Inhibition of Paracoccidioides brasiliensis by ajoene is associated with blockade of phosphatidylcholine biosynthesis

Gioconda San-Blas, Julio A. Urbina, Edgar Marchán, Lellys M. Contreras, Françoise Sorais and Felipe San-Blas

In Paracoccidioides brasiliensis, a dimorphic fungus pathogenic for humans, no significant differences were observed in the phospholipid species of both morphological phases. The species observed were phosphatidylcholine (PC, 30-40%), phosphatidylethanolamine (PE, 27-28%), phosphatidylinerine (16-19%), phosphatidylinositol (13-17%) and sphingomyelin (3-5%). The main fatty acids found in the yeast (Y) phase were palmitate (56%), linoleate (18%) and oleate (15%), while linoleate predominated (61%) in the mycelial (M) phase, followed by palmitate (27%) and oleate (7%). In the Y phase the main free sterol was ergosta-5,22-dien-3β-ol (82%) plus some lanosterol (12%) and ergosterol (6%), while in the M phase, the latter predominated (88%), followed by low levels of ergosta-5,22-dien-3β-ol (12%). Ajoene [(E,Z)-4,5,9-trithiadodeca-1,6,11-triene 9-oxide], a platelet aggregation inhibitor derived from garlic, induced alterations in phospholipid and fatty acid proportions such that PC was reduced to about 18% in both phases and PE increased to 38% (Y phase) or 44% (M phase), suggesting inhibition of PC synthesis. Ajoene also reduced saturated fatty acids (16:0 and 18:0) from 67 to 35% in the Y phase, with a corresponding increase in the unsaturated components. This effect was not seen in the M phase.

Keywords: fungal dimorphism, Paracoccidioides brasiliensis, ajoene, antifungal drugs, fungal lipids

INTRODUCTION

Paracoccidioides brasiliensis is a thermally dimorphic fungus and the causative agent of a prevalent human systemic mycosis in Latin America where it is geographically constrained (Wanke & Londero, 1994). The pathogenic yeast-like (Y) phase grows at 37 °C, while a mycelial (M) phase develops at 23 °C (Lacaz, 1994). Ajoene [(E,Z)-4,5,9-trithiadodeca-1,6,11-triene 9-oxide], a platelet aggregation inhibitor derived from garlic (Apitz-Castro et al., 1983), behaves as an antifungal agent against P. brasiliensis (San-Blas et al., 1989), Aspergillus niger and Candida albicans (Yoshida et al., 1987), Cladosporium carrionii and Fonsecaea pedrosoi (Sánchez-Mirt et al., 1993). Ajoene disturbs the fungal plasma membrane (San-Blas et al., 1989; Sánchez-Mirt et al., 1993), an effect that is more prevalent in the Y phase in P. brasiliensis. Membrane disturbance leads to the deterioration of fungal structures, among them the cell wall, whose synthesis depends on the correct performance of the enzymic machinery located in the plasma membrane (San-Blas & San-Blas, 1994). Ajoene is also capable of blocking the thermally induced transition from the M to the Y phase (San-Blas et al., 1993b), an effect that together with the already mentioned effect on growth, may be of interest in therapy, since the transition process is the first requirement for the establishment of paracoccidioidomycosis in the host (Lacaz, 1994).

Recently, it was shown that ajoene also acts as an antiproliferative drug against the epimastigote and amastigote forms of Trypanosoma cruzi, the causative agent of Chagas disease (Urbina et al., 1993), an effect associated with a specific blockade of the de novo
synthesis of phosphatidylcholine, the main phospholipid species of these cells. Here we report the effects of ajoene on the lipid composition of Y and M phase cells of \textit{P. brasiliensis}. Our results suggest a biochemical mechanism of action similar to that proposed for \textit{T. cruzi}.

**METHODS**

**Fungus and growth conditions.** \textit{P. brasiliensis} strain IVIC Pb73 (ATCC 32071) has been maintained in our laboratory on PYG medium (per litre distilled water: peptone, 5 g; yeast extract, 5 g; glucose, 15 g; final pH 7.0) agar slants for several years. It was grown in PYG liquid medium (200 ml medium in 500 ml Erlenmeyer flasks) after inoculation with 10 ml of a seed culture. The cultures were incubated for 3 d at 37 °C (Y phase) or 23 °C (M phase) with continuous shaking on a gyratory shaker operated at 120 r.p.m. (Sorais-Landiez & San-Blas, 1993). Ajoene (25 mM for the M phase) was added to the culture medium at time zero to inhibit growth by 50% (San-Blas et al., 1989). It was prepared for use as a 100 mM solution in ethanol. Cultures with the addition of equivalent volumes of ethanol in the absence of ajoene were used as controls. Ajoene was the kind gift of Dr R. Apitz-Castro (R. Apitz-Castro & M. K. Jain, US patent 4665088, May 1987).

**Lipid analysis.** Y and M cultures of \textit{P. brasiliensis} were washed three times by centrifugation (Y phase) or filtration (M phase) and total lipids extracted with chloroform/methanol (2:1; v/v). Polar and neutral lipids were separated by silicic acid column chromatography (Larralde et al., 1988). Polar lipids were fractionated by TLC using high performance plates (Merck 5715) with chloroform/methanol/30% ammonia (17:1:O, by vol.) as eluent (Cuzner & Davison, 1967); the different species were quantified by measuring organic phosphate content (Ames & Dubin, 1960). Fatty acids esterified to the lipid fraction were converted to their methyl esters by incubation in the presence of 2% H\textsubscript{2}SO\textsubscript{4} in methanol at 60 °C for 1 h (Urbina et al., 1993). The neutral lipid fraction was analysed by GLC in a Varian 3700 Gas Chromatograph equipped with a flame ionization detector as described previously (Urbina et al., 1993). For structural assignments, this fraction was also analysed in a capillary high resolution column (25 m × 0.20 mm i.d. Hewlett-Packard HP-5 MS column with 5% phenylmethysiloxane, 0.33 mm film thickness) using a Hewlett-Packard 5890 Series II Gas Chromatograph equipped with a 5971A Mass Sensitive Detector. The lipids were injected in ethyl acetate and the column kept at 50 °C for 1 min, followed by a temperature increase to 270 °C at a rate of 25 °C min\textsuperscript{-1} and finally to 300 °C at a rate of 1 °C min\textsuperscript{-1}. The carrier gas (He) flow was kept constant at 1 ml min\textsuperscript{-1}. The injector temperature was fixed at 250 °C and that of the detector at 280 °C.

**RESULTS AND DISCUSSION**

No significant differences were observed in the phospholipid species of either morphological phase of \textit{P. brasiliensis} (Table 1). Phosphatidylcholine (PC) was the main species in both Y and M cells (30-5 and 39.7%, respectively), followed by phosphatidylethanolamine (PE, 27-7 and 28.7%), phosphatidylserine (PS, 15-8 and 19.0%), and phosphatidylinositol (PI, 13-1 and 17.8%). Therefore, their proposed role in \textit{P. brasiliensis} dimorphism (Manocha, 1980) is questioned. These results contrast with those found in \textit{C. albicans} whose M form contains significantly higher levels of PC, PS and PI, and lower levels of PE, compared to the Y form (Goyal & Khuller, 1994).

The predominant fatty acid in control Y cells was palmitate (16:0; 56.2%), followed by linoleate (18:2; 18.2%) and oleate (18:1; 14.9%) (Table 2). In the M phase, linoleate was by far the most abundant component (61.1%), followed by palmitate (26.8%) and much lower levels of oleate (5%). Stearate (18:0) was a minor component in both phases (5.5–10.7%). These results agree with those of Manocha (1980) on the predominance of palmitate (16:0) in the Y phase and linoleate (18:2) in the M phase. As reported in \textit{C. albicans} (Ghanounou et al., 1986; Mishra et al., 1992; Goyal & Khuller, 1994), the ratio of unsaturated to saturated fatty acids (UFA/SFA) was higher in the M form of \textit{P. brasiliensis} (Table 2). The higher levels of SFA in the Y form are probably required to maintain normal basic permeability properties of the plasma membranes at its relatively high growth temperature (Goyal & Khuller, 1994). As in \textit{P. brasiliensis}, the lowering of growth temperature increases fatty acid unsaturation in \textit{Aspergillus niger}, \textit{Penicillium chrysogenum} and \textit{Trichoderma reesei}, but not in \textit{Neurospora crassa} where unsaturation remains unchanged (Suutari, 1995).

Ajoene is an inhibitor of \textit{in vitro} growth of \textit{P. brasiliensis} (San-Blas et al., 1989), although not as potent as sulfonamides (Restrepo & Arango, 1980), azoles and amphotericin B (Restrepo et al., 1984; San-Blas et al., 1993a). It provokes important changes in the plasma membrane, without any noticeable direct effect on the cell wall (San-Blas et al., 1989). Analysis of the cellular phospholipid content showed that ajoene induced a reversal of the relative proportion of the main phospholipid species in both Y and M phases (Table 1), leading to a predominance of PE (38.2 and 43.6%) in the Y and M phases, respectively, in contrast with data from untreated cells. The proportions of PS (14.6 and 20.9%), respectively, PI (13.1 and 18.1%) and sphingomyelin (2.7 and 5.0%) were not significantly affected. Therefore, while ajoene (200 μM) does not induce any marked changes in the ultrastructure of blood platelets from human or other mammalian species (Apitz-Castro et al., 1988), it seems highly selective for the fungal membrane where it modifies the headgroup composition of \textit{P. brasiliensis} phospholipids, similar to the effect of aqueous garlic extracts (AGE) on \textit{C. albicans} (Adetumbi et al., 1986; Ghanounou, 1988). Compositional changes of this type should lead to a very unstable lipid bilayer, consistent with the ultrastructural results obtained previously (San-Blas et al., 1989). Treatment of Y cells with ajoene (Table 2) also produced a drastic reduction in SFA (16:0 and 18:0) (67–35%) with a concomitant increase (33%–65%) of the UFA (18:1 and 18:2), leading to a UFA/SFA ratio similar to that of the M phase. The large increase in UFA in the Y phase could lead to loss of vital cytoplasmic components in the treated cells. On the other hand, no significant modifica-
The results, expressed as mol%, are means ± SEM of three independent experiments.

<table>
<thead>
<tr>
<th>Phospholipid species</th>
<th>Y phase Control + 25 mM ajoene</th>
<th>M phase Control + 50 mM ajoene</th>
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<tbody>
<tr>
<td>PC</td>
<td>30.5 ± 4.1 17.2 ± 2.5</td>
<td>39.7 ± 3.8 19.4 ± 3.6</td>
</tr>
<tr>
<td>PE</td>
<td>27.7 ± 3.4 38.2 ± 4.0</td>
<td>28.7 ± 3.5 43.6 ± 5.0</td>
</tr>
<tr>
<td>PS</td>
<td>19.0 ± 2.4 20.9 ± 2.5</td>
<td>15.8 ± 2.8 14.6 ± 3.0</td>
</tr>
<tr>
<td>PI</td>
<td>17.8 ± 2.3 19.8 ± 2.6</td>
<td>13.1 ± 2.6 18.5 ± 3.0</td>
</tr>
<tr>
<td>Sphingomyelin</td>
<td>5.0 ± 1.2 3.9 ± 1.1</td>
<td>2.7 ± 0.9 3.9 ± 1.0</td>
</tr>
</tbody>
</table>

The results, expressed as mol%, are means ± SEM of three independent experiments.

<table>
<thead>
<tr>
<th>Fatty acid species</th>
<th>Y phase Control + 25 mM ajoene</th>
<th>M phase Control + 50 mM ajoene</th>
</tr>
</thead>
<tbody>
<tr>
<td>16:0</td>
<td>56.2 ± 5.3 32.3 ± 3.6</td>
<td>26.8 ± 1.1 28.7 ± 1.5</td>
</tr>
<tr>
<td>18:0</td>
<td>10.7 ± 3.2 2.6 ± 0.5</td>
<td>5.5 ± 0.3 6.3 ± 1.0</td>
</tr>
<tr>
<td>18:1</td>
<td>14.9 ± 0.7 23.9 ± 3.8</td>
<td>6.6 ± 0.1 5.0 ± 1.7</td>
</tr>
<tr>
<td>18:2</td>
<td>18.2 ± 4.0 41.2 ± 1.9</td>
<td>6.1 ± 1.3 6.0 ± 4.2</td>
</tr>
<tr>
<td>SFA</td>
<td>66.9 ± 6.9 34.9 ± 4.2</td>
<td>32.3 ± 1.5 35.0 ± 2.3</td>
</tr>
<tr>
<td>UFA</td>
<td>33.1 ± 4.5 65.1 ± 5.5</td>
<td>67.7 ± 1.4 65.0 ± 5.2</td>
</tr>
<tr>
<td>UFA/SFA</td>
<td>0.49 ± 0.1 1.87</td>
<td>2.10 ± 1.5 1.86</td>
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previously for the protozoon *T. cruzi* (Urbina et al., 1993), suggesting a common antiproliferative mechanism of ajoene against lower eukaryotes and resulting in a novel mechanism of antifungal action.

ACKNOWLEDGEMENTS


Received 22 October 1996; revised 26 November 1996; accepted 19 December 1996.

G. SAN-BLAS and OTHERS

REFERENCES


