5-Octadecenoic acid: evidence for a novel type of fatty acid modification in schistosomes

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INTRODUCTION

The parasitic blood fluke Schistosoma mansoni is a long-term inhabitant of the mesenteric veins of its final host. The presence of a membrane complex that surrounds the schistosome is a very characteristic adaptation to the continuous contact between the parasite and the immune system of the host. This membrane complex, which consists of two closely apposed lipid bilayers, is synthesized from discoid and multilamellar bodies that are made in cell bodies just below the tegument [1,2]. Maintenance of the integrity of these outer tegumental membranes is essential for the survival of the parasite. Phospholipids, and, in particular, phosphatidylycerine (PC; referring collectively to all diradyl choline glycerophospholipid species), are the main constituents of these membranes [3].

Many important physical and biological properties of membranes are determined by their phospholipid composition. The degree of unsaturation of the fatty acyl constituents of the phospholipids determines the fluidity of the membranes, and therefore plays an important role in events such as endocytosis and exocytosis, sorting of lipids and membrane fusion. Furthermore, after hydrolysis of phospholipids by the appropriate phospholipases, diacylglycerol and fatty acids can serve as important mediators (or precursors thereof) in events such as signal transduction, immune modulation (e.g. via the synthesis of prostaglandins and leukotrienes) and lipid metabolism. Therefore a rigorous characterization of the fatty acids present in the tegumental membranes is essential in order to assess lipid-related functions of the tegument.

Here we demonstrate the presence in the schistosome of the unusual fatty acid, 5-octadecenoic acid [C\textsubscript{18:1\(\text{10:1}\)] which is very abundant in the outer tegumental membrane complex, but absent in the blood of the host and nearly absent in the worm body. This fatty acid, which does not observe the general rule that the first double bond in a fatty acid is introduced in the 9 position [4], was present in one PC molecular species only. According to [4], this was the most abundant phosphatidylcholine species in the outer membrane complex. The specific synthesis by the schistosome of C\textsubscript{18:1\(\text{10:1}\)] and the highly specific localization of this fatty acid to the tegumental membranes suggest an important tegument-mediated role for this lipid.

MATERIALS AND METHODS

Chemicals

All solvents were of HPLC grade and obtained from Labscan (Dublin, Ireland). Hepes was purchased from Gibco (Paisley, Renfrewshire, Scotland, U.K.) All other chemicals, including standards of molecular species and fatty acids, were from Sigma Chemical Co. (St. Louis, MO, U.S.A.) or Baker (Deventer, The Netherlands) and were of the highest purity available. [1\(\text{13}^\text{C}\)]Stearic acid was obtained from Amersham (Amersham, Buckinghamshire, UK).

Parasites

S. mansoni worms were isolated from diethyl ether-anaesthetized...
Isolated peaks were identified with on-line ESI-MS. Note the occurrence of two (C\textsubscript{16:0–18:1})PtdCho species (labelled in bold). Major species were identified as: 1, (C\textsubscript{16:0–22:6})PtdCho; 2, (C\textsubscript{16:0–20:4})PtdCho; 3, (C\textsubscript{16:0–18:2})PtdCho; 4, (C\textsubscript{16:0–18:1})PtdCho; 5, (C\textsubscript{16:0–16:0})PtdCho; 6, (C\textsubscript{16:0–18:1})PtdCho; 7, (C\textsubscript{16:0–16:0})alkylacyl PC; 8, (C\textsubscript{20:1–16:0})PtdCho; and 9, (C\textsubscript{18:0–18:1})PtdCho.

**Isolation of lipid fractions**

Lipids were extracted by the method of Bligh and Dyer [7]. Approx. 80–100 worm pairs were used per HPLC analysis of tegumental phospholipids. Phospholipid classes were separated on HPLC using a 125 mm \times 4 mm Lichrospher 4 \mu m normal-phase column (Merck, Darmstadt, Germany). Elution was performed with a gradient of hexane:propan-2-ol: dichloromethane (20:27:3, by vol.) to hexane:propan-2-ol:dichloromethane:water (20:23:3:4, by vol.) during 10 min, and an additional 10 min elution with the latter solvent. Detection was performed with a Varex MKIII light-scattering detector (Alltech, Deerfield, IL, U.S.A.) operated at a gas flow rate of 2 l\(\text{min}^{-1}\), and the drift tube temperature set to 100 °C. Phosphatidylethanolamine (PE), phosphatidylserine (PS), phosphatidylinositol (PI), sphingomyelin and PC were isolated with the aid of a flow splitter inserted between column and detector.

**Molecular species analysis**

After isolation of the lipid fractions, PC molecular species were separated as described previously [8]. Briefly, PC was eluted isocratically from two Lichrospher RP18 end-capped columns in series using a mobile phase of acetonitrile:methanol:triethylamine (8:11:1, by vol.). The light-scattering detector was set to 1.8 l\(\text{min}^{-1}\) and 100 °C. The positions of the fatty acyls along the glycerol backbone were determined by phospholipase A\textsubscript{2} digestion of individual components collected at the outlet of the column. Free fatty acids were converted into their phenacyl ester derivatives, eluted with acetonitrile from the reverse-phase columns described above, and detected at 242 nm [5,9]. Identification of molecular species eluting from the column was performed with on-line electrospray ionization–MS (ESI–MS), as described below.

**Figure 1** HPLC chromatogram, as monitored by light-scattering detection, showing the separation of molecular species of PC isolated from the outer tegumental membranes of *S. mansoni*

**Figure 2** Negative-ion-mode electrospray mass spectrum of component 6 from Figure 1, identifying (C\textsubscript{16:0–18:1})PtdCho

Fragment ions result from fragmentation at the positions indicated in the structural formula. The positions of palmitate (m/z 255) and oleate (m/z 281) along the glycerol backbone were determined by phospholipase A\textsubscript{2} digestion of the isolated molecular species. The spectrum shown is identical to the mass spectrum recorded during elution of component 4 in Figure 1.
Identification of PC molecular species was performed with a Fisons VG Platform II single quadrupole mass spectrometer (Micromass, Manchester, U.K.), fitted with an electrospray ion source operated at atmospheric pressure and in the negative mode, which was connected to the outlet of the HPLC columns via a flow splitter. Nitrogen was used as nebulizer gas (0.2 l min⁻¹) and as curtain gas (7 l min⁻¹). The capillary voltage was set to 3.0 kV and the cone voltage to 200 V.

Determination of the position of the double bond within the fatty acid was performed with tandem MS on a Jeol JMS SX/SX 102A instrument, equipped with a fast-atom-bombardment (FAB) gun and source used for the generation of the negative precursor ion $M^−$ ($m/z$ 281) from individual PC molecular species that were purified by HPLC. Triethanolamine was used as a matrix. Collision-induced dissociation (CID) spectra of the precursor ion selected by MS-1 were acquired by MS-2. Air was used as the collision gas.

RESULTS

Identification of two isomers of octadecenoic acid (C₁₈:₁) in the tegumental membranes

In Figure 1 the separation of molecular species of PC from the outer tegumental membranes is shown, as monitored by light-scattering detection. Species that eluted from the column were identified by on-line ESI–MS, resulting in a mass spectrum for each component that allowed the identification of the choline headgroup, the two fatty acyl chains and the molecular mass of the component. This resulted in the identification of the main molecular species, as shown in Figure 1. Most notable was the fact that two major molecular species (Figure 1; peaks 4 and 6) displayed identical mass spectra. In Figure 2, this mass spectrum is shown (collected during the elution of component 6 in Figure 1). From the mass of the molecular ion ($m/z$ 758) it can be deduced that the acyl chains of the PC species under investigation have a total of 34 carbon atoms, contain one single double bond and are linked to the glycerol backbone via ester bonds [10]. In the $m/z$ range from 250 to 350 the fatty acid carboxylate anions are present that are lost from the glycerol backbone during fragmentation. The two fatty acids present are C₁₆:₀ ($m/z$ 255) and C₁₈:₁ ($m/z$ 281), which are in agreement with the expected total mass of the acyl chains. The presence of the fragment ions $m/z$ 744 [$M$−15], $m/z$ 699 [$M$−60] and $m/z$ 673 [$M$−86] further confirms that the components under investigation are indeed PC species, since these fragment ions result from typical losses from the choline headgroup ([10]; see structural formulae and fragmentation sites in Figure 2). Peak 4 (Figure 1) co-eluted with an authentic standard of palmitoyl oleoyl PC [C₁₆:₀·C₁₈:₁(PtdCho)].

By phospholipase A₂ digestion of the isolated molecular species, it could be demonstrated that the C₁₆:₀ chain of both components 4 and 6 was esterified at the sn-1 position and that, in both components, the sn-2 position contained the C₁₈:₁ fatty acid. The longer retention time of component 6 compared with component 4 was a result of a difference in the C₁₈:₁ fatty acid: after hydrolysis of the molecular species and conversion of the C₁₈:₁ fatty acids to their phenacyl derivatives, the C₁₈:₁ from component 4 co-eluted with an authentic standard of oleic acid [C₁₈:₁(9)], whereas the C₁₈:₁ from component 6 had a longer retention time on the reverse-phase HPLC column (data not shown).

Figure 3 CID spectrum of $m/z$ 281

Tandem MS was performed on components 4 and 6 (see Figure 1). After FAB ionization, MS-1 was used to select $m/z$ 281 as precursor ion for MS-2. After collision activation of $m/z$ 281, the charge-remote fragmentation of the precursor ion was recorded with MS-2. (A) shows the charge-remote fragmentation of $m/z$ 281, derived from component 4 (Figure 1), identifying C₁₈:₁(9) (oleic acid). (B) shows the charge-remote fragmentation of $m/z$ 281, derived from component 6 (Figure 1), identifying C₁₈:₁(9). Fragmentation sites and the resulting products are indicated in the structure formula. The ion at $m/z$ 263 results from the loss of water from the precursor ion. Fragment ions indicated by • are matrix-derived, and are unrelated to the $m/z$ 281 ion.

Determination of the position of the double bond in the fatty acid

The position of the double bond within the fatty acyl chain was determined by FAB–CID tandem MS. The fatty acid carboxylate anion selected with MS-1 was collisionally activated and the subsequent dissociation of the ion, mainly occurring via charge-remote fragmentation, was recorded by MS-2. The position of the double bond is identified by a gap in the regularly spaced series of fragment ions (Figure 3). This gap results from the fact that no fragmentation occurs at either the double bond or at the two adjacent vinylic bonds [11]. MS-2 spectra are presented of the $m/z$ 281 ion of components 4 (Figure 3A) and 6 (Figure 3B). On the basis of the fragmentation pattern in Figure 3A, the $m/z$ 281 ion from component 4 was identified as C₁₈:₁(9) (oleic acid), which was confirmed by a standard of oleic acid having the same fragmentation pattern (results not shown). All commercially available positional isomers of C₁₈:₁ [C₁₈:₁(10), C₁₈:₁(11), C₁₈:₁(12), C₁₈:₁(9)] gave the typical regularly spaced series of fragment ions, in which a gap identifies the position of the double bond. Therefore the $m/z$ 281 ion from component 6 was identified as C₁₈:₁(10), although an authentic standard of this fatty acid is not available (Figure 3B).
Table 1 Ratios of C18:1(9) to C18:1(10) in lipid fractions of the host and the parasite

<table>
<thead>
<tr>
<th>Sample</th>
<th>C18:1(9):C18:1(10)</th>
</tr>
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<tbody>
<tr>
<td>Hamster blood, total lipids</td>
<td>&lt; 0.001*</td>
</tr>
<tr>
<td>S. mansoni, total lipid</td>
<td>0.007 ± 0.002</td>
</tr>
<tr>
<td>S. mansoni, tegumental phospholipids</td>
<td>0.41 ± 0.07</td>
</tr>
<tr>
<td>S. mansoni, tegumental PS + PI (6%)</td>
<td>&lt; 0.005</td>
</tr>
<tr>
<td>S. mansoni, tegumental sphingomyelin (14%)</td>
<td>&lt; 0.010</td>
</tr>
<tr>
<td>S. mansoni, tegumental PE (29%)</td>
<td>0.16 ± 0.09</td>
</tr>
<tr>
<td>S. mansoni, tegumental PC (50%)</td>
<td>1.4 ± 0.3</td>
</tr>
</tbody>
</table>

Specific localization of C18:1(9) to the tegumental PC fraction

The amount of C18:1(9) in the blood of the host, on which schistosomes feed, was too low to be detected. Within the schistosome, C18:1(9) was not distributed homogeneously, as it was almost exclusively present in the outer tegumental membranes (Table 1). The distribution of C18:1(10) among the phospholipid classes of the outer tegumental membranes was also found not to be homogeneous. The phospholipid composition of the outer tegumental membranes, given in Table 1, is in good agreement with previous data on this membrane complex [3]. Of the major phospholipids in these membranes, sphingomyelin and PE contained only very small amounts of C18:1(9), whereas the amount of C18:1(9) exceeded the amount of C18:1(10) in the PC fraction (Table 1). Desaturation of [1-13C]stearic acid to [1-13C]C18:1(9) could not be demonstrated during in vitro incubations of intact adult schistosomal worm pairs (results not shown).

Discussion

Identification of C18:1(9) in the outer tegumental membranes

C18:1(9) is rarely present in Nature. However, using the combination of a chromatographic procedure that is capable of isolating a single molecular species of PC and subsequent tandem MS, we were able to unequivocally demonstrate the presence of this fatty acid in schistosomes. The spectra of Figure 3 revealed the elimination of C18:1(9) units from the m/z 281 precursor ion, which is characteristic of charge-remote fragmentation of fatty acids [12]. Other types of fragmentation that occur include the loss of water from the carboxy group (resulting in m/z 263) and the loss of C18:1(9) units when fragmentation occurs in the vicinity of a double bond [m/z 58 and m/z 126 in Figure 3(B)]. The observed fragmentations are in good agreement with CID spectra of monounsaturated fatty acids published previously [11].

Implications of the C18:0 double bond

The double bond located at the C18:0 position may be expected to result in considerable metabolic differences between (the common) oleic acid [C18:1(9)] and the isomer identified here [C18:1(10)]. Only fatty acids containing a double bond at C18:0 were released following thrombin or histamine stimulation of vascular endothelial cells [13,14]. A comparable selectivity was seen in several lipases, e.g. the lipase from oilseed rape discriminates against C18:1(9) and C18:0 unsaturated fatty acids, but readily esterifies C18:1(9) or C18:0 unsaturated fatty acids [15]. The activity of pancreatic lipase is less specific, but it is inhibited by a double bond at any position up to C16:0 [16]. A strong indication that, also in schistosomes, there is a fatty acyl specificity mediated by the position of the double bond in the fatty acid lies in the different ratios of C18:1(9):C18:1(10) found in the phospholipids of the outer tegumental membrane complex (Table 1). PC was particularly enriched in C18:1(9); lesser amounts were present in PE, and only trace amounts of C18:1(10). Were present in sphingomyelin, PS and PI. It is not yet known, however, whether this heterogeneous distribution of C18:1(9) is already achieved during the de novo synthesis of the phospholipids, or whether it is introduced by the deacylating/reacylating system, which is very active in schistosomes [5]. It is of interest that Wolf [17] reported on the structural importance of the double bond at C18:0 in channelling fatty acids to the m-2 position of phospholipids (PI in particular), but it could not be shown whether this was a result of de novo synthesis or of deacylation/reacylation.

Origin of the C18:0 double bond

Schistosomes are not capable of either de novo synthesis or β-oxidation of fatty acids and, to date, fatty acid desaturation has not been demonstrated [18,19]. However, the fact that the uncommon C18:1(9) fatty acid is absent in the blood of the host on which schistosomes feed implies that C18:1(9) is synthesized by the parasite itself. This idea is further supported by the very specific localization of this fatty acid in the tegumental membranes. The phospholipids of these membranes are synthesized by highly specialized cells lying directly underneath the tegument, and the enzymes required for the synthesis of C18:1(9) might be expressed in these cells only. The small quantity of C18:1(9) present in schistosomes is likely to explain why no measurable conversion of [1-13C]stearic acid into [1-13C]C18:1(9) occurred. An alternative explanation for the presence of this specific fatty acid in the tegumental membranes, i.e. uptake (and subsequent transport) of undetectable trace amounts of C18:1(9) specifically to the PC fraction of the tegumental phospholipids, seems highly improbable.

In conclusion, we have reported the presence of a fatty acid [C18:1(9)] in schistosomes that is almost exclusively present in the outer tegumental membranes of the parasite. On the basis of the absence of the fatty acid in the host and the highly specific localization within the schistosome, we believe that this fatty acid is synthesized by the parasite, thereby representing a type of fatty acid modification that was previously not known in schistosomes. The function of the fatty acid in the tegumental membranes remains to be resolved.

The expert help of E. Vernooij (Department of Pharmaceutics, Utrecht University, Utrecht, The Netherlands) with the collection and interpretation of ESI-MS data is gratefully acknowledged. This work was supported by the Netherlands Foundation for Chemical Research (SON) and the Life Science Foundation (SLW) of The Netherlands Organization for Scientific Research (NWO).

References


Received 26 May 1998/9 July 1998; accepted 14 July 1998