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Phospholipase C gamma and ERK1/2 Mitogen Activated Kinase Pathways are differentially modulated by *Trypanosoma cruzi* during tissue invasion in human placenta

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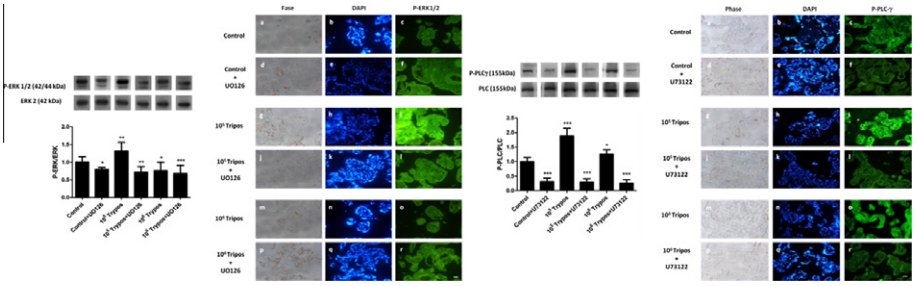
HIGHLIGHTS

- Low concentration of *T. cruzi* induces PLC-γ and ERK1/2 MAPK pathways activation during *ex vivo* infection of human placenta.
- High concentration of *T. cruzi* induces a moderate PLC-γ pathway activation during *ex vivo* infection of human placenta.
- High concentration of *T. cruzi* inhibits ERK1/2 MAPK pathway during *ex vivo* infection of human placenta.
- Inhibition of any of those signal transduction pathways do not prevent the *ex vivo* infection of human placenta by *T. cruzi*.

GRAPHICAL ABSTRACT



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ABSTRACT

Chagas' disease is caused by the haemophlagelated protozoan *Trypanosoma cruzi* (*T. cruzi*). During congenital transmission the parasite breaks down the placental barrier, however studies about the physiopathology of this process are scarce. Different signal transduction pathways are involved during cell invasion of the parasite. However, the possible role of those processes during tissue infection has not been studied. In the present study we analyzed the modulation of two signal transduction pathways, PLC-γ and ERK1/2 MAPK, during *ex vivo* infection of human placental chorionic villi explants.

Chorionic villi from healthy woman placentas were incubated in the presence or absence of 10^5 or 10^6 *T. cruzi* trypomastigotes (DM28c strain) with or without specific inhibitors for each pathway. Effective infection was tested determining parasite DNA by PCR. The activation of PLC-γ and ERK1/2 MAPK signaling pathways was determined by western blotting and immunofluorescence.

The low concentration of *T. cruzi* trypomastigotes activates both signaling pathways; however, the high concentration of parasite induces a modest activation of the PLC-γ pathway and impairs the ERK1/2 MAPK pathway activation. Interestingly, inhibition of any of those signaling pathways did not prevent parasite infection, as it was previously shown in cell cultures. We conclude that both signal transduction pathways are modulated during *ex vivo T. cruzi* infection of human placental chorionic villi explants.

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1. Introduction

The protozoan parasite *Trypanosoma cruzi* (*T. cruzi*) is the etiologic agent of Chagas' disease, an endemic disorder proper of poverty in Central and South America. Ten to sixteen million people are chronically infected with *T. cruzi*, with widely variable clinical manifestations, ranging from absence of symptoms to an inflammatory cardiomyopathy and/or dilatation of the enteric viscera derived from denervation injury (Coura and Dias, 2009; Moncayo and Silveira, 2009).

Typically, *T. cruzi* infection occurs when parasites excreted by the triatomine insect vector contaminate the bite wound or a mucous membrane. In non-endemic areas, transmission may occur via blood transfusion or organ transplantation, as a result of a laboratory accident or congenitally (Epting et al., 2010).

Despite a century of scientific studies, the host–parasite cell biology and its relationship with the pathophysiology of Chagas' disease remains incompletely understood (Epting et al., 2010). In this context, parasite invasion in cell cultures has been studied in some depth (Yoshida et al., 2000; Fernandes and Andrews, 2012; Díaz-Luján et al., 2012), but studies that analyze parasite invasion in tissues and organs are scarce (Duaso et al., 2010; Fretes, 2012).

The *ex vivo* infection of human chorionic villi explants from human term placenta with the parasite constitutes an excellent and easy way to study the mechanism of cellular and tissue infection and invasion (Fretes, 2012). The explants can be kept in culture for several days (Drewlo et al., 2008), where constituent cells are in a more physiological condition than their isolated counterparts in cell culture models. The co-incubation of 10^5 or 10^6 trypomastigotes produces a reproducible infection of the chorionic villi (Duaso et al., 2010). The histopathological changes observed in the explants are similar to those observed in placenta obtained from women with chronic Chagas' disease (Duaso et al., 2012).

The cellular invasion of *T. cruzi* induces calcium (Ca^{2+}) signals and activation of signal transduction pathways both in the parasite and in the host cell (Yoshida, 2006; Yoshida, 2008). The parasite's capacity to generate calcium signals in the host is related to its infectivity (Manque et al., 2003; Yoshida, 2008).

Phospholipase C (PLC) activation is one of the most important signal transduction pathways, leading to the generation of inositol 1, 4, 5 triphosphate (IP_3) and the liberation of Ca^{2+} from intracellular deposits (Rodríguez et al., 1995; Yoshida, 2006). The increase in Ca^{2+} induces recruitment (Andrews, 2002) and fusion of lysosomes (Jaiswal et al., 2002) with the plasma membrane at the site of parasite invasion. Inhibition of the PLC-γ signal transduction pathway prevents the parasite invasion in HeLa cells (Yoshida et al., 2000).

Mitogen activated protein kinases (MAPKs) function as signal transducers from cell surface receptors to transcription factors in the nucleus, which consequently triggers long-term cellular responses. Activation of the components of the MAPK pathway (ERK, p38 MAPK, and JNK) plays an important role in cell proliferation, differentiation and death (Camps et al., 2000; Ramos, 2008). *T. cruzi* induces a significant increase in phosphorylated (activated)-ERK in adipocytes (Nagajyothi et al., 2012), macrophages (Villalta et al., 1998) endothelial cells, smooth muscle cells (Mukherjee et al., 2004), and cardiomyocytes (Huang et al., 2003). Additionally, specific inhibitors of the ERK1/2 MAPK pathway impair parasite infection in different macrophage cell cultures.

Here, we show that PLC-γ and ERK1/2 MAPK pathways are modulated upon ex *vivo* infection of human chorionic villi explants with *T. cruzi* trypomastigotes. However, inhibition of any of those pathways does not alter placental tissue parasite infectivity.

2. Material and methods

2.1. VERO cell culture

Green Monkey (*Cercopithecus aethiops*) renal fibroblast like cells (VERO cells (ATCC® CCL-81)) were grown in RPMI medium enriched with 5% fetal bovine (FBS) serum and antibiotics (penicillin–streptomycin). Cells were grown at 37 °C in a humid atmosphere at 5% CO_2 for 96 h, replacing the medium every 24 h (Salas et al., 2008).

2.2. Infection of VERO cells with T. cruzi and trypomastigote harvesting

After confluence, VERO cells were incubated with a culture of epimastigotes, DM28c strain, in late stationary phase, which increases the percentage of trypomastigotes to approximately 5% (Contreras et al., 1985). Trypomastigotes then invade fibroblasts and replicate intracellularly as amastigotes. After 72 h, amastigotes transform back to trypomastigotes that lyse host cells. Parasites were recovered by low speed centrifugation (500*g*), thus obtaining trypomastigotes in the supernatant and amastigotes in the sediment (Villalta and Kierszenbaum, 1982).

2.3. Placenta and chorionic villi culture

Human term placentas were obtained from uncomplicated pregnancies from vaginal or cesarean delivery. Informed consent for the experimental use of the placenta was given by each patient as stipulated by the Code of Ethics of the Faculty of Medicine, University of Chile. Exclusion criteria for the patient were the following: major fetal abnormalities, placental tumor, intrauterine infection, obstetric pathology, and any other maternal disease. The organs were collected in cold sterile saline-buffered solution (PBS) and processed no more than 30 min after delivery. Their maternal and fetal surfaces were discarded and villous tissue was obtained from the central part of the cotyledons. The isolated chorionic villi were washed with PBS in order to remove blood, cut in approximately 0.5 cm^3 pieces and co-cultured with *T. cruzi* trypomastigotes DM28c strain (1×10^5/ml or 1×10^6/ml), in presence or absence of specific inhibitors for each signal transduction pathway (PLC-γ: U73122 (100 μM); ERK1/2 MAPK: UO126 (10 μM)). Final concentration of the inhibitors solvent, dimethyl sulfoxide (DMSO), was lower than 1%. DMSO alone did not induce activation of either signal transduction pathway nor inhibit the effect of the parasite, see supplementary figure. Incubation was performed for 1 or 24 h in 1 ml of RPMI culture media supplemented with inactivated FBS and antibiotics. Samples for signal transduction pathway analysis were analyzed after 1 h of co-incubation. *T. cruzi* infection was tested after 1 and 24 h by parasite DNA detection using the polymerase chain reaction. All experiments were performed in triplicate in at least three different placentas.

2.4. Western blotting

Tissues were homogenized in a lysis buffer (Tris 10 mM pH 8.0; SDS 1% w/v and protease inhibitor cocktail (Complete Mini (Roche®)) at 4 °C using a Potter-Elvejem homogenizer and centrifuged at 15,000*g* for 20 min to remove debris. Protein concentration was measured in the supernatant by the Bradford assay using Sigma bovine serum albumin as a protein standard. 30 μg of protein were separated in a 10% sodium dodecyl sulfate polyacrylamide gel, blotted onto a nitrocellulose membrane, and probed with monoclonal antibodies against P-PLC-γ (#2821, Cell signaling®; 1:1000 v:v) or P-ERK (#9106, Cell signaling®; 1:2000 v:v). To correct for loading, membranes were stripped and

re-probed with anti PLC-γ (sc-7290 1:2000 v:v) and anti-ERK2 (sc-1647 1:2000 v:v) antibodies. Immunoreactive proteins were detected using enhanced chemiluminiscence reagents according to the manufacturer instructions (Amersham Biosciences UK Ltd.). The films were scanned and the NIH-Image software program V 1.6 (NIH, Bethesda, MD) was employed for densitometric analysis of the bands.

2.5. Immunofluorescence

The placental chorionic villi were fixed in 10% formaldehyde-0.1 M phosphate buffer (pH 7.3) for 24 h, embedded in paraffin wax and cut into 5 μm sections. P-PLC-γ (#8221 Cell signaling®; 1:25 v:v) antibody was applied to each section overnight at 4 °C and P-ERK antibody (sc-16982 Santa Cruz®; 1:50 v:v) was applied to each section for 2 h at 37 °C. The preparations were washed with PBS and incubated with a DyLight™ 488-conjugated secondary antibody (Jackson Inmuno Research Laboratories®; 1:400 v:v) in the presence of 1 μg/ml of 4-6-diamidino-2-phenylindole (DAPI). Afterwards, sections were mounted in VectaShield (ScyTek, ACA), observed in a Nikon Eclipse E400 microscope (Tokio, Japon). Ten fields were selected randomly and the signal intensity was scored as follows: ±, patchy; +, weak; ++, moderate; +++, strong using the NIS-Element F 3.0 software.

2.6. Control of infection by PCR

T. cruzi invasion was tested by parasite DNA detection using the polymerase chain reaction. For this purpose, genomic DNA was extracted from the placental tissue with the Wizard Genomic DNA Purification Kit (Promega) according manufactureŕs instruction. To avoid the presence of adhered, non-interiorized parasites, the explants were washed ten times in PBS previous DNA extraction. A 330 base pair fragment of *T. cruzi* satellite DNA was amplified as described previously (Zulantay et al., 2004). The sequence of the oligonucleotides is the following: forward (5′-AAATAATG-TACGG(T/G)GAGATGCATGA-3′, specific primer 121) and backward (5′-GGTTCGATTGGGGTTGGTGTAATATA-3′ specific primer 122). The PCR product was subjected to electrophoresis in 1.6% agarose gels and stained with ethidium bromide. PCR markers from Promega were employed as molecular weight standards.

2.7. Statistics

Results are expressed as mean ± S.D. The significance of differences was evaluated using ANOVA followed by Dunnett's post-test as indicated.

3. Results

3.1. T. cruzi induces Phospholipase C gamma pathway activation during ex vivo infection of human chorionic villi explants

Human placental chorionic villi were co-incubated with 10^5 or 10^6 *T. cruzi* trypomastigotes DM28c strain in presence or absence of U73122 100 μM for 1 h.

10^5 Trypomastigotes induces a statistically significant ($p \leqslant 0.001$) increase of PLC-γ phosphorylation (88.31 ± 0.265% Fig. 1A) compared to control (not infected chorionic villi). A higher

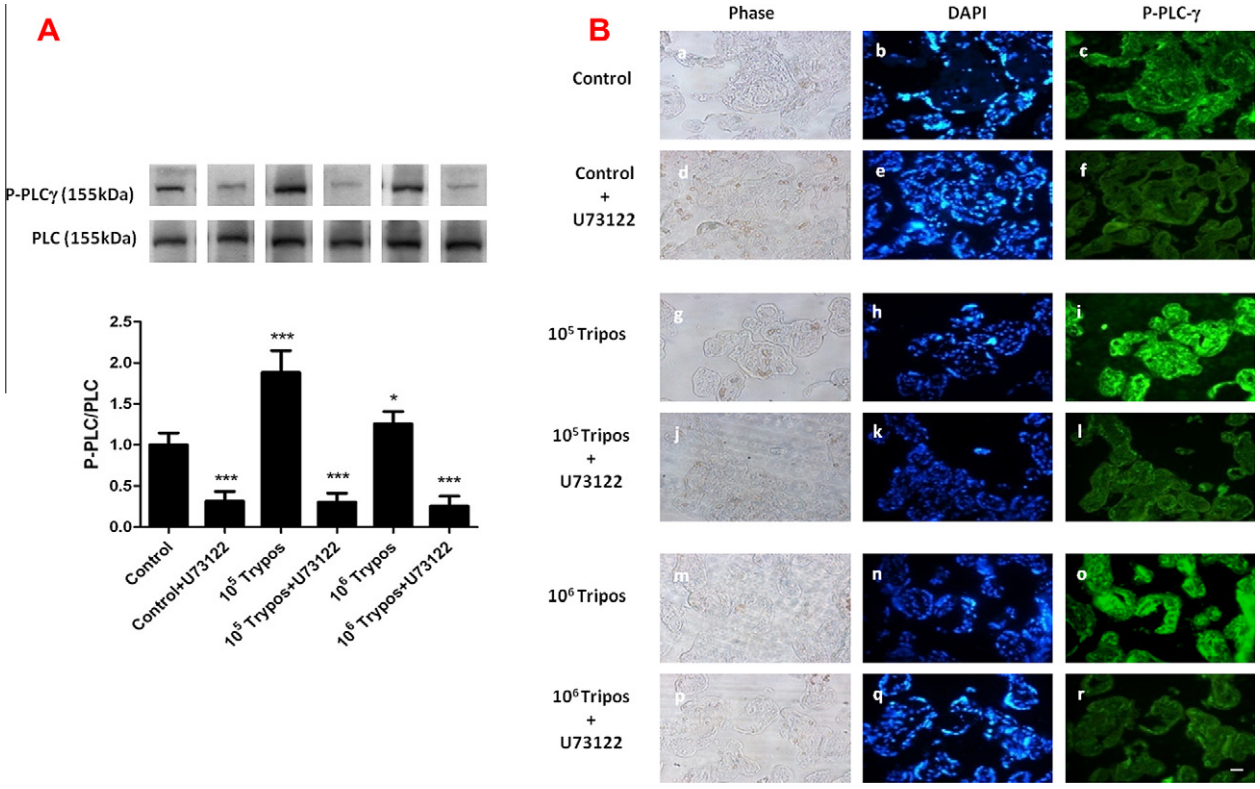

Fig. 1. *T. cruzi induces PLC-γ pathway activation during ex vivo infection of human chorionic villi explants:* Human placental chorionic villi were co-incubated with 10^5 or 10^6 *T. cruzi* trypomastigotes DM28c strain in presence or absence of U73122 (100 μM) during 1 h. In (**A**) (left panel) Western blots for P-PLC-γ and PLC from chorionic villi infected with 10^5 or 10^6 *T. cruzi* trypomastigotes, in the presence or absence of U73122, are shown. The bar graphs represent the ratios of P-PLC-γ over PLC, normalized with respect to the values obtained in control cells, under the different experimental conditions used. All values are given as mean ± S.D. and correspond to at least 3 independent experiments carried out in duplicates or triplicates. $^{*}p < 0.05$, $^{***}p < 0.001$. In (**B**) it is shown that P-PLC-γ immunoreactivity increases in chorionic villi incubated with the parasite (10^5**g-i** and 10^6**m-o**) as compared to control (**a-c**) while the inhibitor U73122 (10^5**j-l** and 10^6**p-r**) diminishes the P-PLC-γ immmunoreactivity under control tissue levels (**a-c**). Tissues were processed for routine immunofluorescence methods. Bar scale: 25 μm.

concentration of parasites (10^6) induces a lower, but statistically significant ($p \leqslant 0.05$) increase of PLC-γ phosphorylation (25.5 ± 0.265% Fig. 1A) as evidenced by Western blot. The increase of PLC-γ phosphorylation in the chorionic villi in presence of the parasite was confirmed by immunofluorescence (Fig. 1B). The trophoblast as well as the villous stroma shows a strong (+++) immunoreactivity (Fig. 1B (g–i; m–o) compared to control chorionic villi (Fig. 1B (a–c)), which present a weak (+) immunoreactivity. The presence of the U73122 inhibitor impairs the PLC-γ phosphorylation in control and in parasite infected explants (Fig. 1A and Fig. 1B (d–f; j–l; p–r).

3.2. T. cruzi modulates ERK1/2 MAPK pathway during ex vivo infection of human chorionic villi explants

Human placental chorionic villi were co-incubated with 10^5 or 10^6 *T. cruzi* trypomastigotes DM28c strain in presence or absence of UO126 10 μM for 1 h.

10^5 trypomastigotes induces a statistically significant ($p \leqslant 0.01$) increase of ERK1/2 phosphorylation (31.54 ± 0.242% Fig. 1A) compared to control (not infected chorionic villi). Contrarily, a higher concentration of parasites (10^6) impairs the ERK1/2 activation, showing ERK1/2 phosphorylation levels lower than control ones (11.29 ± 0.072%; $p \leqslant 0.05$) as evidenced by Western blot. The differential modulation of ERK1/2 phosphorylation (Fig. 1(A)) in the chorionic villi in presence of the parasite was confirmed by immunofluorescence (Fig. 1(B)). Thus, the trophoblast as well as the villous stroma show a strong (+++) immunoreactivity (Fig. 1(B) (g-i)) when challenged with 10^5 parasites as compared to control chorionic villi (Fig. 1(B) (a-c)). Confirming results obtained by western blot, the placental explants challenged with 10^6 trypomastigotes presents a weak (+) immunoreactivity. The ERK1/2 phosphorylation inhibitor UO126 impairs the ERK1/2 activation both in control and in parasite infected explants (Fig. 1(A) and (B) (d–f; j–l; p–r)).

3.3. Inhibition of PLC gamma and ERK1/2 MAPK signaling pathways do not prevent the ex vivo infection of human chorionic villi explants by T. cruzi

It has been previously described, that inhibition of different cell signaling pathway components inhibits parasite endocytosis in different cell cultures (Villalta et al., 1998; Zhong et al., 1998; Yoshida et al., 2000). To determine whether the inhibition of the signaling pathways prevents the *ex vivo* infection of human chorionic villi explants parasite DNA was assayed by PCR in the presence or absence of specific inhibitors of each signaling pathway. The human placental chorionic villi were co-incubated during 24 h with 10^5 or 10^6 *T. cruzi* trypomastigotes DM28c strain in presence and absence of U73122 (100 μM) or UO126 (10 μM), inhibitors for PLC-γ and ERK1/2 MAPK signal transduction pathways, respectively. As shown in Fig. 3 those inhibitors were not able to prevent the presence of the parasite DNA in chorionic villi homogenates. Same results were observed when chorionic villi were incubated for 1 h in the same experimental conditions as described above (data not shown).

4. Discussion

Critical events in the process of *T. cruzi* infection are the initial interactions of the trypomastigote form of the parasite with the

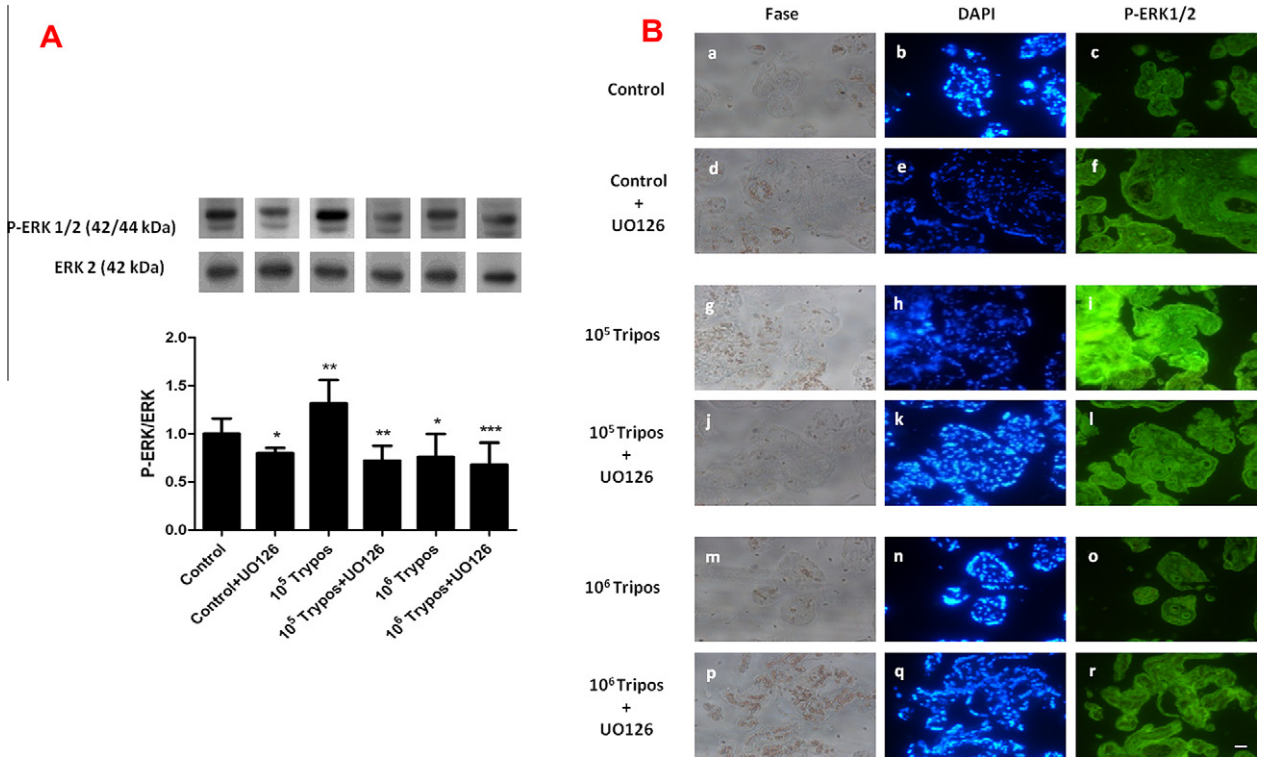

Fig. 2. *T. cruzi modulates ERK1/2 MAPK pathway during ex vivo infection of human chorionic villi explants:* Human placental chorionic villi were co-incubated with 10^5 or 10^6 *T. cruzi* trypomastigotes DM28c strain in presence or absence of UO126 (10 μM) during 1 h. In (**A**) (left panel) Western blots for P-ERK1/2 and ERK from chorionic villi infected with 10^5 or 10^6 *T. cruzi* trypomastigotes, in the presence or absence of UO126, are shown. The bar graphs represent the ratios of P-ERK1/2 over ERK, normalized with respect to the values obtained in control cells, in the different experimental conditions used. All values are given as mean ± S.D. and correspond to at least 3 independent experiments carried out in duplicates or triplicates. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$. In (**B**) is shown that chorionic villi incubated with 10^5 trypomastigotes (**g-i)** show an increase in P-ERK1/2 immunoreactivity compared to control (**a-c**). Contrarily, explants incubated with 10^6 parasites (**m-o**) present a decrease in the immunoreactivity compared to control (**a-c**). Chorionic villi incubated with the parasite and the inhibitor UO126 (10^5**j-l** and 10^6**p-r**) show a P-ERK1/2 immmunoreactivity lower than control tissue (**a-c**). Tissues were processed for routine immunofluorescence methods. Bar scale: 25 μm.

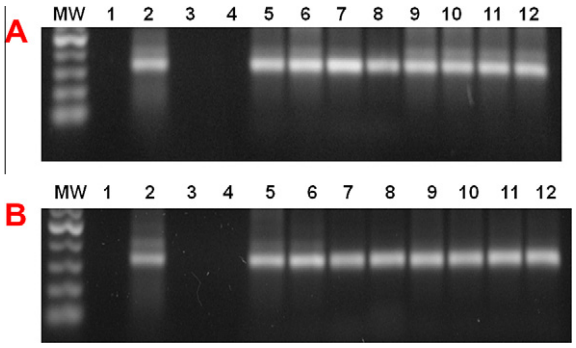

Fig. 3. *T. cruzi DNA detection by PCR in ex vivo infected human chorionic villi:* A 330 base pair fragment of the *T cruzi* satellite DNA was amplified from chorionic villi incubated with 10^5 or 10^6 parasites in presence or absence of P-PLC-γ inhibitor (panel A) or P-ERK1/2 MAPK inhibitor (panel B). Primer oligonucleotide sequences were the following: forward (5′-AAATAATGTACGG(T/G)GAGATGCATGA-3′, specific primer 121) and backward (5′-GGTTCGATTGGGGTTGGTGTAATATA-3′, specific primer122). MW: Molecular weight markers. Lanes 1: negative control without DNA; lanes 2: DM28c trypomastigote DNA; lanes 3: control (not infected) human chorionic villi DNA; lanes 4: control (not infected) human chorionic villi DNA incubated either with U73122 (100 μM) (A) or UO126 (10 μM) (B); lanes 5–6 human chorionic villi incubated with 10^5 trypomastigotes/ml; lanes 7–8 human chorionic villi incubated with 10^5 trypomastigotes/ml and with U73122 (100 μM) (A) or UO126 (10 μM) (B); lanes 9–10 human chorionic villi incubated with 10^6 trypomastigotes/ml; lanes 11–12 human chorionic villi incubated with 10^6 trypomastigotes/ml and with U73122 (100 μM) (A) or UO126 (10 μM) (B).

host mammalian cell. These interactions result in the bidirectional (host and parasite) activation of signal transduction pathways relevant in the pathogenesis of Chagas disease (Villalta et al., 2009; Caradonna and Burleigh, 2011; Fernandes and Andrews, 2012). Among them, PLC-γ and ERK1/2 MAPK pathways have been associated to *T. cruzi* infectivity (Villalta et al., 1998; Yoshida, 2008; Nagajyothi et al., 2012). On the other hand, both signal transduction pathways are involved in placental physiology (Delidaki and M., 2011; Laramée et al., 2002 Nov 29) and physiopathology (Anton 2012). PLC participates in receptor mediated intracellular Ca^{2+} increase in adhesion-competent trophoblast cells during blastocyst implantation (Wang et al., 2007). In human term placenta, parathyroid hormone and parathyroid-related peptide regulates maternal-fetal Ca^{2+} homeostasis by activating Protein Kinase C (PKC). The PKC signaling is mainly regulated by PLC activation (Laramée et al., 2002 Nov 29). PLC-γ activation has been related to several pregnancy complications such as pre-eclampsia and intrauterine growth restriction. In that context, endothelin-1 (ET-1) induced PLC-γ activation is responsible of endoplasmic reticulum (ER) stress, characterized by excessive Ca^{2+} release from internal stores and accumulation of misfolded proteins. ER stress activates pro-inflammatory pathways (Jain et al., 2012) and may induce apoptotic cell death in the trophoblast (Scifres and Nelson, 2009). We have previously shown, that a low concentration of *T. cruzi* induces apoptotic cell death in chorionic villi explants (Duaso et al., 2011), which is the same condition that induces a strong PLC-γ phosphorylation (Fig. 1).

On the other hand, MAPK signaling pathways (ERK1/2, JNK1/2 and p38MAPK) participate in the villous trophoblast maintenance. Specifically, ERK1/2 is involved in trophoblast differentiation (Delidaki and M., 2011). Recently it has been described that lipopolysaccharides activates the MAPK pathway including the ERK1/2 in term syncytiotrophoblasts, trophoblasts and choriodecidua explants, providing evidence that the MAPKs are involved in the placental immune response (Anton et al., 2012). Additionally, the three different MAPKs pathways are all activated by ER stress. Particularly, ERK1/2 activation is an important downstream effector mechanism for cellular protection during ER stress (Hung, 2003). As shown previously, the presence of the parasite in the intervillous space induces severe tissue damage of the chorionic villi (Duaso et al., 2010, 2012). Therefore, the activation of ERK1/2 pathway, in presence of a low concentration of trypomastigotes, may constitute an attempt to protect the tissue. However, the higher concentration of parasites inhibits ERK1/2 activation (Fig. 2) as well as induces a lower phosphorylation of PLC-γ (Fig. 1); these results are compatible with the necrotic cell death observed in that condition (Duaso et al., 2011).

Interestingly, the inhibition of each signal transduction pathway did not impair *T. cruzi* infection of the human chorionic villi (Fig. 3). Considering that the parasite induces several signaling pathways in the host cell, the inhibition of one of them would not be sufficient to impair the invasion. This is in spite of the fact that at least one of the inhibitor used (PLC-γ) has also effect on Phospholipase A pathways (Cinque et al., 2008). Since the "invasion and infection strategy" of the parasite is highly complex and implies not only the activation of the signaling pathways, an effective treatment of the disease may consider the inhibition of more than one signaling pathway (in the host and the parasite) as well as other events during the invasion process such as enzymatic activation or cytoskeleton re-arrangement.

During the first three months of pregnancy, tumor necrosis factor alpha (TNF-α) induces ERK1/2 mediated expression of matrix metalloprotease-9 (MMP-9) in trophoblastic cells (Cohen et al., 2006). The expression and activity of MMPs are fundamental for embryonic implantation in the endometrium (Anacker et al., 2011). Additionally, several protozoan pathogens induce expression and activity of those enzymes in different tissues and organs (Geurts et al., 2012). In that context, we have shown that *T. cruzi* induces expression and activity of MMP-2 and MMP-9 in the infected *ex vivo* chorionic villi explants (Castillo et al., 2012).

Moreover, bacteria colonizing the lower genital tract may ascend to the gestational tissues, triggering an inflammatory response mediated primarily by interleukin-1β (IL-1β), TNF-α, and Interleukin-6 (IL-6). These cytokines stimulate the secretion of additional cytokines and other mediators, such as prostaglandin E_2 (PGE_2) and MMPs, which may participate in the preterm labor frequently observed in that type of infections (Estrada-Gutierrez et al., 2010). Preterm labor has also been described in congenital Chagas' disease (Bittencourt, 1976), probably due to the immune and inflammatory response induced by the parasite.

5. Conclusion

Both signal transduction pathways are modulated during *ex vivo T. cruzi* infection of human placental chorionic villi explants. At present, a role of PLC-γ and the ERK1/2 MAPK pathways as part of the invasion and infection "strategy" of the parasite or as part of local placental response to that pathogen cannot be discarded.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.exppara.2012.10.012.

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