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Age-related NADH oxidase (arNOX) activity is significantly reduced in coelomic fluid of long-lived sea urchins

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Abstract

Age-related NADH oxidase (arNOX) is a cell surface protein shed into the circulation and other body fluids, which generates superoxide. The activity increases with age in human tissues and body fluids (serum, saliva, and perspiration) and is a potential source of age-related oxidative damage. We measured arNOX activity in the coelomic fluid of sea urchin species with different life spans. Coelomic fluid of long-lived sea urchin species *Strongylocentrotus purpuratus* and *Strongylocentrotus franciscanus* exhibited low levels of arNOX compared to the short-lived urchin species *Lytechinus variegatus*. arNOX activity was positively correlated with animal size in *L. variegatus*, whereas with *S. purpuratus* and *S. franciscanus*, arNOX activity and animal size were inversely correlated. The inverse correlation of arNOX activity with life span and decreased levels of arNOX with age in the long-lived species is consistent with a contribution of reduced arNOX activity to slower aging.

Keywords: Sea urchins, Aging, Age-related NADH oxidase (arNOX), *Strongylocentrotus purpuratus*, *Strongylocentrotus franciscanus*, *Lytechinus variegatus*.

Background

Age-related hydroquinone (NADH) oxidase (arNOX) is a recently discovered marker protein associated with aging in man (Morré and Morré 2006a; Morré et al. 2009). A member of the ENOX family of enzymes (Morré and Morré 2003), it is unique in that it appears in man only after age 30 and generates superoxide (Morré et al. 2003a). The protein is shed from the cell surface where it circulates to come in contact with serum lipoproteins (Morré and Morré 2006a) and extracellular matrices (Kern et al. 2010) and membranes to potentially significantly contribute to adverse effects of aging on cardiovascular and skin health.

Sea urchins present an interesting model for investigating the process of aging since different species of sea urchins have very different natural life spans, and some species display extreme longevity and negligible senescence (Bodnar 2009). The red sea urchin (*Strongylocentrotus franciscanus*) is one of the earth's longest living animals, living more than 100 years with no age-related increase in mortality rate or decline in reproductive capacity (Ebert and Southon 2003; Ebert 2008). In contrast, *Lytechinus variegatus* has an estimated life expectancy of only 4 years (Moore et al. 1963; Beddingfield and McClintock 2000), while the most widely studied species of sea

urchin, *Strongylocentrotus purpuratus*, has a maximum life expectancy of more than 50 years (Russell 1987; Ebert 2001, 2007, 2010).

The objective of this study is to investigate the arNOX activity in the coelomic fluid of sea urchin species with different life spans as a potential contributor to the slow aging process in long-lived species.

Methods

Animal collection

Sea urchins were collected in the summer of 2010. Small (46 to 59 mm) and large (75 to 84 mm) *S. purpuratus* were collected near Taylor Island in Barkley Sound, British Columbia (48°49.572′ N, 125°11.823′ W). Small (38 to 50 mm) and large (158 to 165 mm) *S. franciscanus* were collected near Jackscrew Island, British Columbia (48°57′320″ N, 123°35.109′ W). Small (21 to 31 mm) and large (61 to 73 mm) *L. variegatus* were collected at Flatt's Inlet, Bermuda (32°10.375′ N, 64°44.216′ W).

Collection of coelomic fluid

By analogy with arNOX in blood of humans, coelomic fluid was collected and immediately frozen after harvesting from urchins. The coelomic fluid was centrifuged at 6,000×*g* for 5 min to remove coelomocytes, and the resulting cell-free coelomic fluid (cfCF) was used to measure arNOX activity and protein amounts.

Assay of arNOX activity

arNOX activity was assayed from measurements of superoxide production based on a standard method, where the reduction of ferricytochrome *c* by superoxide was monitored from the increase in absorbance at 550 nm with reference at 540 nm (Butler et al. 1982). As a further check for the specificity of the arNOX activity, superoxide dismutase (SOD) was added near the end of the assay to ascertain that the rate returned to baseline. The assay consisted of 150 μl (2 mg/ml) oxidized ferricytochrome *c* solution and 50 μl of cfCF added to 2.5 ml assay buffer (0.15 M NaCl, 2.5 mM KCl, 1.5 mM Na_2HPO_4, 1.5 mM KH_2PO_4, 1 mM $CaCl_2$, 1 mM $MgCl_2$, and 7.5 mM glucose dissolved in deionized water, adjusted to pH 7.4, filtered and stored at 4°C). Rates were determined using a SLM Aminco DW-2000 spectrophotometer (SLM Instruments, Inc., Urbana, IL, USA) in the dual wavelength mode with continuous measurements over 1 min every 1.5 min. After 45 min, 60 μl (containing 60 units) SOD was added, and the assay was continued for an additional 45 min. An extinction coefficient for reduced cytochrome *c* of 19.1/mM/cm was used.

Determination of protein

Protein content was determined using the bicinchoninic acid method (Smith et al. 1985) (Pierce Technology Corporation, Iselin, NJ, USA). Standards were prepared with bovine serum albumin.

Statistical analyses

Means and standard deviations were analyzed for statistical significance using a two-tailed test. r^2 values were determined by linear regression.

Results

arNOX activity as measured by superoxide-induced reduction of ferricytochrome by cfCF of a short-lived species of sea urchin, *L. variegatus,* increased from 1.7 ± 0.9 moles/min/mg/protein for 21- to 31-mm diameter animals to 8.8 ± 3.2 nmoles/min/mg for 61- to 73-mm animals of the same species (Table 1). The specific activity correlated positively with diameter as an index of increasing animal age (r^2 = 0.72) (Figure 1A).

For the longer-lived Strongylocentrotus species, the activity was much reduced especially with the larger-diameter animals being 0.15 and 0.5 nmoles/min/mg protein for *S. purpuratus* and *S. franciscanus*, respectively (Table 1). In contrast to *L. variegatus*, the arNOX specific activity values for both *S. franciscanus* and *S. purpuratus* were negatively correlated with animal diameter, and specific activity declined with animal age (r^2 = 0.62 and 0.69, respectively) (Figure 1B,C). Total arNOX activity in cfCF was closely correlated with size (r^2 of 0.79 for *L. variegatus*, 0.73 for *S. purpuratus*, and 0.90 for *S. franciscanus* (Figure 2A,B,C)). Additionally, arNOX activity of muscle tissue also decreased with test diameter for *S. franciscanus* from 1.93 ± 0.21 nmoles/min/mg protein for a 44-mm diameter animal to 0.77 ± 0.04 nmoles/min^{-}/ mg protein^{-1} for a 146 mm diameter animal.

The arNOX specific activity increased 5.2-fold with size in *L. variegatus* (*t* test, $p < 0.02$), while it decreased 4-fold (*t* test, $p < 0.0001$) and 3.4-fold (*t* test, $p < 0.005$) with size in *S. purpuratus* and *S. franciscanus,* respectively. The specific activity was not significantly different between the small urchins of *L variegatus* and *S. franciscanus*, but was significantly different between the small urchins of *L. variegatus* and *S. purpuratus* (*t* test, $p < 0.0002$). The difference between the large urchins was significant ($p < 0.005$) with arNOX specific activities 58.7-fold and 17.6-fold greater for *L. variegatus* compared to *S. purpuratus* and *S. franciscanus*, respectively.

Discussion

In man, arNOX activity appears in serum, saliva, sweat, skin, and circulating lymphocytes (Morré and Morré 2003, 2006a, 2008; Kern et al. 2010; Morré et al.

Table 1 arNOX specific activity in cell-free coelomic fluid of sea urchins

Ferricytochrome *c* reduction						
	N	**Diameter**	**nmoles/min 50 μl**			**nmoles/min/mg protein**
		(mm)	**Total (*A*)**	**+ SOD (*B*)**	***A* − *B***	
L. variegatus						
Small	34	21 - 31	0.10 ± 0.02	0.08 ± 0.02	0.02 ± 0.01	1.7 ± 0.9
Large	14	61 - 73	0.16 ± 0.03	0.07 ± 0.02	0.09 ± 0.1	8.8 ± 3.2*
S. purpuratus						
Small	10	46 - 59	0.14 ± 0.02	0.12 ± 0.02	0.02 ± 0.05	0.6 ± 0.2**
Large	8	75 - 84	0.13 ± 0.015	0.125 ± 0.02	0.005 ± 0.003	0.15 ± 0.1***
S. franciscanus						
Small	9	38 - 50	0.12 ± 0.03	0.03 ± 0.01	0.09 ± 0.01	1.7 ± 0.4[a]
Large	6	158 - 165	0.09 ± 0.01	0.07 ± 0.002	0.02 ± 0.005	0.5 ± 0.3****

Superoxide dismutase-inhibited ferricytochrome *c* reduction as a measure of superoxide formation (arNOX activity) is given by *A* − *B*, where *A* is the total ferricytochrome *c* reduction and *B* is ferricyanide reduction following the addition of superoxide dismutase. arNOX specific activity is given in nmoles/min/mg protein. Results are averages ± standard deviations. *$p < 0.02$: differences were significant; **$p < 0.0002$, ***$p < 0.0001$, ****$p < 0.005$: highly significant; [a]not significant (NS).

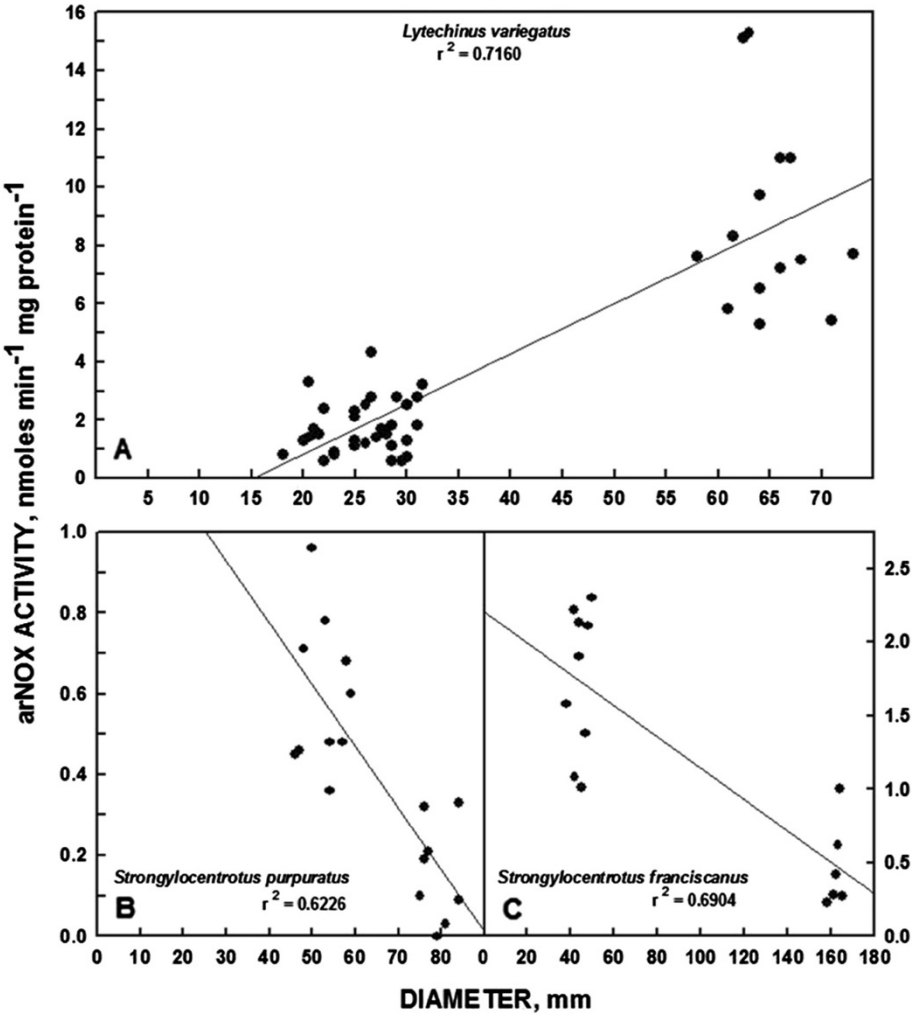

Figure 1 arNOX specific activity as function of test diameter. (**A**) *L. variegatus*. (**B**) *S. purpuratus*. (**C**) S. franciscanus.

2003b) and is associated with serum low density lipoproteins at about age 30 and then increases linearly in activity to about age 65 to 75. The activity correlates with oxidative damage (Morré and Morré 2006a) and with the aging process in general (Morré and Morré 2006b).

The hypothesis under investigation was that if arNOX levels and longevity were inversely correlated, the long-lived sea urchins might exhibit significantly reduced levels of arNOX activity. This expectation was realized as the cell-free coelomic fluid of long-lived species had lower levels of arNOX than the short-lived species. Interestingly, the level of arNOX activity decreased with size/age of the long-lived species which is in contrast to the short-lived species and in humans. This is consistent with the observation that Strongylocentrotus species (*S. franciscanus* in particular) has been shown to exhibit negligible senescence. *S. franciscanus* has been estimated to live for more than 100 years, with the oldest animals estimated to be over 200 years (Ebert and Southon 2003; Ebert 2007, 2008). Moreover, it has been demonstrated that there is no age-related decline in reproduction or increase in mortality rate (Ebert 2008). It has been

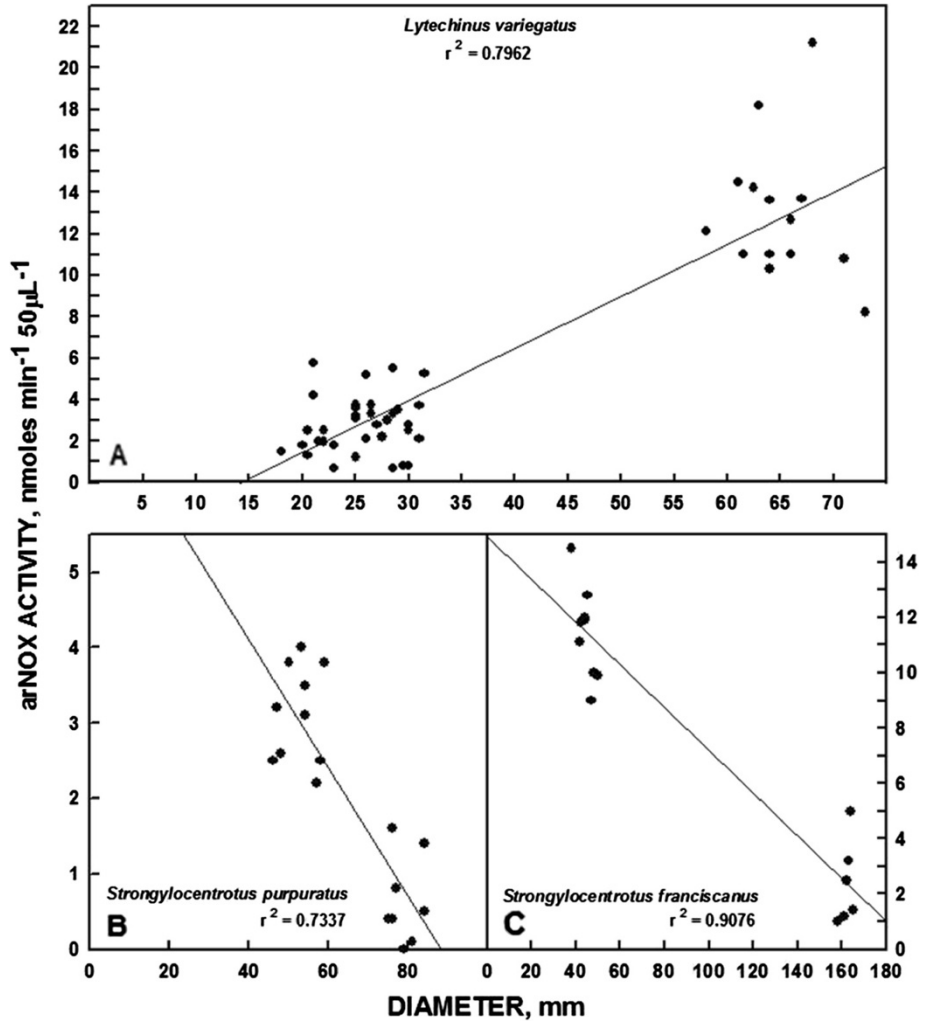

Figure 2 arNOX activity (50 µl cell-free coelomic fluid) as function of test diameter.

suggested that this species does not die of old age but primarily of as prey to predators (Tenger and Levin 1983). It has even been suggested that *S. franciscanus* may be an animal that exhibits negative senescence (Vaupel et al. 2004).

Although *S. purpuratus* has not been analyzed with respect to senescence, growth data derived earlier from urchins at a location near our study site indicated that about 5% of the population could be 50 years old, and about 1% could be as old as 75 years (Ebert 2010). We did not conduct age determinations by growth band counting or other methods in this study. We did, however, select animals at the upper and lower size ranges for the populations under study on the assumptions that the smaller animals were the youngest and the larger animals were the oldest. When compared to growth curves generated at or near our study sites, these animals represent the lower 25% and the upper 35% of the size/age categories for their species (Ebert 1975, 2008, 2010; Moore et al. 1963).

Although *S. franciscanus* has a longer life expectancy than *S. purpuratus*, it had higher arNOX activity, indicating that there was not a strict inverse correlation with age among these long-lived species. There was, however, a significant reduction in

arNOX with age in both species and a significant difference between arNOX levels in the older animals of these species and the short-lived *L. variegatus*. The general inverse correlation of arNOX with longevity and the reduction with age in long-lived species support the idea that reduced levels of arNOX may be a significant contributor to slow the process of aging in long-lived sea urchins.

Conclusion

The inverse correlations between arNOX activity with sea urchin life span and decreased levels of arNOX activity with age in the long-lived species are consistent with a reduction of arNOX activity as an aid to slowing natural aging.

Competing interest
The authors declare that they have no competing interests.

Authors' contributions
ET conducted the enzymatic assays. AB provided oversight for the collection of animals and the interpretation of findings. DMM participated in the design of the study and in the statistical analyses. DJM participated in study design and coordination. All authors read and approved the final manuscript.

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