cycloheximide. Induction of MnSOD mRNA was also observed with TNF-β, interleukin-1α (IL-1α), and IL-1β but not with other cytokines or agents tested. TNF-α induced MnSOD mRNA in all cell lines and normal cells examined in vitro and in various organs of mice in vivo. These effects of TNF-α and IL-1 on target cells may contribute to their reported protective activity against radiation as well as their ability to induce resistance to cell killing induced by the combination of TNF-α and cycloheximide.

TUMOR NECROSIS FACTORS (TNFs) are cytotoxic to some tumor cells but not to normal cells (1), and they also mediate many other biological effects (2). The mechanism of TNF cytotoxicity is unclear but can occur in the absence of RNA or protein synthesis (3–5) and may be at least partially mediated through the generation of hydrogen peroxide (H_2O_2) and oxygen-free radicals such as O_2^- (6). Thus, the susceptibility of a cell to killing by TNF-α might be influenced by its content of antioxidant enzymes such as catalase, superoxide dismutase (SOD), and glutathione peroxidase. SOD protects cells from the toxicity of O_2^-, whereas catalase and glutathione peroxidase scavenge H_2O_2 (7). Eukaryotic cells contain copper-zinc SOD, which is found predominantly in the cytosol, and MnSOD, which is found mainly in mitochondria (8). We examined the effect of TNFs on the expression of antioxidant enzyme mRNAs. Unexpectedly, TNF-α was found to induce mRNA for MnSOD but not for Cu-ZnSOD, catalase, or glutathione peroxidase.

The actions of TNF-α and interferon-γ (IFN-γ) on cells are often synergistic (9). We therefore examined the effect of TNF-α and IFN-γ on the expression of antioxidant enzyme mRNAs in the human A549 lung carcinoma cell line. RNA hybridization showed that catalase, Cu-ZnSOD, and glu-

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