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The known and unknown sources of reactive oxygen and nitrogen species in haemocytes of marine bivalve molluscs

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ABSTRACT

Reactive oxygen and nitrogen species (ROS and RNS) are naturally produced in all cells and organisms. Modifications of standard conditions alter reactive species generation and may result in oxidative stress. Because of the degradation of marine ecosystems, massive aquaculture productions, global change and pathogenic infections, oxidative stress is highly prevalent in marine bivalve molluscs. Haemocytes of bivalve molluscs produce ROS and RNS as part of their basal metabolism as well as in response to endogenous and exogenous stimuli. However, sources and pathways of reactive species production are currently poorly deciphered in marine bivalves, potentially leading to misinterpretations. Although sources and pathways of ROS and RNS productions are highly conserved between vertebrates and invertebrates, some uncommon pathways seem to only exist in marine bivalves. To understand the biology and pathobiology of ROS and RNS in haemocytes of marine bivalves, it is necessary to characterise their sources and pathways of production. The aims of the present review are to discuss the currently known and unknown intracellular sources of reactive oxygen and nitrogen species in marine bivalve molluscs, in light of terrestrial vertebrates, and to expose principal pitfalls usually encountered.

1. Introduction

Reactive oxygen species (ROS) and reactive nitrogen species (RNS) encompass a variety of small radical and non-radical molecules. Although ROS and RNS have a very high deleterious potential, they also display a wide range of physiological functions. From medical and biochemical perspectives, the production of ROS and RNS, as well as their beneficial and detrimental mechanisms are fairly well understood in higher vertebrates [1–5]. Although metabolic pathways of ROS and RNS productions are highly conserved between vertebrates and invertebrates, some uncommon pathways appear to only exist in invertebrates. For the last decade, ROS and RNS have become of great interest across the ecological and environmental sciences. It is now evident that most modifications of standard environmental conditions (*e.g.*, biological and chemical contaminants, pathogens, alteration of physico-chemical variables …) alter ROS and RNS productions and may result in oxidative stress [6–8].

Marine environment, more than any other, undergoes frequent and potentially drastic alterations of standard conditions [9–11]. Because of the degradation of the overall health of marine ecosystems [10,12,13] and in the contexts of massive aquaculture productions [14] and global change [9], oxidative stress is becoming more and more prevalent in marine organisms [6,15]. Within marine environment, invertebrates represent a great majority of all macroscopic life and inhabit all ecosystems, from estuaries to the deep sea. Among marine invertebrates, bivalve molluscs are used as sentinel organisms to monitor pollution and depict the health of ecosystems [16,17]. In fisheries and aquaculture, they represent a substantial part (about 25%) of the global production and economy (FAO data).

In marine bivalve molluscs, haemocytes are involved in various biological functions, including immune response [18]. Haemocytes produce ROS and RNS as part of their basal metabolism [19] as well as in response to endogenous and exogenous stimuli [15,20]. However, the intracellular sources of ROS and RNS are currently poorly deciphered in marine bivalves, potentially leading to misinterpretations.

The aims of the present review are to discuss the currently known and unknown intracellular sources of ROS and RNS in marine bivalve molluscs, in light of terrestrial vertebrates, and to

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expose principal pitfalls usually encountered. Detailed molecular and physicochemical aspects of ROS and RNS generation are not discussed herein (for exhaustive reviews please refer to, *e.g.*, [1,5,21]). The first section summarises the cellular sources of ROS and RNS in terrestrial vertebrates and, for each of them, discusses the current knowledge in marine bivalves. The second section criticises some of the interpretations of ROS and RNS measured in haemocytes of marine bivalves.

2. The multitude of cellular sources of ROS and RNS

Before discussing the multitude of cellular sources, it is helpful to briefly summarise the main ROS and RNS. Superoxide ($O_2^{\bullet-}$) reacts with relatively few biological molecules and has a limited membrane permeability. Superoxide can spontaneously dismutate to hydrogen peroxide (H_2O_2) [22,23]. Hydrogen peroxide is a strong oxidant with high membrane permeability, but most of its oxidation reactions are too slow to be biologically relevant [24]. Hydroxyl radical (•HO) is extremely reactive with most biological molecules but display a very short range of action [25]. At last, hypochlorous acid (HOCl) is also a strong oxidant but is more selective than hydroxyl radical [2]. The production of RNS starts with the generation of nitric oxide (•NO). Nitric oxide is highly reactive and diffuses freely across cell membranes [26]. Reaction between nitric oxide and superoxide forms peroxynitrite ($ONOO^-$). Peroxynitrite is also highly reactive but at a relatively slow rate [27]. This slow reaction rate allows it to react more selectively throughout the cell. Peroxynitrite is able to get across cell membranes but only to some extent. A vast array of other ROS and RNS can also be generated in biological systems. For detailed descriptions, readers are encouraged to refer to exhaustive reviews [28–30].

2.1. Production of ROS in the mitochondrion

In all organisms, mitochondrion generates most of the cellular energy through the production of Adenosine Tri-Phosphate (ATP) by complex V (Fig. 1). Complexes I to IV are called the electron transport chain (ETC). The ETC transports electrons via reduction-oxidation (REDOX) reactions to an oxygen molecule. However, at least in terrestrial vertebrates, about 1.5–2% of the oxygen consumed is converted into superoxide and then hydrogen peroxide [21].

Marine bivalves inhabit environments where oxygen availability can be highly reduced (hypoxia) or even absent (anoxia). Solubility of oxygen depends on water temperature and salinity. Oxygen availability then varies both seasonally and daily. Furthermore, the tidal cycle daily interrupts oxygen availability to intertidal invertebrates. At last, burrowing animals that live in sediment also often experience hypoxia. In order to adapt their metabolism to low oxygen availability, cells of bivalves need to switch to an anaerobic energy metabolism [31,32]. Mitochondria of marine invertebrates able to survive anoxia are classified as "anaerobically functioning" [32]. Anaerobically functioning mitochondria present both an aerobic and an anaerobic pathway (Fig. 1) but display the same enzymatic repertoire as those of aerobically specialised animals [32]. Only the presence of rhodoquinone seems restricted to anaerobically functioning mitochondria [32].

Isolated mitochondria of marine bivalves have the potential to produce ROS *in vitro* (Figs. 1 and 2). For instance, isolated mitochondria of the clams *Laternula elliptica* and *Mya arenaria* [33], the scallops *Aequipecten opercularis* and *Adamussium colbecki* [34] and the clam *Arctica islandica* [35] generated H_2O_2 *in vitro*. However, the actual extent to which ROS generation happens *in vivo* remains largely unknown. We recently suggested mitochondrion as one of the main sources of ROS in haemocytes of the Pacific oyster *Crassostrea gigas*, and reported some uncommon pathways compared with the textbook-type vertebrate models [19].

Although mitochondrial complex I is one of the main sites for the production of ROS in terrestrial vertebrate cells [21], the inhibition of complex I in the oyster *C. gigas* had no effect on ROS production [19]. Furthermore, contrastingly with mammalian cells, the inhibition of complex III, still in *C. gigas*, resulted in a dose-dependent inhibition of ROS production [19]. Accordingly, Abele et al. [36] stated that "$O_2^{\bullet-}$ and H_2O_2 production in marine invertebrates seems to occur mainly during forward electron transport", which mostly involves complex III rather than complex I

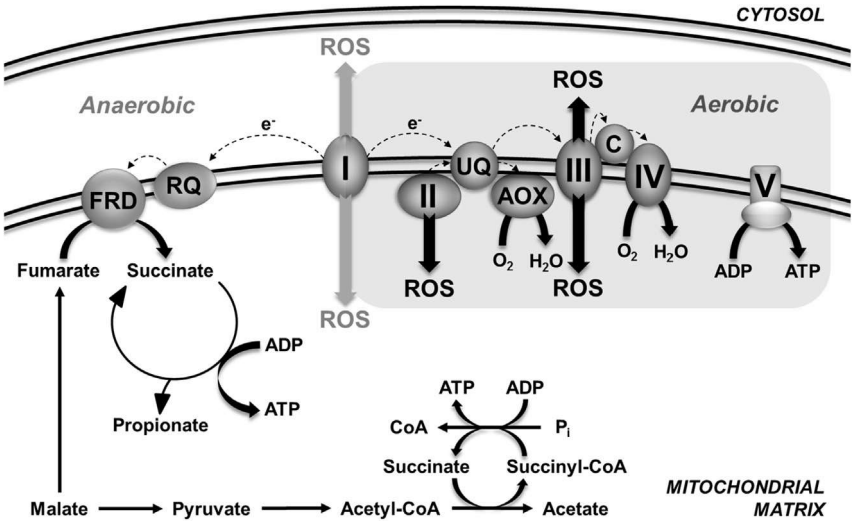

Fig. 1. Sites of reactive oxygen species production and simplified major metabolic pathways in generalized anaerobically functioning mitochondria of a marine bivalve. The light red rectangle and the red particles highlight the electron transfer chain employed during aerobic metabolism. The blue particles highlight the membrane-bound enzymes preferentially employed during anaerobic metabolism. The dotted arrows are the main exchanges of electrons in aerobic and anaerobic metabolisms. Fumarate reduction to succinate is coupled to the electron transfer chain; rhodoquinone (RQ) serves as an electron donor from complex I to fumarate reductase (FRD). Under conditions of prolonged anaerobiosis, propionate is preferentially formed instead of succinate. Known sites of reactive oxygen species (ROS) production are indicated by dark arrows; uncertain site of ROS production is indicated by light grey arrows. Abbreviations: I to V, mitochondrial complexes I to V; AOX, alternative oxidase; C, cytochrome c; FRD, fumarate reductase; RQ, rhodoquinone; UQ, ubiquinone. Adapted from [19,32]. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

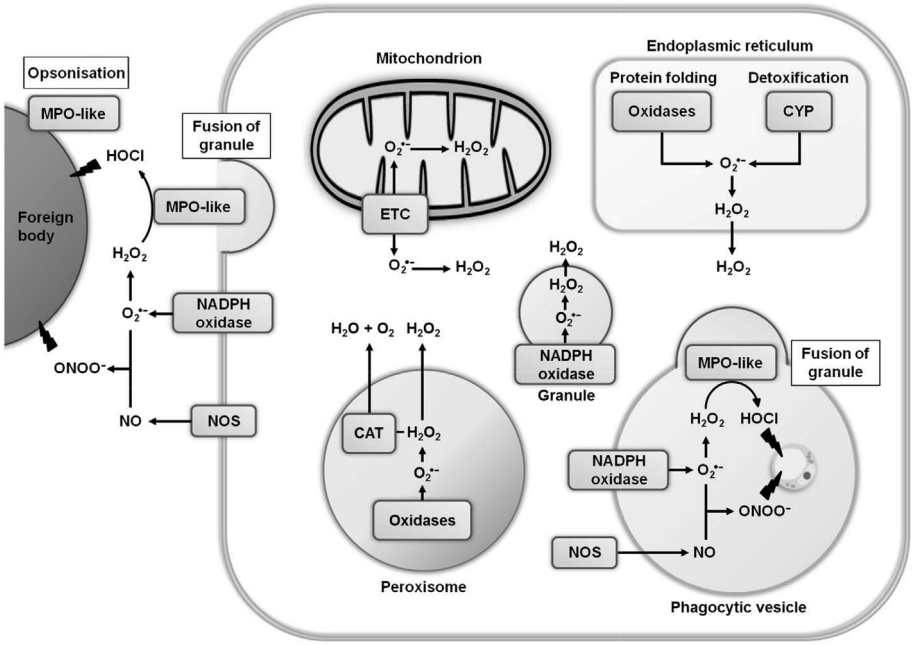

Fig. 2. Major sites of reactive oxygen and nitrogen species generation in generalized haemocyte of marine bivalve. Abbreviations: CAT, catalase; CYP, cytochrome P-450; ETC, electron transfer chain; MPO, myeloperoxidase; NOS, nitric oxide synthase. For details, readers are invited to refer to the text.

(Fig. 1; [37,38]). It is currently unknown whether mitochondrial complex I in haemocytes of the Pacific oyster and, by extension, of intertidal bivalves can produce ROS under certain conditions (Fig. 1). One can however hypothesise this may happen during the decrease in oxygen availability, when energy metabolism gradually switches from aerobic to anaerobic pathways. The molecular characterisation and quantification of the electron transfer chain complexes, as well as their respective involvement in the production of ROS in haemocytes of marine bivalves appears necessary.

2.2. Production of ROS in the endoplasmic reticulum

Although mitochondrion is generally assumed as the primary producer of ROS in vertebrate cells, Brown and Borutaite [39] recently argued that, until now, no convincing experimental evidence supports this postulate. In fact, the endoplasmic reticulum (ER) is an underestimated site of ROS production in vertebrate cells. The ER is the place on the protein production pathway where proteins fold and assemble into complexes [40]. Protein folding requires reactions of oxidation with oxygen as the final oxidant. In vertebrates, accumulating evidence now suggests that ROS are generated as by-products of protein folding in ER [40,41] (Fig. 2).

The ER also contains several cytochromes, collectively known as cytochrome P-450 (CYP), that perform catabolic activity (*i.e.*, breaking down molecules into smaller units in order to release energy) through REDOX reactions, eventually generating superoxide [42,43]. The substrates for CYP catabolic reactions can be organic xenobiotics such as herbicides, insecticides or hydrocarbons [44]. Because of the widespread occurrence of these pollutants in terrestrial and aquatic ecosystems, their detoxification in the ER may then contribute significantly to the oxidative stress of all organisms (Fig. 2).

In marine invertebrates, CYP molecules have been identified in several species, including the mussels *Mercenaria mercenaria* [45] (NCBI Accession AAB66556) and *Mytilus galloprovincialis* [46] (AAC32835). CYP isoforms have also been cloned and characterised in the abalone *Haliotis rufescens* [46] (AAC32833), the polychaete worms *Nereis virens* [47] (AY453407 and AY453508) and *Capitella capitata* [48] (AAS87603 and AAS87604), the crab *Carcinus maenas* [49] (AY328466 and AY328467), the shrimp *Penaeus setiferus* and lobster *Homarus americanus* [46] (AAC32834 and AAC28351), and the sea urchin *Lytechinus anamesis* [46] (AAC32831 and AAC38830). However, in the context of pollution monitoring, only the variations of the expression of these genes were investigated. No information is currently available regarding the enzymatic activity and substrates of these CYP isoforms. Although one can expect CYP isoforms from marine bivalves to perform similar functions as those from terrestrial vertebrates, their actual involvement in detoxification processes is still unknown.

While some authors now admit that ER may be one of the major sources of ROS in marine invertebrates, experimental evidence remains limited. To our best knowledge, the only available data were obtained during the mid- and late-nineties by Livingstone and colleagues: isolated microsomes (*i.e.*, vesicle-like artifacts reformed *in vitro* from pieces of ER) from the mussel *Mytilus edulis* generate superoxide, hydrogen peroxide and hydroxyl radical *in vitro* [50,51]. In these microsomes, the NADH-dependent production of ROS was higher than the NADPH-dependent, which is the opposite of what happens in vertebrates. Thus, the probable detoxification-related generation of ROS in ER, the scarcity of available data and the uncommon pathways of ROS production in ER justify a further effort to understand these mechanisms in haemocytes of marine bivalves.

2.3. Production of ROS in the peroxisomes

In higher vertebrates, along with mitochondrion and ER, peroxisome is also a major intracellular site of oxygen consumption and subsequent generation of ROS. Peroxisome is defined as an organelle which contains at least one H_2O_2-producing oxidase and one H_2O_2-degrading enzyme (catalase) [52]. This definition implies that ROS are naturally produced in peroxisomes (Fig. 2). Although the extent by which peroxisomes generate ROS varies between species and also between organs [53], this production may be

everything but negligible. For instance, about 35% of all hydrogen peroxide formed in a rat liver arises from peroxisomes [54]. The main H_2O_2-producing oxidases in peroxisomes are the family of acyl-CoA oxidases, the xanthine oxidase and the urate oxidase [52,53].

Peroxisomes exist in the cells of marine invertebrates and were microscopically observed in various species such as the mussels *M. edulis* [55] and *M. galloprovincialis* [56], the polychaete worm *Hediste diversicolor* [57] and in some gastropod species [58]. Observation of peroxisomes was also indirect through the detection of peroxisomal enzymatic activities in, for instance, the mussel *M. galloprovincialis* [59], the sea urchin *Tetrapigus niger* [60] and the polychaete worm *Arenicola marina* [61]. However, in marine bivalves, only the pollutant-induced proliferation of peroxisomes has been studied yet [62]. The molecular nature and capacity to generate ROS of peroxisomal enzymes are currently totally unknown in marine bivalves. To our best knowledge, the only available molecular data in marine bivalves are the partial mRNA sequence of the palmitoyl-CoA oxidase (Acyl-CoA oxidase) from the mussel *M. galloprovincialis* (EF525542; Unpublished). Consequently, the expression, enzymatic activities, capacity to produce ROS and response to pollutants of peroxisomal proteins remain to be discovered in haemocytes of marine bivalves.

2.4. Production of ROS by the NADPH-oxidase family

While in mitochondria, ER and peroxisomes ROS are generated as by-products of other biological reactions, NADPH-oxidases are enzymes entirely dedicated to the production of ROS. The family of NADPH-oxidases comprises trans-membrane proteins which transfer electrons across biological membranes. Usually, the final electron acceptor is oxygen and the product of the reaction is superoxide [5]. Since the discovery of the first member of the NADPH-oxidase family (NOX2) in human phagocytes, the family has grown and is now constituted, at least in higher vertebrates, of seven NOX isoforms (NOX1-5 and DUOX1 and 2) [5]. While some features are invariable among the whole NADPH-oxidase family (*e.g.*, electron transfer to oxygen), all members display their own characteristics, being, for instance, a tissue-specific localisation or particular mechanisms of activation. Thanks to their condition-specific ROS generation, NADPH-oxidases play key roles in numerous physiological mechanisms such as host defence, cellular signalling, gene expression, cell growth, cell death and oxygen sensing [5].

Because it was the first identified, the phagocyte oxidase NOX2 is usually considered, perhaps wrongly, as the archetype NADPH-oxidase. NOX2 is a molecular complex with two subunits permanently bound to the membrane: $gp91^{phox}$ (firstly referred to as NOX2 before the discovery of other constituting subunits) and $p22^{phox}$ [3,5]. In absence of cellular activation, the other components ($p40^{phox}$, $p47^{phox}$, $p67^{phox}$ and rac) remain in the cytosol and the enzyme is dormant. Upon cellular activation, the cytosolic components associate to the membrane-bound complex to form the functional NOX2. The active NADPH-oxidase then generates superoxide that dismutates spontaneously, or by the action of a superoxide dismutase, to hydrogen peroxide [2]. Membrane-bound components of NOX2 can be found at the plasma membrane (*i.e.*, around the cell), the membrane of phagocytosis vesicles (which originates from the plasma membrane), and the membrane of cytoplasmic granules (Fig. 2). Activated NOX2 bound to the plasma membrane releases ROS to the extracellular milieu (ecROS), whereas activated NOX2 located on an intracellular membrane (phagocytic vesicle or granule) releases intracellular ROS (icROS) [3]. The phagocytic vesicle is commonly regarded as the typical site for icROS formation in phagocytic cells. However, one need to keep in mind that production of icROS can also occur in absence of phagocytosis, triggered by stimuli such as the fusion between granules [63].

The existence of NADPH-oxidases in haemocytes of marine bivalves was first suggested by the use of chemical inhibitors [64,65]. Unfortunately, most of these studies should be carefully re-analysed because of the misuse of Diphenyleneiodonium chloride (DPI) as a specific inhibitor of NADPH-oxidases. While DPI truly inhibits NADPH-oxidases, it also inhibits numerous sources of ROS including mitochondrial complexes I and II, and also nitric oxide synthase [66,67]. Nonetheless, good or not, these results inspired further transcriptomic studies to characterize the genes encoding for NADPH-oxidases in some marine invertebrate species. In the shrimp *Marsupenaeus japonicus*, full-length cDNA of two NADPH-oxidases were cloned. One is a NOX homologue [68] (MjNox; AB594770) while the other one is a DUOX homologue [69] (MjDuox; AB744213). MjNox is highly expressed in the lymphoid organ, hepatopancreas and haemocytes whereas MjDuox seems more specific of gills tissue. MjNox and MjDuox are inducible and, at least, involved in the immune response. A DUOX homologue was also cloned and characterized in the eggs of the sea urchin *Lytechinus variegatus* [70] (Udx1; AY747667). Udx1 is activated by the entry of a spermatozoid in the egg, and is necessary for the physical block to polyspermy. Finally, a gene encoding for a DUOX homologue was also discovered in the genome of the oyster *C. gigas* [71] (CgDuox; EKC37173) but no information is available about the expression and function(s) of CgDuox.

The proteins MjDuox, Udx1 and CgDuox, obtained by conceptual translations, share a high degree of similarity, with percentages of identity ranging from 31 to 42% (ClustalW2 multiple alignments). However, a high degree of similarity and identity does not necessary mean these proteins share functional similarities. For example, the percentages of identity between the seven human NADPH-oxidase isoforms range from 24 to 59% (ClustalW2 multiple alignments). While isoforms such as NOX2 and NOX4 share 37% of identity, they differ for their tissue expression, cytosolic components and functions [5]. Thus, although transcriptomic studies have proven highly valuable, functions of NADPH-oxidases in marine bivalves still cannot be inferred. The characterisation of regulatory components, tissue specific expression and condition-specific activation(s) now become primordial to eventually understand the involvement of NADPH-oxidases in the immune response and other biological processes of marine bivalves.

2.5. Production of ROS by the myeloperoxidase

Even in higher vertebrates, the mechanisms by which ROS kill pathogens is still a matter of debate [72]. Because of its weak reactivity, superoxide anion generated by NADPH-oxidases is usually considered as not much involved in microbial killing. Instead, phagocytic cells may utilise hypochlorous acid (HOCl) which seems much more efficient as bactericide [2,72]. Hypochlorous acid is generated by combined action of hydrogen peroxide (dismutated from superoxide) and the secreted enzyme myeloperoxidase (MPO) [2,3,72,73]. In absence of stimulation, MPO is restrained to cytoplasmic granules and remains inactive because of the absence of hydrogen peroxide. Fusion of granules with phagocytic vesicle participates to the production of icROS while fusion with plasma membrane releases MPO to the extracellular milieu, participating to the generation of ecROS (Fig. 2).

In haemocytes of marine bivalves, MPO has been always investigated through the detection of its enzymatic activity, generally by the use of tetramethylbenzidine (TMB) oxidation. Authors have reported TMB oxidation in various marine invertebrates, such as the mussel *M. edulis* [74], the pen shell *Pinna nobilis* [75], the sea cucumber *Apostichopus japonicus* [76] or the

shrimp *Penaeus monodon* [77]. However, while Schlenk et al. [74] confirmed the pH profile and subcellular distribution of the activity to conclude about the presence of MPO, TMB oxidation in other referenced studies was detected from whole cells and may then result from numerous other cellular and extra-cellular peroxidases. The factual proof for the existence of MPO in marine bivalves would come from the characterization of MPO homologue gene(s) and protein(s) but they have not yet been identified.

A peroxidase belonging to the same protein family as mammalian MPO however exists in crustacean haemocytes and is referred to as peroxinectin [78,79]. Surprisingly, peroxinectin is absent from all other phyla within marine invertebrates. Similarly to MPO, peroxinectin is not only a peroxidase but also a cell adhesion ligand, and both functions are independent [78–81]. Peroxinectins are stored in semi-granular and granular crustacean haemocytes and released to the extracellular milieu upon cellular activation [79]. As a cell adhesion protein, peroxinectin can bind to foreign surfaces and enhance phagocytosis or encapsulation process (*i.e.*, opsonisation). Peroxinectin may then cooperate with NADPH-oxidases to produce ecROS in order to destroy the foreign body [79,82] (Referred to as MPO-like in Fig. 2).

Although MPO homologues have not yet been identified, and may not exist, in haemocytes of marine bivalves, MPO-like peroxidases may fulfil similar roles. The search for molecules displaying MPO-like functional domains along with the study of their expression and functions might allow for the discovery of MPO alternatives in marine bivalves.

2.6. Production of RNS by the nitric oxide synthase

In terrestrial vertebrates, nitric oxide (NO) is generated by the nitric oxide synthase (NOS). Three NOS isoforms exist: neuronal NOS (nNOS), endothelial NOS (eNOS) and inducible NOS (iNOS), originally identified in macrophages but expressed in virtually any cell [1,83,84]. While nNOS and eNOS are constitutively expressed, iNOS gene expression is induced in phagocytes by inflammatory signals [1]. Furthermore, activity of constitutive NOSs is strictly dependent on the intracellular free Ca^{2+} whereas the activity of iNOS is Ca^{2+}-independent. At last, constitutive NOSs generate small quantities of NO for only few minutes while iNOS produces two to three orders of magnitude more NO than with the constitutive NOS, for hours to days [1]. These high amounts of NO produced by iNOS exert cytotoxic and antimicrobial effects on the immune system [1,83,84] (Fig. 2).

Historically, NO was first considered as a highly toxic and environmentally damaging pollutant but, by the end of the 1980s, interest in NO as involved in various physiological functions grew rapidly. In 1992, NO was named “Molecule of the Year” and the Nobel Prize in physiology and medicine was then awarded for the identification of NO as a signalling molecule in cardiovascular system [1]. This mediatisation may explain the popularity of NO and NOS among scientists, including in the field of marine invertebrates. Indeed, NOS homologues have been cloned and characterized in numerous marine invertebrate species.

Early detection of NOS in marine invertebrates relied on cyto- and histo-chemistry. NOS activity was detected in tissues of a variety of marine invertebrates such as the gastropods *Pleurobranchaea californica* [85] and *Clione limacine* [86], the starfish *Marthasterias glacialis* [87], the sea urchins *Lytechinus pictus* [88] and *Strongylocentrotus purpuratus* [89], or the horseshoe crab *Limulus polyphemus* [90]. Thanks to the advent of molecular biology, NOS isoforms have been cloned and characterized in some marine invertebrates during the last decade, including the bivalves *Azumapecten farreri* [91], *C. gigas* [71] and *Crassostrea virginica* [92]. However, as previously discussed, although genetic and phylogenetic analyses are necessary, the functional characterisation of NOS isoforms remains the key to eventually understand the physiologic and stress-related production of RNS in marine bivalves.

3. Principal pitfalls in analyses of ROS and RNS production

Various techniques are currently available to measure ROS and RNS in haemocytes and isolated organelles. However, their significance and precautions of use are sometimes unfamiliar. Fluorescent probes, for example, can be excellent indicators of reactive species but many precautions must be taken [93]. The main pitfall commonly encountered is the lack of selectivity of the probe, while used to measure specific reactive species. Fluorescein probes such as (Dichloro)dihydrofluorescein H_2DCFDA (or $DCFH_2$) and its derivatives are very commonly used but are actually not very useful for detection of the two most common ROS: $O_2^{\bullet-}$ and H_2O_2. H_2DCFDA-derived probes detect numerous ROS and RNS, including $ONOO^-$, HOO^-, HO^-, H_2O_2 and $O_2^{\bullet-}$ [30,94,93]. Unfortunately, these probes show their lowest reactivity toward superoxide and hydrogen peroxide, as well as a pH-dependence that can result in a leakage from the cells under certain conditions [95]. Fluorescent probes have been designed to study ROS and RNS in cells from terrestrial animals. haemocytes of marine bivalve molluscs are maintained in seawater or osmotically-modified culture medium. The presence of salts may modify the activity of probes. For instance, DCFH-DA in seawater spontaneously fluoresces (Lambert, personal communication), which may alter detection of extracellular ROS production. Numerous kinds of probes are commercially available but they all come with drawbacks researchers need to keep in mind to know what they are measuring in their experiments. The objective of the present review is not to provide an exhaustive list of probes so readers are highly encouraged to refer to recent reviews [95–98].

In the field of marine bivalves, misinterpretations may arise from the negative synergy between limited knowledge and misuse of techniques. A common illustration is the assumption that unstimulated ROS production in haemocytes (*i.e.*, without phagocytosis of particles or chemical stimulus) arises from NADPH-oxidases, and reflects the immune capacities of the cells (*e.g.*, [99,100]). As discussed herein, even in immune cells, basal ROS are more likely to happen from mitochondria, ER and peroxisomes (Fig. 2). Phagocytosis and microbicidal capacities must not be inferred from basal cellular levels of ROS. A second usual concern is the misuse of the concept of “oxidative burst” to describe ROS production in unstimulated immune cells of marine invertebrates (*e.g.*, [101,102]). First, whatever the organism, the notion of “burst” is, as per its definition, inappropriate to qualify a basal ROS production. Second, in stimulated phagocytes of higher vertebrates, “oxidative burst” is a rapid, transient, production of huge amounts of ROS [5]. In vertebrate phagocytes, oxidative burst generally occurs within few minutes [103,104]. In haemocytes of marine bivalves, delayed increased ROS production happens between 15 and 70 min post-stimulation and remains moderate compared to vertebrate models (*e.g.*, [105,106]). In marine bivalves, the involvement of ROS in microbicidal activity is still uncertain. A further effort is necessary to understand the roles of reactive species in microbicidal mechanisms.

4. Conclusion

In haemocytes of marine bivalves, unravelling the sources of ROS and RNS appears necessary. The recent development of all the “-omic” techniques and computational approaches in marine sciences, along with the availability of more and more complete

genomes will certainly prove helpful to comprehend the specificities of ROS and RNS generation in marine bivalves. However, characterisation of the functions and activities of putative sources will also be necessary to eventually understand the biology of ROS and RNS in marine bivalves.

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