Theory Biosci.
DOI 10.1007/s12064-012-0159-y

ORIGINAL PAPER

A kinetic approach to assess oxidative metabolism related features in the bivalve *Mya arenaria*

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Received: 23 January 2012 / Accepted: 6 July 2012

Abstract Electron paramagnetic resonance uses the resonant microwave radiation absorption of paramagnetic substances to detect highly reactive and, therefore, short-lived oxygen and nitrogen centered radicals. Previously, steady state concentrations of nitric oxide, ascorbyl radical (A·) and the labile iron pool (LIP) were determined in digestive gland of freshly collected animals from the North Sea bivalve *Mya arenaria*. The application of a simple kinetic analysis of these data based on elemental reactions allowed us to estimate the steady state concentrations of superoxide anion, the rate of A· disappearance and the content of unsaturated lipids. This analysis applied to a marine invertebrate opens the possibility of a mechanistic understanding of the complexity of free radical and LIP interactions in a metabolically slow, cold water organism under unstressed conditions. This data can be further used as a basis to assess the cellular response to stress in a simple system as the bivalve *M. arenaria* that can then be compared to cells of higher organisms.

Keywords Kinetic approach · Ascorbyl radical · Labile iron pool · Superoxide anion · Unsaturated fatty acids

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Introduction

Oxygen free radicals (ROS) are highly reactive oxidants/reductants and mainly regarded as hazardous species whose production in cellular and extracellular systems has to be tightly controlled by antioxidants and radical scavenging biochemical reactions. Recently, the importance of radical species in cellular signaling and in the maintenance of homeostatic conditions has been recognized. Arguably, some radicals, such as the very short-lived and extremely hazardous hydroxyl radical (·OH), are still regarded as highly reactive and dangerous, but many other more stable species have been postulated as signaling molecules for cellular growth or as oxidants that assure an appropriate oxidation state of cellular compartments and the biochemical structures and elements they contain. According to the present view, a basal amount of ROS is formed at all times in all aerobic cells, and the steady state concentration of ROS in each cell or compartment depends on the formation rate of the radical, its reactivity and the concentration of available reaction partners. Several decades ago, Chance et al. (1979) have calculated the steady state concentration of ·OH, based on measurements of superoxide anion ($O_2^{\cdot-}$) and hydrogen peroxide (H_2O_2) production rates in rat liver cells assessed using biochemical techniques. More recently, Antunes et al. (2007) included nitric oxide (NO) into the picture for rat tissues, and lately, by applying electronic paramagnetic resonance (EPR) spectroscopy techniques, Galleano et al. (2002) and Galatro et al. (2006) developed a simple kinetic study to estimate ascorbyl radical (A·) steady state concentration in rat plasma and photosynthetic cells, respectively.

Similar analyses for marine invertebrates, such as benthic bivalves, are missing but would be interesting from an evolutionary perspective. Bivalves belong to the cold

Published online: 25 July 2012

blooded (ectothermic) evolutionary early fauna with low metabolic rates (lower mitochondrial density than mammals) and many of them also lack O_2 carrying proteins (Terwilliger 1998; Terwilliger et al. 1988). Among them, *Mya arenaria* was shown to lack respiratory pigments such as hemocyanin (Abele-Oeschger 1996; Alyakrinskaya 2002). In birds and mammals the phospholipid content of membranes from all tissues sampled, except brain, showed a significant decline in the proportion of polyunsaturated fatty acids (PUFA) and a significant increase in the proportion of monounsaturated fatty acids (MUFA) (Brand et al. 2003) with increasing body mass. Moreover, the proportion of PUFA and MUFA in the membranes also affects the vulnerability to peroxidative damage (Buttemer et al. 2010). Thus, ectotherms with lower metabolic rates than mammals and birds can be anticipated to be less vulnerable to membrane peroxidation. Further, as a non-hemoglobin carrying species, the compartmentalization and overall content of Fe in *M. arenaria* is assumed to be lower than in vertebrates (Winzerling and Law 1997). Thus, for a comparison of ROS steady state levels in an evolutionary context, the contribution of Fe, unsaturated membrane lipids (RH) that can generate the reactive species peroxyl (ROO·), alkoxyl (RO·) and alkyl (R·) radicals, and NO should be included as parts of the oxidative-nitrosative network of the cell.

Fe has been central to life since the early days of the evolution and is involved in many biological reactions, mainly participating in electron transfer (cytochromes, cytochrome-oxidase) reactions, since it can attain a wide range of redox potentials (+771 to −500 mV) depending on its coordination with different ligands (Galatro et al. 2007). For its multiple tasks, Fe is incorporated into a heme moiety (such as in catalase) or alternatively bound to enzymes in form of non-heme moieties or Fe–S motifs, as in the electron transport chain components (Cairo et al. 2002). Besides the bound Fe-fraction there is a fraction of the total cellular Fe content called the labile Fe pool (LIP). The LIP is experimentally defined as a low-molecular-weight pool of weakly chelated Fe that is involved in a dynamic flux of solutes passing rapidly through the cell. It represents only a minor fraction of the total cellular Fe and is estimated to amount to 3–5 % for rat liver cells (Kakhlon and Cabantchik 2002). The LIP is assumed to consist of both forms of ionic Fe (Fe^{2+} and Fe^{3+}) bound to citrate and other organic ions, phosphates (ATP, AMP), carbohydrates and carboxylates, nucleotides and nucleosides, polypeptides, and phospholipids (Petrat et al. 2002; Wang and Pantopoulos 2011). The actual composition of the intracellular ligands participating in the LIP formation in different cell types in *M. arenaria* and other organisms remains obscure. Besides the importance of this readily available Fe for cellular growth and metabolism, the LIP (also defined as the source for catalytically active available Fe in the cells) is also held responsible for the generation of extremely reactive species such as OH·, which can cause lipid peroxidation, DNA strand breaks and degradation of several biomolecules (Harrison and Arosio 1996). Defense against the toxic effect of Fe and O_2 mixtures is provided in mammals and invertebrates by two specialized Fe-binding proteins: the extracellular transferrins (Tfs) and the intracellular (and extracellular in invertebrates) ferritins (Ft) (Winzerling and Law 1997). Moreover, some cellular constituents such as ascorbate (AH^-) can reduce Fe^{3+} to Fe^{2+} generating catalytically active Fe that generates ·OH and A·. Finally, NO is a regularly occurring radical intermediate of many reactions and also a cellular signaling molecule capable of binding Fe (Schneppensieper et al. 2001). NO has been shown to act as intracellular or transcellular signal and as cytotoxic host defense compound (Moncada et al. 1991). NO is generated in mammalian cells and tissues from L-arginine (L-A) by the enzyme nitric oxide synthase (NOS, EC 1.14.13.39) (Knowles 1997). NOS-like enzyme activity has been detected in marine, freshwater and terrestrial molluscs, including three gastropod subclasses (prosobranchs, opisthobranchs and pulmonates) (Jacklet 1997), and recently in the bivalve *M. arenaria* (González et al. 2008). Basically, pro- and antioxidant processes are the same across marine invertebrates and mammalian systems. However, the specific conditions for radical formation, a key feature in stress physiology and aging, are different and highly variable among aquatic cold blooded organisms (Lesser 2006).

The soft shell clam *M. arenaria* from the European Wadden Sea is a typical representative of the group of infaunal marine molluscs. It is a benthic filter feeder that colonizes intertidal and subtidal areas where it digs up to 40 cm deep into the sediment. *M. arenaria* takes up O_2 and food particles (algae and detritus) from surface waters through a long siphon, and is exposed to hypoxia-reoxygenation as tidal flats emerge and are again flooded by the tides. As a particle feeder *M. arenaria* is not totally selective, ingests considerable amounts of sediment that may contain Fe and other trace metals together with the targeted food particles which then are taken up into the tissues. The amount of trace elements absorbed into the tissues from digestion of ingested food particles largely depends on the amounts available in the environment (Estévez et al. 2002). However, Fe can also be taken up in dissolved form (González et al. 2010). As the Wadden Sea receives nutrients and trace metals from river discharge and land run-off, dissolved and particulate matter can depend on many factors, including water O_2 levels and rain regimes, and be variable in space and over time. The sand flat where the animals for this study were collected had 7.5 mg Fe/g dry weight (0.8 %) (Estévez et al. 2002).

Changes in O_2 supply in addition to changes in temperature and food supply as a consequence of tidal changes result in quite drastic metabolic adaptations of the bivalves, including changes between aerobic and anaerobic metabolism which, in turn, affect conditions for radical formations in the cells. To maintain low hemolymph and tissue oxygenation, the P_{O_2} in molluscan mantle cavity water is lowered against normoxic (21 kPa) seawater P_{O_2}, but balanced high enough to meet the energetic requirements in a given species (Abele et al. 2010). Adjustment of mantle cavity P_{O_2} to lower than ambient values through controlled pumping prevents high O_2 gradients between bivalve tissues and surrounding fluid, limiting O_2 flux across the body surface. Under anoxic conditions (closed shells) the P_{O_2} in this species was reported as 0 kPa (Abele et al. 2010). Oxygen levels ranging considerably above 3 kPa within a tissue can accelerate the formation rate of hazardous ROS, and oxidative stress is assumed to increase linearly with tissue O_2 concentration. In *M. arenaria* the mantle cavity P_{O_2} is almost always maintained as low as 0.4 kPa (2.62–0.37 kPa) against fully oxygenated experimental conditions (Abele et al. 2010) and this situation is understood as unstressed condition.

In this paper, recent data will be summarized to integrate information obtained for the content of ROS and reactive nitrogen species (RNS) and Fe in digestive gland (DG) homogenates of the bivalve *M. arenaria*. A basic kinetic approach was applied to calculate the steady state concentration of the main parameters related to the cellular oxidative and nitrosative metabolism of the bivalve cells under physiological control conditions, i.e., the situation in the unstressed animal maintained in similar conditions to its natural environment. The relative proportion of Fe^{2+}/Fe^{3+} in the LIP, the unsaturated lipids and the $O_2^{\cdot-}$ steady state concentration were estimated based in experimental measurements of oxidative parameters.

Results

Free radical generation rates and steady state concentrations in *M. arenaria* DG

Experimental parameters used for assessing the kinetic model were obtained from animals maintained in unstressed conditions similar to their natural environment (control conditions). In all cases, animals were kept in two aquaria with fully aerated natural seawater of 23–26 % PSU, and at 10 °C for at least 1 week prior to the experiments, to ensure that animals were healthy and not stressed from sampling. The bottom of the aquaria was covered with pebble stones to allow the bivalves to burrow as in their natural habitat. They were fed live phytoplankton twice a week. These animals tolerate salinities between 4 and 35 % (Lassig 1965; Newell and Hidu 1986; Castagna and Chanley 1973; Brousseau 1978). In the Wadden Sea the temperatures fluctuate during the year between 0 and 18 °C (Anderson 1978). Any deviation in the natural parameters may lead to stress conditions, e.g., drastic changes in temperature or salinity, lack of O_2, toxics presence, etc.

Estimation of the relative proportion of Fe^{2+} and Fe^{3+} in the LIP

AH^- is an important antioxidant in numerous biological systems. It acts as an antioxidant by donating electrons in a two steps oxidation reaction which generates A· as an intermediate and dihydroascorbic acid (DHA) as end product (Hubel et al. 1997) according to Fig. 1.

The direct detection of A· employing EPR in complex biological systems is possible since both, AH^- and DHA, are spectroscopically silent (Hubel et al. 1997). Moreover, A· has an extraordinary long lifespan (50 s) as compared to other radical species (Buettner and Jurkiewicz 1993), rendering its identification at room temperature feasible.

Applying a simple kinetic analysis the following rate expressions can be formulated for the velocity of generation of A·($V_{A\cdot\ \mathrm{generation}}$) (Eq. 1).

$$\begin{aligned} v_{A\cdot\ \mathrm{generation}} &= \mathrm{d}[A\cdot]/\mathrm{d}t = v_1 + v_2 \\ &= (\mathrm{d}[A\cdot]/\mathrm{d}t)_{\mathrm{Fe}} + (\mathrm{d}[A\cdot]/\mathrm{d}t)_{\mathrm{radicals}} \end{aligned} \quad (1)$$

In Eq. 1, the expression $v_1 = (\mathrm{d}[A\cdot]/\mathrm{d}t)_{\mathrm{Fe}}$ stands for the generation rate of A· from the oxidation of AH^- catalyzed by Fe^{3+}. A· is, moreover, generated by the reaction of AH^- with free radicals by second order reactions $v_2 = (\mathrm{d}[A\cdot]/\mathrm{d}t)_{\mathrm{radicals}}$ according to Eq. 2.

$$\begin{aligned} v_2 = {} & (\mathrm{d}[A\cdot]/\mathrm{d}t)_{O_2^{\cdot-}} + (\mathrm{d}[A\cdot]/\mathrm{d}t)_{\cdot\mathrm{OH}} + (\mathrm{d}[A\cdot]/\mathrm{d}t)_{\mathrm{R}\cdot} \\ & + (\mathrm{d}[A\cdot]/\mathrm{d}t)_{\mathrm{tocopherol}\cdot} \end{aligned} \quad (2)$$

Taking into account the low steady state concentrations of the radicals in non-stressed biological systems, the non-specificity of the reactions of free radicals with many biomolecules, and the fact that many of the apparent rate constants of the reactions indicated in Eq. 2 are lower than 10^5 (Galatro et al. 2006), it can be concluded that the main reaction responsible for A· generation is the one catalyzed by Fe^{3+} (reaction 1).

$$AH^- + Fe^{3+} \xrightarrow{k_1} A\cdot + Fe^{2+} \quad \text{(reaction 1)}$$

Ft plays a dual role in LIP homeostasis regulating the amount of catalytically active Fe in the cytosol. When the cells are exposed to high Fe concentrations Ft is buffering cellular Fe content by binding Fe to protect cells against its toxicity. At low Fe concentrations, however, Ft releases Fe

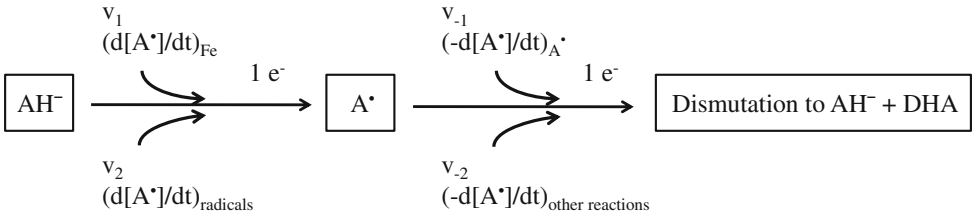

Fig. 1 Scheme of generation and consumption rate of A· in *M. arenaria*. The total generation rate of A· depends on the rate of the Fe and radicals reactions (v_1 and v_2, respectively) that refers to the rate of the reaction with other cellular radicals such as $O_2^{\cdot-}$, ·OH, R· and tocopherol· with AH^-. The rate of consumption of A· is related to the rate of regeneration of AH^- by the dismutation reaction of A· to AH^- and DHA (v_1) and by other reactions of less importance (v_2)

from its protein core to satisfy the cellular requirements. In vitro experiments have shown that Fe mobilization from Ft occurs (Winzerling and Law 1997). However, since Fe is stored as Fe^{3+}, appropriate reductants are required (Funk et al. 1985). Ft isolated from DG of *M. arenaria* was exposed to 1 mM AH^- and Fe was released, suggesting the importance of this mechanism for cellular supply of Fe (González, unpublished data). The Fe forming the LIP actively participates in the catalysis of many free radical reactions, among them the autoxidation of AH^- that generates A· (Graziano and Lamattina 2005). The generation of A·, as described by reaction 1, has an estimated rate constant (k_1) of 30 M^{-1} s^{-1} (Galleano et al. 2002).

Measurable amounts of A· are detectable in DG of *M. arenaria* also under physiological control conditions (Table 1). According to Williams (1982), the ratio Fe^{2+}/Fe^{3+} in mammalian cells is 10^{10} and also for *Escherichia coli* Woodmansee and Imlay (2002) reported that Fe in the LIP is mostly present in the reduced form. Contrary, in yeast, Srinivasa et al. (2000) found more oxidized Fe in the LIP. Thus, the ratio Fe^{2+}/Fe^{3+} of the LIP seems to differ between organisms with different metabolic strategies.

The fluorometric calcein assay can be employed to measure the total amount of LIP in cells and tissues including both, the reduced (Fe^{2+}) and oxidized (Fe^{3+}) form of Fe. Using this method, the total amount of LIP in human erythroleukemia cells was estimated to be 0.50×10^{-6} M (Breuer et al. 1995). This value was even lower than the typical values reported for bacteria and yeast (10^{-5} M) where the LIP is measured by EPR, which detects only Fe^{2+} (Srinivasa et al. 2000; Keyer and Imlay 1996). The intracellular pH of *M. arenaria* DG homogenate was determined to be 6.53 ± 0.01, suggesting that in this tissue the proportion of Fe^{2+} would be higher than of Fe^{3+} which would precipitate in the range of neutral pH (unpublished data). We estimated the total amount of LIP (Fe^{2+} and Fe^{3+}) in *M. arenaria* DG under physiological control conditions by the calcein assay [Table 1 (González et al. 2008)]. Moreover, when we measured the LIP employing EPR, the value we obtained (146 ± 10 pmol/mg FW, González et al. 2008) was not significantly different from the value recorded with the calcein-fluorimetric technique. The hypothesis that Fe^{2+} is the dominant form of Fe in *M. arenaria* DG LIP is corroborated by estimating Fe^{3+} based on the A· steady state concentration, approximated as a function of A· generation and disappearance rates, and compared to the measured amount of total LIP.

According to reaction 1, the rate of generation A·, catalyzed by Fe^{3+}, can be defined by the Eq. 3

$$v_{\text{A}\cdot\text{ generation}} = k_1[Fe^{3+}][AH^-] \quad (3)$$

$[AH^-]$ as given in Table 1, is (0.60 ± 0.02) × 10^{-6} M. To approximate $[Fe^{3+}]$, the net formation rate of A· radical over time must be calculated. This, in turn, requires calculation of the rate of A· disappearance according to Fig. 1 and Eq. 4, formulated for the consumption of A·.

Table 1 Oxidative and nitrosative parameters experimentally assessed in DG of *M. arenaria*

Parameters	Experimental values	References	Transformed units
LIP	(118 ± 9) (pmol/mg FW)	González et al. (2008)	(0.15 ± 0.01) × 10^{-6} (M)
$[AH^-]$	(478 ± 12) (pmol/mg FW)	González et al. (2008)	(0.60 ± 0.02) × 10^{-6} (M)
[A·]	(0.04 ± 0.01) (pmol/mg FW)	González et al. (2008)	(50 ± 13) × 10^{-12} (M)
d[LR·]/d*t*	(10 ± 2) (pmol/mg prot min)	Estévez et al. (2002)	(21 ± 4) × 10^{-12} (M s^{-1})
[NO]	(99 ± 3) (pmol/mg FW)	González et al. (2008)	(12.0 ± 0.4) × 10^{-8} (M)
d[NO]/d*t*	(1.8 ± 0.2) (pmol/mg FW min)	–[a]	(37 ± 4) × 10^{-12} (M s^{-1})

[a] González PM (2011) Ph.D. thesis, University of Buenos Aires, School of Pharmacy and Biochemistry, Argentina, pp 95, unpublished result

$$v_{\text{A}\cdot\text{ disappearance}} = v_{-1} + v_{-2} = -\text{d}[\text{A}\cdot]/\text{d}t = (-\text{d}[\text{A}\cdot]/\text{d}t)_{\text{A}\cdot} + (-\text{d}[\text{A}\cdot]/\text{d}t)_{\text{other reactions}} \quad (4)$$

Consumption of A· occurs mainly through its dismutation (see reaction 2)

$$2\text{A}\cdot \xrightarrow{k_2} \text{AH}^- + \text{DHA}. \quad \text{(reaction 2)}$$

The rate of disappearance of A· could be calculated with Eq. 5, where $k_2 = 2 \times 10^6$ M^{-1} s^{-1} at pH = 7 (Galleano et al. 2002; Buettner and Jurkiewicz 1993; Asada and Takahashi 1987).

$$v_{\text{A}\cdot\text{ disappearance}} = 2k_2[\text{A}\cdot]^2 \quad (5)$$

The disappearance rate of A· by other less important reactions is summarized in the expression $(-\text{d}[\text{A}\cdot]/\text{d}t)_{\text{other reactions}}$, including termination reactions with other free radicals or A· reduction at the plasma membrane presumably mediated by cytochrome b (Horemans et al. 1994; Smirnoff 2000). The steady state [A·] is a dynamic value, set by the relationship between the rate of generation and decay of this species. The rate of disappearance of A· can be approximated as 1×10^{-14} M s^{-1} from the A· content measured by EPR in *M. arenaria* DG (Table 1). This value is lower than the A· disappearance rate estimated for rat plasma (1.3×10^{-10} M s^{-1}, Galleano et al. 2002).

Among other features, the significantly higher content of AH^- in plasma of rats as compared to *M. arenaria* DG, might contribute to this difference. Fish as well as invertebrates, lack the ability of generating AH^-, since the enzyme L-gulonolactone oxidase, involved in the catalysis of the end pathway of the conversion of glucose to AH^- (Chatterjee 1973a), is missing. The route of synthesis of AH^- in molluscs was studied by many authors. Chatterjee (1973b) was unable to detect the production of AH^- in homogenate of hepatopancreas of the freshwater snail *Pila* sp. Further, in the abalone *Haliotis discos hannai* and *Haliotis tuberculata*, Mai (1998) observed a content of AH^- of 219 ± 4 and 316 ± 6 pmol AH^-/mg FW, respectively, that decreased by 50 and 60 %, respectively, after feeding the abalone with an AH^- deficient diet during 100 days. For the Kuruma prawn, *Marsupenaeus japonicus*, whole body tissue, Moe et al. (2004) reported a significantly lower AH^- content (7.4 ± 0.3 pmol AH^-/mg FW) than the values obtained for *M. arenaria*. Thus, although molluscs do not synthesize AH^-, they absorb it from their algal diet which is usually high in AH^- (Chapman and Chapman 1980).

Under physiological control conditions there should be no significant change in A· concentration over time $(\text{d}[\text{A}\cdot]/\text{d}t = 0)$ and, thus, generation and disappearance rate of A· are equal (1×10^{-14} M s^{-1}). Going back to Eq. 3 and replacing k_1, $[\text{AH}^-]$, and $v_{\text{A}\cdot\text{ disappearance}}$ by their values, the $[\text{Fe}^{3+}]$ was calculated as 6×10^{-10} M. González et al. (2010) developed a model of Fe overload in *M. arenaria* by exposing the animals to 500 μM Fe in the water during 17 days. Using the kinetic model shown here for day 9 of Fe overload and knowing the [A·], by Eq. 5 the rate of disappearance of A· can be estimated as 1.4×10^{-11} M s^{-1}. Further, the calculated $[\text{Fe}^{3+}]$ was 7.6×10^{-7} M. This increase in $[\text{Fe}^{3+}]$ by three orders of magnitude as compared to unstressed conditions, indicates that exposure to high concentrations of dissolved Fe substantially affects the internal state concentration of catalytically active Fe. This fact increases the risk of oxidative damage since endogenous control systems of Fe uptake are overwhelmed. Interestingly, the increase of the LIP in the DG from 3.8 ± 0.4 to 14 ± 1 ng Fe/mg FW at day 0 and 9 of exposure to Fe overload, respectively (González et al. 2010), correlates with a proportional increase of the lipid peroxidation content of thiobarbituric reactive substances (57 ± 8–157 ± 14 pmol TBARS/mg FW at day 0 and 9, respectively). This underlines that LIP values need to be kept within a narrow and secure range of concentration to prevent toxic effects of Fe.

In DG of *M. arenaria* the LIP was $(0.15 \pm 0.01) \times 10^{-6}$ M, considering a water content in the tissue of 80 % and the density of the hydrophilic media of approximately 1 mg/ml. Thus, $[\text{Fe}^{3+}]$ indeed appears significantly lower concentrated than $[\text{Fe}^{2+}]$, suggesting that the Fe^{2+} forming the LIP is the one that actively participates in the catalysis of ROS production and the oxidation of biomolecules commonly referred to as oxidative stress.

Unsaturated lipids (RH) steady state concentration

Peculiarities of membrane lipids in marine organisms, particularly high contents of PUFA (Joseph 1982), is a characteristic which renders the DG highly susceptible to Fe-induced oxidative damage. The wide range of TBARS concentrations reported from different bivalve DG samples can be attributed to variable lipid contents and unsaturation levels. For example, DG of the bivalves *Mytella guyanensis*, *Pecten jacobaeus* and *Mytilus galloprovincialis* showed values of 120 ± 20, 23 ± 2 and 11 ± 3 pmol TBARS/mg FW, respectively (Viarengo et al. 1995, 1999; Aloísio Torres et al. 2002). Data from DG of *M. arenaria* (27 ± 7 pmol TBARS/mg FW) (Lesser 2006) were similar to those reported for the bivalves *Adamussium colbecki* and *Perna perna* (45 ± 7 and 55 ± 18 pmol TBARS/mg FW, respectively) (Viarengo et al. 1995; Alves de Almeida et al. 2007). The main process able to start the free radical chain reaction that leads to TBARS accumulation in vivo is the decomposition of lipid hydroperoxides.

The hydroperoxides are affected by homolysis and decomposition (Pryor 1976), and the Haber–Weiss reaction, in which Fe catalyzes the decomposition of H_2O_2 yielding ·OH, is a very fast reaction even at temperatures below 25 °C (Walling 1957). Many in vitro studies suggest that peroxo- and peroxy-compounds formed by the oxidation of Fe through its reaction with ·OH (McKnight and Hunter 1965) are able to act as initiators of the lipid radical (LR·) chain reaction with high rate constants. Regarding propagation and termination reactions, it should be recognized that in complex systems such as it might occur in the cell, more than one type of radical is present and a variety of terminations are possible. On the other hand, the relative importance of propagation reactions 3 and 4

$$\mathrm{LR}\cdot + \mathrm{O_2} \rightarrow \mathrm{LROO}\cdot \qquad \text{(reaction 3)}$$

$$\mathrm{LROO}\cdot + \mathrm{RH} \rightarrow \mathrm{LROOH} + \mathrm{LR}\cdot \qquad \text{(reaction 4)}$$

differs with O_2 partial pressures. At low O_2 pressures, reaction 3 will be slow and the concentration of LR· will be greater than that of LROO·, under these conditions, the reaction between two LR· will be the most important termination reaction (Pryor 1976). However, at high O_2 pressures the most important termination process depends on the reaction between two molecules of LROO·. As previously indicated, under environmental normoxic conditions *M. arenaria* changes internal O_2 pressure conserving a very low shell water P_{O_2} for most of its lifetime in the sediment (3 kPa as a maximum with a median P_{O_2} of 0.4 kPa) (Abele et al. 2010). Thus, the effect of the self-propagated cycle of the chain reaction is considered as a minor contributing factor to the overall process. On the other hand, a possible over estimation of the rate of peroxidation based on Fe^{2+} catalysis might occur but its effect cannot be approximated. This assumption could, however, underestimate the reactions that consume RH. Taking into account these considerations, and assuming a constant rate of the lipid peroxidation initiation reaction (k_5) with a value close to the diffusional limit (10^9 M^{-1} s^{-1}) (reaction 5), the cellular rate of generation of LR· ($v_{LR\cdot\ generation}$) in the presence of Fe as the catalyzer of the reaction is defined by Eq. 6.

$$\mathrm{RH} \xrightarrow[\mathrm{Fe^{2+}+H^+}]{k^5} \mathrm{LR}\cdot \qquad \text{(reaction 5)}$$

$$v_{\mathrm{LR}\cdot\ \mathrm{generation}} = k_5[\mathrm{Fe^{2+}}][\mathrm{RH}] \qquad (6)$$

The $[Fe^{2+}]$ can be considered as the total amount of the LIP experimentally determined as described above. This assumption slightly overestimates the resulting $[Fe^{2+}]$. The [RH] represents the concentration of PUFA susceptible to damage in DG cells. Detectable amounts of LR· were measured in DG of *M. arenaria* even under control physiological conditions (Estévez et al. 2002). With the appropriate changes of units, the rate of generation of LR· was determined as $(21 \pm 4) \times 10^{-12}$ M s^{-1}. Thus, going back to Eq. 6 and replacing the experimental values by values shown in with Eq. 7, the steady state concentration of PUFA that become target of oxidative damage catalyzed by Fe in DG from *M. arenaria* was estimated as 1.4×10^{-13} M.

$$[\mathrm{RH}] = (21 \pm 4) \times 10^{-12}\,\mathrm{M\,s^{-1}}/10^{9}\,\mathrm{M^{-1}\,s^{-1}}(0.15 \pm 0.01) \times 10^{-6}\,\mathrm{M} \qquad (7)$$

This information for *M. arenaria* could be valuable for future interspecies comparisons. *Laternula elliptica* from Antarctica shares with *M. arenaria* several physiological features, but is constantly exposed to extreme conditions, including an active uptake of Fe from ablation of volcanic rocks (Ahn et al. 1996). Thus, a significant alteration in the content of RH could be expected from a similar analysis to that reported here.

Interaction between the oxidative and the nitrosative metabolism: estimation of the steady state concentration of $O_2^{\cdot-}$ in M. arenaria

The mitochondria, which are thought to consume over 90 % of the cellular O_2 in unstressed cells, are considered the major sites of aerobic cellular ROS production (Abele et al. 2007). Studies with isolated mitochondria of marine invertebrates including *M. arenaria* revealed that mitochondrial electron transport converts around 0.1–2 % of the O_2 consumed in vitro (21 kPa oxygenation) to $O_2^{\cdot-}$ (Abele et al. 2002, 2007). The extent to which this happens in vivo and the real rate of escape of radicals and H_2O_2 to the cytoplasm are still under debate (St-Pierre et al. 2002). Moreover, ROS are also produced by the microsomal systems of the endoplasmic reticulum (Winston et al. 1996; Regoli et al. 2000), and by various enzymatic oxidase reactions (Murphy 2009). The basic components and mechanisms of oxidative stress and defense in marine invertebrates are the same as in higher organisms (Buttemer et al. 2010). However, many water breathers and especially many bivalves and worms burrowing in the bottom sediment are oxyconforming species, in which O_2 consumption varies as a function of the environmental O_2 partial pressure (Abele et al. 1998). As most of these species are susceptible to higher environmental O_2 they colonize sedimentary, low O_2 environments (Massabuau and Abele 2012). On the other hand, NO, a free radical molecule, can be formed from endogenous or exogenous NO donors (such as *S*-nitrosoglutathione, GSNO, sodium nitroprusside, SNP and diethylenetriamine NONOate, DETA NONOate) or from L-A by NOS (Knowles 1997).

Isoforms of NOS have been isolated from fish (Olsson and Holmgren 1997; Nilsson and Söderström 1997) and invertebrates, and are partly sequenced (Olsson and Holmgren 1997; Jacklet 1997; Martinez 1995; Moroz et al. 1996). Data on NO signaling in diverse phyla suggest that a common ancestor had the ability to use NO signaling and that it conferred high adaptive value in the species evolution (Jacklet 1997). Among the physiological functions ascribed to NO in marine invertebrates and fish, its neurotransmitter function and its role in cellular immune defense are outstanding (Philipp et al. 2012). In marine and freshwater molluscs, NO signaling is involved in muscle contraction and relaxation, mucus secretion and excretion, and in triggering feeding behavior (Moroz et al. 1996). However, high concentrations of NO have cytotoxic effects as they inhibit a number of cellular processes such as DNA synthesis and mitochondrial respiration (Lizasoain et al. 1996; Moncada and Bolaños 2006; Bolaños and Heales 2010). Some of these effects may be direct and others arise from the reaction of NO with $O_2^{\cdot-}$ to generate peroxynitrite ($OONO^-$) (Beckman et al. 1990; Tarpey et al. 2004), indicating that O_2 and N_2 radicals are highly interactive at the cellular level (Taha et al. 1992) (Fig. 2). This feature can be used to estimate $O_2^{\cdot-}$ steady state concentration employing a simple kinetic approach.

Data included in Table 1 show the steady state [NO] and the rate of generation of NO ($v_{NO\ generation}$) in DG of *M. arenaria* over the spring-summer period when both the water temperature and the bivalve metabolic activity are significantly higher than during the rest of the year (Lewis and Cerrato 1997). González et al. (2008) confirmed the role of NOS-like enzymes in the generation of NO in *M. arenaria* by EPR and specific inhibitors (such as *N*ω-nitro-L-arginine methyl ester hydrochloride, L-NAME and *N*ω-nitro-L-arginine, L-NA). However, since neither addition of the inhibitor nor heating of the tissue, which is supposed to remove enzymatic activity, completely

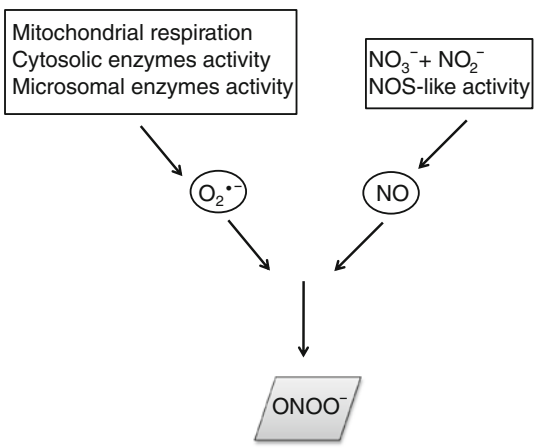

Fig. 2 Interaction between the oxidative and nitrosative metabolism in *M. arenaria*

blocked the generation rate of NO, the existence of other non-enzymatic sources of production is suggested. Non-enzymatic NO production from acidic reduction of nitrite has been described for invertebrate tissues with the following reductive sequence (Benjamin et al. 1994) (reactions 6, 7, 8).

$$NO_2^- + H^+ \rightarrow HNO_2 \quad \text{(reaction 6)}$$

$$2HNO_2 \rightarrow H_2O + N_2O_3 \quad \text{(reaction 7)}$$

$$N_2O_3 \rightarrow NO + NO_2 \quad \text{(reaction 8)}$$

The reduction of NO_2^-, and even other not yet described generation sources that are favored at low pH and low O_2 tension down to complete anoxia (Schreiber 2009; Zweier et al. 1999), seem also to be involved in marine invertebrates. The general expression for the NO generation rate can be written as follows (Eq. 8).

$$v_{NO\ generation} = \left(\frac{d[NO]}{dt}\right)_{NOS-like} + \left(\frac{d[NO]}{dt}\right)_{NO_2^-} + \left(\frac{d[NO]}{dt}\right)_{other\ sources} \quad (8)$$

For *M. arenaria* DG, the rate of NO generation by NOS-like activity in the presence of saturating concentrations of L-A and NADPH is 1.8 ± 0.2 pmol/min mg FW (Table 1). This value is two orders of magnitude below the value for rat liver, 285 ± 40 pmol/min mg FW (Galleano et al. 2001). Since in DG of *M. arenaria*, NO_2^- content under physiological conditions is under the detection limit (2–4 μM, Tarpey et al. 2004) this component in Eq. 8 can be discarded.

Other sources of NO generation could include non-enzymatic reactions such as the decomposition of endogenous complexes like, e.g., GSNO. In other organisms and tissues mononytrosyl-Fe (MNIC) or dinytrosyl-Fe (DNIC) complexes were identified as possible transport forms of NO in the cytosolic compartment, the formation of which depended on the presence of NO and catalytically active Fe (Vanin et al. 1998). However, the relative importance of these production pathways for NO still needs to be established for *M. arenaria*. Analysis of the disappearance of NO is a complex matter, as NO interaction with its main biological targets (O_2, $O_2^{\cdot-}$, Fe, GSH, organic radicals) needs to be considered. Autoxidation in the presence of O_2 in aqueous media mainly produces NO_2^- in a series of elemental reactions that could be summarized in a complex global reaction scheme (reaction 9) (Czapski and Goldstein 1995; Robbins and Grisham 1997).

$$4NO + O_2 + 2H_2O \xrightarrow{k_9} 4H^+ + 4NO_2^- \quad \text{(reaction 9)}$$

Kinetic studies have shown that this reaction is of first order with respect to O_2 and second order with respect to NO (Czapski and Goldstein 1995), according to Eq. 9.

$$v = k_9[O_2][NO]^2 \quad k_9 = 6 \times 10^6\ M^{-2}\ s^{-1} \tag{9}$$

The O_2 concentration in the water overlying the sediment at the Wadden Sea is around 300 μM under fully oxygenated conditions (de Beer et al. 2005), and this concentration can thus, be considered as the maximum O_2 concentration in the DG of *M. arenaria*. Since O_2 solubility can be calculated as the product of k_H and P_{O_2}, and considering a $k_H = 3.5 \times 10^{-4}$ M atm^{-1} (Chang 1999), at the physiological P_{O_2} (0.004 atm), oxygen solubility is approximately 1.4 μM. The NO steady state concentration is $(12.0 \pm 0.4) \times 10^{-8}$ M (Table 1), and the autoxidation rate of NO under fully oxygenated conditions, calculated from Eq. 9 was 2.6×10^{-11} M s^{-1}, moreover under most usual physiological conditions autoxidation rate of NO would be approximately 1.2×10^{-13} M s^{-1}. As a consequence, NO autoxidation can be theoretically discarded as an important pathway of NO elimination in *M. arenaria* hemolymph or DG under in vivo conditions.

The values of the rate constants for the formation of compounds such as MNIC and DNIC with different substrates are reported in the literature. According to Vanin et al. (2004), the k for the formation of DNIC-cysteine as assessed by EPR is $(1.0 \pm 0.2) \times 10^5$ M^{-2} s^{-1} whereas the estimation by optical measurements yields a k of $(2.0 \pm 0.1) \times 10^4$ M^{-2} s^{-1}. The formation of MNIC-cysteine assessed by EPR yields a k of 30 ± 5 M^{-1} s^{-1}, and for the formation of MNIC-*S*-nitroso glutathione $k = 3.0 \pm 0.3$ M^{-1} s^{-1}. Moreover, the rate of formation of the complex DNIC-GSH that depends on the concentrations of GSH, Fe^{2+} and NO, is ten times slower than the formation of DNIC-cysteine (Vanin et al. 2004). Finally, the constant rates for NO reactions with the majority of metallic centers ($k \sim 10^7$ M^{-1} s^{-1} for Fe^{2+}-hemo, k $\sim 10^2$–10^7 M^{-1} s^{-1} for Fe^{3+}-hemo) are at least two orders of magnitude lower than the k_{10} value for the reaction between NO and $O_2^{\cdot-}$ (reaction 10) which is close to the diffusion limit ($k_{10} = 6.7 \times 10^9$ M^{-1} s^{-1}) (Huie and Padmaja 1993).

$$NO + O_2^{\cdot-} \xrightarrow{k_{10}} ONOO^- \qquad \text{(reaction 10)}$$

Based on these kinetic considerations, $ONOO^-$ formation likely represents the main pathway for NO disappearance in this biological system, and the generation rate of $ONOO^-$ can be calculated according to Eq. 10.

$$v_{ONOO^-\ \text{generation}} = v_{NO\ \text{disappearance}} = k_{10}[NO][O_2^{\cdot-}] \tag{10}$$

Assuming that NO is in a steady state condition, the rate of NO generation can be assumed to equal the rate of its disappearance. Thus, according to Eq. 10 and based on the experimentally determined NO generation rate (37×10^{-12} M s^{-1}) and the NO steady state concentration (12×10^{-8} M) in *M. arenaria* DG, it becomes possible to approximate $O_2^{\cdot-}$ steady state concentration. The calculated $[O_2^{\cdot-}]$ is 4.5×10^{-14} M in *M. arenaria* DG under physiological control conditions, which lets the steady state concentration of $O_2^{\cdot-}$ in *M. arenaria* range three orders of magnitude lower than in mammalian cells (10^{-11} M, Cadenas and Davies 2000). Keeping the steady state of $[O_2^{\cdot-}]$ in this low range corresponds to the lower metabolic rate calculated for *Mya truncata* (1.4 μmol O_2/g FW h, Camus et al. 2003) as compared to mammals (30 μmol O_2/g FW h for rat, Randall et al. 1997). Moreover, by employing Eqs. 9 and 10, shown here, and the experimentally determined [NO] at day 9 of 500 μM Fe overload (González et al. 2010), the NO disappearance rate was estimated to calculate $[O_2^{\cdot-}]$. At day 9 of Fe exposure the calculated value for $[O_2^{\cdot-}]$ was 1.5×10^{-14} M. This value suggests that, at least under environmental stress by Fe overload, the endogenous mechanisms to control $[O_2^{\cdot-}]$ in steady state are operative. However, further studies are needed to determine if the higher content of catalytically active Fe could favor Haber–Weiss reactions leading to an increase in [·OH] steady state concentration in *M. arenaria*. Through the combination of experimental and estimated data, this simple kinetic analysis allows us to integrate the reactions of the oxidative and nitrosative metabolism for cells of an aquatic organism. This study can be a useful tool to approximate the dynamics of ROS interactions in the bivalve in response to changing environmental conditions.

Conclusions

The simple kinetic approach presented here using EPR experimental data to describe the oxidative status in the DG of *M. arenaria* is, to our knowledge, the first complete scenario obtained for a marine invertebrate. Figure 3 summarizes the steady state concentration of unsaturated lipids and $O_2^{\cdot-}$, taking into account the availability of labile Fe in the cell. It also summarizes the Fe availability in the cell and its allocation in marine invertebrates. Fe reaches the cell by endocytosis through transferrin-dependent receptors (TfR) and via transporters of divalent metals of low affinity (DMT) (Gunshin et al. 1997). Huebers et al. (1982) reported that a protein that binds Fe in *Cancer magister* fulfills the criteria to be identified as Tf. Little is known about the exportation mechanism of Fe from the cells, but Fe ions could leave the cells as thiol- and/or NO-complexes (DNIC and MNIC) (Kruszewski 2004). Ft, the main storage protein, seems to play an important role in bio-mineralization. On the other hand, it is known that hemocytes have the capacity of removing metal ions from the hemolymph and transfer them to the excretion sites. Winston et al. (1996) reported the presence of Ft in *Mytilus edulis* hemocyte cells which are considered to be major Fe-storage cells in invertebrates (Ahearn et al. 2004).

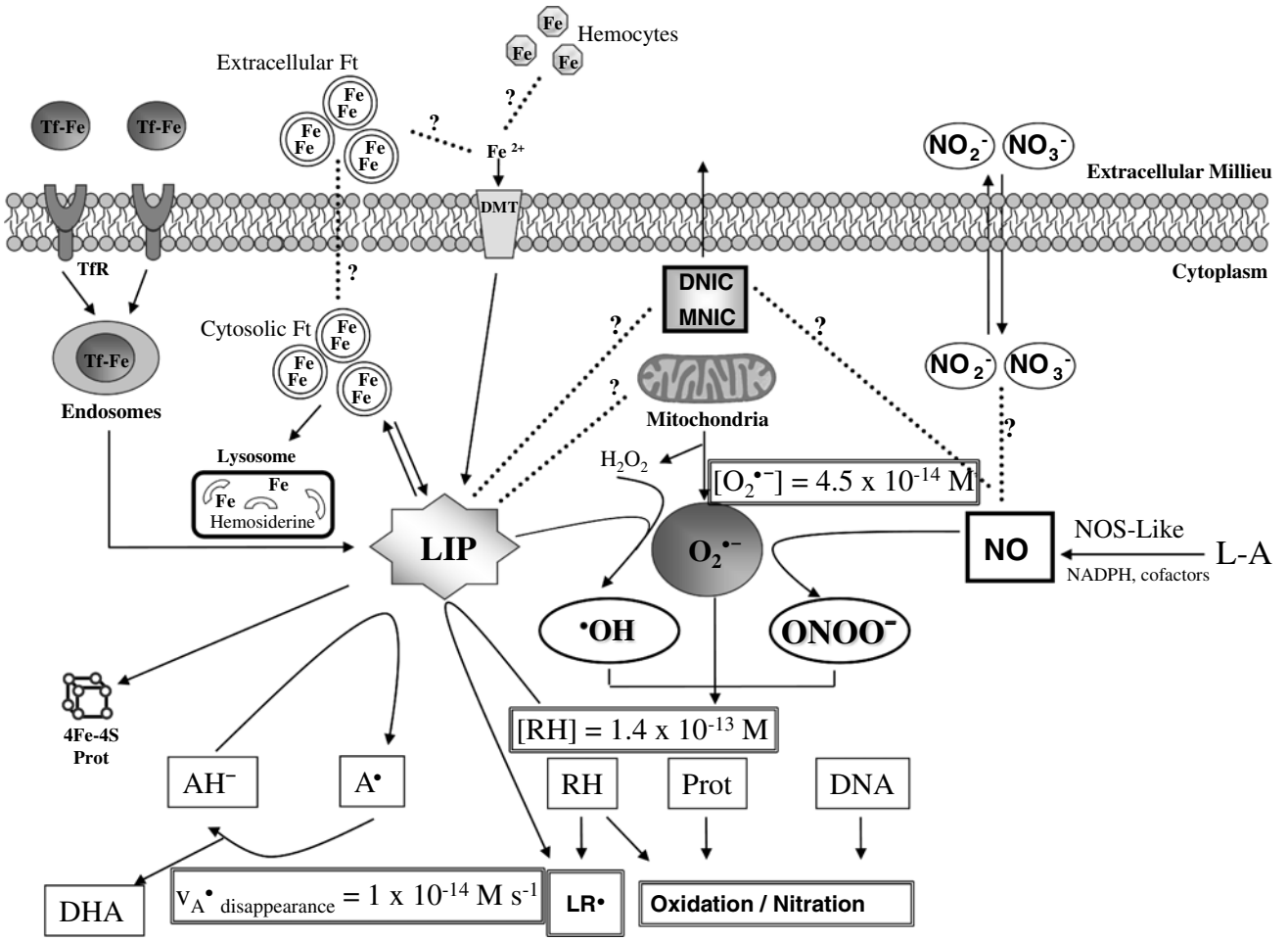

Fig. 3 General diagram for a marine invertebrate cell showing oxidative and nitrosative metabolism and Fe role. The estimated steady state concentration of $O_2^{\cdot-}$, RH, and the disappearance rate of A· for *M. arenaria* are included

It was also suggested that Fe is stored in lysosomes of marine animals (Fowler et al. 1975). An important point in Fig. 3 is the possible link between the labile Fe, ROS and RNS in invertebrates. Since EPR is the method of choice for the study of paramagnetic molecules (molecules with an unpaired electron), the data used for estimation of steady state concentration are highly specific, and their application in the kinetic study seems appropriate. Importantly, the steady state concentration for $O_2^{\cdot-}$ in *M. arenaria* resulted in three orders of magnitude lower than in mammalian cells, which relates to the lower metabolic activity and the lower body temperature of invertebrates as compared to mammals, among other factors.

The Wadden Sea receives high amounts of suspended material (Puls et al. 1997) together with a parallel import of nitrogen and phosphorous containing nutrients (Radach and Lenhart 1995; Beddig et al. 1997) that might affect NO_3^- content in the water and in *M. arenaria* shell water. In spite of the lack of previous data on the uptake of NO_3^- into molluscan tissues, it could be interesting to investigate how such changes might affect the steady state concentration of RNS in *M. arenaria* hemolymph and tissues, for physiological, biochemical and even ecological purposes. The approach we present here opens the possibility of a holistic analysis that could be helpful to understand the complex pathways that control the responses of marine organisms to environmental challenges. This analysis intends to adequately combine observations linked to the biological chemistry, the physics and the physical chemistry with the aim to improve the knowledge of critical pathways in living organisms.

Acknowledgments This study was supported by grants from the University of Buenos Aires, National Council for Science and Technology (CONICET) (PIP 1171) and DAAD-CONICET collaborative visit. S.P. is career investigator from CONICET and P.M.G. is a postdoctoral fellow from CONICET.

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