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A marine diatom-derived aldehyde induces apoptosis in copepod and sea urchin embryos

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Summary

The diatom-derived aldehyde 2-*trans*-4-*trans*-decadienal (DD) was tested as an apoptogenic inducer in both copepod and sea urchin embryos, using terminal-deoxynucleotidyl-transferase-mediated dUTP nick-end labelling (TUNEL), DNA fragmentation profiling (laddering) and an assay for caspase-3 activity. DD induced TUNEL positivity and DNA laddering, but not caspase-like activation, in copepod embryos spawned by females fed for 10–15 days the diatom diet *Thalassiosira rotula* Meunier (*in vivo*), or when newly spawned eggs were exposed for 1 h to 5 μg ml^{-1} DD (*in vitro*). To our knowledge, this is the first time that evidence for an apoptotic process in copepods has been obtained by cytochemical (TUNEL) and biochemical (DNA fragmentation) approaches. The absence of caspase-like activity in copepod embryos suggests that caspase-independent programmed cell death occurs in these organisms. In sea urchin embryos, DD induced apoptosis and also activated a caspase-3-like protease. The saturated aldehyde decanal induced apoptosis at higher concentrations and after a longer incubation period than DD, indicating that α,β-unsaturation of the molecule, coupled with the aldehyde group, is responsible for the greater biological activity of DD. Since diatoms are an important food source for marine herbivores such as copepods and sea urchins, these findings may help explain why unsaturated aldehydes often induce reproductive failure, with important ecological consequences at the population level.

Key words: apoptosis, unsaturated aldehyde, copepod, sea urchin, embryo, caspase-3, diatom.

Introduction

Diatoms are unicellular algae, ubiquitously present in marine and freshwater habitats, that provide the bulk of the food sustaining top consumers and important fisheries. However, recent evidence has shown that certain diatom diets can seriously impair the egg hatching viability of zooplankton crustaceans such as copepods. Thus, while diatoms may be a nutritive food source for copepod larval growth, they often reduce hatching success, with birth-control effects at the population level (Ianora et al., 2003). This biological model is new and has no equivalent in marine plant–herbivore systems, since most negative plant–animal interactions are the consequence of repellency or poisoning, but never reproductive failure. The secondary metabolites responsible for these effects were recently identified as 2-*trans*-4-*cis*-7-*cis*-decatrienal, 2-*trans*-4-*trans*-7-*cis*-decatrienal and 2-*trans*-4-*trans*-decadienal, unsaturated aldehydes that block cell division in copepod and sea urchin embryos and arrest the proliferation of human carcinoma cells (Miralto et al., 1999).

The production of aldehydes is induced by damage of diatom cells, as would occur during grazing by copepods (Pohnert, 2000). Pohnert showed that this mechanism of cell defence is initiated by phospholipases, with a drastic increase in the amount of free polyunsatured eicosanoids in the first minutes after wounding. Using fluorescent probes, he suggested that the main enzyme activity responsible for initiation of the aldehyde-generating lipase–lipoxygenase–hydroperoxide lyase cascade is phospholipase A2 (PLA) (Pohnert, 2002).

In terrestrial environments, the production of aldehydes and other oxylipins in plants has often been associated with a wound-activated mechanism (Matsui et al., 2000; Rosahl, 1996), involving the sequential action of the above-mentioned enzymes: a lipase, lipoxygenase (LOX) and hydroperoxide lyase (HPL) (Blée, 1998). The aldehydes thus generated show pheromonal, bactericidal and fungicidal activities, providing in many cases a chemical defence against pathogens and herbivorous insects (Pohnert and Boland, 2002). It has also been suggested that in copepods such compounds are an activated chemical defence by diatoms to deter future generations of potential grazers.

Here, we examine for the first time the effects of the diatom-derived aldehyde decadienal (DD) on the apoptotic machinery of copepod and sea urchin embryos and compare the biological

activity of this unsaturated fatty aldehyde with the saturated aldehyde decanal. Apoptosis, or programmed cell death (PCD), is the result of complex signal transduction pathways leading to gene-mediated cell death. PCD is an evolutionarily conserved process, present in both the animal and plant kingdom. Apoptotic events induce morphological and biochemical alterations including cell shrinkage, disintegration through blebbing and activation of specific caspases that lead to enzymatic breakdown of DNA (Lockshin et al., 1998). PCD plays an essential role in physiological processes such as differentiation (Jacobson et al., 1997) and immune system regulation (Krammer, 2000; Nagata, 1997). Extensive apoptosis is active in the female germline of many species, ranging from worms to humans. Indeed, the functional life-span of the female gonads is defined by the size and rate of depletion of oocytes enclosed within follicles in the ovaries at birth. The physiological goal for germline apoptosis resides in the removal of defective cells unable to develop into fertile eggs, and to provide essential nutrients to the surviving oocytes (reviewed in Buszczak and Cooley, 2000; Morita and Tilly, 1999). Thus, any factor that disrupts the normal production of female gametes is a potential threat to reproductive performance. Potential hazards to gonadal function might be derived from environmental sources, and particularly affect females since, unlike males, females are born with an irreplaceable number of germ cells in their ovaries at the time of birth. For example, exposure of women to potentially damaging agents, such as anti-cancer drugs, industrial chemicals or even cigarette smoke, can have dramatic and irreparable effects on the ovary by accelerating the natural process of germ cell depletion (Tilly, 1998).

The results presented here demonstrate that the diatom-derived aldehyde DD triggers an apoptotic mechanism in copepod and sea urchin embryos. These results are discussed in the context of laboratory findings on the toxic effects of diatoms on potential grazers such as copepods and sea urchins.

Materials and methods

Copepod embryo collection

Zooplankton were collected in the Gulf of Naples, from February to April 2000, using a 200 µm plankton mesh net, and transported within 1 h to the laboratory. Adult females of the copepod *Calanus helgolandicus* (Claus) were sorted and incubated individually in 100 ml crystallizers containing 0.22 µm filtered sea water (FSW) and the diatom *Thalassiosira rotula* (THA) at final concentrations ranging from 10^4 to 10^5 cells ml^{-1} for 10–15 days. Each day, females were transferred to new containers with FSW and fresh algae. Embryos were collected daily and fixed overnight with 2–4% paraformaldehyde in phosphate buffer solution (PBS), pH 7.4 and 0.2 mol l^{-1} NaCl.

A second group of *C. helgolandicus* females were incubated in 100 ml crystallizers containing the control algae *Prorocentrum minimum* (PRO), a dinoflagellate that does not impair copepod embryogenesis (Turner et al., 2001). Embryos produced by females fed PRO for 24 h were collected at the 8- to 32-blastomere stage and fixed as described above, or incubated for 1 h in 5 µg ml^{-1} of DD or in 5 µg ml^{-1} decanal. DD and decanal solutions were prepared by diluting commercial compounds (Sigma-Aldrich, Milan, Italy) in methanol to obtain 2 mg ml^{-1} stock solutions. Copepod embryos were also incubated for 1 h in 0.5% methanol to exclude unspecific effects of this solvent.

Copepod embryos used for DNA fragmentation experiments were obtained from females fed PRO for 24 h, and incubated in DD as described above.

Sea urchin embryo collection

Sea urchins *Paracentrotus lividus* (Lamarck) were collected by SCUBA diving in the Gulf of Naples and transported to the laboratory. Soon after their arrival, living organisms were injected with 0.5 mol l^{-1} KCl to induce gamete ejection. Spawned eggs were allowed to settle and were then washed three times with FSW and diluted to a final concentration of 3000 eggs ml^{-1}. Concentrated sperm were collected and diluted immediately prior to fertilization in FSW. Fertilization occurred in FSW for enzymatic bioassays and in FSW with 1 mmol l^{-1} ATA (3-amino-1,2,4-triazole; Sigma-Aldrich) for immuno-fluorescence staining (Buttino et al., 1999). 90 min after fertilization, when the third mitotic division was completed, embryos were incubated in 5 ml tissue culture wells containing 2, 5 and 10 µg ml^{-1} (final concentration) of DD or decanal, prepared as described above. A different group was used as controls. To test the effect of the solvent, another group of embryos was incubated in 0.5% methanol, which was the highest concentration of solvent used in the incubation experiments. The percentage of embryos presenting membrane blebbing was determined every 30 min for each concentration of DD, decanal and methanol.

Fluorescence labelling and confocal microscopy

C. helgolandicus embryos, incubated in DD, decanal or methanol, were washed three times in FSW before fixation in 2–4% paraformaldehyde overnight. Fixed embryos were then rinsed several times in PBS and immediately stained with TUNEL (terminal-deoxynucleotidyl-transferase-mediated dUTP Nick End Labelling; Roche Diagnostics GmbH, Mannheim, Germany), or stored at 4°C in PBS containing 0.02% NaN_3, until fluorescence labelling.

Before TUNEL staining, copepod embryos were incubated for 24 h in 250 µl of 1 U ml^{-1} chitinase enzyme (EC3.2.1.14; Sigma-Aldrich) dissolved in 50 mmol l^{-1} citrate buffer, pH 6, at 25°C, to permeabilize the chitinous wall. After rinsing several times in PBS, embryos were incubated for 2 h in 0.1% Triton X-100 at room temperature, rinsed in PBS containing 1% BSA, and further incubated for 90 min in TUNEL solution at 37°C. To obtain TUNEL-positive samples, embryos were incubated for 10 min in 50 mmol l^{-1} Tris-HCl, pH 7.5, 10 mmol l^{-1} $MgCl_2$, 0.1% dithiothreitol, containing 250 µg ml^{-1} DNase I (grade II from bovine pancreas; Boheringer GmbH, Mannheim, Germany) at room

temperature. Negative controls were obtained by incubating embryos in label solution only, as recommended by the manufacturers of the TUNEL kit.

8-blastomere sea urchin embryos, incubated for 30, 60, 90 and 120 min in DD or in decanal, for 30 and 120 min in 0.5% methanol and controls, were gently forced through a Pasteur pipette to remove the fertilization envelope, rinsed three times in FSW, and fixed for 1 h in 4% paraformaldehyde dissolved in PBS and 0.2 mol l^{-1} NaCl, at room temperature. Fixed sea urchin embryos were washed several times in PBS, pH 7.4, to remove paraformaldehyde and then incubated for 1 h in a solution of 0.1% Triton X-100 and 0.1% sodium citrate, at 4°C. After washing in PBS containing 1% bovine albumin serum (BSA, Sigma-Aldrich) samples were incubated at 37°C for 90 min in TUNEL solution in a humidified chamber, in the dark. Control embryos were fixed and stained as described above. Before staining with TUNEL, a group of embryos that were not incubated in DD were used as positive and negative controls. Positive and negative controls were obtained as described above for copepod embryos.

Whole-mount sea urchin and copepod embryos were observed with an inverted Zeiss LSM-410 confocal laser scanning microscope equipped with a 40× water immersion objective (NA 1.2). Each image was acquired with an Argon 488 nm wavelength (λ) laser to detect TUNEL fluorescence (green), and with a 633 nm λ laser to visualize samples in transmitted light. Images were reconstructed three-dimensionally using the Zeiss software.

DNA extraction and fragmentation

DNA was extracted from *C. helgolandicus* embryos incubated for 1, 3 and 6 h with 5 μg ml^{-1} of DD, and their untreated controls, after initial digestion with chitinase as follows. Embryos stored at –80°C were thawed and resuspended in 25 μl of 50 mmol l^{-1} citrate buffer, pH 6.0, before addition of an equal volume of chitinase dissolved in the same buffer to a final concentration of 10 U ml^{-1}. Incubation was continued for 16 h at 25°C, followed by addition of a second amount of enzyme (10 U ml^{-1}) and incubation for a further 2 h. Subsequently, genomic DNA was extracted using a commercially available kit (Nuclespin nucleic acid purification kit), following the manufacturer's instructions (Clontech, Palo Alto, CA, USA). The total amount of DNA recovered from 300–800 embryos was 1–4 μg. Amplification of the 16S rRNA gene by polymerase chain reaction (PCR) was carried out as a positive control for DNA extraction, as reported by Bucklin et al. (1995). Using 100–200 ng of template, and the 16SAR (5′-CGCCTGTTTAAC-AAAAACAT-3′) and 16SBR primers (5′-CGGTTTGAA-CTCAGATCACGT-3′), PCR was performed on a MJ (Waltham, MA, USA) apparatus (Bucklin et al., 1995).

DNA fragmentation was measured directly on recovered DNA by loading samples of extracted DNA (approx. 1 μg) onto 2% agarose gels, and analysis by ethidium bromide staining. Alternatively, DNA samples were subjected to LM (ligation mediated)-PCR, a technique designed for the detection of nucleosomal ladders in apoptotic cells (Staley et al., 1997). A commercially available kit (Clontech) was used to visualise ladders that were undetectable by other methods. Briefly, fragmented DNA was ligated with adaptor primers and these were amplified using PCR and a 24-mer primer. The resulting ladder was visualized on an agarose gel stained with ethidium bromide.

Assay for caspase-like activity

Approximately 3000 sea urchin embryos were incubated for 30, 60, 90 and 120 min in 5 or 10 μg ml^{-1} DD, rinsed three times in FSW, transferred to a 0.5 ml tube and centrifuged for 3 min at 1700 ***g***. The pelleted embryos were stored at –80°C until the enzymatic assay was performed. Copepod embryos were collected 1, 3, 6 and 12 h after incubation with 5 μg ml^{-1} DD, and prepared using the same protocol as sea urchin embryos. Frozen embryos were resuspended in lysis buffer (50 mmol l^{-1} Tris-Cl, pH 7.6, 150 mmol l^{-1} NaCl, 5 mmol l^{-1} EDTA, 1% Nonidet NP-40, 0.5 mmol l^{-1} dithiothreitol, 10% glycerol, 100 μg ml^{-1} phenylmethanesulfonyl fluoride, plus a cocktail of protease inhibitors: 'Complete'; Boehringer) and sonicated twice for 20 s at 20% of the maximum potency with a Sonfier 250 (Branson Ultrasonic Corporation, Danbury, CT, USA). The FlorAce Apopain Assay Kit (Bio-Rad Laboratories, Hercules, CA, USA) was used to detect caspase-3-like activity. The kit contains a fluorescent substrate, the acetylated peptide Ac-DEVD-AFC (carbobenzoxy-Asp-Glu-Val-Asp-7-amino-4-trifluoromethyl coumarin), which releases the fluorescent AFC moiety after enzymatic hydrolysis. Samples (40 μl) were incubated with 55 μmol l^{-1} Ac-DEVD-AFC substrate at 37°C for 2 h. Apopain included in the kit was used as a positive control for caspase-3 activity. The caspase-3-specific inhibitor Z-DEVD-FMK was employed to determine and quantify the non-specific caspase-3 activity in the extracts, following the manufacturer's instructions (Bio-Rad Laboratories). AFC fluorescence was detected using a Perkin–Elmer spectrophotometer LS50B at 395 nm excitation and 540 nm emission wavelengths. Protein concentrations were determined using the method of Bradford (1976). To detect other caspase-like enzymes in the homogenate samples, a caspase-family fluorimetric substrate set (Biovision, purchased from Alexis Corporation, Lausen, Switzerland) was used under the same conditions. The set contained substrates for caspases 1, 2, 5, 6, 8 and 9.

Results

Apoptosis is generally assessed by using different assays, including measurements of morphological and biochemical parameters, in conjunction with cytochemical staining (TUNEL). Copepod embryos are not transparent when viewed by light microscopy, however, so simple morphological analysis cannot be considered adequate as a test for apoptotis.

Two approaches were employed to verify the ability of DD to induce apoptosis in copepods. (1) *In vivo* experiments. *C. helgolandicus* embryos were obtained from females fed the

diatom THA for 10–15 days, the time necessary to induce about 35% hatching failure (Chaudron et al., 1996). (2) *In vitro* experiments. Newly spawned embryos from females fed the control diet PRO were directly exposed to 5 μg ml^{-1} DD. In *in vivo* experiments, immunofluorescence assays revealed that 30±12.3% (mean ± S.D., *N*=200) of the embryos were positively stained with TUNEL (Fig. 1). Fig. 1A shows a TUNEL-positive embryo in which apoptotic nuclei appear green. Chromatin is condensed without any apparent apoptotic bodies characteristic of late apoptosis; however, distribution of the nuclei is asymmetrical and, in transmitted light, the embryo appears abnormal with an irregular shape (Fig. 1B). It is known that *C. helgolandicus* females fed the non-diatom algae PRO produce almost 100% viable embryos (Poulet et al., 1994), but induction of apoptosis was observed in 61.7±22.5% (mean ± S.D.) of PRO-fed embryos incubated for 60 min with 5 μg ml^{-1} DD (Fig. 1C,D). Fig. 1E,F shows a control embryo produced by *C. helgolandicus* females fed PRO in which TUNEL staining is negative with nuclei appearing dark and symmetrically distributed (Fig. 1E). The green fluorescent background was obtained by amplifying the brightness to highlight unstained nuclei. Positive controls, incubated with DNase, have fluorescent nuclei, like the DD-incubated embryos (Fig. 1G,H). After 1 h of incubation, decanal induced apoptosis in 23.7±19.4% (mean ± S.D., *N*=100) embryos. After incubation in methanol the percentage of TUNEL-positive embryos was 14.0±9.9% (mean ± S.D., *N*=100), similar to that observed in untreated embryos (data not shown).

Since treatment of *C. helgolandicus* embryos with DD revealed an apoptogenic phenotype, we tried to detect DNA fragmentation in embryos that had been treated for 1–6 h with 5 μg ml^{-1} of DD. Initially, DNA recovery was very low, probably due to the external chitinous wall of the embryos, but this problem was overcome by solubilisation with chitinase before beginning the DNA extraction protocol, allowing us to obtain enough DNA for further analysis. As a positive control, we amplified the 16S rRNA gene using specific primers, and obtained a single specific fragment of approximately 460 bp (Fig. 2A) similar to that reported by other authors (Bucklin et al., 1995; Lindeque et al., 1999). Subsequently, these DNA samples were analysed for DNA fragmentation. Fig. 2B shows an example DNA ladder obtained from copepod embryos that had been treated with DD. For each length of treatment, the same amount of DNA isolated from untreated and DD treated embryos was loaded on agarose gels. Table 1 summarizes the results obtained from copepod embryos incubated for different times with DD. Incubation for 1 h in the presence of 5 μg ml^{-1} of DD was sufficient to induce clear, detectable DNA fragmentation into oligonucleosomal DNA fragments compared to untreated embryos (no detectable fragmentation).

The effects of DD were tested on sea urchin embryos at the 8-blastomere-cell stage and the results compared with those obtained for copepod embryos. Progression through apoptosis was observed by morphological cell changes, chromatin condensation and degradation, and by activation of caspases. Changes in cell morphology, such as cell shrinkage and membrane blebbing, were analysed by light microscopy after incubation at three different DD concentrations (Fig. 3). A concentration of 2 μg ml^{-1} induced blebbing in >20% of embryos 120 min after incubation and in 50% of embryos after

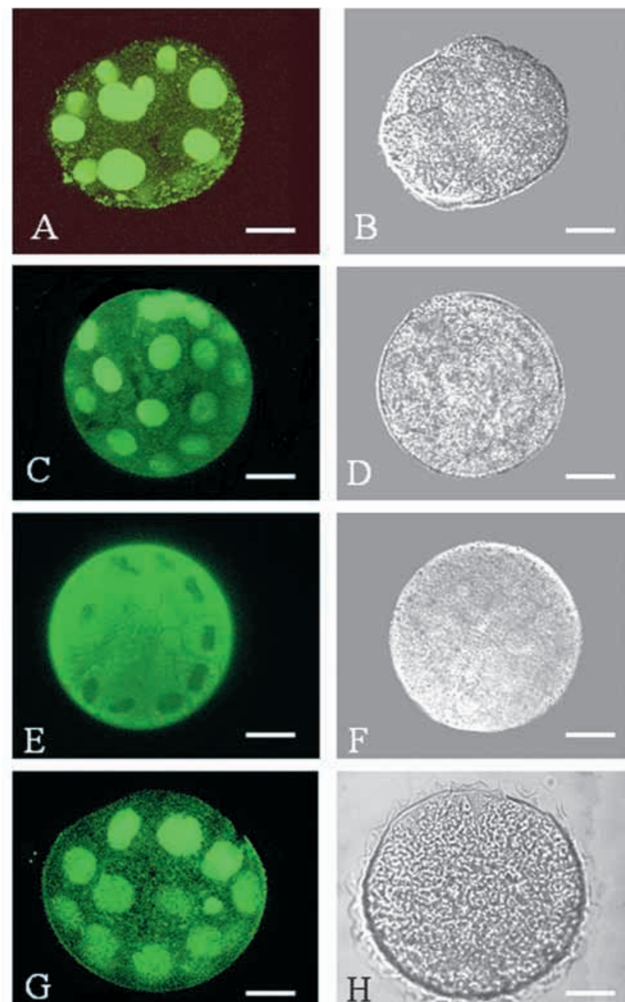

Fig. 1. *Calanus helgolandicus* embryos after TUNEL staining and observed by confocal laser scanning microscopy in fluorescence (A,C,E,G) and transmitted (B,D,F,H) light. (A) Fluorescent, three-dimensional image of an embryo produced by females fed for 10 days with the diatom *Thalassiosira rotula*. Nuclei are positively stained (green) by the TUNEL. Bar, 39.7 μm. (B) The same embryo as in A observed in transmitted light. Bar, 42.1 μm. (C) Fluorescent three-dimensional image of a *C. helgolandicus* embryo produced by female fed non-diatom *Prorocentrum minimun* (PRO) algae for 24 h then incubated for 1 h in 5 μg ml^{-1} DD. Nuclei (green) are positively stained by TUNEL. Bar, 40 μm. (D) Embryo in C observed in transmitted light. (E) Three-dimensional image of the embryo produced by female fed 24 PRO. Nuclei are not stained in green and appear as black shadows. Bar, 42.2 μm. (F) The same embryo as in E observed in transmitted light. Bar, 40.3 μm. (G) Three-dimensional fluorescent image of a TUNEL-positive control embryo obtained by female fed for 24 h with PRO and incubated in DNase to simulate apoptosis. Nuclei are stained in green as in A and C. Bar, 34.6 μm. (H) The same embryo as in G observed in transmitted light. Bar, 34.6 μm.

Table 1. *DNA fragmentation determined from copepod embryos treated with decadienal*

Sample	Time of treatment with DD (h)	Ladder intensity*
1	1	++
5	1	+
18	1	+++
31	Ctrl	ND
6	3	++
12	3	++++
9	Ctrl*	ND
22	6	++++

DD, decadienal (5 µg ml^{-1}).

*Increasing numbers of + symbols indicate increasing DNA ladder intensity.

Ctrl, untreated embryos.

ND, not detectable.

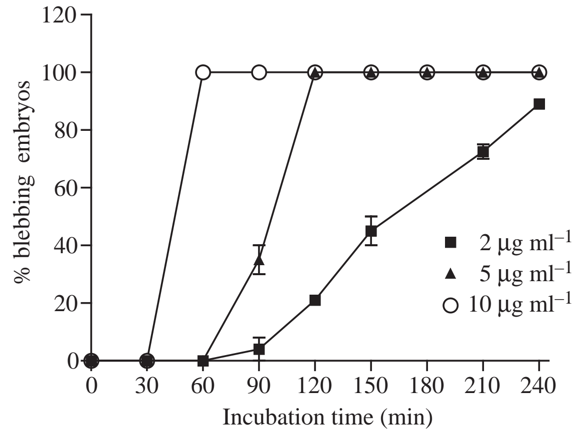

Fig. 3. Induction of membrane blebbing following DD treatment. Sea urchin embryos were treated with the indicated concentrations of DD and the percentage of cell blebbing was determined every 30 min. Values (means ± S.D.; N=600) are the results of three different experiments.

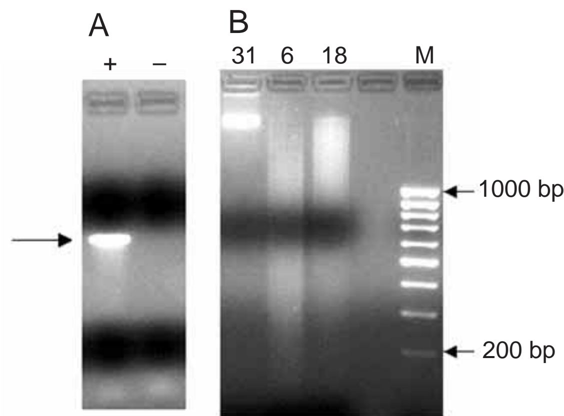

Fig. 2. (A) PCR amplification of DNA isolated from copepod embryos using the 16SAR and 16SBR primers, as reported in Materials and methods. The arrow indicates the presence of a single PCR product of approximately 430 bp. The presence (+) or absence (–) of template DNA is shown above. (B) DNA laddering of two DNA samples (6, 18) isolated from copepod embryos treated with 5 µg ml^{-1} of DD for 3 and 1 h, respectively. Sample 31 represents an untreated control. Semiquantitative analysis of the fragmentation extent is reported in Table 1. M, markers (DNA Ladder 100; from top to bottom: 1000, 900, 800, 700, 600, 500, 400, 300, 200 bp).

150 min. After 240 min, >90% of embryos showed vesciculated membranes. A concentration of 5 µg ml^{-1} of DD induced blebbing in 50% of embryos after almost 100 min, and 100% after 120 min incubation. All embryos incubated in 10 µg ml^{-1} DD showed plasma membrane blebbing 45 min after incubation. At all concentrations tested, mitotic divisions were arrested and embryos remained blocked at the 8-blastomere stage. In contrast, cell division in sea urchin embryos incubated in decanal was arrested only at concentrations >10 µg ml^{-1} and blebbing was never observed. Methanol incubation had no effect on cell division (data not shown).

TUNEL-positivity was detected using laser confocal microscopy. Fig. 4A shows an untreated sea urchin embryo at the 16-blastomere stage, fixed 3 h after fertilization. Green fluorescence, due to TUNEL labelling, was not evident, indicating the absence of DNA fragmentation. Fig. 4B shows the same embryo in transmitted light with symmetrically dividing cells. Fig. 4C,D shows the effect of 90 min incubation with 5 µg ml^{-1} DD: the embryo was blocked at the 8-blastomere stage; nuclei appeared green when observed for TUNEL fluorescence, indicating they had undergone apoptosis. Although the transmitted light image showed an apparently normal embryo with 8 symmetrical blastomeres (Fig. 4D), the fluorescent image clearly showed that nuclei had been subjected to DNA fragmentation, identified by single strand breaks. After 120 min incubation with 5 µg ml^{-1} DD, embryos appeared morphologically abnormal, with small, condensed fragments of green fluorescent chromatin (TUNEL) (Fig. 4E). Parallel transmitted light images revealed a very abnormal embryo (Fig. 4F).

Fig. 5 shows that the number of apoptotic embryos increased with time of incubation in 5 µg ml^{-1} DD. In fact, more than 40% of the embryos were positive for TUNEL after 1 h incubation in DD; the percentage increased to about 80% after 90 min of incubation. In control embryos, a physiological apoptotic process occurred in less than 20% of the embryos (N=200). The same results were obtained when embryos were incubated in methanol. The percentage of TUNEL-positive embryos incubated in 5 µg ml^{-1} decanal is similar to the controls until 90 min of incubation, after which it increased to 43.3±2.26% (mean ± S.D.).

As a biochemical marker for apoptotic processes in sea urchin embryos treated with DD, we tested the ability of DD to activate caspase-like enzymes using several commercially available kits developed for vertebrate caspases. None of the caspases 1, 2, 4, 6, 8 and 9 were active in our experiments (G. Romano, unpublished data). We detected caspase-3-like

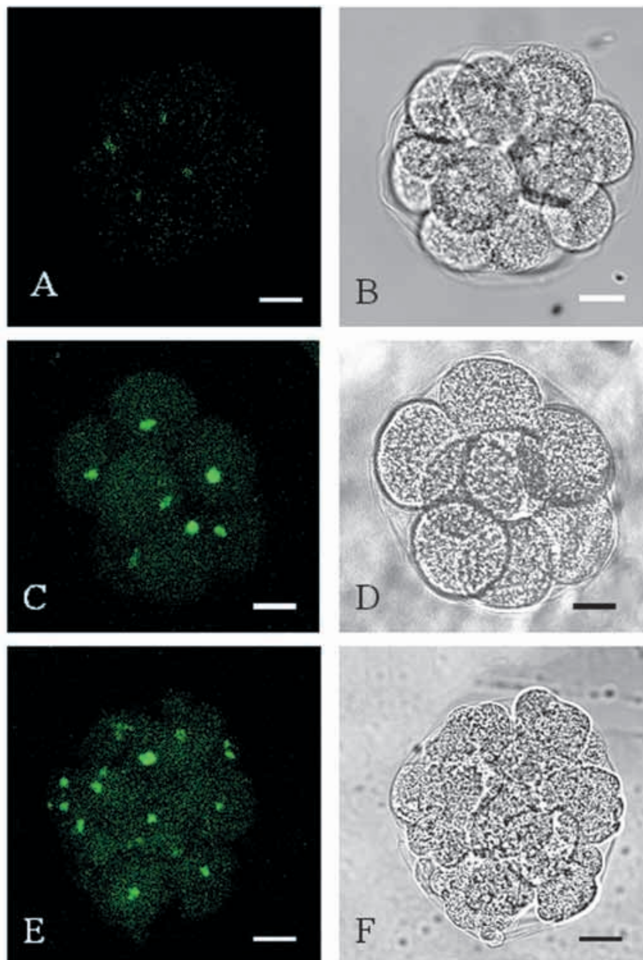

Fig. 4. Sea urchin embryo observed by confocal laser scanning microscopy in fluorescent (A,C,E) and in transmitted (B,D,F) light. (A) Fluorescent three-dimensional image of the untreated embryo (16-blastomere stage) stained with TUNEL. Bar, 30.6 µm. (B) The same embryo as in A observed in transmitted light. Bar, 28.2 µm. (C) Fluorescent three-dimensional image of the sea urchin embryo incubated at the 8-blastomere stage in 5 µg ml^{-1} DD for 60 min and stained with TUNEL. Bar, 29.8 µm. (D) The same embryo as in C observed in transmitted light. Bar, 26.8 µm. (E) Fluorescent three-dimensional image of the sea urchin embryo incubated at the 8-blastomere stage in 5 µg ml^{-1} DD for 120 min and stained with TUNEL. Bar, 30.6 µm. (F) The same embryo as in E observed in transmitted light. Bar, 28.9 µm.

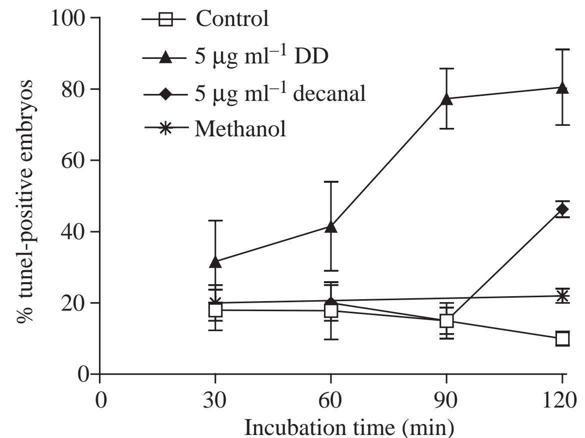

Fig. 5. Percentage of TUNEL-fluorescent apoptotic sea urchin embryos observed by confocal laser scanning microscopy. 8-blastomere embryos were incubated in 5 µg ml^{-1} DD or 5 µg ml^{-1} decanal for 30, 60, 90 and 120 min, and then stained with TUNEL. See Materials and methods for details. Values (means ± S.D.; *N*=200) are the results of three different experiments.

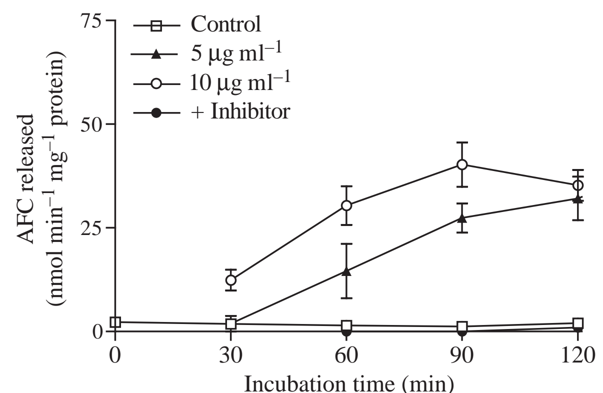

Fig. 6. Caspase-3-like activity in sea urchin embryos. Caspase-3-like activity was measured as described in Materials and methods. The amount of the fluorescent AFC moiety released during the reaction was related to the incubation time in DD. Values (means ± S.D.) are the results of three different experiments.

activity only in sea urchin embryos after 60 min of exposure to 5 µg ml^{-1} of DD (Fig. 6), with maximum activity after incubation for 120 min. After this time, embryos appeared degenerated (Fig. 4). When the assay was performed in the presence of a caspase-3 specific inhibitor (ZVAD-FMK), release of the AFC moiety fell to zero, indicating that the proteolytic activity measured was attributable specifically to a sea urchin caspase-3-like enzyme. Treatment of the embryos with a higher concentration of DD (10 µg ml^{-1}) induced an earlier and more pronounced activation of caspase-3 activity, suggesting a dose-dependent effect between DD and enzyme activation. Caspase-3-like activity was also tested on copepod embryos but we were unable to detect any activity (data not shown).

Discussion

In the present paper we show, for the first time, that the diatom-derived aldehyde DD induces apoptosis in copepod and sea urchin embryos. DD belongs to a family of α,β-unsaturated aldehydes that originate from enzymatic lipid peroxidation when cells are mechanically broken or otherwise damaged (d'Ippolito et al., 2003; Pohnert and Boland, 2002). Molecules of this category are particularly reactive due to the characteristic structural features of α,β-unsaturation (Kirichenko et al., 1996; Refsgaard et al., 2000) that render

them more reactive towards various biological macromolecules, most of which are associated with the induction of apoptosis (Tang et al., 2002). By contrast, saturated aldehydes are more stable and relatively non-toxic (Bruenner et al., 1994; Kruman et al., 1997; Spiteller, 2001). Recently, Adolph et al. (2003) reported that the antiproliferative activity of diatom-derived fatty aldehydes on sea urchin embryos depends on the degree of unsaturation and length of the chain, and that saturated aldehydes were biologically less active than unsaturated aldehydes of the same chain length. We here confirm that the apoptogenic activity of an unsaturated diatom aldehyde (decadienal) is greater than that of a saturated aldehyde (decanal) in both sea urchin and copepod embryos. In fact, exposure of sea urchin embryos to 5 $\mu g\ ml^{-1}$ decanal induced apoptosis only after 120 min whereas embryos treated with the same concentration of DD underwent apoptosis after 30 min. In copepods, following 1 h of incubation in 5 $\mu g\ ml^{-1}$ of decanal, 24% of embryos had apoptotic nuclei, whereas after incubation in DD the proportion increased to 61.7%. Also Tosti et al. (2003) reported that DD acted specifically as a fertilization channel inhibitor in ascidian oocytes at micromolar concentrations, while formaldehyde and acetaldehyde did not affect current fertilisation even at concentrations 1 or 3 orders of magnitude higher, respectively.

DD was previously reported to induce apoptosis in mammalian tumour cells (Miralto et al., 1999). Here we demonstrate that DD also induces apoptosis in copepod and sea urchin embryos at micromolar concentrations. In copepods, DNA fragmentation was detected both in embryos spawned by females fed the diatom THA, and in embryos spawned by females fed the control diet PRO and then incubated directly in DD. This is the first time that DNA fragmentation has been detected in copepods using two independent but complementary techniques, such as laddering and TUNEL bioassays. Similarly to the observation in copepods, DD induced TUNEL positivity and blebbing in 8- to 16-blastomeres of sea urchin embryos. Mitotic divisions were arrested at all DD concentrations tested, whereas apoptogenic phenotype appearance was time- and dose-dependent. Interestingly, in both systems, the appearance of the apoptotic phenotype followed the same kinetics, peaking after 90–120 min after exposure to DD, which suggests possible similarities between the two apoptogenic pathways.

Programmed cell death (PCD) is a process that is generally classified into two main categories, depending on whether a class of proteolytic enzymes known as caspases are activated or not. In the so-called caspase-dependent cell death, activation of these enzymes is absolutely required to induce late and irreversible apoptotic processes, such as DNA fragmentation and cell blebbing (Krammer, 2000; Lockshin et al., 1998). By contrast, in the caspase-independent PCD, caspase activity is not required (Mathiasen and Jaattela, 2002). In the present study, we did not detect caspase activity in copepod embryos treated with DD using either enzymatic assays or immunoblotting as evidence of proteolytic activation of caspases (data not reported), which suggests that the PCD was caspase-independent. By contrast, we could detect caspase-3 activity in sea urchin embryos treated with DD using a commercially available kit designed for the mammalian version of caspase-3. We clearly measured increased caspase-3 activity in sea urchin embryos 60 min after addition of DD. Caspase-3 activity was tested for two reasons: because it is conserved in animals ranging from worms (*Caenorhabditis elegans*) to humans, and because caspase-3 is a downstream member of the caspase cascade, so its enzymatic activity is enhanced compared to most upstream caspases, such as caspase-8. At the present time, we cannot exclude the possibility that the assay employed was not sensitive enough for copepods, or that the substrate used for caspase-3 activity was not specific for the copepod enzyme. Future work will help clarify this aspect.

Activation of caspases in sea urchins has also been reported for embryos exposed to staurosporine (Voronina and Wessel, 2001). These authors observed differences in apoptotic response between oocytes, eggs and early sea urchin embryos, suggesting that activation of apoptotic pathways may differ depending on the developmental stage. Although we did not characterised sea urchin caspase-3 activity in detail, we can speculate that it is probably structurally different from its vertebrate homologs, since the K_m for the peptide used as a substrate (Ac-DEVD-AFC) appeared relatively higher than that of the human enzyme used as a positive control (G. L. Russo, unpublished data). We also tried to detect other caspase-like activity in sea urchins using substrates available for mammalian caspases 1, 2, 5, 6, 8 and 9, without any success (G. Romano, unpublished data). The time course of caspase-3 activation correlated perfectly with the appearance of apoptotic nuclei determined by TUNEL. This is unusual in a caspase-dependent PCD process, where caspase-3 activation normally precedes DNA fragmentation and blebbing. Other studies have reported that caspases may be activated even if not required for the progression of apoptosis (Johnson et al., 1999), and DNA fragmentation may be caused by mechanisms other than caspase-3 activation of DNase (Hamilton et al., 1998). Hence increased caspase-3 activity may be an epiphenomenon, not the cause, of late events such as DNA laddering and cell blebbing. The correspondence of these two events, caspase-3 activation and blebbing, in sea urchin embryos may indicate the presence of a caspase-independent PCD. This observation strengthens the hypothesis that DD also induces a caspase-independent apoptosis in copepod embryos, where, in fact, caspase activity was not found.

Recently Pohnert and Boland (1996) and Pohnert et al. (2002) reported the presence of an aldehydic compound with similar reactive unsaturation and biological activity to DD, the oxoacid 12-oxo-5-*cis*-8-*cis*-10-*trans* dodecatrienoic acid, in benthic diatoms, and showed that this molecule blocked cell division in sea urchin embryos. Hence sea urchins, which actively feed on benthic diatoms, are potentially exposed to the effects of unsaturated reactive compounds, as are copepods that have already been shown to graze on pelagic diatoms containing DD and other α,β-unsaturated aldehydes (Ianora et

al., 2003 and references therein). The concentration of diatom cells used in our *in vivo* experiments are of the same order of magnitude reached in bloom conditions at sea (Miralto et al., 2003), suggesting that diatom blooms can potentially impact the reproductive fitness of grazers, with important consequences at the population level. Clearly more investigations are needed to clarify the molecular targets of diatom-derived α,β-unsaturated aldehydes, to better understand their antiproliferative activity.

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References

Adolph, S., Poulet, S. A. and Pohnert, G. (2003). Synthesis and biological activity of alpha,beta,gamma,delta- unsaturated aldehydes from diatoms. *Tetrahedron* **59**, 3003-3008.

Blée, E. (1998). Phytooxylipins and plant defence reactions. *Prog. Lipid Res.* **37**, 33-72.

Bradford, M. M. (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* **72**, 248-254.

Bruenner, B. A., Jones, A. D. and German, J. B. (1994). Maximum-entropy deconvolution of heterogeneity in protein modification – protein adducts of 4-hydroxy-2-nonenal. *Rapid Commun. Mass Spect.* **8**, 509-512.

Bucklin, A., Frost, B. W. and Kocher, T. D. (1995). Molecular systematics of six *Calanus* and three *Metridia* species (Calanoida: Copepoda). *Mar. Biol.* **121**, 655-664.

Buszczak, M. and Cooley, L. (2000). Eggs to die for: cell death during *Drosophila* oogenesis. *Cell Death Differ.* **7**, 1071-1074.

Buttino, I., Miralto, A., Ianora, A., Romano, G. and Poulet, S. A. (1999). Water-soluble extracts of the diatom *Thalassiosira rotula* induce aberrations in embryonic tubulin organisation of the sea urchin *Paracentrotus lividus*. *Mar. Biol.* **134**, 147-154.

Chaudron, Y., Poulet, S. A., Laabir, M., Ianora, A. and Miralto, A. (1996). Is hatching success of copepod eggs diatom density-dependent? *Mar. Ecol. Prog. Ser* **144**, 185-193.

d'Ippolito, G., Romano, G., Caruso, T., Spinella, A., Cimino, G. and Fontana, A. (2003). Production of octadienal in the marine diatom *Skeletonema costatum*. *Organic Lett.* **5**, 885-887.

Hamilton, R. F., Li, L., Eschenbacher, W. L., Szweda, L. and Holian, A. (1998). Potential involvement of 4-hydroxynonenal in the response of human lung cells to ozone. *Am. J. Physiol. Lung Cell. Mol. Physiol.* **18**, L8-L16.

Ianora, A., Poulet, S. A. and Miralto, A. (2003). The effects of diatoms on copepod reproduction: a review. *Phycologia* **42**, 351-363.

Jacobson, M., Weil, M. and Raff, M. (1997). Programmed cell death in animal development. *Cell* **88**, 347-354.

Johnson, K. L., Grubb, D. R. and Lawen, A. (1999). Unspecific activation of caspases during the induction of apoptosis by didemnin B in human cell lines. *J. Cell. Biochem.* **72**, 269-278.

Kirichenko, A., Li, L., Morandi, M. T. and Holian, A. (1996). 4-Hydroxy-2-nonenal-protein adducts and apoptosis in murine lung cells after acute ozone exposure. *Toxicol. Appl. Pharmacol.* **141**, 416-424.

Krammer, P. (2000). CD95's deadly mission in the immune system. *Nature* **407**, 789-795.

Kruman, I., BruceKeller, A. J., Bredesen, D., Waeg, G. and Mattson, M. P. (1997). Evidence that 4-hydroxynonenal mediates oxidative stress-induced neuronal apoptosis. *J. Neurosci.* **17**, 5089-5100.

Lindeque, P. K., Harris, R. P., Jones, M. B. and Smerdon, G. R. (1999). Simple molecular method to distinguish the identity of *Calanus* species (Copepoda: Calanoida) at any developmental stage. *Mar. Biol.* **133**, 91-96.

Lockshin, R. A., Zakeri, Z. and Tilly, J. L. (1998). *When Cells Die: A Comprehensive Evaluation of Apoptosis and Programmed Cell Death*. New York: Wiley-Liss.

Mathiasen, I. S. and Jaattela, M. (2002). Triggering caspase-independent cell death to combat cancer. *Trends Mol. Med.* **8**, 212-220.

Matsui, K., Kurishita, S., Hisamitsu, A. and Kajiwara, T. (2000). A lipid-hydrolysing activity involved in hexenal formation. *Biochem. Soc. Trans.* **28**, 857-860.

Miralto, A., Barone, G., Romano, G., Poulet, S. A., Ianora, A., Russo, G. L., Buttino, I., Mazzarella, G., Laabir, M., Cabrini, M. et al., (1999). The insidious effect of diatoms on copepod reproduction. *Nature* **402**, 173-176.

Miralto, A., Guglielmo, L., Zagami, G., Buttino, I., Granata, A. and Ianora, A. (2003). Inhibition of population growth in the copepods *Acartia clausi* and *Calanus helgolandicus* during diatom blooms. *Mar. Ecol. Prog. Ser* **254**, 253-268.

Morita, Y. and Tilly, J. L. (1999). Oocyte apoptosis: like sand through an hourglass. *Dev. Biol.* **213**, 1-17.

Nagata, S. (1997). Apoptosis by death factor. *Cell* **88**, 355-365.

Pohnert, G. (2000). Wound-activated chemical defense in unicellular planktonic algae. *Angew. Chem. Int. Edit.* **39**, 4352-4354.

Pohnert, G. (2002). Phospholipase A(2) activity triggers the wound-activated chemical defense in the diatom *Thalassiosira rotula*. *Plant Physiol.* **129**, 103-111.

Pohnert, G. and Boland, W. (1996). Biosynthesis of the algal pheromone hormosirene by the freshwater diatom *Gomphonema parvulum* (Bacillariophyceae). *Tetrahedron* **52**, 10073-10082.

Pohnert, G. and Boland, W. (2002). The oxylipin chemistry of attraction and defense in brown algae and diatoms. *Nat. Prod. Rep.* **19**, 108-122.

Pohnert, G., Lumineau, O., Cueff, A., Adolph, S., Cordevant, C., Lange, M. and Poulet, S. (2002). Are volatile unsaturated aldehydes from diatoms the main line of chemical defence against copepods? *Mar. Ecol. Prog. Ser.* **245**, 33-45.

Poulet, S. A., Ianora, A., Miralto, A. and Meijer, L. (1994). Do diatoms arrest embryonic development in copepods? *Mar. Ecol. Prog. Ser.* **111**, 79-86.

Refsgaard, H. H. F., Tsai, L. and Stadtman, E. R. (2000). Modifications of proteins by polyunsaturated fatty acid peroxidation products. *Proc. Natl. Acad. Sci. USA* **97**, 611-616.

Rosahl, S. (1996). Lipoxygenases in plants – Their role in development and stress response. *Z. Naturforsch.* **51c**, 123-138.

Spiteller, G. (2001). Lipid peroxidation in aging and age-dependent diseases. *Exp. Gerontol.* **36**, 1425-1457.

Staley, K., Blaschke, A. J. and Chun, J. (1997). Apoptotic DNA fragmentation is detected by a semiquantitative ligation-mediated PCR of blunt DNA ends. *Cell Death Differ.* **4**, 66-75.

Tang, D. G., La, E. H., Kern, J. and Kehrer, J. P. (2002). Fatty acid oxidation and signaling in apoptosis. *Biol. Chem.* **383**, 425-442.

Tilly, J. L. (1998). Molecular and genetic basis of normal and toxicant-induced apoptosis in female germ cells. *Toxicol. Lett.* **102-103**, 497-501.

Tosti, E., Romano, G., Buttino, I., Cuomo, A., Ianora, A. and Miralto, A. (2003). Bioactive aldehydes from diatoms block the fertilization current in asciadian oocytes. *Mol. Reprod. Dev.* **66**, 72-80.

Turner, J. T., Ianora, A., Miralto, A., Laabir, M. and Esposito, F. (2001). Decoupling of copepod grazing rates, fecundity and egg-hatching success on mixed and alternatine diatom and dinoflagellate diets. *Mar. Ecol. Prog. Ser.* **220**, 187-199.

Voronina, E. and Wessel, G. M. (2001). Apoptosis in sea urchin oocytes, eggs, and early embryos. *Mol. Reprod. Dev.* **60**, 553-561.