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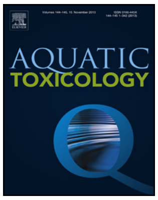

Diatom-derived oxylipins induce cell death in sea urchin embryos activating caspase-8 and caspase 3/7

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ABSTRACT

Diatoms are an important class of unicellular algae that produce bioactive secondary metabolites with cytotoxic activity collectively termed oxylipins, including polyunsaturated aldehydes (PUAs), hydroxyacids (HEPEs), oxo-acids and epoxyalcohols. Previous results showed that at higher concentrations, the PUA decadienal induced apoptosis on copepods and sea urchin embryos via caspase-3 activation; at lower concentrations decadienal affected the expression levels of the *caspase-8* gene in embryos of the sea urchin *Paracentrotus lividus*. In the present work, we studied the effects of other common oxylipins produced by diatoms: two PUAs (heptadienal and octadienal) and four hydroxyacids (5-, 9- 11- and 15-HEPE) on *P. lividus* cell death and caspase activities. Our results showed that (i) at higher concentrations PUAs and HEPEs induced apoptosis in sea urchin embryos, detected by microscopic observation and through the activation of caspase-3/7 and caspase-8 measured by luminescent assays; (ii) at low concentrations, PUAs and HEPEs affected the expression levels of *caspase-8* and *caspase-3/7* (isolated for the first time here in *P. lividus*) genes, detected by Real Time qPCR. These findings have interesting implications from the ecological point of view, given the importance of diatom blooms in nutrient-rich aquatic environments.

1. Introduction

Echinoderms play a key role in the maintenance of the integrity of the ecosystem where they live (Hereu et al., 2005), particularly in their early planktonic life stages (most critical for their survival). They are constantly exposed to a number of pollutants (Bellas et al., 2008a; Rosen et al., 2008). Sea urchins have been adopted as an excellent model system to study the ecotoxicological response of marine invertebrates to environmental pollutants (Geraci et al., 2004; Bonaventura et al., 2005; Cakal Arslan and Parlak, 2007; Bellas et al., 2008b; Nahon et al., 2008). Echinoderms are world-wide in their distribution and are extremely important in structuring benthic marine communities. Maintenance of these animals and obtaining ripe gametes for experimentation is relatively easy, development is sensitive to several kinds of environmental pollutants, and results can be obtained in a short time frame (Kobayashi and Okamura, 2005). The transparent embryo enables easy observation of malformation, making it possible to detect sub-lethal effects of pollutants on multicellular body formation at an early stage in development. To date, the stressors that have been examined using the sea urchin as a model include physico-chemical changes in the water, such as acidic pH (Dupont et al., 2010), hypoxia (Kodama et al., 2010), UV (Lesser et al., 2003; Schröder et al., 2005; Bonaventura et al., 2005, 2006; Lister et al., 2010a, 2010b; Russo et al., 2010), X-rays (Matranga et al., 2010; Bonaventura et al., 2011), and chemicals such as antifouling agents/pesticides (Garaventa et al., 2010; Aluigi et al., 2010), endocrine disruptors (Sugni et al., 2010; Horiguchi et al., 2010) and metals (Pinsino et al., 2014; Kiyomoto et al., 2010). Natural toxins represent a major source of stress for marine organisms. In this regard, diatoms are particularly noteworthy, traditionally

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regarded as a good food for primary consumers of plankton, consisting mainly of small crustacean copepods. However, their beneficial role in supporting marine food chains has been challenged with the discovery that some diatom species produce secondary metabolites with cytotoxic activity (Miralto et al., 1999). These cytotoxic compounds are the end-products of a lipoxygenase/hydroperoxide lyase metabolic pathway (Fontana et al., 2007) initiated by damage to algal cells, including short chain polyunsaturated aldehydes (PUAs) and other oxygenated fatty acid degradation products such as hydroxides, oxo-acids and epoxyalcohols, collectively termed oxylipins. Oxylipins from marine diatoms are known to be disruptive to reproductive and developmental processes of several marine invertebrates (Caldwell, 2009). Miralto et al. (1999) also demonstrated that the PUA decadienal induced apoptosis in mammalian tumor cells. In invertebrates, Romano et al. (2003) demonstrated that at higher concentration decadienal induced apoptosis in the copepod *Calanus helgolandicus* and in sea urchin *Paracentrotus lividus* embryos via caspase-3 activation. Subsequently, Romano et al. (2010) extended these studies, incubating newly fertilized eggs from *P. lividus* in a range of concentrations (1.32–5.26 μM) lower than those inducing cell blockage (6.58 μM). At these lower concentrations decadienal was able to induce embryonic malformations in a concentration-dependent manner, whereas at higher concentrations embryos showed blebbing associated with apoptosis. More recently, molecular studies at low concentration have revealed that decadienal affected the expression levels of the *caspase-8* gene in *P. lividus* embryos (Marrone et al., 2012).

Programmed cell death or apoptosis is a physiological process of cell elimination, playing a key role in development and in tissue homeostasis in invertebrates and vertebrates. Apoptosis plays two major roles during development, removing damaged cells in embryogenesis and sculpting tissues during morphogenesis and metamorphosis (Hirata and Hall, 2000; Lockshin and Zakeri, 2002). This evolutionary conserved form of cell suicide is defined by characteristic morphological features, which include cell shrinkage, chromatin condensation, membrane blebbing, and internucleosomal DNA fragmentation (Ellis et al., 1991; Clarke and Clarke, 1996; Nagata, 1997; Green, 1998; Messmer and Pfeilscifter, 2000; Stennicke and Salvesen, 2000; Wang, 2001). Apoptosis is coordinated by members of the caspase family of cysteine proteases. Caspases have been found in many multicellular organisms, and their role in programmed cell death is highly conserved.

Sea urchin embryos use a typical protective strategy against many kinds of stressful agents (Casano et al., 1998; Gianguzza et al., 2000; Roccheri et al., 1981, 1988, 1993, 2000, 2001; Casano et al., 2003) using the synthesis of stress proteins to cell self-defence. A prolonged exposure to stress induces a toxicity level that may activate apoptotic processes as a part of the defence strategy (Roccheri et al., 2004; Agnello et al., 2007; Campanale et al., 2011; Russo et al., 2014).

It has indeed been shown that sea urchin embryos possess the machinery for apoptosis when exposed to different agents (Sato and Yazaki, 1999; Voronina and Wessel, 2001). Previous studies on cell death in *P. lividus* sea urchin embryos showed that at the stages of gastrula and pluteus, treatment with combined phorbol ester (TPA) followed by an increase in temperature had a clear apoptotic effect, as revealed by DNA analysis and other apoptotic signals. In this study apoptosis was also found in some cells of non-treated plutei (Roccheri et al., 1997). Roccheri et al. (2002) later studied whether programmed cell death was a natural physiological process occurring in sea urchin embryos/larvae due to remodelling and cellular homeostasis during metamorphosis, as observed in the tadpole tail and intestine (Shi et al., 2001). *P. lividus* embryos at the early pluteus stage underwent spontaneous apoptosis. Observations of larval stages closer to metamorphosis confirmed that apoptosis was a physiological event necessary for development to adulthood. In particular, larvae at different developmental stages showed apoptotic cells in the oral and aboral arms, intestine, ciliary band and both apical and oral ganglia. Moreover, the number of apoptotic cells decreased in later larva stages, possibly because a smaller number of cells needs to be eliminated as the organism approaches metamorphosis (Roccheri et al., 2002).

The sequenced genome of the sea urchin *Strongylocentrotus purpuratus* permits an investigation into which parts of the vertebrate apoptotic toolkit are also present in other invertebrate deuterostomes (e.g. sea urchins). Furthermore, the availability of the recently sequenced genome of the sea anemone *Nematostella vectensis* (Sullivan et al., 2006), a cnidarian that provides an outgroup of the bilateria, allows us to investigate which parts of the apoptotic toolkit evolved prior to the emergence of bilaterians. Robertson et al. (2006) showed that the complexity of the genomic toolkit for apoptosis in sea urchins is comparable to, but qualitatively different from that of vertebrates, and greater than that of sea anemones, which is in turn greater than that of arthropods or nematodes. Apoptotic programmed cell death is infrequent and not obligatory during early embryogenesis of the purple sea urchin *S. purpuratus* (Vega Thurber and Epel, 2007). Moreover, homologues of death domain-linked TNFR family members, previously thought to be confined to vertebrates (Bridgham et al., 2003), are present in both sea urchins and sea anemones, indicating that the absence of this family in nematodes and arthropods is in fact due to gene loss. Furthermore, sea urchins have an unusually large number of caspases: there are 31 caspase genes in *S. purpuratus* and only 14 in vertebrates (Robertson et al., 2013). More specifically, while the set of effector caspases (caspases-3/7 and caspase-6) in sea urchins is similar to that found in other basal deuterostomes, signal-responsive initiator caspase subfamilies (caspases-8/10 and 9) have undergone echinoderm-specific expansions. In addition, there are two groups of divergent caspases, one distantly related to the vertebrate interleukin converting enzyme (ICE)-like subfamily, and a large clan that does not cluster with any of the vertebrate caspases. The complexity of proteins containing an anti-apoptotic BIR domain and of Bcl-2 family members approaches that of vertebrates, and is greater than that found in protostome model systems (*Drosophila* or *Caenorhabditis elegans*). Finally, the presence of Death receptor homologues, previously known only in vertebrates, in both *S. purpuratus* and *N. vectensis* suggests that this family of apoptotic signalling proteins evolved early in animals and was subsequently lost in the nematode and arthropod lineage(s).

In the work reported here, we tested if other oxylipins were able to induce apoptosis, focusing our attention on the proteases caspase-8, 3/7, because of their key roles in the activation of the apoptotic machinery. In addressing this aim, sea urchin embryos of *P. lividus* were treated with two different classes of diatom-derived oxylipins: i) two ecologically important, but relatively unknown, PUAs, heptadienal and octadienal, in comparison with the better-known PUA, decadienal; ii) four hydroxyacids, 5-, 9-, 11 and 15-HEPE, which represent the most abundant secondary metabolites in diatoms. The experiments were performed at higher concentrations to reveal induction of apoptosis by PUAs and HEPEs, measuring the activities of caspase-3/7 and caspase-8 by luminescent assays. Experiments were also performed at low concentrations to follow the expression levels of *caspase-8* and *caspase-3/7* genes by Real Time qPCR on treated embryos, to understand if these genes were switched on by PUAs and HEPEs. These experiments assume important ecological significance. In fact, in aquatic ecosystems a considerable proportion of the primary production from phytoplankton blooms sinks to the sediment and most benthic communities below the photic zone are entirely dependent on such imported organic matter (see below for further details).