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RESEARCH PAPER

Early events in the perception of lipopolysaccharides in the brown alga *Laminaria digitata* include an oxidative burst and activation of fatty acid oxidation cascades

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Abstract

This study provides evidence that bacterial lipopolysaccharides can be strong triggers of early events of defence reactions in the brown algal kelp *Laminaria digitata*, constituting the first report of a biological activity of this class of macromolecules in a marine alga. The early events include an oxidative burst, release of free saturated and unsaturated fatty acids (FFAs) and accumulation of oxylipins such as 13-hydroxyoctadecatrienoic acid and 15-hydroxyeicosapentaenoic acid. The formation of reactive oxygen species can be inhibited by diphenylene iodonium, suggesting that the source is an NAD(P)H oxidase and is similar to the oxidative burst in neutrophils and terrestrial plants. In addition and besides triggering an oxidative burst, the hypolipidemic drug clofibrate also induces the release of FFAs, to a lesser extent than lipopolysaccharides, but it does not induce oxylipin production. Other strong inducers of the oxidative burst in *Laminaria* such as oligoguluronates could not induce the release of FFAs nor oxylipin production. These results suggest that different signalling pathways are involved in the induction of the oxidative burst and oxylipin production.

Key words: Clofibrate, endotoxin, fatty acids, lipase, LPS, oxidative burst, oxylipin, *Salmonella*, *Marinobacter*.

Introduction

A growing body of evidence indicates that a major common feature of innate immunity in animals and higher plants is the capability to recognize invariant pathogen-associated molecular patterns (PAMPs) that are characteristic of micro-organisms but that are not found in potential hosts (Medzhitov and Janeway, 2002; Nürnberger *et al*., 2004). PAMPs include cell wall components of micro-organisms such as peptidoglycans, lipoteichoic acid (LTA) of Gram-positive bacteria, and lipopolysaccharides (LPS) of Gram-negative bacteria. However, despite the considerable knowledge about the role of surface macromolecules such as LPS and LTA in inflammatory reactions in mammals, there have been few investigations about their potential activities in other organisms, in particular, in terrestrial plants and even less in marine organisms. In mammalian inflammatory reactions, LPS and LTA are widely established as inducers of cytokine production, nitric oxide (NO) release, the oxidative burst (Farnell *et al*., 2003; He *et al*., 2003; Remer *et al*., 2003), and metabolite formation of the arachidonic acid cascade such as prostaglandins, by inducing enzymes for their synthesis such as cyclooxygenases (Chen *et al*., 2001; Ichitani *et al*., 2001; López-Urrutia *et al*., 2001). Their role in activating the eicosanoid pathway has also been recognized in other animal systems such as insects (Bedick *et al*., 2000; Miller and Stanley, 2004). Among the studies involving marine

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Abbreviations: APCI, atmospheric pressure ionization source; BSTFA, *N,O*-bistrimethylsilyltrifluoroacetamide; DAF-FM DA, 4-amino-5-methylamino-2,7-difluorofluorescein diacetate; DMSO, dimethyl sulphoxide; DPI, diphenylene iodonium; EPR, electron paramagnetic resonance; FFAs, free fatty acids; 15-HEPE, 15-hydroxy-5*Z*,8*Z*,11*Z*,13*E*,17*Z*-hydroxyeicosapentaenoic acid; 15-HETE, 15-hydroxy-5*Z*,8*Z*,11*Z*,13*E*-eicosatetraenoic acid; 13-HODE, 13-hydroxy-9*Z*,11*E*-octadecadienoic acid; 13-HOTrE, 13(S)-hydroxy-9*Z*,11*E*,15*Z*-octadecatrienoic acid; LPS, lipopolysaccharide; LTA, lipoteichoic acid; NO, nitric oxide; PAMPs, pathogen-associated molecular patterns.

animals, LPS have been found to induce DNA synthesis in crab haemocytes (Hammond and Smith, 2002), to induce the expression of an aggregation factor in fish leucocytes (Mulero *et al*., 2001), to trigger an oxidative burst in fish macrophages (Cook *et al*., 2001), to activate bactericidal activity in mussels (Hernroth, 2003), and to induce (2′-5′) oligoadenylate synthetase activity in sponges (Grebenjuk *et al*., 2002).

The vast majority of investigations concerning related structures in plants refer to lipo-chito-oligosaccharidic Nod factors of rhizobial bacteria in their legume plant hosts prior to the establishment of symbiosis (for a review, see Cullimore *et al*., 2001), with only a few mentioning a role of LPS in the induction of defence mechanisms (Dow *et al*., 2000; Reitz *et al*., 2000). In many different plants, pretreatment with LPS can prevent the hypersensitive response induced by avirulent bacteria, a phenomenon that has been termed localized induced resistance (LIR) (Erbs and Newman, 2003). LPS have been recognized as elicitors of innate immunity in plants (Silipo *et al*., 2005). Only recently, the induction of an oxidative burst (Meyer *et al*., 2001; Gerber *et al*., 2004) and the activation of phosphorylation cascades (Gerber and Dubery, 2004) by LPS from bacterial plant pathogens have been shown in terrestrial plants. The oxidative or respiratory burst is a central element of eukaryotic defence. It was initially discovered in human macrophages (Baldridge and Gerard, 1933). Decades later, its role in terrestrial plants was recognized (Doke, 1983*a*, *b*). Only very recently was it demonstrated that one of the most prominent features of animal innate immunity, LPS-mediated NO production, is apparent in higher plants in response to a variety of LPS from animal or plant pathogens and from rhizobacteria (Zeidler *et al*., 2004). At the gene expression level, the effects of LPS on higher plant cells involve the transcriptional activation of an array of defence or stress-associated genes, including glutathione S-transferases, cytochrome P450, and many PR proteins, as well as the enzymes involved in the octadecanoid pathway, leading to the synthesis of the cyclopentanone hormone jasmonic acid (Zeidler *et al*., 2004).

By contrast, nothing was known about the signals which mediate the activation of cell-based induced defence responses in seaweeds until recently (Potin *et al*., 2002). Marine algae, which emerged as independent phyla as early as the crown diversification of eukaryotes (Baldauf, 2003), evolved into several lineages about 1.3 billion years ago (Yoon *et al*., 2004), from which three phyla have acquired multicellularity. Endosymbiosis between a eukaryotic host and a cyanobacterium gave rise to Rhodophytes (red algae), from which green plants (green algae and land plants) have diverged. Phaeophyceae (brown algae) belong to thesamelineageastheunicellularBacillariophyceae(diatoms) and oomycetes, stemming from a secondary endosymbiosis between a plastid-less protist and an ancestral unicellular red alga (Baldauf, 2003).

Populations of brown algae (Phaeophyceae) are plagued by various pathogens, including bacteria (Sawabe *et al*., 1998, 2000), fungi, and oomycetes (Küpper and Müller, 1999), plasmodiophoraleans (Maier *et al*., 2000), viruses (Müller *et al*., 1998), and brown algal, pathogenic endophytes (Ellertsdóttir and Peters, 1997). Sporophytes of Laminariales, an order of brown algae with one of the most complex morphologies, have recently been shown to recognize fragments of their major cell polysaccharide, alginate, and to react with a rapid oxidative burst (Küpper *et al*., 2001). The oxidative burst in *Laminaria digitata* (Hudson) Lamouroux has been shown to play a crucial role in controlling the growth of epiphytic, potentially pathogenic bacteria (Küpper *et al*., 2001, 2002), producing hydrogen peroxide concentrations sufficient markedly to reduce bacterial growth and to reduce the number of microorganisms on algal surfaces. Still, it is not clear so far whether recognition of bacteria relies solely on endogenous elicitors such as oligoguluronates, released from the algal cell wall during bacterial attack, or whether brown algae can also recognize exogenous, bacterial elicitors. Whilst there is a certain body of knowledge on bacterial elicitors in higher plants (reviewed by Wojtaszek, 1997), there is only one report from a marine alga, the red agarophyte *Gracilaria conferta* (Weinberger and Friedlander, 2000), mentioning low-molecular weight (700–1500 Da) peptide elicitors, without their exact structure being known.

In this study, an attempt was made first to draw comparisons regarding the recognition of conserved principles of eliciting molecules, a key signal transduction event, the activation of an oxidative cascade of polyunsaturated fatty acids, and a conserved eukaryotic defence response, the oxidative and nitric oxide burst. Therefore, young *L. digitata* thalli were challenged with a number of both LTA and LPS preparations from bacterial pathogens to humans or other mammals, as well as with LPS from a marine bacterium. Compounds originating from non-marine organisms were chosen in order to compare the response of *Laminaria* sporophytes with the well-established responses of mammalian cells.

Materials and methods

Experimental cultures

Unialgal sporophytes were prepared by mating a compatible pair of gametophytes (♀: CCAP 1321/1; ♂: CCAP 1321/2) of *Laminaria digitata* from Helgoland (German Bight). Cultures were grown in Provasoli ES medium (Starr and Zeikus, 1987) prepared from artificial seawater (WIMEX, Krefeld, Germany, adjusted to 30‰ salinity), or natural Atlantic open-ocean water. They were illuminated with daylight-type fluorescent lamps at an irradiance of 9 $\mu E\ m^{-2}\ s^{-1}$ for 10 h d^{-1} and kept at 10 ±1 °C. Cultures were transferred to fresh medium at 1- or 2-week intervals.

Chemicals

Lipopolysaccharide (LPS) preparations from *Escherichia coli* (strain 0113), *Pseudomonas fluorescens* (wild-type strain), *Klebsiella*

pneumoniae (wild-type strain), and *Shigella dysenteriae* (wild-type strain) were from RIBI ImmunoChem Research Inc. (Montana, USA), whilst LPS from *Salmonella abortus equi* and *Salmonella minnesota* (strain 1114) originated from Sigma (Saint Quentin Fallavier, France). Purified LTA from *Bacillus subtilis* and *Staphylococcus aureus* and LPS from *Marinobacter hydrocarbonoclastiscus* (Gauthier *et al*., 1992) were prepared as described previously [Morath *et al*. (2001) for preparation of LPS and LTA; Hickford *et al*. (2004) for culture of *M. hydrocarbonoclasticus*]. Both LPS and LTA stock solutions were sonified prior to application to destroy micelles of these amphiphilic molecules, in order to render them accessible to potential receptors on the algal surface.

Diphenylene iodonium (DPI) was obtained from Sigma and dissolved in dimethyl sulphoxide (DMSO) to obtain stock solutions (100- or 1000-fold concentrated, respectively).

All fatty acids were from Sigma and dissolved in dimethyl sulphoxide (DMSO) to obtain stock solutions (100- or 1000-fold concentrated), depending on the experimental needs. 15-Hydroxy-5*Z*,8*Z*,11*Z*,13*E*-eicosatetraenoic acid (15-HETE), 15-hydroxy-5*Z*,8*Z*,11*Z*,13*E*,17*Z*-eicosapentaenoic acid (15-HEPE), 13-hydroxy-9*Z*,11*E*-octadecadienoic acid (13-HODE), and 13(S)-hydroxy-9*Z*,11*E*,15*Z*-octadecatrienoic acid (13-HOTrE) were purchased from Cayman (Ann Arbor, MI, USA). Clofibrate was from Sigma and was dissolved in DMSO to 3 mg ml^{-1} for stock solutions. The silylating reagent *N*,*O*-bistrimethylsilyltrifluoroacetamide (BSTFA; 1% in trimethylchlorosilane) was from Pierce Europe (Ound-Beijerland, The Netherlands). All solvents were from Merck (Darmstadt, Germany).

Hydrogen peroxide measurements

The concentration of hydrogen peroxide in the medium around algal thalli was determined using the luminol chemiluminescence method (Glazener *et al*., 1991) with a LUMAT LB 9507 luminometer (EG&G Berthold, Bad Wildbach, Germany), as described by Küpper *et al*. (2001). Typically, 400 µl aliquots (out of 10 ml sea water with of young *Laminaria* sporophytes 20–50 mg FW) were taken for one measurement. In the luminometer, 50 µl of 20 units ml^{-1} of horseradish peroxidase (Boehringer Mannheim, Meylan, France), dissolved in pH 7.8 phosphate buffer) and 100 µl of 0.3 M luminol (5-amino-2,3-dihydro-1,4-phthalazinedione; Sigma) solution were added to the sample. Chemiluminescence was recorded immediately after the last injection with a signal integration time of 10 s. For calculating the concentration of H_2O_2 present in the samples, calibration with a standard curve was carried out at least once during any series of measurements.

Extraction of free fatty acids (FFAs) and of their oxygenated derivatives

Algal samples were ground in liquid nitrogen. The frozen powder was transferred and homogenized with 2 ml of ethyl acetate. Lipids were extracted by mixing on a rotary shaker for 1 h at 4 °C and then 1 ml of ice-cold water was added. The mixture was kept for 5 min on ice and then centrifuged at 4 °C and 3500 *g*. After recovery of the organic phase, ethyl acetate was evaporated under a stream of argon. The residue was dissolved in ethanol and divided into two parts. One part was used for the characterization of major FFAs and of their oxygenated derivatives by LC/MS analysis. The other part was used to confirm structural assignments by GC/MS analysis after derivatization of metabolites and quantification of FFAs.

LC/MS analyses

Oxygenated derivatives of fatty acids were resolved and characterized by RP-HPLC coupled to a Navigator LC/MS mass spectrometer (Finnigan, Manchester, UK), equipped with an atmospheric pressure ionization source (APCI) running on negative ion mode, as detailed by Adas *et al*. (1998). Oxidized fatty acids were analysed by RP-HPLC (SpectraSystem P400 with UV detector UV1000), using a 5 µm Ultrasphere C18 column 250×4.6 mm (Beckman, France). The mobile phase (0.2% acetic acid in water/acetonitrile) programme began isocratically with a 40:60 mixture (v/v) for 35 min followed by a 5 min linear gradient to 5:95 (v/v) mixture for 30 min at a flow rate of 1 ml min^{-1}, in order to elute lipophilic compounds, i.e. fatty acids and sterols, before returning to the initial conditions. Negative ions were monitored by a full scan from 60 to 600 *m/z*. The source heater was at 150 °C and the APCI heater at 350 °C with a cone voltage of 45 V to increase fragmentation. Detection of oxidized fatty acids was achieved by monitoring their expected carboxylate anions $[M\text{-}H]^-$. For complete identification, co-chromatography using standard compounds was employed to compare LC peak retention times and mass spectra obtained. A UV detector at 234 nm was used to monitor products of lipoxygenase-like reactions containing conjugated double bonds. These compounds were then quantified with standard curves obtained with reference compounds by integrating the peak surfaces at 234 nm. Statistical significance was tested by Student's *t* test.

GC/MS analyses

GC/MS analyses were carried out on an HP 5890 Series II gas chromatograph equipped with a fused silica capillary column (HP-5MS 5% phenyl methyl siloxane; 30 m×0.32 mm IP; film thickness, 0.25 µm) and combined to a quadrupole mass-selective detector (HP 5971A; Agilent Technology). Mass spectra (EI mode) were recorded at 70 eV. Double-bond positions were assigned for polyunsaturated fatty acids after dimethyloxazolination (DMOX derivatives) with 2-amino-2-methyl-1-propanol for 2 h at 180 °C. Structural assignments of major oxylipins were confirmed after methylation with ethereal diazomethane and silylation with BSTFA containing 1% trimethylchlorosilane for 30 min at 60 °C as detailed by Le Quere *et al.* (2004). Compounds were dissolved in 100 µl of hexane and 2 µl were injected in the splitless mode at 60 °C. After 5 min at 60 °C, the oven temperature was increased to 200 °C at 50 °C min^{-1}, and then linearly ramped to 280 °C at 2 °C min^{-1} that became stable for 10 min before returning to initial conditions. Fatty acids were quantified as methyl esters from standard curves obtained by measuring the peak surfaces of authentic standards. Statistical significance was tested by Student's *t* test.

NO detection by fluorescence and electron paramagnetic resonance (EPR) spectroscopy

For NO detection by confocal laser-scanning microscopy, 4-amino-5-methylamino-2,7-difluorofluorescein diacetate (DAF-FM DA) was dissolved in DMSO to produce 10 mM stock, which was frozen as aliquots. Young *L. digitata* thalli were cross-sectioned using razor blades, and cross-sections were placed in the dark for 15 min in Petri dishes containing 5 ml of 0.22-µm-filtered seawater and 5–50 µl of DAF-FM DA from the stock solution. Cross-sections were removed from the DAF-FM DA loading solution, rinsed with 22-µm-filtered seawater, and affixed to the bottom of a Petri dish containing 2 ml of seawater for microscopy and treated with 100 µg ml^{-1} LPS (0.1% DMSO for control) as described above. Settings and laser parameters of the IX 70/Fluoview inverted microscope (Olympus, Tokyo) were as described previsously (Küpper *et al*., 2001).

For EPR analysis of NO, algal samples were frozen and ground in liquid nitrogen. About 0.5 g frozen powder was transferred and homogenized with 1.2 ml of buffered solution (50 mM HEPES, pH 7.6) for 2 min. The mixture was centrifuged at 13 200 *g* for 2 min. The supernatant was added to 300 µl of freshly made $[Fe(II)\text{-}(DETC)_2]$ solution [2 M $Na_2S_2O_4$, 3.3 mM diethyldithiocarbamate (DETC), 3.3 mM $FeSO_4$, 33 mg ml^{-1} BSA; Tsuchiya *et al*., 1996], incubated for 2 min at room temperature and frozen again in liquid nitrogen. EPR measurements were performed on

a Bruker ELEXSYS X-band spectrometer under the following conditions: temperature 140 K, microwave power, 20 mW; modulation amplitude, 3 G; scan rate, ~2.5 G s^{-1}; time constant, 164 ms.

Results

LPS induce a strong but late oxidative burst in L. digitata *sporophytes*

Treatment of young sporophytic thalli of *L. digitata* with LPS from *Salmonella abortus equi* induced a strong release of hydrogen peroxide into the surrounding sea water medium (Fig. 1). Similar, though weaker, responses were observed with LPS from *Escherichia coli*, *Pseudomonas fluorescens*, the oil-degrading marine bacterium *Marinobacter hydrocarbonoclasticus*, and the human pathogen *Klebsiella pneumoniae* (data not shown).

The threshold concentration for triggering a response was found between 1 and 10 μg ml^{-1} LPS. Maximum hydrogen peroxide concentrations were typically reached more than 60–90 min after addition of LPS to the algae, corresponding to a maximum of approximately 2–3 μmol g^{-1} FW (not shown) with further H_2O_2 release often still continuing 2 h after the initial addition. Within this time span, its amplitude reached or exceeded the level of maximum peroxide release triggered by oligoguluronates.

By contrast, LTA from *Staphylococcus aureus* at 10 μg ml^{-1} (Morath *et al*., 2001) and LTA from *Bacillus subtilis* at either 1 or 2 μg ml^{-1} had no observable effect.

DPI at 10 μM inhibited the response to LPS from *Salmonella abortus equi*, reducing the peak release of hydrogen peroxide 105 min after the addition of LPS by over 75% compared with the steady-state release (data not shown).

Likewise, clofibrate (100 μM) triggered an oxidative burst in *Laminaria*, with maximum H_2O_2 concentrations reached by 30–120 min after addition of the compound (not shown).

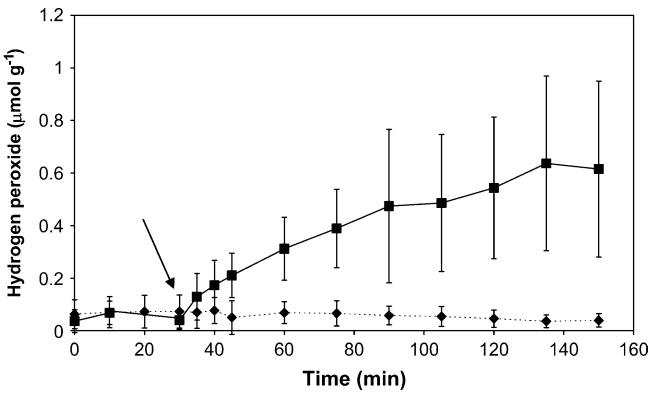

Fig. 1. Bacterial lipopolysaccharides trigger an oxidative burst in *Laminaria*. *Laminaria* seedlings were kept in seawater (control, dotted line) for 0–120 min and treated with LPS from *Salmonella abortus equi* (10 μg ml^{-1}; squares). Results are the mean of triplicate experiments and expressed (±standard deviation) in μmol H_2O_2 g^{-1} FW. Addition of LPS at *t*=30 min is marked by an arrow.

NO was not detected in L. digitata *sporophytes in response to a challenge with LPS*

Based on analyses by confocal microscopy with the fluorescent, NO-sensitive probe, DAF-FM DA, NO production was not detected after challenging with LPS from *Salmonella abortus equi*. Then, a highly specific method was used for NO detection in plants and animals, which is EPR measurements with ferrous and mononitrosyl dithiocarbamate [$Fe^{2+}(DETC)_2$] or other dithiocarbamate derivatives for spin trapping of NO. Despite great care to preserve the sensitivity and stability of the complexed NO (Tsuchiya *et al*., 1996), no significant differences between the spectra recorded for control sporophytes and LPS-treated plantlets were observed (data not shown). It should be noted, that the EPR signal for sodium nitroprusside, an NO donor, was particularly stable in brown algal aqueous extracts.

Challenge with LPS induces the release of FFAs and accumulation of oxylipins

In order to assess the effect of LPS, oligoguluronates, and clofibrate on the activation of the fatty acid metabolism of *L. digitata* sporophytes, LC/MS and GC/MS analyses were carried out to measure the level of FFAs released and to characterize the chemical structure of generated oxylipins. The results of the treatment of algae with LPS preparations from both *Salmonella abortus equi* and *Marinobacter hydrocarbonoclasticus* (Fig. 2) were very similar. Compared with controls, the overall amount of a number of FFAs was strongly increased after LPS treatment. In particular, significant amounts of free myristic acid (C14:0), palmitic acid (C16:0), palmitoleic acid (C16:1), stearic acid (C18:0), and oleic acid (C18:1) were detectable 30 min after treatment, although the level of linoleic acid (C18:2), linolenic acid (C18:3), octadecatetraenoic acid (C18:4), arachidonic acid (C20:4), and eicosapentaenoic acid (C20:5) was strongly increased slightly later (60 min treatment; Fig. 2). The most pronounced increases mainly concerned the level of polyunsaturated fatty acids such as C20:5, C20:4, C18:3, and C18:2; for example, the concentrations of free C20:4 and C20:5 were increased 10-fold and 4.5-fold, respectively, 60 min after treatment with LPS from *Marinobacter*. Interestingly, treatment with clofibrate, a mammalian hypolipidemic drug and a plant anti-auxin, also induced the release of FFAs; however, mainly of polyunsaturated FFAs including C18:3, C18:4, and C20:4. By contrast, the treatment with oligoguluronates did not significantly increase the level of FFAs or of oxylipins.

Concomitantly, four peaks showing the retention times (R_t) and the expected molecular masses [M-H](−) of

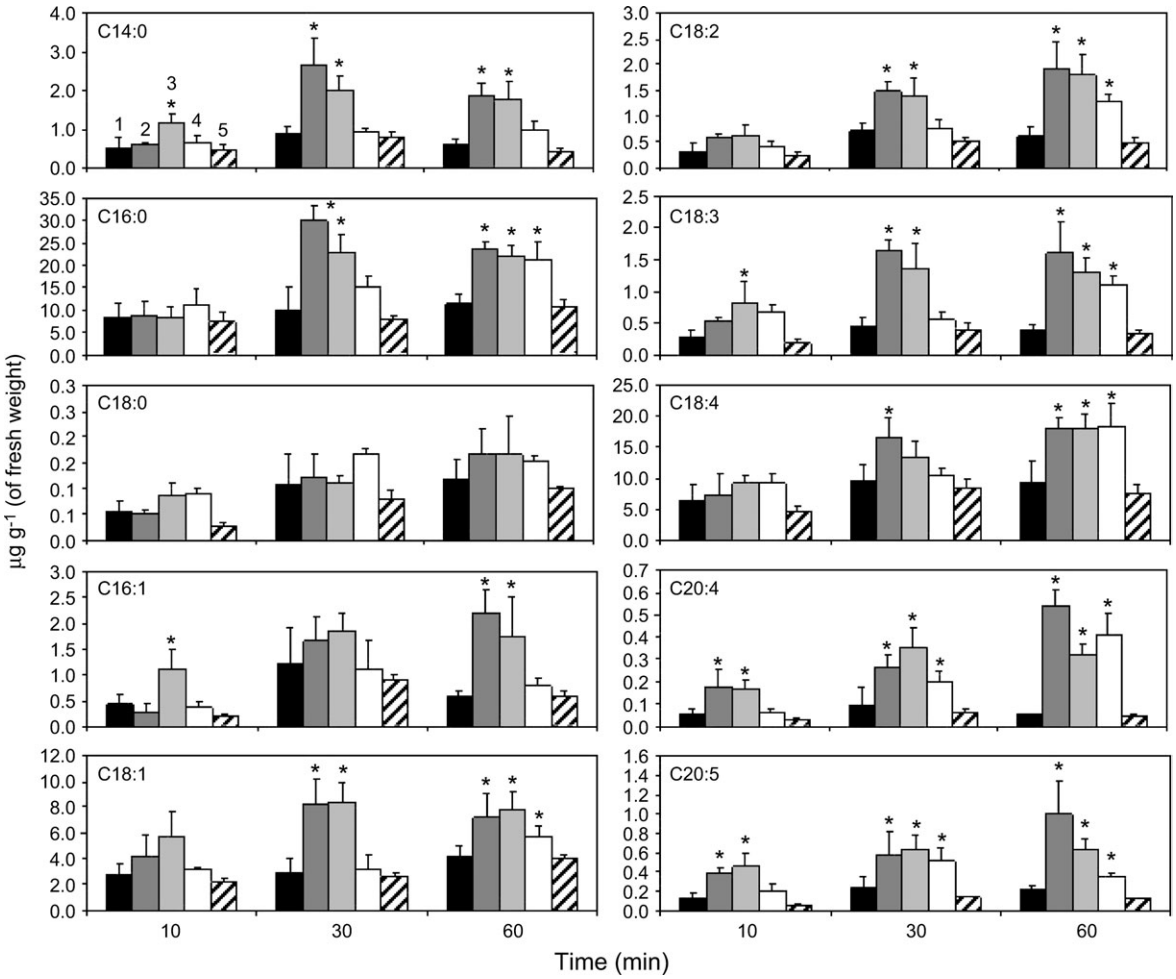

Fig. 2. Effects of lipolysaccharides, oligoguluronates, and clofibrate on the levels of FFAs in *Laminaria. Laminaria* seedlings were kept in seawater (control) (1) for 10, 30, and 60 min and treated with: LPS from *Salmonella abortus equi* (10 µg ml^{-1}) (2); LPS from *Marinobacter hydrocarbonoclasticus* (10 µg ml^{-1}) (3); oligoguluronates (150 µg ml^{-1}) (4), and clofibrate (100 µM) (5) for the same periods of time as the control. Free saturated and unsaturated fatty acids from C14:0 to C20:5 were quantified by GC/MS. Results are the mean of triplicate experiments and expressed (±standard error) in µg g^{-1} FW. *, $P < 0.05$ (Student's *t* test).

hydroxylated fatty acids were structurally characterized by APCI(⁻)-LC/MS and GC/MS as Me/TMS derivatives. The mass spectra of the two major peaks displayed high intensity signals at 293 *m/z* and 317 *m/z*, respectively, and an informative fragment at 195 *m/z* and 219 *m/z*, respectively. These data were very similar to those of authentic 13-hydroxyoctadecatrienoic acid (13-HOTrE; R_t 15.2 min) and 15-hydroxyeicosapentaenoic acid (15-HEPE; R_t 17.1 min), respectively. In addition, two minor peaks with molecular masses [M-H](⁻) at 295 *m/z* and 319 *m/z* were characterized as 13-hydroxyoctadecadienoic acid (13-HODE; R_t 16.5 min) and 15-hydroxyeicosatetraenoic acid (15-HETE; R_t 18.5 min), respectively. 13-HOTrE and 15-HEPE, the two major hydroxy fatty acids detected after treatment with LPS from *Salmonella* and *Marinobacter*, were quantified (Fig. 3). A significant increase in the concentration of 13-HOTrE and 15-HEPE was observed 60 min after treatment with LPS from *Marinobacter* and *Salmonella*. By contrast, although treatment with clofibrate seemed to induce an early release of FFAs including C18:3 and C20:5, the level of hydroxylated derivatives was not significantly modified by this treatment. Treatment with oligoguluronates did not impact the level of 13-HOTrE and 15-HEPE significantly when compared with controls.

Discussion

It is shown here that challenging *L. digitata* sporophytes with LPS from various sources resulted in the activation of a DPI-sensitive oxidative burst and the rapid release of FFAs (Fig. 2), with a concomitant accumulation of oxidized derivatives of linolenic and eicosapentaenoic acid (Fig. 3). Up to now there have been no reports about an oxidative burst in the context of LPS in any algal lineage. A few reports mention induction of systemic acquired

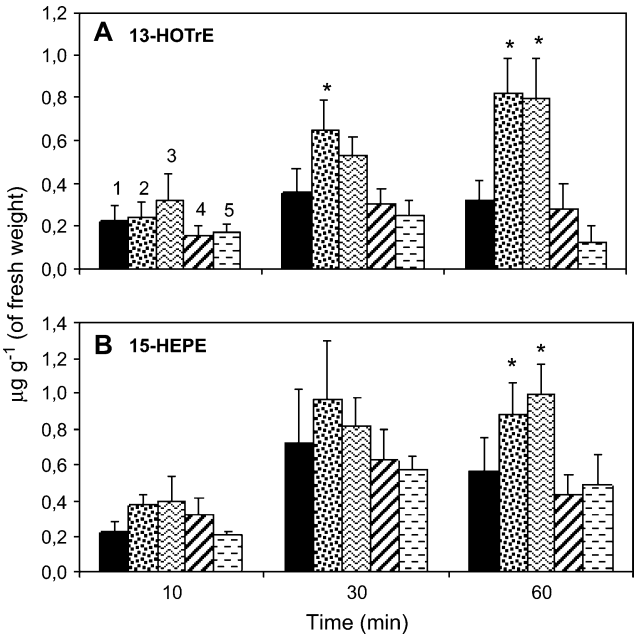

Fig. 3. Effects of lipolysaccharides, oligoguluronates, and clofibrate on the synthesis of oxidized fatty acids in *Laminaria. Laminaria* seedlings were kept in seawater (control) (1) for 10, 30, and 60 min and treated with: LPS from *Salmonella abortus equi* (10 µg ml^{-1}) (2); LPS from *Marinobacter hydrocarbonoclasticus* (10 µg ml^{-1}) (3); oligoguluronates (150 µg ml^{-1}) (4), and clofibrate (100 µM) (5) for the same periods of time as the control. (A) 13-hydroxyoctadecatrienoic acid (13-HOTrE) and (B) 15-hydroxyeicosapentaenoic acid (15-HEPE) were quantified by APCI(−)-LC/MS. Results are the mean of triplicate experiments and expressed (±standard error) in µg g^{-1} FW. 13-Hydroxyoctadecatrienoic acid (13-HOTrE) and 15-hydroxyeicosapentaenoic acid (15-HEPE) were not significantly increased by clofibrate (100 µM). Oligoguluronates (150 µg ml^{-1}) were without effect. *, $P < 0.05$ (Student's *t* test).

resistance (Reitz *et al*., 2000) by LPS in terrestrial plant-pathosystems, whilst others found a suppression of the hypersensitive reaction by bacterial LPS (Newman *et al*., 1997). Contrary to Newman *et al*. (2001), LPS was recently found to trigger an oxidative burst in terrestrial plants by Meyer *et al*. (2001).

The initial response within the first 30 min after elicitation reached <25% of the maximum H_2O_2 emission elicited by oligoguluronates (Küpper *et al*., 2001). The peak hydrogen peroxide emission triggered by LPS from *Salmonella* or *Marinobacter* (2–3 mmol g^{-1} FW) is in a similar range to the H_2O_2 production observed after elicitation by oligoguluronates (Küpper *et al*., 2001, 2002); however, the main difference is that the oxidative burst observed after challenging *L. digitata* with LPS occurs considerably later than the response to oligosaccharides (Küpper *et al*., 2001). This could be entirely due to physicochemical reasons such as the time needed to establish distribution equilibrium of these amphiphilic compounds between the surrounding medium and the cell membrane, or this lag phase could be attributed to biochemical conversions of metabolites and induction of enzymes upstream of hydrogen peroxide production, or both. It is worth noting that *Laminaria* exhibits a highly co-ordinated hydrogen peroxide emission to LPS, clofibrate (this study), and oligoguluronates (Küpper *et al*., 2001, 2002), contrasting with other macroalgae, typically of less complex morphology, that show high steady-state release rates for hydrogen peroxide (Collén and Pedersén, 1996; Küpper *et al*., 2002).

It should be noted here that, even though a burst of NO is now known as an important transduction step in the LPS-mediated innate immunity in mammals and land plants (Zeidler *et al*., 2004), attempts to detect NO production in *L. digitata* in response to LPS treatment remain unsuccesful. The lack of NO production in LPS-challenged *L. digitata* may reflect differences in the transduction pathways of LPS perception in brown algae on the one hand and animals and green plants on the other.

Taken together, the present results demonstrate that components of the outer membranes of Gram-negative bacteria can be considered as exogenous elicitors in marine algae. As the results of stimulation with LPS from *Salmonella abortus equi* highlight, recognition of these bacterial surface structures is not restricted to their natural mammalian hosts where they induce violent inflammatory responses, including an oxidative burst (Chen *et al*., 2001; Ichitani *et al*., 2001). This study shows that recognition occurs equally in a phylogenetically distant lineage that separated from the ancestors of animals early in eukaryotic evolution. Even though close relatives of *M. hydrocarbonoclasticus* are often found associated with dinoflagellate microalgae (Green *et al.*, 2004), the bacteria which produce the LPS investigated here are not pathogenic to *Laminaria*. These observations suggest that brown algae express unspecific receptors by which they can recognize common bacterial structures. Also Zeidler *et al*. (2004) had observed that the terrestrial plant model, *Arabidopsis thaliana*, can recognize LPS, not only from bacterial plant pathogens but also from a range of mammalian pathogens as well. It appears worthwhile to search for the presence of Toll-like receptors in this context (Qureshi *et al*., 1999).

LPS are interesting candidates to study enzymes and metabolites involved in the oxidative cascade of polyunsaturated fatty acids in brown algae; they are well known to be potent triggers of eicosanoid pathways in animal inflammatory reactions (Vafeas *et al*., 1998). More specifically, among their multiple effects, they induce cyclooxygenase in arterial endothelium (Chen *et al*., 2001), cytochrome P450-dependent fatty acid hydroxylases (CYP4A subfamily), and acyl-CoA oxidase in mammals (Barclay *et al*., 1999). Further upstream in the signalling chain, the role of LPS in the activation of phospholipases, especially PLA2 (Forehand *et al*., 1993; Jiang *et al*., 2003), is well recognized in mammalian systems. In this regard, the present study constitutes one of the few reports of an activation of lipase-like enzyme activities in plant-like organisms by LPS. Furthermore, the present study revealed

that LPS induces oxidative reactions leading to hydroxy derivatives of octadecanoid and eicosanoid fatty acids in *L. digitata*. 13-HOTrE and 15-HEPE, the major products detected, have already been described in *Laminaria* by Gerwick *et al.* (1993) as hydroxy fatty acids derived from a putative ω-6 lipoxygenase metabolism. These two compounds, generated by lipoxygenases from C18:2 and C20:5 in mammals, have been reported to be anti-inflammatory mediators (Miller *et al*., 1990). Their synthetic pathway in *L. digitata* in response to LPS treatment is not clearly identified. First, a non-enzymatic generation such as by the reactive oxygen species released during the oxidative burst cannot be excluded, even though no significant amounts of metabolites of the highly oxidizable arachidonic acid were present in the lipid extract of *L. digitata*. Oligoguluronates, previously described inducers of an intense oxidative burst in *L. digitata* sporophytes (Küpper *et al*., 2001), did not induce 13-HOTrE and 15-HEPE synthesis. From this observation, it can at least be concluded that the increase of 15-HEPE and 13-HOTrE seems to be independent of the observed oxidative burst. Further analyses will be required to examine strictly the oxidative reactions leading to the synthesis of these hydroxy fatty acids during LPS treatment on *L. digitata*.

In conclusion, the comparison between phyla as distant as mammals, green plants, and brown algae, that separated during the so-called crown diversification of higher eukaryotes about 1.3 billion years ago (Yoon *et al*., 2004), appears worthwhile, since it might allow conclusions to be drawn about conserved principles in the defence mechanisms of eukaryotes in general, regarding recognition of pathogens, signal transduction, and responses. The results of the present study show that defence pathways exist in brown algae, which are similar to those known from animals and land plants. The features that are obviously conserved between such distant lineages include recognition of pathogenesis-related macromolecular patterns, eicosanoid and cyclopentanone signalling (Bouarab *et al*., 2004), and the capacity to produce an oxidative burst. These are probably evolutionary ancient features and it is suggested that they have been a prerequisite for eukaryotic evolution.

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