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IMMUNOLOGY AND HOST-PARASITE INTERACTIONS - ORIGINAL PAPER

Bioactive lipids regulate *Trypanosoma cruzi* development

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Abstract

Trypanosoma cruzi is the etiological agent of Chagas disease. These parasites undergo dramatic morphological and physiological changes during their life cycle. The human-infective metacyclic trypomastigotes differentiate from epimastigotes inside the midgut of the Triatominae insect vector. Our group has shown that the saliva and feces of *Rhodnius prolixus* contains a lysophospholipid, lysophosphatidylcholine (LPC), which modulates several aspects of *T. cruzi* infection in macrophages. LPC hydrolysis by a specific lysophospholipase D, autotaxin (ATX), generates lysophosphatidic acid (LPA). These bioactive lysophospholipids are multisignaling molecules and are found in human plasma ingested by the insect during blood feeding. Here, we show the role of LPC and LPA in *T. cruzi* proliferation and differentiation. Both lysophospholipids are able to induce parasite proliferation. We observed an increase in parasite growth with different fatty acyl chains, such as C18:0, C16:0, or C18:1 LPC. The dynamics of LPC and LPA effect on parasite proliferation was evaluated in vivo through a time- and space-dependent strategy in the vector gut. LPC but not LPA was also able to affect parasite metacyclogenesis. Finally, we determined LPA and LPC distribution in the parasite itself. Such bioactive lipids are associated with reservosomes of *T. cruzi*. To the best of our knowledge, this is the first study to suggest the role of surrounding bioactive lipids ingested during blood feeding in the control of parasite transmission.

Keywords *Trypanosoma cruzi* · Lysophosphatidylcholine · Lysophosphatidic acid · *Rhodnius prolixus*

Introduction

Chagas disease is caused by *Trypanosoma cruzi* that turns into its infective form in the final stage of its life cycle inside the digestive system of their Triatominae vectors in a process termed as metacyclogenesis. Metacyclogenesis involves the transformation of epimastigotes into the human-infective metacyclic trypomastigotes. Triatomines ingest a large amount of blood, which is quickly followed by diuresis and then slow digestion. The changes caused by blood feeding in the intestinal environment trigger a series of cellular responses that induce metacyclogenesis of *T. cruzi* (Lopes et al. 2010; Ribeiro et al. 2014). This transition involves changes in cell morphology, displacement of flagellum, and large

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transcriptional activity that ceases only after the formation of metacyclic trypomastigotes (Ferreira et al. 2008). Epimastigote adhesion to the intestine occurs in various regions of the vector gut, through the flagellum, and more specifically in the rectum. It is a prerequisite for the differentiation of the parasite and involves the interaction between the molecules present on parasite surface and in the rectal epithelium of the vector (Kollien and Schaub 1998, 2000).

The primary changes in parasite biology during its passage through the intestine occur in the rectum, and it is there where the highest number of metacyclic trypomastigotes is found (Kollien and Schaub 1998, 2000). Many studies have revealed specific aspects of the modulation of cellular and molecular changes in this phase of the parasite's life cycle. Detailed analysis of proteomics and phosphoproteomics revealed dozens of marker proteins involved in this process (Parodi-Talice et al. 2007; De Godoy et al. 2012). About 50% of the identified proteins are unique to specific stages of the parasite. Isoforms of protein kinase C and their activation by oleic acid have been shown previously, but the source and trigger signals for the activation of this pathway remain unknown (Belaunzarán et al. 2009). Parasites that overexpress intracellular signaling proteins, such as GTPase Rho (TcRho1), generate fewer metacyclic trypomastigotes (De Melo et al. 2004; Dos Santos et al. 2012). There is also evidence that the presence of free heme and the redox environment play an important role in controlling the cell cycle of the parasite during metacyclogenesis (Souza et al. 2009; Paes et al. 2011; de Almeida Nogueira et al. 2011; Mesquita et al. 2015). The release of digestion products, such as hemoglobin, heme, and lipids, produces a huge flow of metabolic molecules. Thus, a large portion of the modulatory signals regulating metacyclogenesis arise from the events occurring in parallel to it. The integration of induction signals and parasite responses along the metacyclogenesis still deserve a more specific biochemical approach. This will allow defining of the molecular events involved, their main effectors, and their connections with the microenvironment in which it occurs.

We have been trying to identify and determine the mechanism of action of molecules involved in the metabolism of various phases of the cycle of transmission and pathogenesis of Chagas disease, particularly in those stages that occur within the invertebrate vector, *R. prolixus* (Corrêa et al. 2008; Mesquita et al. 2008, Silva-Neto et al. 2012; Nogueira et al. 2015, dos Ximenes et al. 2015). Our group has been studying the molecular and physiological details of lipid metabolism during blood meal digestion (Atella et al. 2000; Grillo et al. 2007; Bittencourt-Cunha et al. 2013; Ribeiro et al. 2014; Mesquita et al. 2015). Digestion-derived lipids are synthesized de novo and transferred to lipophorin, the major hemolymphatic lipoprotein. These lipids are transported to other organs such as fat body, where they are stored in the insect ovary to ensure the development of oocytes and embryos (Atella et al. 2005, 2006; dos Ximenes et al. 2015). In the intestinal epithelium, fatty acids from digestion are used for the synthesis of triacylglycerols (TAGs), diacylglycerols (DAG), and phospholipids (Grillo et al. 2007; Ribeiro et al. 2014). In contrast, phospholipids are synthesized in the enterocytes, in part used for the synthesis of perimicrovillar membranes present in the gut lumen where they may have an important role in the interaction with *T. cruzi* (Bittencourt-Cunha et al. 2013).

Lysophospholipids, such as lysophosphatidylcholine (LPC), sphingosylphosphorylcholine (SPC), lysophosphatidic acid (LPA), and sphingosine-1-phosphate (S1P), regulate a large number of cellular processes (Liu et al. 2009; Sevastou et al. 2013). LPC is normally generated in biological membranes and is a derivative of phosphatidylcholine formed by the loss of a fatty acid acyl chain. Such reaction is mediated by the action of phospholipase (PLA2). LPC is particularly present during the deposition and infiltration of inflammatory cells and deposition of atheroma (Schmitz and Ruebsaamen 2010). It is mainly produced by the hydrolysis of oxidized phospholipids associated with LDL (Schmitz and Ruebsaamen 2010; Drzazga et al. 2014). Lysophosphatidic acid (LPA) is produced from LPC by the activation of platelets, adipocytes, and neuronal cells in humans. Several enzymes are involved in the synthesis of this lipid; among them, a lysophospholipase D, named Autotaxin (Ferry et al. 2008), converts LPC to LPA, and this later bioactive lipid has demonstrated high specificity to receptors coupled to G proteins by activating the proliferation and migration of cancer cells and angiogenesis (Houben and Moolenaar 2011). LPA induces a high rate of cellular responses through the activation of G protein and protein signaling cascades, such as MAPK, PLC, and Rho, for active cell proliferation in cancer cells (Harrison et al. 2013), but no information is available for parasites.

Our group showed for the first time the presence of phospholipids and lysophospholipids in the saliva and feces of *R. prolixus*, the vector of Chagas disease, in South America. Such molecule acts as an enhancer of blood feeding because it inhibits platelet aggregation as well as increases the production of nitric oxide (NO) thus acting as an inducer of vasodilation in endothelial cells (Golodne et al. 2003). We have later demonstrated that LPC also acts as an enhancer of *T. cruzi* transmission once it blocks parasite-induced production of NO in a TLR-dependent pathway (Mesquita et al. 2008; Carneiro et al. 2013). Thus, LPC plays an important role in insect feeding process and parasite infection, due to its ability to ensure blood fluidity and modulate the chemical and signaling environment for host cell infection by the parasite (Silva-Neto et al. 2012).

Despite efforts to understand the role of LPC in the establishment of *T. cruzi* infection in the vertebrate host, the effect of these bioactive lipids, present in the triatomine digestive tract, on the parasite development has not been demonstrated

yet. In the present study, we investigated the effects of LPA and LPC on *T. cruzi* development. Thereby, we evidence that LPA and LPC promote parasite proliferation in vitro and in vivo, while only LPC has an influence on metacyclogenesis. Additionally, we localized the distribution of both bioactive lipids inside parasite.

Material and methods

Reagents

Lysophosphatidic acid (LPA), lysophosphatidylcholine (LPC), Autaxin inhibitor S32826, and protease inhibitor cocktail (AEBSF, aprotinin, leupeptin, 82 bestatin, pepstatin A, and E-64) were purchased from Sigma Fine Chemicals (St. Louis, MO, USA). LPC 16:0, LPC 18:0, LPC 18:1, TopFluor LPA, and TopFluor LPC were purchased from Avanti Polar Lipids, Inc. (Avanti Polar Lipids, Inc., Alabama, USA).

Fetal calf serum delipidation

Lipid extraction from fetal calf serum (FCS) (Cultilab Ltda., Campinas, SP, Brazil) without protein precipitation was described by Cham and Knowles (1976); however, it was performed according to the procedure described by De Cicco et al. (2012). Briefly, 5.0 mL of FCS, 10.0 mL of a mixture of di-isopropyl ether (DIPE) and n-butanol (60:40 *v*/*v*), and 5.0 mg of ethylenediaminetetraacetic acid (EDTA) were added to Falcon tubes in this order. The tubes were then fastened on a suspension rotator to provide end-over-end rotation at 28–30 rpm for 30 min at room temperature. After extraction, the mixture was centrifuged at 2000 rpm for 2 min to separate the aqueous and organic phases. The aqueous phase was removed from the organic phase by careful suction with a needle and glass syringe. The remaining solvents were harvested using nitrogen gas (N_2). The delipidated fetal calf serum (dFCS) was sterilized by filtration using a 0.22-μm membrane (Millex-GV, Millipore S.A., Molsheim, France).

Parasites

T. cruzi epimastigotes (Y strain) were cultivated for 3–4 days at 28 °C in liver infusion tryptose (LIT) medium supplemented with 10% FCS, Cultilab Ltda., Campinas, SP, Brazil) or 10% delipidated FCS (dFCS). Cultures were kept at an initial density of 10^7 cells/mL in 10 mL of medium. Cell density was measured by direct counting in a Neubauer chamber.

Effect of lysophospholipids and Autaxin inhibitor on parasite proliferation

Parasites were incubated in LIT supplemented with 10% FCS. For in vitro assays, after 4 days in culture, *T. cruzi* epimastigotes were washed twice with LIT and resuspended in the same medium. On the following day, parasite concentration was adjusted to 1×10^7 cells/mL. Cells were then incubated with LIT supplemented with 10% dFCS and in the presence or absence of LPA 1 μM or LPA 10 μM, LPC 1 μM or LPC 10 μM, LPC 1 μM with S 32826 10 μM, or LPC 10 μM in the presence of S32826 10 μM. To determine the influence of LPC with different fatty acid chains, parasites were incubated in LIT supplemented with 10% dFCS and in the presence or absence of LPC 1 μM (16:0, LPC 18:0, LPC 18:1). Cell densities were measured by direct counting in a Neubauer chamber. In vivo assays were also conducted to evaluate the effect of lysophospholipids and Autaxin on parasite proliferation. For these assays, on the 3rd/4th day of parasite growth in LIT supplemented with 10% FCS, *T. cruzi* epimastigotes at an initial concentration of 1×10^7 cells were washed twice in PBS and resuspensed in rabbit blood (Silva-Cardoso et al. 2018) that had been previously added in the presence or absence of LPA 5 mM, LPC 5 mM, S 32826 5 mM or LPC 5 mM, and S 32826 5 mM. Treated blood with parasites, LPA, LPC, and S 32826 was offered to starved *Rhodnius prolixus*, triatominae adult females. On the 1st, 3rd, 7th, and 14th days after feeding, insect guts were dissected and homogenized with 200 μL PBS. Protozoa found in each gut compartment were enumerated in a Neubauer chamber.

Effect of lysophospholipids on metacyclogenesis

T. cruzi epimastigotes Dm 28c clone were maintained for 5–6 days in liver infusion tryptose with yeast (LITB) medium supplemented with 10% FCS, at 28 °C. After epimastigotes were washed twice with PBS and incubated for 2 h in triatomine artificial urine (TAU), the parasites were incubated in TAU supplemented with L-proline (TAUP) (Contreras et al. 1988) and LPA 1 μM or LPC 1 μM at 28 °C. Epimastigote and metacyclic trypomastigote density was measured by counting in a Neubauer chamber.

Lipid analysis

For lipid analysis, samples of LIT, LITB, FCS, and dFCS were subjected to lipid extraction as described by Bligh and Dyer (1959) using methanol/chloroform/distilled water (2:1:0.8 *v*/*v*). The lipid extracts were analyzed by one-dimensional high thin-layer chromatography on Silica Gel (E. Merck, Darmstadt, Germany) for the detection of phospholipids using a chloroform/methanol/acetic acid/distilled water mixture

(50:37.5:3.5:2 *v*/*v*). PC (phosphatidylcholine), LPC (lysophosphatidylcholine), LPA (lysophosphatidic acid), PA (phosphatidic acid), PE (phosphatidylethanolamine), PI (phosphatidylinositol) (Sigma-Aldrich Co, USA) were used as standards. The lipids were visualized using a charring reagent ($CuSO_4$) after heating at 200 °C for 20 min (Ruiz and Ochoa 1997). Chromatography plates were then digitalized using Epson EcoTank L380 scanner.

For the analysis of LPC or LPA in the insect gut content, 100 *R. prolixus* adult females were taken from a colony maintained at 28 °C and 70% relative humidity in Hatisaburo Masuda's laboratory at UFRJ, Rio de Janeiro, Brazil. Insects were dissected 3 days after feeding, and all intestines were homogenized in 200 μL of PBS containing 20 μL of protease inhibitor cocktail. Further, the homogenate was centrifuged at 3000 rpm for 10 min to collect the supernatant. The pellet was resuspended with 1 mL PBS and was centrifuged again to collect the supernatant. All supernatants were subjected to lipid extraction as described by Bligh and Dyer (1959) using methanol/chloroform/distilled water mixture (2:1:0.8 *v*/*v*). The lipid extracts were analyzed by one-dimensional thin-layer chromatography (TLC) on Silica Gel (E. Merck, Darmstadt, Germany) for phospholipids using chloroform/methanol/acetic acid/distilled water mixture (50:37.5:3.5:2 *v*/*v*). PC, LPC, LPA, PA, PE, and PI were used as standards. The lipids were visualized using iodine vapor. The LPA and LPC spots were dissected from the silica gel and re-extracted using methanol/chloroform/distilled water (2:1:0.8 *v*/*v*). Samples were then subjected to drying using nitrogen to be subsequently analyzed by gas chromatography (GC) coupled with mass spectrometric detection (GC-MS). For quantitative analysis and structural assignment, LPA and LPC were separated in a capillary high-resolution column in an Agilent Technologies 7890A gas chromatographer equipped with mass-sensitive detector Agilent Technologies 5975C inert XL MSD. Lipids were dissolved in ethyl acetate and injected into the column at an initial temperature of 50 °C (1 min), followed by a temperature increase to 270 °C at a rate of 20 °C/min and further raised to 300 °C at a rate of 1 °C/min. The carrier gas (He) flow was kept constant at 1 mL/min. Injector temperature was 250 °C, and the detector was kept at 280 °C (Pereira et al. 2015).

LPC and LPA uptake by epimastigotes

Parasites (1×10^7 cells) were washed in LIT medium and resuspended in LIT supplemented with 1 μM TopFluor LPC or 5 μM TopFluor LPA for 30 min at 4 °C or 28 °C. After the parasites were washed in PBS and fixed in 4% formaldehyde in PBS for 20 min at room temperature. Cell suspensions were adhered to 0.1% poly-L-lysine-coated glass coverslips for 20 min and mounted with Prolong Gold with DAPI (Life Technologies, Grand Island, NY, USA). To observe reservosome localization, parasites were kept in serum-free medium containing transferrin coupled to CF555 (Tf-CF555) for 30 min at 28 °C and then treated with fluorescent lipid analogs as described before. Samples were mounted with Prolong Gold with DAPI (Life Technologies, Grand Island, NY, USA). Images of random areas were acquired using a Leica TCS SPE confocal laser scanning microscope equipped with DMI4000 B inverted microscope, 63× oil immersion objective.

Statistical analysis

Statistical analyses were conducted using GraphPad Prism 3 software (GraphPad Software, Inc., San Diego, CA). Data are presented as means ± standard deviation (SD). Data were analyzed using a two-way analysis of variance (ANOVA), and differences between groups were assessed using the Bonferroni post-test. The level of significance was set at $P < 0.05$.

Results

T. cruzi is classically cultivated in the presence of FCS in which lipids, particularly phosphatidylcholine (PC) and lysophosphatidylcholine (LPC), are commonly available (Fig. S1). Delipidation removes all lipids from FCS as showed previously by our group. De Cicco et al. (2012) demonstrated the total removal of neutral lipids from FCS. Herein, we observed that the same occurred with phospholipid and lysophospholipids (Fig. S1A). We also analyzed LIT or LITB medium by TLC and both mediums were found to be lipid free (Fig. S1A). Parasites' proliferation profile in the presence of delipidated FCS (dFCS) is similar to the culture with serum lipids; however, the substitution of FCS by a dFCS affected parasite proliferation (Fig. S1B).

We evaluated *T. cruzi* proliferation in the presence and in the absence of bioactive lipids LPC, LPA, and in the presence of S32826, an autotaxin inhibitor (ATX) which blocks the production of LPA from LPC. Figure 1 shows that both bioactive lipids induced the proliferation of the parasites in a time-dependent fashion. The inclusion of an S32826 also affects parasite proliferation; after day 4, some decrease in proliferation is noted. Thus, both LPC and LPA are able to induce parasite proliferation.

These parasites produce different forms of LPC with a high diversity in the acyl chain, such as C16:0-, C18:0, C18:1, and C18:2-LPC (Gazos-Lopes et al. 2014). The ability of such forms to induce platelet aggregation varied according to the structure, which indicates that each form of LPC may interact with different receptors on cell surface. The digestive system of the bug is also a significant source of LPC and LPA which display different acyl chains. We found, mainly, 32.99%, 21.37%, and 34.59% of C16:0-, C18:1-, and C18:0-LPC and 12.79%, 31.59% and

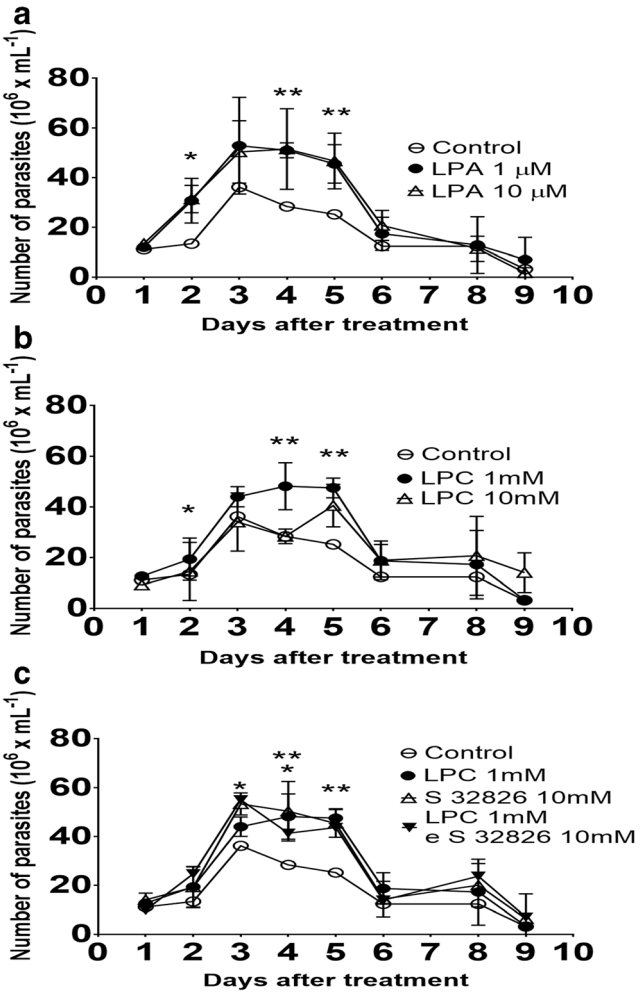

Fig. 1 *T. cruzi* proliferation with LPA, LPC, or ATX Inhibitor—*T. cruzi* epimastigotes were maintained for 9 days in LIT supplemented with 10% dFCS in the **a** absence (control) or presence of LPA 1 μM or 10 μM, **b** absence (control) or presence of LPC 1 μM or 10 μM, **c** absence (control) or presence of LPC 1 μM, autotaxin inhibitor S32826 10 μM, or LPC 1 μM in the presence of S32826 10 μM. Values are mean ± S.E.M. of three independent experiments as determined by two-way ANOVA test, $*P < 0.05$ and $**P < 0.01$

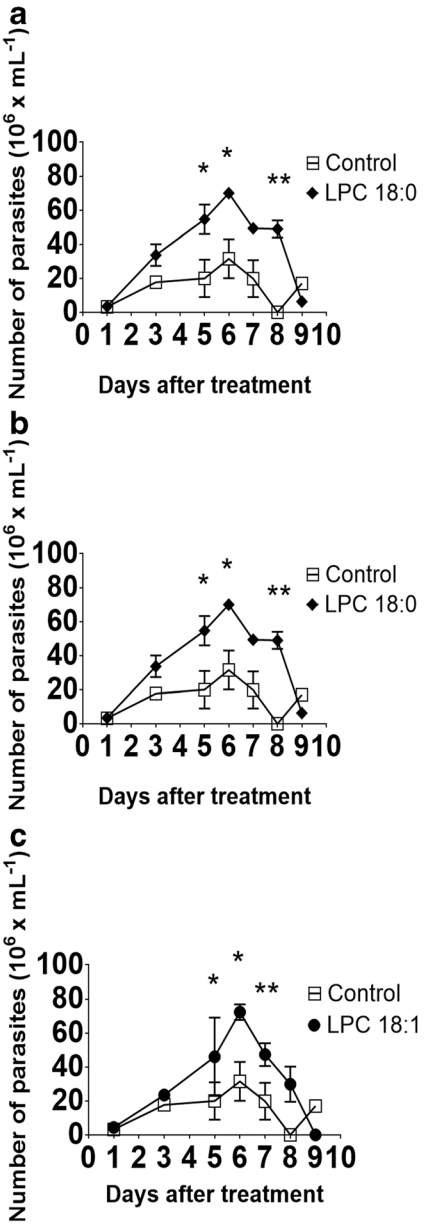

Fig. 2 *T. cruzi* proliferation in the presence of different fatty acid chains—*T. cruzi* epimastigotes were maintained for 9 days in LIT supplemented with 10% dFCS in the **a** absence (control) or presence of LPC 18:0 1 μM, **b** absence (control) or presence of LPC 16:0 1 μM, **c** absence (control) or presence of LPC 18:1. Values are mean ± S.E.M. of three independent experiments as determined by two-way ANOVA, $*P < 0.05$, $**P < 0.01$, and $***P < 0.001$

25% of C14:0-, C16:0-, and C18:0-LPA chains after 3 days of feeding (Tables S1 and S2). Such results indicated that mechanism of action of LPC is determined by some specific aspects of its structure. Therefore, we next tested the effect of different structurally related LPC forms on parasite proliferation. Figure 2 shows that all forms were able to induce parasite proliferation in a similar fashion which suggests that the activated signaling pathway must be common to all of them.

Because parasite proliferation and differentiation occur in the bug digestive system, we fed *R. prolixus* insects with LPC, LPA, and LPC in the presence of the ATX inhibitor. The different portions of the digestive system (crop, midgut, and rectum) were dissected at different points after the consumption of an infected-blood meal and the number of parasites in each compartment evaluated. Figure 3 shows that LPA but not LPC was able to increase the total number of parasites in the crop over the levels obtained in the control on the 3rd (Fig. 3d), 7th (Fig. 3g), and 14th day (Fig. 3j) of infected-blood ingestion. Similar results were observed in the midgut on the 7th (Fig. 3h) and 14th day (Fig. 3k) of blood ingestion. These results indicate that, under in vivo conditions, LPA is the major bioactive lipid regulating the proliferation of parasites. We also tested the hypothesis of these bioactive lipids controlling *T. cruzi* metacyclogenesis in vitro. LPC or LPA

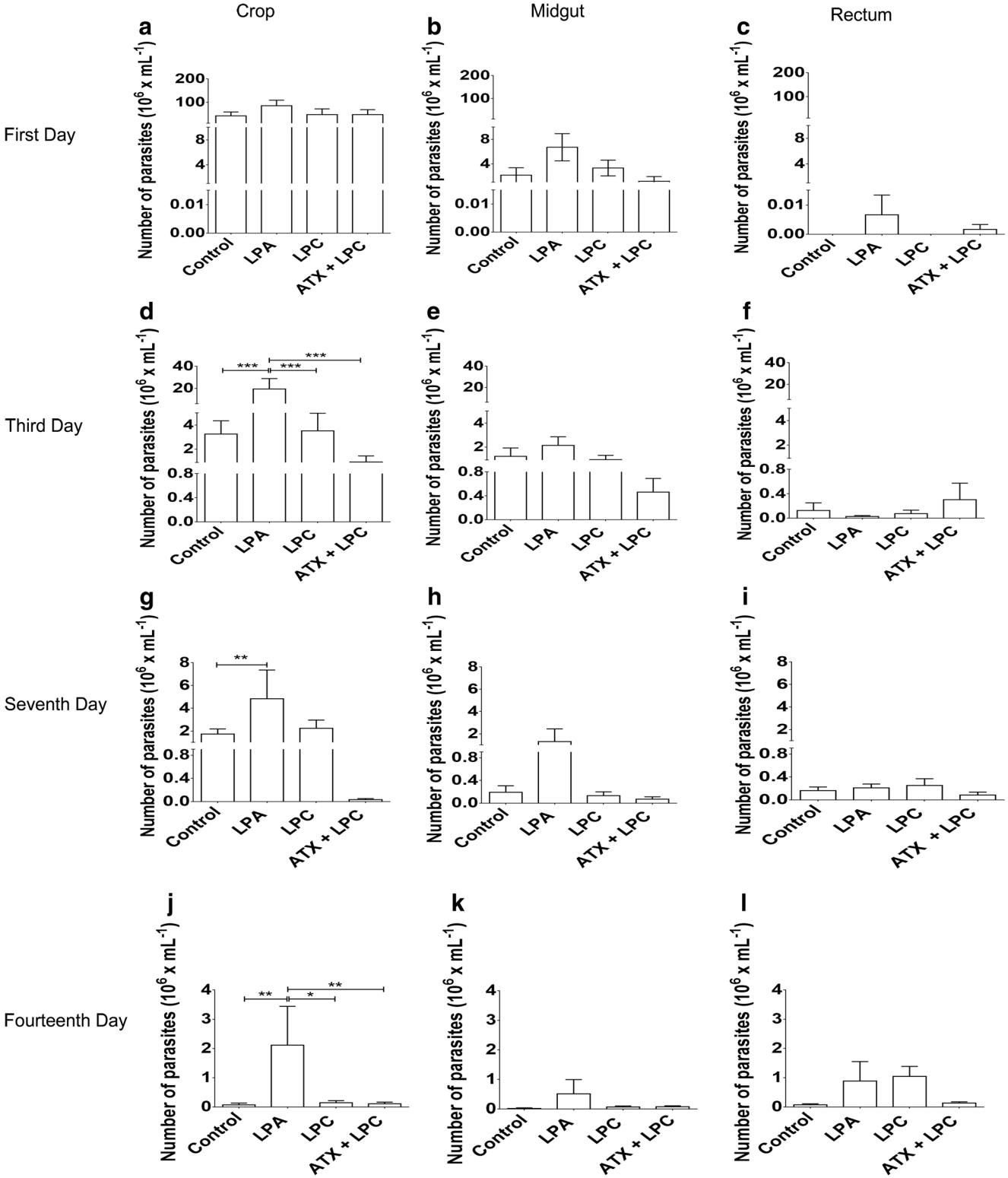

Fig. 3 In vivo proliferation of *T. cruzi* in insects fed with a diet supplemented with LPA, LPC, or ATX inhibitor (S32826)—*R. prolixus* females were fed with blood treated either with LPA 5 mM, LPC 5 mM, or LPC 5 mM in the presence of S32826 5 mM. The number of parasites present in the crop (**a**, **d**, **g**, **j**), midgut (**b**, **e**, **h**, **k**), and rectum (**c**, **f**, **i**, **l**) were counted on the 1st (**a**, **b**, **c**), 3rd (**d**, **e**, **f**), 7th (**g**, **h**, **i**), and 14th day of blood feeding (**j**, **k**, **l**). Values are mean ± S.E.M. of three independent experiments as determined by two-way ANOVA, $***P < 0.001$

was added to TAUP differentiation inducer medium, and the number of metacyclic trypomastigotes was obtained. LPC but not LPA was able to enhance the number of metacyclic parasites in vitro by 30% (Fig. 4).

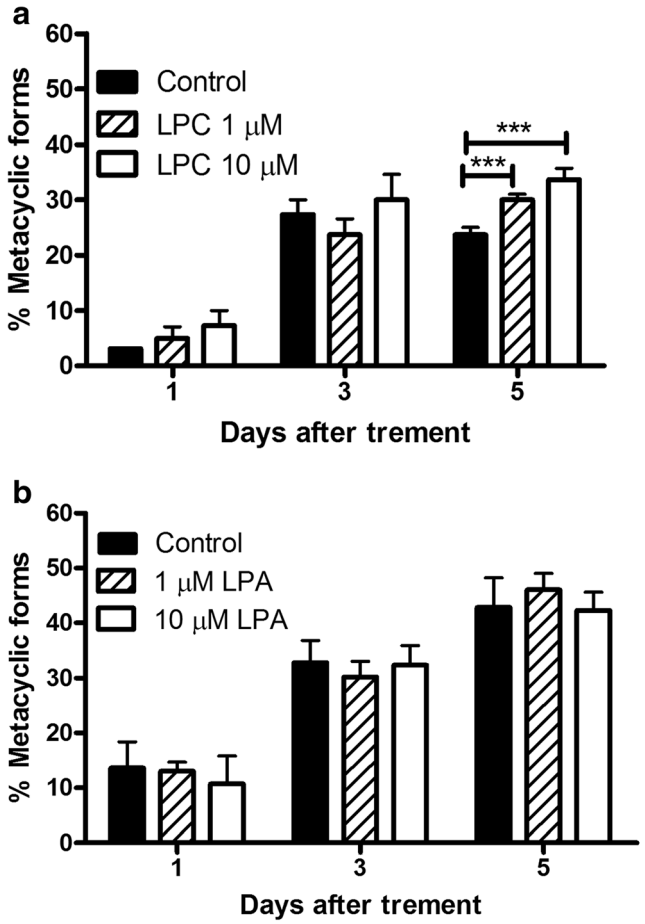

Fig. 4 In vitro metacyclogenesis of *T. cruzi* in the presence of LPC or LPA—differentiation of *T. cruzi* from epimastigote to metacyclic trypomastigote form was evaluated in the absence (control) or presence of LPC or LPA. **a** Differentiation of *T. cruzi* from epimastigote to metacyclic trypomastigote form. Epimastigotes were maintained in the absence (control) or in the presence of LPC 1 μM or LPC 10 μM. **b** Differentiation of *T. cruzi* from epimastigote to metacyclic trypomastigote form. Epimastigotes were maintained in the absence (control) or presence of LPA 1 μM or LPA 10 μM. Cell proliferation (represented as a percentage) was calculated based on absolute values of the treated parasites related to the control group. Values are mean ± S.E.M. of three experiments; two-way ANOVA; ***$P < 0.001$

To determine if LPC or LPA added in vitro are in fact interacting with the parasites, we investigated the incorporation of these lipids using fluorescent analogs by *T. cruzi*. Thus, we observed the distribution of LPA and LPC in epimastigotes (Fig. 5). Figure 5a (LPA) and Figure 5e (LPC) show fluorescent lipids incorporated into the plasma membrane when parasites were kept at 4 °C. Further, we evaluated the uptake of the lipid analogs by endocytosis and we used Tf-CF555 to be stored in endocytic vesicles and reservosomes. Figure 5b, f shows Tf-CF555 at the posterior end. The lipid analogs were also observed inside compartments placed at the posterior side after 30 min of endocytosis (Fig. 5c, g). These results indicate that lipid analogs were internalized by epimastigotes being delivered via an endocytic route. Figure 5d, h show colocalization of Tf-CF555 within the endocytic compartments.

Discussion

Despite the research for development of new safe and effective strategies to control Chagas disease, effective measures are not available yet. Current available drugs do not always eliminate the parasite and eventually cause severe adverse reactions which prevent the continuation of treatment. Furthermore, vaccines to prevent infection have not been successful so far. Thus, the molecular detailing of parasite development, including within its passage through the invertebrate host, may shed light on novel strategies to block *T. cruzi* transmission. Lysophospholipids are multidirectional molecules coordinating several signaling pathways and their effects on the different organisms involved in the transmission of Chagas disease have been described (Silva-Neto et al. 2016).

Lysophospholipids for a long time were considered only as membrane components essential to mediating synthesis of various phospholipids and to embedding proteins into cell membranes. It is now clear that they are necessary to maintain homeostasis of many physiological processes, including vascular development. (Drzazga et al. 2014). Trypanosomatidae protozoa require exogenous lipid sources, such as fatty acids or serum lipoproteins, for their metabolism. Elucidation of the roles of key nutrients present in the serum is essential for understanding the proliferation of parasites. Our group showed the importance of lipids and low-density lipoprotein (LDL) from culture medium for *Leishmania amazonensis* survival (De Cicco et al. 2012). To evaluate the effect of lysophospholipids present in culture medium on *T. cruzi* proliferation, parasites were grown on medium supplemented with delipidated fetal bovine serum (dFCS). We observed that there are phosphatidylcholine (PC) and lysophosphatidylcholine (LPC) in FCS and delipidation removes all lipids of FCS; we also observed that LIT or LITB medium are lipid-free (Fig. S1A).

To the best of our knowledge, our group is the first to show the presence of LPC in the saliva and feces of *R. prolixus*, a vector of Chagas disease (Golodne et al. 2003). The effect of such molecule during blood feeding included the inhibition of platelet aggregation as well as an increase in the production of nitric oxide (NO), hence acting as an inducer of vasodilation in endothelial cells and an enhancer of blood feeding (Golodne et al. 2003). Moreover, regarding its effect on parasite surface, LPC signaling may occur by binding to the PAF receptor with induction of a cascade of intracellular signaling by PKC, MAPKs, and CK2 (Silva-Neto et al. 2002). LPA is produced by activated platelets, activated adipocytes, neuronal cells, and other cell types as well as by multiple enzymatic pathways, such as lysophospholipase D (lyso PLD) (Ishii et al. 2004). LPA acts through G protein coupled receptors

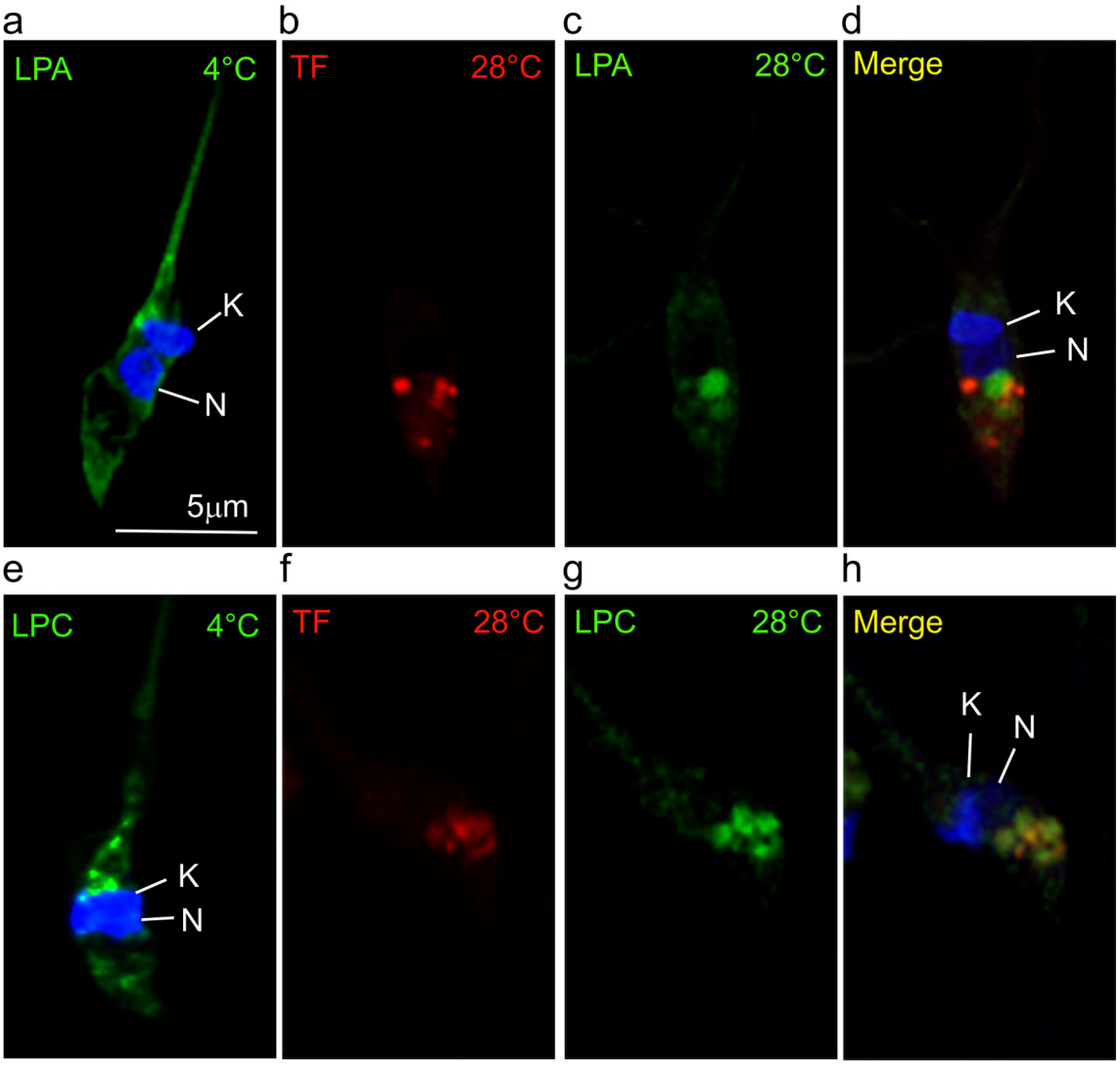

Fig. 5 LPA/LPC internalization in epimastigotes. Parasites incubated with LPA or LPC at 4 °C demonstrate both lipid analogs all over the plasma membrane (**a**, **e**). Alternatively, epimastigotes were pretreated with TF-CF555 to load reservosomes at 28 °C (**b**, **f**) followed by incubation with lipid tracers. LPA and LPC were incorporated and observed at posterior region inside endocytic compartments (**c**, **d**, **g**, and **h**) or clearly colocalized with reservosomes (**h**). DAPI was used to stain nucleus (N) and kinetoplast (K). Bar 5 μm.

(GPCRs) and also induces different cellular responses, such as cell proliferation, survival, and angiogenesis (Liu et al. 2009). In the present study, we have described the effects of LPC and LPA in the proliferation of *T. cruzi* in vitro. Both lysophospholipids led to parasite proliferation enhancement (Fig. 1a, b). ATX, a lysophospholipase D which produces LPA from LPC, signals cells via a set of five GPCRs (East et al. 2010). To verify the effect of this process on parasite growth, we grew parasites in the presence of ATX S32826 inhibitor, and we observed an increment of parasite proliferation probably because of LPC accumulation. This is because, in the presence of the ATX inhibitor only, the proliferation occurred in a higher control ratio (Fig. 1c) (Gazos-Lopes et al. 2014).

LPA displays several physiological actions on blood pressure, platelet activation, and smooth muscle contraction. LPA concentration in human serum is estimated to be 1–5 μM, and it circulates bound to albumin, LDLs, or other proteins, which possibly protect LPA from rapid degradation. Alternatively, LPC is present as a component of oxidized LDL. The physiological concentration of LPC in body fluids, including blood, varies between 5 and 180 μM (Ishii et al. 2004). LPC was found not to be exclusively of mammalian origin; once *T. cruzi* synthesizes a PAF-like phospholipid capable of aggregating platelets, it is also capable of synthesizing at least five species of LPC, C16:0-, C18:0, C18:1-, C18:2-, and C22:6-LPC. The most abundant species are C18:2- and C18:1-LPCs (Gazos-Lopes et al. 2014). Because LPC is produced by the parasite and the vector insect (Golodne et al. 2003), and it is present with LPA in the vertebrate host blood; it was intriguing to determine their role on parasite biology. In the present work, the fatty acid composition of insect midgut

content was determined by GC-MS analysis. We identified eight LPA species and five LPC species, C18:0-, C16:0-, and 18:1- LPC, in the midgut content after 3 days of blood feeding. Tables 1S and 2S show the different fatty acid chain composition of LPA and LPC from the intestinal contents of *R. prolixus*, so these lysophospholipids may be derived from the blood used to feed the insects or even produced by the parasite itself (Golodne et al. 2003). We showed an increase in *T. cruzi* in vitro proliferation growth in the presence of all LPC species tested (Fig. 2). After this result, we can conclude that LPC of the intestinal content is involved with the density of parasites, and its fatty acid chains may have the same influence on parasite proliferation. Further analyses are required for the identification of binding receptors for each LPA and LPC species.

During the interaction of *T. cruzi* and its insect vector, the nutritional state of the bug must be considered because it directly affects parasite development within the intestinal tract (Kollien and Schaub 1998). We fed *R. prolixus* with infected blood in the presence and LPC, LPA, and ATX inhibitor with LPC and observed a major positive effect of LPA in vivo on proliferation in most of the tested days (1st, 3rd, 7th, and 14th) after feeding (Fig. 3). These differential results observed between LPC and LPA effects in vivo are probably because of their action on distinct signaling pathways. Additionally, the intestine content composition varies with the number of days passed after blood meal, which also may interfere with lysophospholipids dynamics and action.

Therefore, the development of *T. cruzi* in the vector is affected by several factors (Kollien and Schaub 1998). For instance, *T. cruzi* synthesizes a lipid with PAF-like activity. That putative PAF-like phospholipid is able to trigger the differentiation of *T. cruzi* epimastigotes into metacyclic trypomastigotes in vitro (Gomes et al. 2005; Gazos-Lopes et al. 2014). Therefore, in the context of biochemical changes that the parasite experiences inside the vector digestive tract, we showed that LPC but not LPA acts on *T. cruzi* metacyclogenesis in vitro, increasing the number of metacyclic trypomastigotes by 30% (Fig. 4).

We determined that LPC and LPA are in fact internalized by the parasite utilizing their fluorescently labeled counterparts (Atella and Shahabuddin 2002). We showed that LPC and LPA are localized in the endocytic compartments (Fig. 5). Since endocytosis of *T. cruzi* epimastigotes occurs almost exclusively by the cytostome-cytopharynx complex (Porto-Carreiro et al. 2000), which distinguish them from other trypanosomatids, cargo is concentrated in cytostome entry and it is internalized through the cytopharynx (de Souza et al. 2009). From there, vesicles bud off and fuse with early endosomes and then macromolecules reach reservosomes placed at the posterior end of the cell. These lysophospholipids are localized in these endocytic organelles, which may indicate that they are recognized by the parasite and follow distinct endocytic and metabolic pathways. Unexpectedly, TcK2, the *T. cruzi* serine threonine kinase, is localized in reservosomes and it is activated by depletion of heme levels leading to its autophosphorylation and activation. TcK2 phosphorylates eIF2α and translation is inhibited, favoring metacyclogenesis (Tonelli et al. 2011; da Silva et al. 2015). Similarly, the uptake of LPA could exercise influence in parasite proliferation in the beginning of infection, while it could be responsible with LPC for events associated with metacyclogenesis by the end of the digestive tract of the insect vector. In conclusion, we determined that LPC and LPA are important for proliferation of *T. cruzi* in vitro and in vivo as well as parasite differentiation into metacyclic trypomastigote forms. We also showed that C18:0-, C16:0-, and 18:1-LPC species produced by the parasite are also present in the vector bug midgut. All these LPC species exhibited the same influence on the parasite in vitro proliferation. However, only LPC increased the rate of parasite metacyclogenesis.

Our group had demonstrated the role of LPC as an anti-hemostatic and immunomodulatory factor, part of the group of pharmacological active molecules injected with the vector bite. Herein, we attributed another important role to this lysophospholipid along with LPA concerning epimastigote proliferation in vitro and in vivo and their differentiation into metacyclic trypomastigotes. Efforts with the goal of identifying the receptors present in the parasite recognized by LPA and LPC should be made to develop drugs directed against parasite transmission.

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Compliance with ethical standards

Ethics approval and consent to participate All the animal care and experimental protocols were conducted in accordance with the guidelines of the institutional animal care and use committee (Comissão de Avaliação do Uso de Animais em Pesquisa da Universidade Federal do Rio de Janeiro, CAUAP-UFRJ) and the NIH Guide for the Care and Use of Laboratory Animals (ISBN 0-309-05377-3). The protocols were approved by CEUA-UFRJ (Comissão de Ética no Uso de Animais da Universidade Federal do Rio de Janeiro) under registry number #115/13. Technicians at the animal facility of the Institute of Medical Biochemistry Leopoldo de Meis (UFRJ) performed the entire rabbit husbandry under strict guidelines to ensure careful and consistent handling of the animals.

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