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Research Paper

Inorganic Polyphosphates Regulate Hexokinase Activity and Reactive Oxygen Species Generation in Mitochondria of *Rhipicephalus (Boophilus) microplus* Embryo

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Abstract

The physiological roles of polyphosphates (poly P) recently found in arthropod mitochondria remain obscure. Here, the possible involvement of poly P with reactive oxygen species generation in mitochondria of *Rhipicephalus microplus* embryos was investigated. Mitochondrial hexokinase and scavenger antioxidant enzymes, such as superoxide dismutase, catalase, and glutathione reductase were assayed during embryogenesis of *R. microplus*. The influence of poly P_3 and poly P_{15} were analyzed during the period of higher enzymatic activity during embryogenesis. Both poly Ps inhibited hexokinase activity by up to 90% and, interestingly, the mitochondrial membrane exopolyphosphatase activity was stimulated by the hexokinase reaction product, glucose-6-phosphate. Poly P increased hydrogen peroxide generation in mitochondria in a situation where mitochondrial hexokinase is also active. The superoxide dismutase, catalase and glutathione reductase activities were higher during embryo cellularization, at the end of embryogenesis and during embryo segmentation, respectively. All of the enzymes were stimulated by poly P_3. However, superoxide dismutase was not affected by poly P_{15}, catalase activity was stimulated only at high concentrations and glutathione reductase was the only enzyme that was stimulated in the same way by both poly Ps. Altogether, our results indicate that inorganic polyphosphate and mitochondrial membrane exopolyphosphatase regulation can be correlated with the generation of reactive oxygen species in the mitochondria of *R. microplus* embryos.

Key words: inorganic polyphosphate; reactive oxygen species; arthropod; mitochondria; scavenger antioxidant enzymes.

Introduction

Inorganic polyphosphates (poly P) are long chains of a few to several hundred phosphate residues linked by phosphoanhydride bonds. Polyphosphates have been found in all cell types examined to date and have been demonstrated to play diverse roles depending on the cell type and circumstances [1, 2]. The biological roles played by polyphosphates have been most extensively studied in prokaryotes and unicel-

lular eukaryotes, where they have been shown to regulate many biochemical processes including the metabolism and transport of inorganic phosphate, cation sequestration and storage [1], membrane channel formation [3, 4], cell envelope formation and bacterial pathogenesis[5, 6], regulation of gene and enzyme activities [7] and activation of Lon proteases [8].

Conversely, poly P functions have not been extensively investigated in higher eukaryotes, although some functions have been described such as the activation of TOR kinase [9], involvement in blood coagulation [10], and apoptosis [11-13]. Regarding mitochondrial metabolism, mammalian mitochondrial poly P production is directly linked to their energetic state [14], as the level of poly P regulates the level of cellular ATP [14] and Ca^{2+} accumulation [15]. The interest in mitochondrial poly P is focused on two aspects: poly P as a macroenergetic compound with the same energy of hydrolysis of the phosphoanhydride bond as ATP, and the fact that, according to the endosymbiotic theory, mitochondria originated from ancient prokaryotic cells; thus, it would be intriguing to discover whether or not mitochondria have preserved polyphosphate functions such as the regulation of energy metabolism [16, 17]. Recently, we have demonstrated that electron flux and redox states may exert some influence and be influenced by the activity of membrane exopolyphosphatase (PPX), the enzyme that splits P_i off the end of a poly P chain, suggesting that it plays a role in energy supply during *R. microplus* embryogenesis [18].

The metabolism of free hexoses begins by phosphorylation in a reaction catalyzed by the hexokinase (ATP: hexose-6-phosphotransferase, E.C. 2.7.1.1; HK). This enzyme has been characterized in several organisms [19-24]. Four distinct hexokinase isozymes are reported for mammalian tissues and are named types I–IV. These isozymes have an internal repeat sequence in their N- and C- terminal halves that is found in mammals, insects and nematodes [20, 25, 26]. In arthropods the binding mechanism of HK to the outer mitochondrial membrane is not fully established.

The mitochondrial electron transport system (ETS) represents one of the major sources of cellular reactive oxygen species (ROS) such as superoxide (O2•–) and hydrogen peroxide (H_2O_2), providing continuous generation of these toxic products [27]. The scavenger activity of antioxidant enzymes constitutes an essential mechanism directed against the primary ROS generated by mitochondrial ETS. Superoxide dismutase (SOD) rapidly converts $O_2^{\bullet-}$ to H_2O_2, protecting the mitochondrial iron-sulfur cluster-containing enzymes from $O_2^{\bullet-}$ attack [28]. This enzyme is present in the matrix (Mn-SOD) and in the intermembrane space (Zn-SOD) [29-32]. The abundance of this enzyme, as well as its presence in both mitochondrial compartments, attests to the importance of removing mitochondrially-generated $O_2^{\bullet-}$ [33]. Catalase (CAT) acts by decomposing H_2O_2 into H_2O and O_2, avoiding the production of hydroxyl radicals ($HO^{\bullet}$) in the presence of transition metals. The presence of CAT in mitochondria is of great importance, as decomposition of H_2O_2 protects these organelles against intra- and extra-mitochondrially generated H_2O_2 [34, 35]. Glutathione reductase (GR) is the enzyme that regenerates reduced glutathione in the mitochondrial matrix. Reduced glutathione can scavenge ROS non-enzymatically or by serving as an electron-donating substrate to several enzymes involved in ROS-detoxification [36].

Ticks are vectors of parasites that cause hemoparasitic diseases and are endemic in many cattle production areas [37]. The present study focuses on *Rhipicephalus microplus,* which causes heavy economic losses to bovine herds, particularly in tropical regions; thus, major efforts have been directed toward developing immunoprophylactic tick-control tools [38, 39]. *R. microplus* has only one host throughout its three life stages, which is usually a bovine, and has a long feeding period (approximately 21 days). Female ticks, after engorgement, drop off the host and initiate oviposition approximately three days later. Being an oviparous animal, embryogenesis occurs in the absence of exogenous nutrients; maternal nutrients are packaged into oocytes and stored mostly as yolk granules. Hatching occurs approximately 21 days after egg laying and the emerging larvae can survive for several weeks before finding a host, using the remaining yolk as their only energy source [40, 41].

The aim of this study was to investigate the possible involvement of poly P in reactive oxygen species generation in mitochondria of *R. microplus* embryos. Such findings could reveal an important role for poly P metabolism in arthropods.

Materials and Methods

Ticks and reagents

Ticks were obtained from a colony maintained at the Faculdade de Veterinária, Universidade Federal do Rio Grande do Sul (UFRGS), Brazil as previously described [39]. *R. microplus* (Acarina, Ixodidae) ticks from the Porto Alegre strain, free of parasites, were reared on calves obtained from a tick-free area. Engorged adult females were maintained in Petri dishes at 28°C and 80% relative humidity upon completion of oviposition, which starts approximately three days after adult ticks drop off calves. Animals were treated

in compliance with the UFRGS review committee for animal care. The reagents were purchased from Sigma (USA), Amersham Biosciences (USA), Invitrogen (USA) and Merck (Germany).

Isolation of Mitochondria

The cell fractionation procedure used required large amounts of fresh eggs (at least 2 g) to obtain functionally active mitochondrial fractions. Mitochondria were isolated by differential centrifugation, following the procedure described by [42]. Eggs were homogenized in a buffer containing 0.5 M sucrose, 100 µM leupeptin, 100 nM pepstatin, 1 mM PMSF, 10 mM EGTA, 1% bovine albumin (fat acid free) and 50 mM HEPES, pH 7.4. The homogenate was centrifuged at 500 X g for 5 min. The supernatant was carefully removed and centrifuged at 2,000 X g for 10 min to yield a nuclear pellet. Then, the supernatant was submitted to another centrifugation at 7,000 X g for 15 min and the mitochondrial pellet that resulted was re-suspended in a storage buffer containing 120 mM KCl, 1 mM EGTA, 0.2% bovine albumin, and 10 mM Tris HCl, pH 7.4. Isolation of the mitochondrial membrane fraction was performed by sonication of freshly prepared mitochondria three times for 20 s at the maximal output using an MSE ultrasonic disintegrator. The suspension was centrifuged for 10 min at 12,000 × *g* to remove unbroken mitochondria. The supernatant was centrifuged at 100,000 × *g* for 60 min to yield the mitochondrial membrane fraction and the pellet was re-suspended in a buffer containing 10 mM Tris HCl, pH 7.4[18]. Protein was determined by the Folin-Lowry method using bovine serum albumin as a standard [43].

Determination of mitochondrial hexokinase activity

The activity of mitochondrial hexokinase was determined based on [27]. A 0.1 mg/mL dilution of mitochondrial protein was used and mitochondrial hexokinase was determined by NADH formation; the absorbance was followed at 340 nm at 37°C using a molar extinction coefficient of 6.22 M^{-1}. The assay medium contained 10 mM Tris HCl, pH 7.4, 5 mM glucose, 10 mM $MgCl_2$, 1 mM β–NAD^+, 2 units/ml G6PDH (glucose-6-phosphate dehydrogenase) from *Leuconostoc mesenteroides* and 50 mM Ap5A (P1,P5-di(adenosine 50)-pentaphosphate), in a final volume of 1 mL. The reaction was started by adding 1 mM ATP.

Exopolyphosphatase Assay

The reaction mixture consisted of 50 mM Tris HCl buffer (pH 7.4) and 5 mM $MgCl_2$, using 5 mM $polyP_3$ as the substrate. Reactions were performed at 37°C. The P_i formed during the reaction was spectrophotometrically determined as described by[44], adding a solution of 0.5% ammonium molybdate, 0.35 M sulfuric acid, 0.5% sodium dodecyl sulfate, and 10% ascorbic acid. Measurements of absorbance at 750 nm were performed after 15 min. One unit of enzyme activity (U) was defined as the quantity of enzyme liberating 1 µmoL of Pi per min. PPX activity during mitochondrial respiration was measured using a reaction mixture consisting of 50 mM Tris HCl buffer (pH 7.4), 120 mM KCl, 1 mM EGTA, 5 mM $MgCl_2$, and 0.2 mM adenosine diphosphate (ADP) in the absence of any Pi source. $PolyP_3$ (0.5 µM) was used as a substrate for PPX activity and 5 mM pyruvate was used as an oxidative substrate. Potassium cyanide (KCN, 1 mM) and 20 µg/mL heparin were used to inhibit cytochrome oxidase and PPX activities, respectively. The reaction was performed at 28 °C for 15 min [42].

Spectrofluorometric measurements of mitochondrial H_2O_2 generation

Mitochondrial release of H_2O_2 was assessed by the Amplex Red oxidation method [45]. Mitochondria (0.2 mg protein/ mL) were incubated in buffer containing 10 mM Tris HCl, pH 7.4, 0.32 M mannitol, 8 mM inorganic phosphate, 5 mM $MgCl_2$, 0.08 mM EDTA, 1 mM EGTA, 1 mM ATP, 10 mM succinate and 0.2 mg/mL fatty acid-free bovine serum albumin supplemented with 10 mM Amplex Red and 2 units/mL horseradish peroxidase. After 5 min incubation, the fluorescence (Ex: 563nm; Em: 587nm) was measured using a Cary Eclipse spectrofluorometer. The total H_2O_2 released was corrected for non-specific oxidation of Amplex Red measured in the absence of horseradish peroxidase. The maximal rate (100%) of mitochondrial H_2O_2 formation was assumed to be the difference between the rate of H_2O_2 formation in the absence of oxidative substrate and that measured after the addition of succinate.

Determination of Mn-SOD activity

The mitochondrial fraction (20 µg/mL) was used to determine Mn-SOD activity using an indirect competition assay between SOD and an indicator molecule, nitroblue tetrazolium [46]. The reaction mixture contained 13 mM methionine, 75 µM nitroblue tetrazolium, 100 mM ethylenediamine tetraacetic acid (EDTA), and 2 µM riboflavin in phosphate buffer (50 mM, pH 7.4) to a final volume of 1 mL at 25°C; the change in absorbance was observed at 560 nm. One unit of SOD was defined as the amount of enzyme needed to inhibit the reduction of nitroblue tetrazolium (NBT) by 50%. Sodium cyanide (5 mM) was used to inhibit Cu/ZnSOD activity.

Determination of CAT activity

CAT activity was determined according to the method of Aebi[47]. The mitochondrial fraction (50 mg/mL) was added to phosphate buffer (50 mM, pH 7.0) containing 15 mM H_2O_2 as substrate; the change in absorbance was noted at 240 nm at 25°C using an extinction coefficient of 43.6 $M^{-1}cm^{-1}$. The specificity of CAT activity to degrade H_2O_2 was confirmed by inhibiting the activity with aminotriazole (20 mM), a compound that is a specific catalase inhibitor [48].

Determination of GR activity

GR activity was measured by monitoring the oxidation of β-NADPH [49]. The reaction mixture contained 1 mM GSSG and 0.1 mM β-NADPH in phosphate buffer (0.1 M, pH 7.0) in a final volume of 1 mL at 30°C. The reaction was initiated by adding the mitochondrial fraction (50 mg/mL) to the cuvette and following the decrease in absorbance at 340 nm at 30°C. One unit of GR was equivalent to the oxidation of 1 mmol of NADPH per min at pH 7.0 at 30°C.

Statistical analysis

Results were expressed as mean ± standard error (± SE) and one-way ANOVA was used for statistical analysis, followed by the post hoc Tukey test. Data were considered statistically significant when $P < 0.05$.

Results and Discussion

Although the first evidence for the presence of poly P in mammalian cells was obtained a long time ago [50], relatively few studies have addressed its physiological roles in animal cells [1, 9-12, 14, 18, 42, 51, 52].

Early *R. microplus* embryonic stages are similar to those of mosquitoes [53]. Tick embryogenesis is characterized by the formation of a cellularized blastodermal cell layer up to day 4. Thereafter, the embryo starts segment formation and initiates organogenesis [24, 54]. Previously, we provided evidence that mitochondrial membrane PPX plays a role in energy metabolism in *R. microplus* during embryo development [18]. Here, we demonstrate that inorganic polyphosphate and mitochondrial membrane PPX regulation can be correlated to the generation of reactive oxygen species in mitochondria during *R. microplus* embryo development.

Regulation of mitochondrial hexokinase by inorganic polyphosphate

Mitochondria from tick embryos were previously characterized by our research group [18, 42, 51]. Mitochondria from tick embryos were isolated and the mitochondrial hexokinase activity was analyzed during *R. microplus* embryogenesis. The activity was higher (320.7 ± 50) during embryo cellularization, on the 3rd day of development (Figure 1A). The profile was not altered after normalization for mitochondrial recovery using the specific activity of F_1F_o APTase as a specific mitochondrial marker instead of mitochondrial protein (data not shown). Cytoplasmic hexokinase activity during *R. microplus* embryogenesis has already been determined by da-Silva [55], and showed a distinct profile. Our results suggest that a mitochondrial hexokinase isoform exists, because higher levels of activity were observed in mitochondria during early embryogenesis, while higher levels were observed in the cytoplasm near larval eclosion [55] indicating different roles for these isoforms during embryo development. The day corresponding to the peak of activity (day 3) was used to analyze the influence of poly P_3 and poly P_{15} on mitochondrial hexokinase. Both poly Ps inhibited mitochondrial hexokinase activity by up to 90% at a 20 µM concentration (Figure 1B and 1C).

To confirm the relationship between mitochondrial hexokinase and poly P metabolism, the effects of glucose-6-phosphate, a hexokinase reaction product, on mitochondrial membrane PPX activity was evaluated. In fact, mitochondrial membrane PPX activity was stimulated by about 40% when using 2 mM glucose-6-phosphate (Figure 2). The observed decrease in mitochondrial hexokinase activity by poly P and the increase in mitochondrial membrane PPX activity by glucose-6-phosphate indicate a co-regulation between these enzymes.

A portion of basal mitochondrial respiration results from the consumption of oxygen that is promoted by ADP recycling by mitochondrial kinases [56]. We have previously demonstrated that poly P can be used as a P_i donor for adenosine-5′-triphosphate (ATP) synthesis in ticks [42]. To obtain further insight into the relationship between these enzymes during mitochondrial respiration, mitochondrial membrane PPX activity was measured using pyruvate as the substrate and poly P_3 as the only source of P_i. Poly P_3 was used in this assay because the affinity of mitochondrial membrane PPX for $polyP_3$ is 10 times stronger than for $polyP_{15}$ [18]. During this assay, the addition of small amounts of ADP (0.2 mM) induces state 3 followed by state 4, when all of the ADP was converted to ATP. Thus, during state 3, a balance exists between P_i released by PPX and ATP synthesis, because PPX is measured by the amount of P_i. Membrane PPX activity increased by a factor of three during mitochondrial respiration when pyruvate and ADP were added, and increased significantly when glucose-6-phosphate was added. Heparin, a PPX inhibitor, and KCN, a mitochondrial elec-

tron transport inhibitor, were used as controls. Heparin completely inhibited the membrane PPX activity and the stimulatory effects disappeared after mitochondrial respiration inhibition by KCN (Figure 3). This increase did not occur without ADP addition, indicating that PPX is stimulated during state 3 and the velocity of P_i release is higher than the rate of ATP synthesis [18].

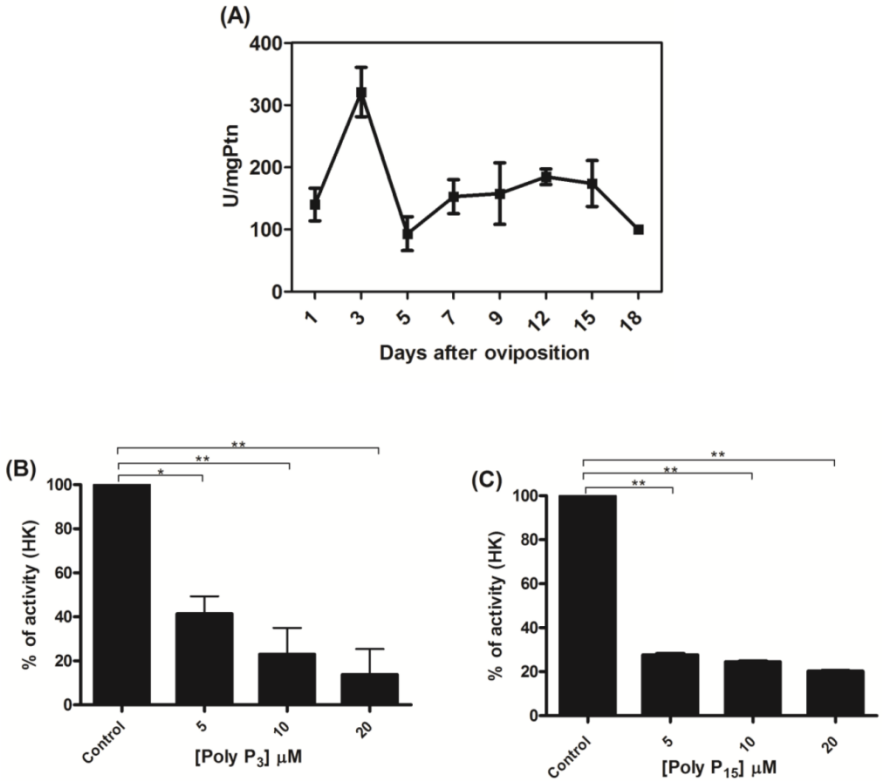

Fig 1. Activity profile of mitochondrial hexokinase and regulation by polyphosphate. (**A**) Specific HK activity was measured in mitochondria on different days after oviposition and is represented as units per milligram of mitochondrial protein. The HK activity was normalized for mitochondrial recovery using the specific activity of F_1F_o ATPase as a mitochondrial marker, instead of mitochondrial protein, and the same activity profile was obtained (data not shown). (**B**) Mitochondria were isolated from eggs on the 3rd day of embryogenesis and HK activity was determined in the presence of 5, 10 and 20μM poly P_3. (**C**) Mitochondria were isolated from eggs on the 3rd day of embryogenesis and HK activity was determined in the presence of 5, 10 and 20μM of poly P_{15}. Data are the mean±S.E. of three independent experiments, in triplicate. *p < 0.05; **p < 0.001.

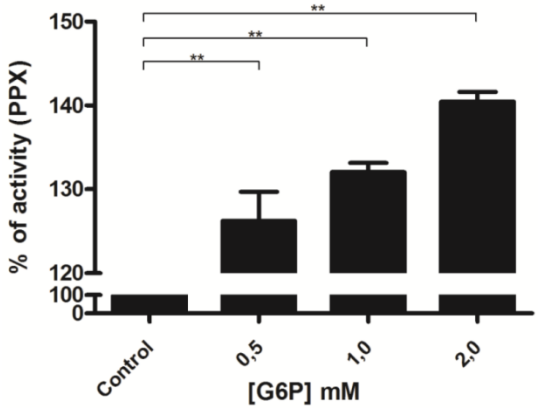

Fig 2. Regulation of membrane mitochondrial PPX by glucose-6-phosphate. Membrane mitochondrial PPX activity was measured in mitochondria from eggs on the 9th day of development using poly P_3 as a substrate in the presence of 0.5, 1.0 and 2.0 mM glucose-6-phosphate. Data are the mean±S.E. of three independent experiments, in triplicate. **p < 0.001.

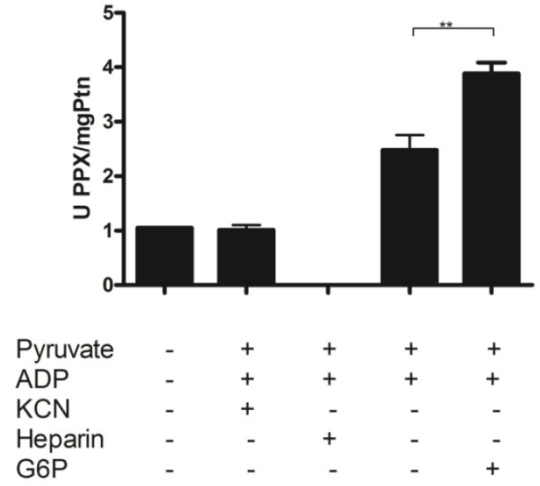

Fig 3. Regulation of mitochondrial PPX activity during mitochondrial respiration. PPX activity was measured in egg mitochondria on the 9th day of development during mitochondrial respiration, using pyruvate as the oxidative substrate, polyP_3 as the PPX substrate, KCN as an inhibitor of the respiratory chain, and Heparin as a PPX inhibitor. The specific activity is represented as units per milligram of mitochondrial protein. Data are the mean±S.E. of three independent experiments, in triplicate. **p < 0.01.

The rate of mitochondrial ROS production is highly dependent on mitochondrial membrane potential and inversely related to the availability of ADP used to drive ATP synthesis [57, 58]. In this context, it was demonstrated that mitochondrial kinases such as hexokinase play a key preventive antioxidant role, avoiding mitochondrial ROS generation[56, 59]. Thus, ADP-producing enzymes would maintain lower mitochondrial membrane potentials and ROS levels in mitochondria [27]. We further confirmed the hypothesis that poly P is correlated with reactive oxygen species generation in mitochondria by investigating whether poly P affects H_2O_2 generation in a situation where mitochondrial hexokinase also is active. First, it was observed that the addition of 2-deoxyglucose (2-DOG) decreased H_2O_2 generation by activating mitochondrial hexokinase. Then the addition of poly P_3 and poly P_{15} restored the H_2O_2 levels nearly to control levels by inhibiting mitochondrial hexokinase. When PPX and mitochondrial hexokinase were stimulated at the same time, the H_2O_2 generation was lower using poly P_3; on the other hand, no significant effect was observed with poly P_{15} (Figure 4). Our group recently demonstrated that membrane mitochondrial PPX has a Km of 0.2 µM and Vmax of 2.4 µmol/mg protein.min for poly P_3, and a Km of 2.2 µM and Vmax of 1.1 µmol/mg protein.min for poly P_{15} [18]. These kinetics parameters clarify the reason why, during the time of incubation (5 min) when PPX was stimulated by G6P, the effect on H_2O_2 generation was only observed using poly P_3 as a substrate. These results reinforce the hypothesis of co-regulation between membrane mitochondrial PPX and mitochondrial hexokinase, and confirm the involvement of reactive oxygen species generation in mitochondria by poly P.

Based on Fig. 3 and Fig. 4, we investigated the hypothesis that polyphosphate (poly P_3 and poly P_{15}) would also affect scavenger antioxidant enzymes.

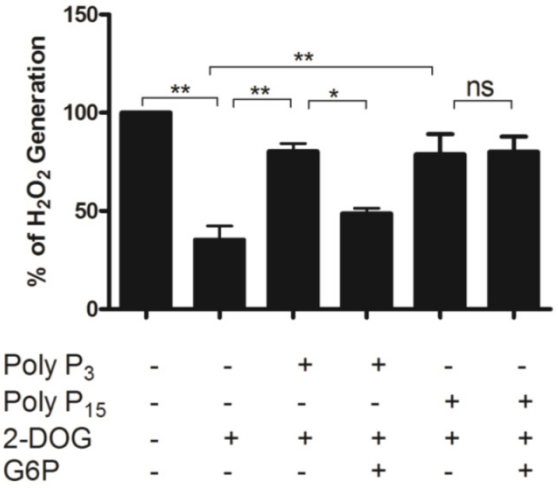

Fig 4. Hydrogen peroxide generation in mitochondria. H_2O_2 generation was measured in egg mitochondria (0.2 mg protein/ mL) on the 9th day of development after addition of 10 mM succinate and 1 mM ATP with or without 10µM Poly P_3, 10µM Poly P_{15}, 10 mM 2-DOG or 2mM G6P. Data are the mean±S.E. of three independent experiments, in triplicate. *p < 0.05; **p < 0.001.

Regulation of scavenger antioxidant enzymes by inorganic polyphosphate in mitochondria

We determined the specific activities of scavenger antioxidant enzymes in mitochondrial preparations from eggs during *R. microplus* embryogenesis. The Mn-SOD, CAT and GR activities were higher during embryo cellularization (3rd day of development), at the end of embryogenesis (15th day of development) and during embryo segmentation (7th day of development), respectively (Figure 5A, 5B and 5C). The profile was not altered after normalizing for mitochondrial recovery using the specific activity of F_1F_o APTase as a specific mitochondrial marker instead of mitochondrial protein (data not shown). These results reveal that, during embryogenesis, at least one scavenger antioxidant enzyme shows high levels of activity.

In fact, the rapid developmental kinetics observed in embryogenesis probably requires a readily available energetic support. Our group showed an increase in oxygen consumption as well as rapid sugar mobilization and lipid reservation until the 12th day of development, a period that includes most cell proliferation and reorganization events [54]. As the

energy demand is high during these steps, pathways involved in energy transduction are increased, thus explaining the high oxygen consumption. However, a transient imbalance in energy demand may lead to an oxidative burst, releasing huge amounts of ROS. Our results are in line with these observations, as Mn-SOD, CAT and GR activities in the mitochondrial fraction were activated during cellularization and embryo segmentation (Figure 5), while only CAT activity remained high after this phase of development. Some of these mechanisms against ROS have previously been characterized in arthropods[48, 60-64]. Antioxidant activity increases in the ovaries in order to protect the *Rhodnius prolixus* embryo [62]. In *R. microplus,* mitochondrial CAT activity was higher close to larval eclosion (Figure 5C) and in *D. melanogaster* it was observed that decreased CAT expression made the eggs fragile, so the embryos died immediately after eclosion [65].

To further investigate if scavenger antioxidant enzymes are regulated by poly P, the influence of different concentrations of poly P_3 and P_{15} on Mn-SOD, CAT and GR were analyzed in mitochondrial fractions. All of the enzymes were stimulated by poly P_3. Increasing concentrations of poly P_3 increased Mn-SOD, CAT and GR activities by a factor of 10, 3 and 3, respectively (Figure 6A, 6B and 6C). However, the effect was different using poly P_{15}. Mn-SOD activity, which was the most stimulated by poly P_3, was not affected by poly P_{15}, CAT activity was stimulated only by 20 μM Poly P_{15} and GR was the only enzyme that was stimulated in the same way by both poly Ps (Figure 7A, 7B and 7C). These results suggest that regulation by poly P is dependent on the chain length, and SOD, CAT and GR have different sensitivities to this. As can be seen from Figures 1B and 1C, in the presence of mainly poly P_3, hexokinase activity was inhibited and Mn-SOD, CAT and GR activities were increased, suggesting a compensatory mechanism of regulation between mitochondrial hexokinase and scavenger antioxidant enzymes by poly P.

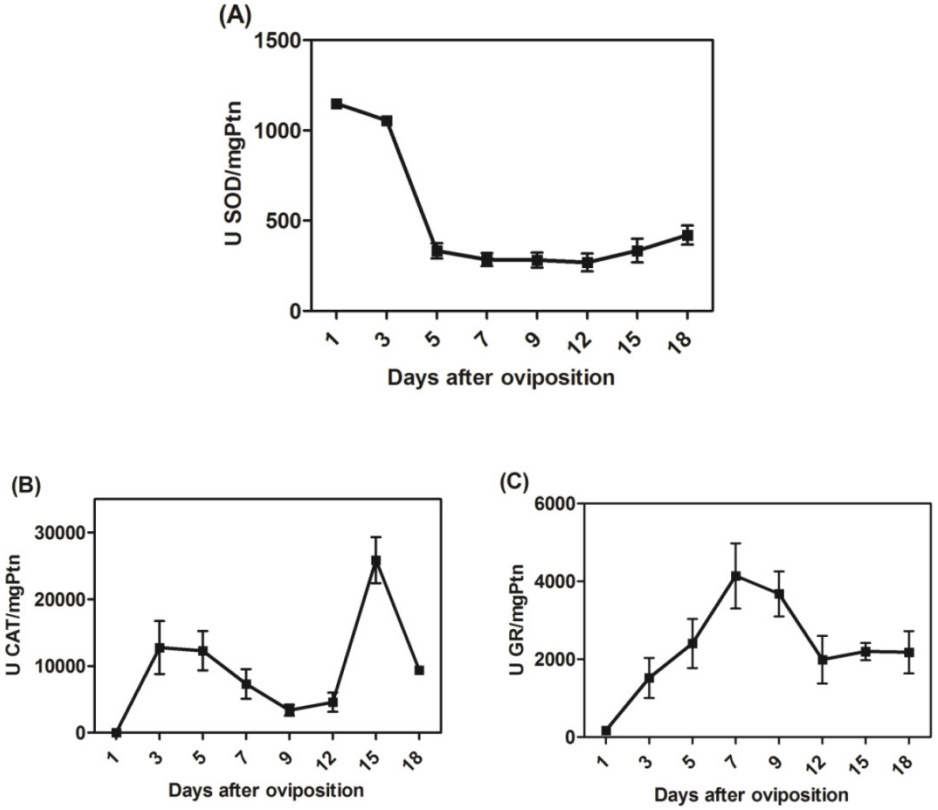

Fig 5. The activities of mitochondrial scavenger antioxidant enzymes. (**A**) SOD-specific activity was measured in mitochondria on different days after oviposition and is represented as units per milligram of mitochondrial protein. (**B**) CAT-specific activity was measured in mitochondria on different days after oviposition and is represented as units per milligram of mitochondrial protein. (**C**) GR-specific activity was measured in mitochondria on different days after oviposition and is represented as units per milligram of mitochondrial protein. All enzyme activities were normalized for mitochondrial recovery using the specific activity of F_1F_o ATPase as a mitochondrial marker instead of mitochondrial protein; the same activity profiles were obtained in both instances (data not shown).

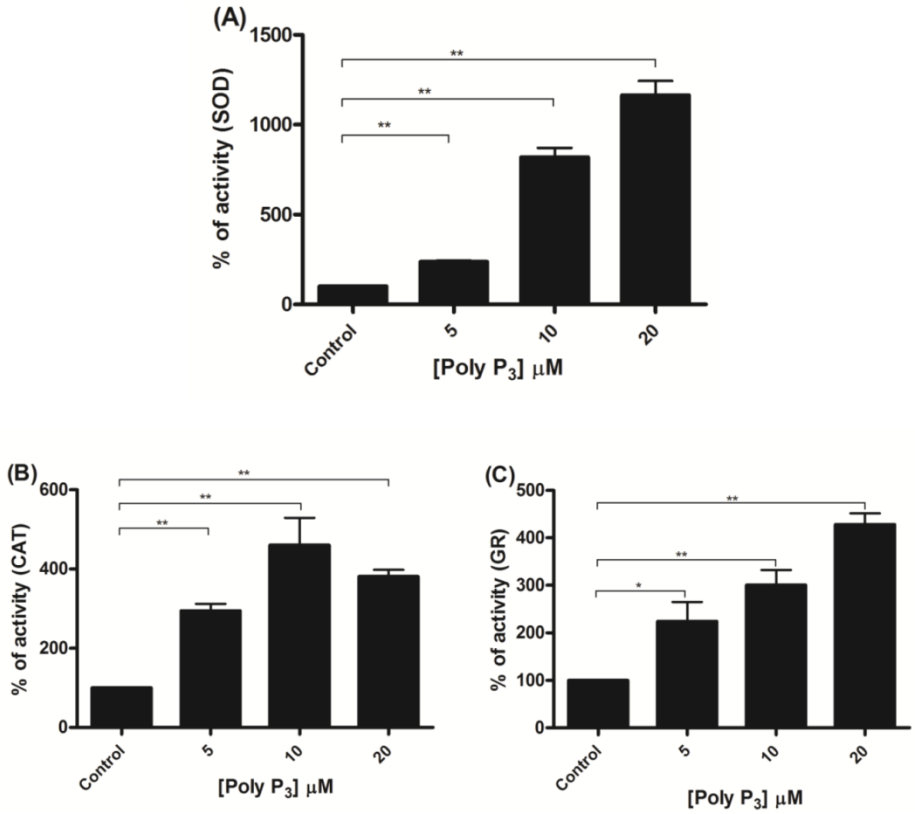

Fig 6. Regulation of scavenger antioxidant enzymes by poly P_3. (**A**) Egg mitochondria on the 3rd day of embryogenesis were isolated and SOD activity was determined in the presence of 5, 10 and 20 μM poly P_3. (**B**) Egg mitochondria on the 15th day of embryogenesis were isolated and CAT activity was determined in the presence of 5, 10 and 20 μM poly P_3. (**C**) Egg mitochondria on the 7th day of embryogenesis were isolated and GR activity was determined in the presence of 5, 10 and 20 μM poly P_3. Data are the mean±S.E. of three independent experiments, in triplicate. *p < 0.05; **p < 0.001.

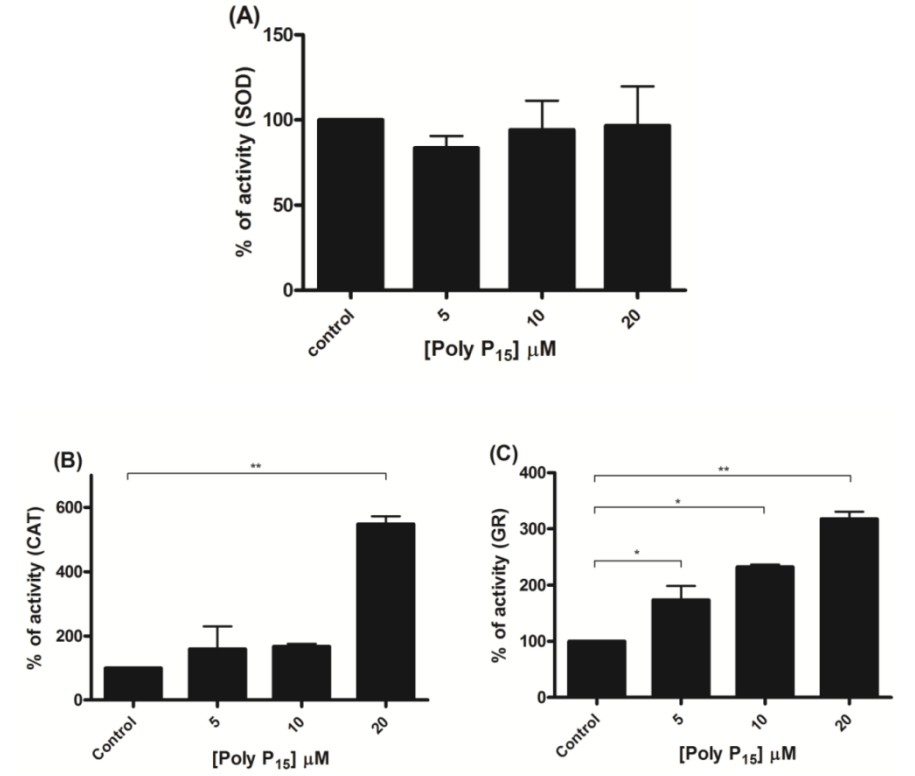

Fig 7. Regulation of scavenger antioxidant enzymes by poly P_{15}. (**A**) Egg mitochondria on the 3rd day of embryogenesis were isolated and SOD activity was determined in the presence of 5, 10 and 20 μM poly P_{15}. (**B**) Egg mitochondria on the 15th day of embryogenesis were isolated and CAT activity was determined in the presence of 5, 10 and 20 μM poly P_{15}. (**C**) Egg mitochondria on the 7th day of embryogenesis were isolated and GR activity was determined in the presence of 5, 10 and 20μM poly P_{15}. Data are the mean±S.E. of three independent experiments, in triplicate. *p < 0.05; **p < 0.001.

Conclusions

The schematic diagram in figure 8 summarizes the major findings reported in this work. First, poly P inhibited mitochondrial hexokinase activity, a situation that increases ROS generation, which is inversely related to the availability of ADP used to drive ATP synthesis. In addition, in a compensatory way, poly P increases the activities of scavenger antioxidant enzymes, providing compelling evidence that poly P plays a role in mitochondrial ROS metabolism during *R. microplus* embryogenesis.

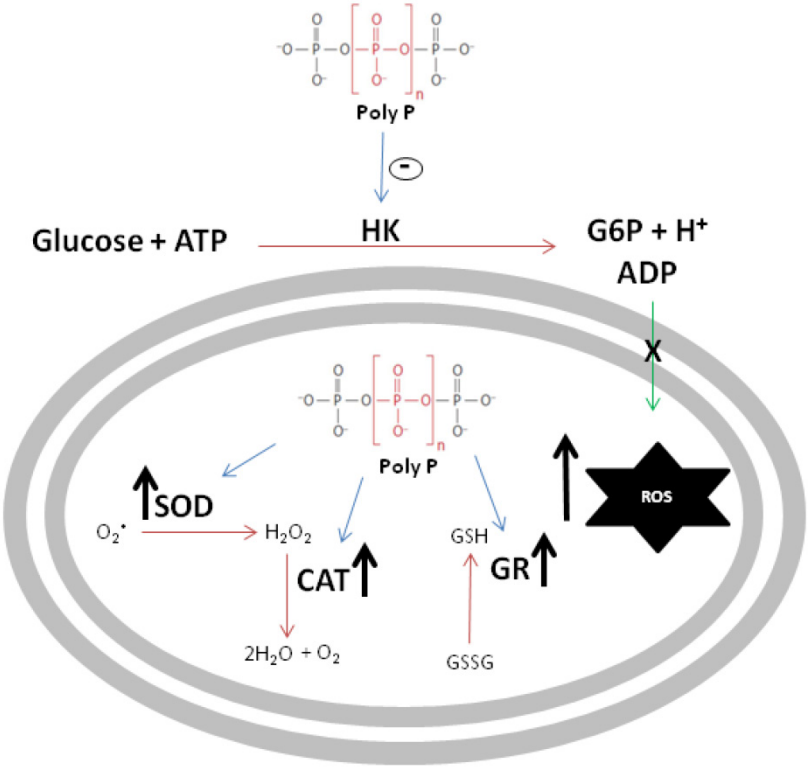

Figure 8. Schematic representation of polyphosphate involvement in reactive oxygen species generation. Black arrows indicate an increase in enzymatic activity or ROS generation, blue arrows indicate poly P modulation of enzymes and green arrow indicates ADP recycling promoted by HK. Poly P represents polyphosphate, HK is mitochondrial hexokinase, ROS denotes reactive oxygen species, SOD is superoxide dismutase, CAT represents catalase and GR denotes glutathione reductase.

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Conflict of Interest

The authors declare no conflict of interest.

Competing Interests

The authors have declared that no competing interest exists.

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