Lipoxygenase Inhibitors Shift the Yeast/Mycelium Dimorphism in *Ceratocystis ulmi*

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The yeast-mycelium dimorphism in *Ceratocystis ulmi*, the causative agent of Dutch elm disease, was switched by gossypol, nordihydroguaiaretic acid, and propylgallate. In each case the mycelial form was converted to the yeast form. These compounds are recognized lipoxygenase inhibitors. Inhibitors of cyclooxygenase and thromboxane synthetase did not cause mycelia to shift to the yeast form. We suggest the following two-part hypothesis: (i) that lipoxygenase is a target for antifungal antibiotics and (ii) that many phytoalexins (antimicrobial compounds of plant origin) are targeted toward fungal lipoxygenases. In addition, in a study to determine potential lipoxygenase substrates, a fatty acid analysis indicated that *C. ulmi* conidiospores contained high levels of oleic, linoleic, and linolenic acids but no arachidonic acid.

Lipoxygenases use molecular oxygen to oxidize polyunsaturated fatty acids (PUFAs) to their hydroperoxide derivatives and are designated either 5-, 12-, or 15- depending on the site of hydroperoxide formation. These compounds are widely distributed in plants and animals and recently have been identified in fungi as well. There is evidence that lipoxygenases occur in *Achlya ambisexualis* (12), *Fusarium oxysporum* (28), *Lagenidium giganteum* (15), *Saccharomyces cerevisiae* (29), *Saccharomyces vini* (18), *Saprolegnia ferax* (11), and *Saprolegnia parasitica* (9, 11). The lipoxygenase-PUFA interaction can be developmentally significant. In the genera *Achlya* (12) and *Saprolegnia* (11), the lipoxygenase products are associated with vegetative (mycelial) growth rather than with sexual reproduction. In contrast, in *L. giganteum* (15) the lipoxygenase products are necessary for oosporogenesis.

The arachidonic acid cascade (8, 23, 24) describes the release of arachidonic acid ($C_{20:4}$) from membrane phospholipids by phospholipase A_2 and its subsequent metabolism via cyclooxygenase to prostaglandins and thromboxanes or via lipoxygenases to leukotrienes. Because of their pharmacological importance, many natural-product and synthetic lipoxygenase and cyclooxygenase inhibitors have been identified (8), and some of these compounds have been shown to be effective in fungi (12, 15). Well-known compounds in this category include aspirin, acetaminophen, and ibuprofen. In this study we examined a series of cyclooxygenase and lipoxygenase inhibitors for their effect on *Ceratocystis ulmi*, the dimorphic fungus which causes Dutch elm disease. Three known lipoxygenase inhibitors caused mycelia to shift to the yeast form in *C. ulmi*. Our data suggest that fungal lipoxygenases may be important targets for both natural and synthetic fungicides.

The dimorphic capacity of *C. ulmi* is important for its pathogenicity toward elm trees. All vascular wilt fungi are dimorphic (27). *C. ulmi* switches to the yeast morphology in infected trees (19), and after inoculation the extent of colonization depends on the vertical movement of the yeast phase in infected xylem (19). Below we suggest that the presence of antifungal phytoalexins contributes to the morphological choice of *C. ulmi* during pathogenesis.

MATERIALS AND METHODS

Fungal growth. *C. ulmi* NRRL 6404 was grown at 26°C in a defined liquid medium which contained (per liter) 20 g of glucose, 6 g of $Na_2HPO_4 \cdot 7H_2O$, 4 g of KH_2PO_4, 0.5 g of $MgSO_4 \cdot 7H_2O$, 1 mg of $CuSO_4 \cdot 5H_2O$, 1 mg of $ZnSO_4 \cdot 7H_2O$, 1 mg of $MnCl_2$, 1 mg of $FeSO_4$, 20 μg of biotin, 200 μg of pyridoxine-HCl, 200 μg of thiamine-HCl, and a nitrogen source at a concentration of 10 mM; the pH of the medium was 6.5. The media designated GPP and GPR contained L-proline and L-arginine, respectively, as the nitrogen sources. Flasks were inoculated with conidiospores to give a final concentration of 2×10^7 spores per ml, were aerated by rotary agitation, and were examined microscopically after 18 h.

Gas-liquid chromatography of fatty acids. Cultures in flasks were harvested after 5 days of growth in GPR medium, and the conidiospores were separated from the mycelia by filtration. For the fatty acid analysis we used the procedures of Ogg et al. (25). The conidiospores were extracted with 10 ml of chloroform-methanol (2:1) containing 1 mg of butylated hydroxytoluene to minimize autooxidation of PUFAs. The phospholipids were purified by thin-layer chromatography on silica gel plates and were transmethylated by refluxing for 1 h in acidified methanol. The fatty acid methyl esters were extracted three times in petroleum ether, concentrated, and injected into a model 5890 gas-liquid chromatograph (Hewlett-Packard) equipped with a type SP-2330 capillary column, a flame ionization detector, and a model 3396A integrator (Hewlett-Packard). Chromatograms were run isothermally at 190°C with helium as the carrier gas. Fatty acid methyl esters were tentatively identified by comparing their retention times with the retention times of authentic standards (Sigma Chemical Co., St. Louis, Mo.). The resulting identifications were confirmed by gas chromatography-mass spectrometry, using a model 5790 gas chromatograph interfaced with a model 5970 electron impact mass selective detector (Hewlett-Packard) operated at 70 eV (25).

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TABLE 1. Fatty acid composition of phospholipids from conidiospores of *C. ulmi*

Fatty acid	% in *C. ulmi* conidiospores[a]
$C_{14:0}$	0.1
$C_{14:1}$	0.1
$C_{16:0}$	10.6
$C_{16:1}$	2.6
$C_{16:2}$	0.1
$C_{18:0}$	0.9
$C_{18:1}$	23.8
$C_{18:2}$	50.5
$C_{18:3}$	9.0
$C_{20:0}$	0.1
$C_{20:2}$	0.1
$C_{22:0}$	tr
Total	97.9

[a] Values are the averages of 12 measurements (six samples run in duplicate).

TABLE 2. Effects of lipoxygenase and cyclooxygenase inhibitors on *C. ulmi*

Compound[a]	Concn (μM) that resulted in conversion of 50% of the mycelia to the yeast form[b]	
	Conidiospore inoculum	Blastospore inoculum
Lipoxygenase inhibitors		
Gossypol	20	ND[c]
Nordihydroguaiaretic acid	40	60
Propylgallate	80	50
Butylated hydroxyanisole	Inhibitory (280 μM)	Inhibitory (400 μM)
1-Naphthol	Inhibitory (140 μM)	Inhibitory (250 μM)
Cyclooxygenase inhibitors		
Acetaminophen	No effect	No effect
Aspirin	No effect	No effect
Flurbiprofen	No effect	No effect
Indomethacin	No effect	No effect
Salicylhydroxamic acid	No effect	No effect
Thromboxane synthetase inhibitors		
Imidazole	Inhibitory (4 mM)	Inhibitory (2–4 mM)
Metyrapone	No effect	No effect
General antioxidants		
Butylated hydroxytoluene	No effect	No effect
Maleic acid	No effect	No effect

[a] Inhibitor classifications as described in references 8, 23, 24, and 30. Stock solutions for all of the compounds except imidazole and maleic acid were prepared in ethanol. The maximum ethanol concentration in any test flask was 1%. Controls consisting of 0.5 and 1.0% ethanol in GPR medium were run for each experiment.

[b] The values for nordihydroguaiaretic acid and propylgallate are averages from six experiments; the values for the other compounds are averages from three or more experiments. Inhibitory means that no development occurred at concentrations greater than the concentration indicated in parentheses within 18 h. No effect means that concentrations up to 100 μg/ml still allowed $\geq$90% germination.

[c] ND, not done.

RESULTS

Fatty acid composition of *C. ulmi*. Lipoxygenases require PUFAs as substrates for activity. Table 1 shows that *C. ulmi* conidiospores were rich in linoleic acid ($C_{18:2}$) and linolenic acid ($C_{18:3}$); these acids accounted for ca. 59.5% of the fatty acids in the phospholipid fraction. Significantly, no $C_{20:3}$, $C_{20:4}$, or $C_{20:5}$ fatty acid was detected either in the polar lipids (Table 1) or in any other cellular lipid fraction (13). Arachidonic acid ($C_{20:4}$) was completely absent in conidiospores (Table 1), as well as in blastospores and in cells growing in both the yeast form and the mycelial form (13). This absence of arachidonic acid is in accordance with the suggestion (7, 35) that fungal PUFA distribution patterns are divided along taxonomic lines; the lower fungi, including *Achlya parasitica* and *A. ambisexualis*, have arachidonic acid as a major fatty acid (35), whereas the higher fungi, such as *C. ulmi*, synthesize only two types of PUFAs, linoleic and α-linolenic acids (7). The high level of linoleic and linolenic acids in *C. ulmi* is similar to the level reported for *Ceratocystis coerulescens* (31) (64.1% $C_{18:2}$ and $C_{18:3}$ in the polar lipids).

Lipoxygenase inhibitors. *C. ulmi* normally grows in the mycelial form in arginine-containing GPR medium (17). However, when we added three 5-lipoxygenase inhibitors (gossypol, nordihydroguaiaretic acid, and propylgallate), the cells grew instead with the yeast morphology (Table 2). None of the cyclooxygenase inhibitors, thromboxane synthetase inhibitors, or general antioxidants influenced morphology when they were present at concentrations up to 100 μg/ml. Virtually identical results were obtained with conidiospore and blastospore inocula (Table 2). Finally, we noted that the imidazole-inhibited cells had an unusual appearance because of the marked accumulation of phase-bright intracellular lipid bodies.

DISCUSSION

We found that three lipoxygenase inhibitors, gossypol, nordihydroguaiaretic acid, and propylgallate, cause mycelia to shift to the yeast form in the dimorphic fungus *C. ulmi*. Evidence that the fungal lipoxygenases are important developmentally leads to questions concerning the chemical identity and mode of action of the lipoxygenase product(s). The fungal lipoxygenase products identified so far include three trihydroxyeicosatrienoic acids (9), as well as their epoxy

alcohol precursors (10, 12). These lipoxygenase products are excreted from the cells. In addition, the structures of many of the extracellular lipids produced by yeasts (32) (e.g., 8,9,13-trihydroxydocosanoic acid) suggest that the synthesis and excretion of these lipids might be lipoxygenase mediated.

The lipoxygenase products that have been identified so far from *Saprolegnia parasitica* (9, 10) and *A. ambisexualis* (12), both of which are classified among the lower fungi, are eicosanoids (C_{20}) that are derived from arachidonic acid ($C_{20:4}$), while *C. ulmi* does not contain any $C_{20:3}$, $C_{20:4}$, or $C_{20:5}$ fatty acids (Table 1). This absence of potential C_{20} substrates in *C. ulmi* should not be a problem because lipoxygenases in general exhibit a rather broad substrate specificity (8, 29). For instance, in addition to arachidonic acid, linoleic acid ($C_{18:2}$) and eicosapentaenoic acid ($C_{20:5}$) are good substrates for the *Saprolegnia parasitica* lipoxygenase (10). Thus, it seems likely that *C. ulmi* follows the plant-higher fungus lipoxygenase pattern, with linoleic and linolenic acids as substrates, rather than the animal-lower fungus pattern, with arachidonic acid as the substrate. In this regard, plants synthesize jasmonic acid and other hormone-like substances from linolenic acid (33, 34), while the importance of PUFAs to *C. ulmi* has been shown by the observation that on a defined agar medium exogenous linoleic acid

causes *C. ulmi* to produce two reproductive structures (2, 20), perithecia (sexual) and synnema (asexual). At least one phytopathogenic fungus, *Botryodiplodia theobromae* (1, 22), is known to produce jasmonic acid. We have preliminary data based on comigration during high-performance thin-layer chromatography that methyl jasmonate is also produced by *C. ulmi* (13).

The discussion below concerns lipoxygenase as a previously unrecognized target for antifungal antibiotics. Many lipoxygenase inhibitors are themselves natural products of plants (8, 21). For instance, nordihydroguaiaretic acid occurs in the resinous exudates of many plants; it was first isolated from the perennial evergreen shrub *Larrea divaricata* (21). Similarly, gossypol is the predominant pigment that is found in glands distributed throughout leaves, stem and root cortices, and floral parts of most cotton varieties (3); it can accumulate in the xylem at concentrations as high as 250 μM (3). *C. ulmi* is a pathogen of elm trees. Do these naturally occurring lipoxygenase inhibitors act as plant defense compounds by interfering with fungal development? For gossypol the answer is clearly yes. Bell (3) showed that gossypol (50 to 250 μM) inhibited spore germination in eight different fungi, while Keen et al. (14) showed that gossypol could be inhibitory to *Verticillium albo-atrum* (now *Verticillium dahliae*), one of the vascular wilt fungi.

Phytoalexins are defined as low-molecular-weight antimicrobial compounds that are both synthesized by and accumulated in plants after exposure to microorganisms (16, 26). Phytoalexins inhibit mycelial growth, conidial germination, and sporulation (4). Significantly, the antifungal mode of action of these compounds is unknown. Gossypol is listed as both a phytoalexin (3, 4) and a lipoxygenase inhibitor (8). As a working hypothesis, we suggest that many or most phytoalexins act as lipoxygenase inhibitors. This suggestion is based primarily on a comparison of the chemical structures of phytoalexins and lipoxygenase inhibitors (e.g., Table 1 of Dixon [5] versus Tables 2, 4, and 7 of Fitzsimmons and Rokach [8]); prominent on both lists are members of the flavonoids, caffeic acid phenolics, and polyacetylenes. We are currently examining other known phytoalexins to determine whether they too act as lipoxygenase inhibitors.

Our suggestion regarding the fungicidal action of lipoxygenase inhibitors is exactly the opposite of the lipid peroxidation mode of action proposed by Edlich and Lyr (6) for two other groups of fungicides. The 3,5-dichlorophenyl-containing dicarboximide fungicides and the aromatic hydrocarbon fungicides are thought to promote unwanted lipid peroxidation of intracellular phospholipids, especially in the inner mitochondrial membranes which are rich in unsaturated fatty acids. The two modes of action described above appear to be complementary; they are not in conflict.

ACKNOWLEDGMENTS

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