Lipids Shed into the Culture Medium by Trypomastigotes of *Trypanosoma cruzi*

Rosalia Agusti, Alicia S Couto, Maria Júlia M Alves*, Walter Colli*/+, Rosa M de Lederkremer

Cihidecar, Departamento de Química Orgánica, Facultad de Ciencias Exactas y Naturales, Universidad de Buenos Aires, 1428, Buenos Aires, Argentina *Departamento de Bioquímica, Instituto de Química, Universidade de São Paulo, Caixa Postal 26077, 05599-970 São Paulo, SP, Brasil

Trypomastigote forms of *Trypanosoma cruzi* were metabolically labeled with [14C]-ethanolamine and [3H]-palmitic acid. Lipids shed to the culture medium were analyzed and compared with the parasite components. Phosphatidylcholine and lysophosphatidylcholine accounted for 53% of the total incorporated precursor. Interestingly, phosphatidylethanolamine and its lyso derivative lysophosphatidylethanolamine, although present in significant amounts in the parasites, could not be detected in the shed material. Shed lipids were highly enriched in the desaturated fatty acids C16:1 and C18:1 when compared to the total fatty acid pool isolated from the parasites.

Key words: [14C]-ethanolamine and [3H]-palmitic acid incorporation - phospholipids - desaturated fatty acids

Lipidic components and their derivatives have been lately related to important biological functions in different systems. In this respect there is an increasing interest in the study of lipids from trypanosomatids as chemotaxonomic and/or chemotherapeutic targets (Docampo & Pignataro 1991, Machado de Domenech et al. 1992, Schneider et al. 1994, Racagni et al. 1995).

Some years ago, we began a systematic study of the lipids of the trypomastigote stage. We have already characterized neutral and zwitterionic lipids (Uhrig et al. 1997) and inositolphospholipids (Uhrig et al. 1996), among others (Couto et al. 1985, Uhrig et al. 1992). The shedding of different lipidic components in vesicles has been reported (Gonçalves et al. 1991). Qualitative and quantitative differences between the shed components and the lipids remaining in the parasite were observed (Couto et al. 1991).

The characterization of shed components may help to explain the multiple manifestations of the disease, including autoimmune processes. In this work, we describe neutral and zwitterionic lipids shed to the culture medium. Metabolic incorporation of [14C]-ethanolamine and [9,10 (n)-3H]-palmitic acid were used as tracers in order to compare the labeled lipids with the parasite composition.

**MATERIALS AND METHODS**

*Parasites -* Trypomastigotes of *T. cruzi* (Y strain) were obtained from infected LLC-MK2 epithelial cell monolayers maintained in Dulbecco’s modified Eagle medium (DME) containing 2% fetal calf serum (FCS). Parasites were collected on the fifth day after infection (Andrews & Colli 1982).

*Parasite labeling -* Parasites (1.5 x 10^9) were resuspended at a density of 8 x 10^7 cells/ml in DME containing 2% of fetal calf serum, 20 mM Hepes and metabolically labeled with [9,10(n)-3H]-palmitic acid (Amersham, Buckinghamshire, England, 54 Ci/mmol, 41 mCi/ml) for 2, 4 and 6 h at 37°C. The homogeneity of palmitic acid was tested before the labeling experiment by RPTLC. Incorporation of [1,2,14C]-ethanolamine (NEN, Boston, USA, 3 mCi/ mmol) was performed with 1 x 10^9 trypomastigotes, Parasites were resuspended at a density of 10 x 10^7 cells/ml in DME, 2% FCS, 20mM Hepes, and were incubated with [1,2,14C]-ethanolamine, 5 mCi/ml, for 6 h at 37°C.

After incubation, microscopic observation showed that all parasites remained viable. Trypomastigotes were harvested and the culture...
medium was filtered through Millipore (0.22 µm) and freeze-dried.

Lipid extraction - Parasites and the correspond- 
ing dried medium were twice extracted with chloroform:methanol 2:1 and 1:1 (v/v). The extracts were separated by centrifugation, pooled, dried in vacuo and fractionated on DEAE-Sephadex A-25 (acetate form, 10 x 0.5 cm) as previously described (Couto et al. 1991). Lipids that do not interact with the resin were eluted with chloroform:methanol:water (65:25:4, by vol.). Non-bound lipids were labeled with [1⁴C]-ethanolamine were resus-
pended in water and passed through a C₁₈-clean up cartridge (Worldwide monitoring PA, USA). Salts and free radioactive precursor were eluted with water and lipids were recovered with methanol.

As a control, a sample of the [³H]-palmitic acid labeled extract obtained from the parasites was in-
cubated for 6 h in DME containing 2% of FCS, 20 mM Hepes. The mixture was extracted and frac-
tionated as above.

TLC and fluorography - Phospholipids and 
lipid standards were purchased from Sigma. Thin layer chromatography (TLC) was performed on silica gel 60 precoated plates (Merck) using the fol-
lowing solvent systems: A) chloroform:methanol:water (15:30:4, 100 ml), and strongly acidic lipids were eluted with chloroform:methanol :0.8 M sodium acetate (15:30:4, 100 ml). Non-bound lipids labeled with [³H]-palmitic acid were resus-
pended in water and passed through a C₁₈-clean up cartridge (Worldwide monitoring PA, USA). Salts and free radioactive precursor were eluted with water and lipids were recovered with methanol.

Reverse-phase TLC was performed on RP-18 F-254 precoated plates (Merck) using acetonitrile: 
acetic acid (1:1, v/v) (solvent system E).

In all cases, radioactive samples were located by fluorography at -70ºC using EN³HANCE (NEN) 
and Kodak-X-Omat AR films. Radioactivity was determined in a 1214 RackBeta Wallac liquid scin-
tillation counter.

Analysis of fatty acids - Free fatty acids were 
extracted from the TLC plate with chloroform and 
the corresponding methyl esters were obtained by 
treatment with BF₃/methanol (20% in methanol, 
Merck, 1 ml) at 80°C for 1 h in a screw cap test tube 
(Manku 1983). Labeled fatty acid methyl esters were 
analyzed by RP-TLC in solvent E.

Hydrogenation - Labeled fatty acid methyl es-
ters were subjected to hydrogenation with palla-
dium on activated carbon (palladium content 10%, 
Aldrich), using an hydrogen pressure of 3 atm. The 
reaction was performed for 4 to 5 h with shaking at 
room temperature (Kates 1986). A sample of linoleic 
acid methyl ester (C₁₈:2) was treated under the same 
conditions as a reaction control.

Phosphatidylinositol phospholipase C diges-
tion - The acidic lipids were suspended in 0.5 ml of 
50 mM Tris/HCl, pH 7.4 and incubated for 90 min at 
37°C with 0.1 units of phosphatidylinositol-phos-
pholipase C (PI-PLC) from Bacillus thuringiensis 
in the presence of 0.1% deoxycholate (Menon 1994).

RESULTS

Trypomastigote forms of T. cruzi were meta-
bolically labeled with [³H]-palmitic acid. Preliminary 
trials have shown that different incorporation times 
resulted in the same lipid pattern. Thus, incorpora-
tion for 6 h was adopted throughout the experi-
mental work in order to optimize incorporation of the 
label. The culture medium was separated by 
centrifugation, filtered by Millipore and freeze-dried.

The material obtained was extracted with 
chloroform:methanol. The lipidic extract was frac-
tionated on a DEAE-Sephadex (AcO⁻ form) column 
and the unbound fraction was analyzed by TLC 
and compared with a fraction obtained by the same 
procedure from labeled parasites (Fig. 1a). The to-
tal pattern of both fractions is significantly differ-
ent, confirming that shed components do not origi-
nate from lysis of parasites. The addition of para-
sites freshly labeled extracts to the same medium 
used for the incorporation did not lead to the con-
version of the pattern of Fig. 1 (lane 1) to that of 
lane 2, indicating that lipid modifications were not 
produced by any residual enzyme activity in FCS.

The culture medium of trypomastigote forms 
metabolically labeled with [¹⁴C]-ethanolamine was 
also analyzed. No radioactive PE was detected and 
only a trace of labeled LPE was shown (Fig. 1b, 
lane 1). Shed [³H]-palmitic acid labeled lipids were 
run in the same TLC only for comparison (Fig. 1b, 
lane 2). No PC could be detected when labeling 
with ethanolamine, as trypomastigotes do not 
biosynthesize this phospholipid by sequential me-
thylation of PE (Uhlig et al. 1997) as is usual in 
other systems (Vance 1990).

Phosphatidylcholine (PC) and lysophosphati-
dylcholine (LPC) were detected in the shed lipids labeled 
with [³H]-palmitic acid. As PC and lysophosphati-
dylinositol-phosphatidylinositol-phospholipase C (PI-PLC) from Bacillus thuringiensis 
in the presence of 0.1% deoxycholate (Menon 1994).
in three fractions (I, II and III) and rechromato-
graphed in solvent B (Fig. 3). As expected, no
triacylglycerol (TAG) was detected confirming no
lysis of parasites. All fractions contained free fatty
acids, which were methylated and analyzed by
RPTLC in comparison with the same sample sub-
jected to hydrogenation (Fig. 4). Spots correspond-
ing to $C_{16}0$, $C_{18}0$, $C_{16}1$ and $C_{18}1$ were detected.
Unsaturated fatty acids disappeared by hydroge-
nation. The $C_{16}1$ is the most abundant radioactive
fatty acid. Of course, it must be appreciated that
not all $C_{16}0$ is of endogenous origin since some
contamination with the original labeled precursor
is unavoidable.

Acidic lipids recovered from the bound frac-
tion of the ion-exchange chromatography were also
analyzed by TLC in solvent D (not shown). Spots
migrating as the reported inositolphospholipids
(Uhrig et al. 1996) were found. As expected, all of
them resulted sensitive to PI-PLC digestion.

**DISCUSSION**

In every stage of the *T. cruzi* cycle, the charac-
terized antigenic structures present a lipidic moiety
that anchors the protein to the membrane (Güther
et al. 1992, Couto et al. 1993, Almeida et al. 1994,
Acosta Serrano et al. 1995, Bertello et al. 1996,
Ferguson 1997, Agusti et al. 1997) and antigens
released by the parasites have been described as
potential targets for immunoidentification (Dzbenski

Circulating antigens either free or in the form of
immunocomplexes may play an important role in
the immunopathology of Chagas disease, contrib-
uting to processes of autoagression or immuno-
suppression (Petry & Eisen 1989). The knowledge
of the intimate mechanism of surface antigen shed-
ding would contribute to the understanding of these
phenomena. In this direction we have already de-
scribed the shedding of lipidic components to the
culture medium of the infective forms (Couto et al. 1991), supporting reports for the spontaneous release of antigens in vesicles (Gonçalves et al. 1991). In the present work, we have found a low content of radioactive neutral lipids in the culture medium in contrast with the results obtained with induced vesiculation of epimastigote forms (Da Silveira & Colli 1981). Furthermore, PC and PE are the main components in epimastigote vesicles and the composition of lipids in trypomastigote forms is 3.1% PE, 2.6% LPE, 13.5% PC and 5.1% LPC (Uhrig et al. 1997). However, radioactive PE could not be detected in the shed fraction with neither of the precursors used.

It is well known that lipids and mainly phospholipids, regulate the membrane fluidity and prepare the bilayer for sheltering and maintenance of active membrane proteins (Singh et al. 1996). This may be related to the shedding of various important proteins of the trypomastigote stage such as SAPA (Affranchino et al. 1989, Pollevick et al. 1991, Agusti et al. 1997) or Tc-85 (Couto et al. 1993, Abuin et al. 1996) both being anchored by a glycosylphosphatidylinositol (GPI) to the surface membrane. It has been speculated that GPI-anchored proteins partition preferentially in plasma membrane domains enriched in certain membrane constituents. The functional consequences of this association are not clear, but perhaps it is important in membrane remodelling events such as vesiculation and some forms of endocytosis (Whitlow et al. 1993). The fact that only certain lipid components of the parasite membrane, namely PC, LPC and free fatty acids, are present in the shed material would highlight the selectivity of this process.

The most striking finding was the presence of C_{16:1} and C_{18:1} free fatty acids in shed lipid fraction. Although unsaturated fatty acids have been detected free and as components of PC and TAG (Uhrig et al. 1997) when cold trypomastigotes were analysed (cf. also Kaneda et al. 1986, Leon et al. 1989, Esteves et al. 1989),
they were not found when labeling with $[^{3}H]$-palmitic acid, as shown in Fig. 4, lane 1.

A well characterized phenomenon associated with the acclimation of organisms to changes is the regulation of the molecular motion or “fluidity” of membrane lipids via unsaturation of the fatty acids. This phenomenon depends on the activation of desaturases activities (Vigh et al. 1993) and may become appreciable and critical for the shedding of certain antigens. It must be stressed also that the fatty acids from trypomastigote GPIs are mostly unsaturated (Ferguson 1997) and, at least in part, could have been released by activated acyl hydrolases.

Further investigation will be necessary to elucidate whether desaturation in fact occurs during or after shedding as well as the degree of contribution of GPI-anchor bound unsaturated fatty acids to the pool of shed lipids.

REFERENCES


