Biomédica 2010;30:32-8

ARTÍCULO ORIGINAL

Synthesis of pyruvate: ferredoxin oxidoreductase and alcohol dehydrogenase E enzymes during *Giardia intestinalis* excystation

Carlos Alberto Niño, Moisés Wasserman

Laboratorio de investigaciones Básicas en Bioquímica, Departamento de Química, Universidad Nacional de Colombia, Bogotá D.C., Colombia.

Introduction. *Giardia intestinalis* is a unicellular parasite of worldwide distribution. It causes an intestinal illness known as giardiasis, and it is probably the earliest diverging eukaryotic microorganism. Previously, changes have been reported in the expression of mRNAs at several stages of the life cycle; however specific enzymatic activity changes have not been explored.
Objective. The expression of pyruvate ferredoxin oxidoreductase (PFOR) and alcohol dehydrogenase E (ADHE) enzymes was measured in cyst and trophozoite stages, and during the excystation process.
Materials and methods. Recombinant proteins were generated for PFOR and ADHE to be used as antigens in the production of polyclonal antibodies for the detection of native proteins by Western Blot. The enzymatic activity of ADHE and glutamate dehydrogenase (GDH) was evaluated by spectrophotometric assays.
Results. PFOR (139 kDa) and ADHE (97 kDa) proteins were detected in trophozoites, but not in cysts. During excystation, ADHE protein was detected after the first phase of induction, but the PFOR protein appeared only after the second phase. This indicated that both proteins were synthesized during excystation, although at different times. ADHE enzymatic activity was present only in trophozoites and not in cysts whereas GDH activity was detected in both stages.
Conclusion. These results conclusively showed that PFOR and ADHE enzymes were translated during the excystation process and is strong evidence that active protein synthesis was occurring during excystation.

Key words: *Giardia lamblia*, cell differentiation, alcohol dehydrogenase.

Síntesis de las enzimas de piruvato, oxidorreductasa de ferredoxina y deshidrogenasa E de alcohol durante el desenquistamiento (*excystacion*) de *Giardia intestinalis*

Introducción. *Giardia intestinalis* es un parásito unicelular diseminado mundialmente. Causa una enfermedad intestinal conocida como giardiosis y, probablemente, es el microorganismo eucarionte más tempranamente divergente.
Objetivo. En el presente estudio se determinó la expresión de las enzimas de piruvato oxidorreductasa ferredoxina (PFOR) y deshidrogenasa E de alcohol (ADHE) en los estadios de quiste y trofozoíto, y durante el proceso de desenquistamiento (*excystacion*). Previamente se habían demostrado cambios en sus ARNm.
Materiales y métodos. Se generaron proteínas recombinantes de PFOR y ADHE que fueron usadas como antígenos para la producción de anticuerpos policlonales. Éstos se emplearon para la detección de las proteínas nativas por *Western blot*. Evaluamos la actividad enzimática de ADHE y de la glutamato deshidrogenasa (*glutamate dehydrogenase*, GDH) por ensayos espectrofotométricos.
Resultados. Las proteínas PFOR (139 kDa) y ADHE (97 kDa) se detectaron en trofozoítos, pero no en quistes. Durante el desenquistamiento (*excystacion*), la proteína ADHE se detectó sólo después de la primera fase de inducción y la proteína PFOR sólo después de la segunda fase. Esto indica que ambas proteínas son sintetizadas durante el desenquistamiento (*excystacion*), aunque en un momento diferente. La actividad enzimática de ADHE está presente sólo en los trofozoítos y no en los quistes, mientras que la actividad de la enzima GDH se detectó en trofozoítos y quistes.
Conclusiones. Nuestros resultados muestran de forma concluyente que las enzimas PFOR y ADHE son traducidas en el proceso de desenquistamiento (*excystacion*) y esto demuestra que existe un proceso activo de síntesis de proteínas durante él.

Palabras clave: *Giardia lamblia*, diferenciación celular, alcohol deshidrogenasa.

Giardia intestinalis is the pathogen that causes giardiasis in humans. This unicellular parasite is considered one of the earliest diverging eukaryotic microorganisms. It is widely distributed, but is found mainly in places with deficient sanitation conditions. *Giardia intestinalis* has two functional, structural, and morphologically differentiated life stages known as cyst and trophozoite (1). Transition from one stage to the other implies a differentiation processes that apparently involves not only morphological changes, but also a regulated change in gene expression (2-4). *Giardia* cysts have four nuclei and a thick cell wall that allows them to survive to harsh external environmental conditions, including low temperatures and hypotonic conditions. On the other hand, the trophozoite has two nuclei and requires anaerobic conditions and the host's well regulated temperature to survive and multiply (1).

Giardia's life cycle begins with the vertebrate host ingesting cysts from contaminated water or food. The exposure of cysts to gastric acid and proteases during their transit through the stomach triggers the differentiation process known as excystation. This process ends when the cysts enter the small intestine, where the trophozoites emerge and adhere to the duodenum and jejunum surfaces and multiply by binary fission. Some trophozoites travel along the intestinal tract and are induced to encyst, probably triggered by the high pH, the presence of bile salts, and a low cholesterol concentration (5). These cysts are excreted in the stools and thereby become available for ingestion by another host to complete the cycle (1,6,7).

Excystation is a key step in the infective process. However, little is known about this stage, especially about the molecular processes involved in its regulation. Paget *et al.* (1998) (8) demonstrated that when excystation is initiated, an increase in oxygen uptake occurs; this increases exponentially during the following 30 minutes and is linked to an increase in metabolic rate. Are the associated enzymes synthesized *de novo* during excystation or are these enzymes carried over from the trophozoite stage? Hetsko *et al.* (1998) (3) approached the question by using the mRNA differential display method which showed complex transcriptional changes during the excystation process. They found that some transcripts profusely appeared, disappeared or changed. However, they did not identify the mRNAs, and therefore were unable to establish whether the corresponding proteins were synthesized during the process.

In a previous paper, the transcriptional changes of genes encoding were evaluated during *G. intestinalis* excystation (4), including pyruvate: ferredoxin oxidoreductase (PFOR), acetyl-CoA synthetase (ACS), alcohol dehydrogenase E (ADHE) and glutamate dehydrogenase (GDH) enzymes. These enzymes are essential for the parasite's carbohydrate metabolism (1,9,10). In trophozoites, transcripts of the four enzymes were detectable, whereas in cysts only GDH and ACS mRNA were found. During excystation, PFOR and ADHE transcripts appeared after the first induction phase and ACS and GDH mRNAs increased throughout the process. These results suggested that excystation is not only a process of mechanical rearrangement of components, but also a stage where a well regulated pattern of gene expression begins, accompanied by significant changes in transcription and possibly in mRNA degradation (4). The mRNA of enzymes not present in the cyst is synthesized after the induction phase (acid phase which simulates the passage of the cyst through the host's stomach). Although a differential pattern in the mRNA levels was observed for the enzymes that were characterized, the question remained as to whether active protein synthesis had occurred.

The present study was designed to answer two questions: (1) have the two fully identified proteins, pyruvate ferredoxin oxidoreductase (PFOR) and alcohol dehydrogenase E (ADHE) proteins demonstrated differentiation-dependent behavior in their expression, and (2) are these enzymes synthesized *de novo* during excystation. PFOR catalyzes the oxidation of pyruvate to acetyl-CoA (11) and the ADHE bifunctional enzyme catalyzes acetyl-CoA reduction to acetyldehyde and later to ethanol (12). These proteins were selected because their genes have been described as transcriptionally active and regulated during excystation (4). Recombinant proteins were generated for PFOR and ADHE to be used as antigens in the generation of polyclonal antibodies. By means of immunoenzymatic techniques, the presence of PFOR and ADHE proteins was evaluated in cells

Correspondencia:
Moisés Wasserman, Laboratorio de investigaciones Básicas en Bioquímica, Departamento de Química, Universidad Nacional de Colombia, Unidad Camilo Torres, carrera 50 No. 27-70 bloque 10, nivel 4, Bogotá D.C., Colombia.
Phone: (571) 3165000 ext 18367.
mwassermannl@unal.edu.co

Recibido: 05/02/08; aceptado:29/07/09

and in protein extracts of trophozoites and cysts, and during *G. intestinalis* excystation.

Materials and methods

Trophozoite culture, cyst production and excystation

Trophozoites of the *G, intestinalis* WB-C6 strain were grown in Diamond's TYI-S-33 modified medium (13), and then incubated at 34°C in 16 ml borosilicate tubes with a 15° inclination. Cysts were obtained from clinical samples as well as from *in vitro* encystation of trophozoite cultures (14). Clinical samples were kept at 4°C until purification using the procedure already reported (4). The cysts for immunodetection assays were preserved at -70°C until they were used. Samples destined for excystation assays were preserved at 4° C and the assay was done within 24 hours after isolation. *In vitro* encystation was done according to the procedure described by Kane *et al.* (14).

The excystation phases were studied with clinical samples because excystation of *in vitro*-derived cysts is not efficient. The two-phase excystation method (15) used in the current study is much more efficient with cysts from fecal samples than with *in vitro* produced cysts. In phase I, the induction phase, cysts were exposed for 1 h at 37ºC to an acid solution (25 mM $NaHCO_3$, 12 mM KCl, 40 mM NaCl, 6 mM $CaCl_2$), pH 1.6. The phase II, the excystation phase, was achieved by incubation in TYI-S-33 medium for 1 h at 37°C.

Recombinant proteins production and purification

Using PCR, PFOR and ADHE gene fragments of 1056 bp (2224-3280 bp region) and 1040 bp (790-1830 bp region) respectively, were amplified using WB-C6 genomic DNA. Primers were designed using the PRIMER 3 software, based on the *G. intestinalis* sequences reported in GenBank:

PFOR GenBank L27221 (size of gene 10422 bp)

GI-PFOR-S3: 5'-**AAGCTT**GATAGTTGAGTCCCA GG GTG-3´

GI-PFOR-AS5: 5'-**GGATCC**CATGATCGTGTTTAT GCGTC-3'

ADHE GenBank U93353 (size of gene 3330 bp)

GI-ADHE-S3: 5'-**AAGCTT**GACGAGACGTGTGAC TAC-3'

GI-ADHE-AS3: 5'-**GGATCC**CTCATACAGAAGC CT AAC-3'

Restriction sites were added for Hind III (AAGCTT) and BamH I (GGATCC). The components of the PCR mixture reaction were as follows: PCR buffer (50 mM KCl, 10 mM Tris-HCl pH 9.0 and 0.1% Triton®X-100), 200 μM 4dNTP, 0.5 μM sense and antisense primer, 2.5 U of Taq DNA polimerase, 100 ng of *G. intestinalis* DNA); deionized water was added to reach a final volume of 25 μl. The amplification conditions were as follows: 94°C for 5 min, 30 cycles of 94°C for 45 sec, 55°C for 45 sec, 72°C for 1 min, and a final extension at 72°C for 7 min. The products were visualized by horizontal electrophoresis in 1.5% agarose gels in TBE 0.5X. PCR products were purified using Promega's Wizard® Kit PCR Preps DNA Purification System. To produce the p-GEM-T-PFOR-Gi and p-GEM-T-ADHE-Gi clones, the purified products were ligated to the pGEM-T (Promega) vector and transfected by electroporation with a constant 12.5 kV/cm pulses of 5 msec in an 80 μl suspension of *Escherichia coli* JM109 competent cells. Plasmid DNA was purified and inserts were removed by double digestion with *Hind*III and *Bam*HI restriction enzymes; digestion products were purified with Promega's Magic Clean-Up System and ligated to Promega's PinPoint Xa expression vector.

Synthesis of the recombinant proteins was effected by incubation with IPTG (100 μM), biotine (2 μM) and ampiciline 100 μg/ml in LB medium at 37°C, with constant agitation for 5 additional hours. The bacteria were homogenized with a teflon piston homogenizer and later subjected to a treatment with lysozyme (1 mg/ml), deoxycholate (0.1 %), 200 U of DNAse I and 5 mM $MgCl_2$. The cellular lysate was centrifuged at 10,000 x g for 15 min at 4°C. The supernatant was used as cell extract; the pellet corresponded to debris and cell remnants. The supernatant and the pellet were subjected to SDS-polyacrylamide gel electrophoresis (PAGE), blotted to polyvinylidene fluoride (PVDF)-cellulose membranes and were enzymatically detected (streptavidin-alkaline phosphatase) to check for the presence of biotinylated fusion proteins. Since the recombinant proteins in the pellet were found in the form of inclusion bodies, the purification was done with the procedure reported by Coligan *et al.* (16). Briefly, the pellet was washed four times with a Tris-HCl 100 mM pH 7.0, EDTA 5 mM, DTT 5 mM, urea 2M and Triton X-100 2% p/v solution, and finally an extraction was done using a Tris-HCl 50 mM pH 7.0, EDTA 5mM, guanidine-HCl 8M and DTT 5mM solution. The extract was centrifuged at 20,000 x g for 20 minutes at 4°C. The supernatant

was dialyzed against 1 liter of a phosphate buffered saline solution (PBS); this was changed 3 times, every 5 hours.

Polyclonal antibodies production

Pure recombinant proteins were used as antigens in the generation of polyclonal antibodies in New Zealand rabbits. Four immunizations were done (days 0, 15, 30 and 45). The amount of recombinant protein for immunizations was 100 ug. The first inoculation was done with complete Freund's adjuvant, and the subsequent three with incomplete Freund's adjuvant. Serum was obtained by centrifuging the coagulated blood at 800 x g for 10 min (17). An initial immunoglobulin fractionation was done by precipitation with ammonium sulfate at 33% saturation followed by an ion-exchange chromatography on DEAE-Sepharose CL-6B (Amersham) to obtain the IgGs.

Immunodetection of recombinant proteins

Fifty ng of recombinant protein purified was analyzed by SDS-PAGE electrophoresis in 10% polyacrylamide gel and was blotted to a PVDF-cellulose membrane for immunodetection using the purified IgGs 1:5000, an anti-IgG peroxidase conjugated rabbit secondary antibody 1:2000 and the 4-chloro-1-naphthol detection system.

Immunodetection of PFOR and ADHE proteins in cysts and trophozoites

Detection was done by electrophoresis of samples of complete trophozoites, of *in vivo* and *in vitro*-derived cysts ($1x10^6$ per lane), and of trophozoite extracts (50 μg per lane), treated with sample buffer (Tris-HCl 50 mM pH 6.8, DTT 100 mM, SDS 2% buffer, bromophenol blue 0.1% and glycerol 10%). For each of the excystation phases, whole parasites were stimulated with the solutions described above. Extracts were obtained from trophozoite cultures or from cysts collected as previously described. They were resuspended in 500 μl extraction buffer (sucrose 250 mM, Tris-HCl 25 mM pH 7.5, and protease inhibitors) (12), and cells were lysed by sonication applying five 30-second pulses (20% power; W-225 200-watt/20 kHz sonicator) at 4°C. The cells were observed on the microscope to check for complete lysis, and later the suspension was centrifuged at 10,000 x g for 20 min at 4°C. The supernatant was collected, and the protein concentration was quantified by the Bradford method. The aliquots were stored at –20°C until further use.

The extracts and the whole cells were analyzed by SDS-PAGE in 8% polyacrylamide gels, in buffer Tris 25 mM, glycine 192 mM, SDS 1%, pH 8.3 (Mini-V Vertical gel 8 10 BRL™). The gel was blotted to a PVDF-cellulose membrane (20 V for 20 h in Tris 100 mM, glycine 192 mM, SDS 1%, methanol 10%, pH 8.3 solution). The membrane was blocked by incubation in TBST-milk (Tris-HCl 20 mM pH 7.5, NaCl 150 mM and milk 5%) for an hour at room temperature with constant agitation. It was later incubated for 1 h with the antibody diluted (1:100 for PFOR and 1:500 for ADHE) in TBST (Tris-HCl 20 mM pH 7.5, NaCl 150 mM and Tween 20 0.1% v/v), washed 3 times with TBST-milk and incubated for 1 h with a biotinylated rabbit anti-IgG (Promega) 1:2000 in TBST, and washed again 3 times in TBST-milk. The membrane was then incubated for 30 min with Promega's streptavidin-alkaline phosphatase conjugate 1:3000 in TBST; washed 3 times with TBST, and, finally, developed with NBT-BCIP.

Immunodetection of PFOR and ADHE proteins during the excystation process

Giardia intestinalis cysts from clinical samples were purified as described previously (15). These cysts were used to detect possible changes in PFOR and ADHE expression during excystation. Three stages were examined: (1) the non-stimulated cysts, (2) immediate after the induction phase, i.e., exposure to Bingham's solution, pH 1.6 for 1 h at 37°C, and (3) complete excystation, i.e., after further incubation in TYI-S-33 medium for 1 h at 37°C. Each treatment was done on $2x10^6$ cysts. Samples corresponding to each of the 3 points were analyzed by SDS-PAGE electrophoresis and blotted to a PVDF membrane for immunodetection with the antibodies.

Enzyme activity assays for ADHE and glutamate dehydrogenase (GDH)

The spectrophotometric assays for ADHE and GDH were conducted as in previous reports for *Giardia* (12,18). GDH enzyme activity was evaluated as a positive control, since its mRNA is present in both stages and during the differentiation process (4). Eighty μg of trophozoite protein extract was used for measuring ADHE activity. For cysts, 80, 160 and 240 μg of protein extract were used during the long reaction times required (up to 5 hours). ADHE activity was evaluated by the oxidation of NADH to NAD^+ during the formation of acetyldehyde from acetyl-CoA (12). The reaction mixture was 0.1 mM acetyl-CoA, 0.2 mM NADH in 50 mM glycine/NaOH

pH 8.5 buffer. A unit was defined as the quantity of enzyme that catalyzes the oxidation of 1 μmol of NADH per min. GDH activity was determined by the oxidation of NADH to NAD^+ during the formation of glutamate from α-ketoglutarate and NH_4Cl (18); the reaction mixture was α-ketoglutarate 20 mM, NH_4Cl 20 mM, 0.1mM NADPH in 100 mM potassium phosphate buffer pH 7.3 in a final volume of 500 μl. A unit was defined as the quantity of enzyme that catalyzes the oxidation of 1 μmol of NADPH per min. Reactions were monitored by absorbance changes at 340 nm. Assays were done in duplicate. Specific activity was expressed as activity units per mg of protein.

Results

Immunodetection of the PFOR and ADHE proteins in G. intestinalis *trophozoites and cysts*

PFOR and ADHE recombinant proteins were generated and purified as described in the previous section. Figure 1 shows the recombinant proteins purified and the immunoblot with the pure IgGs that recognized them. For the analysis of the native protein expression, the immunodetection was done using protein extracts, complete cells, trophozoites or cysts. The results for the extracts are shown in figure 2. Anti-PFOR antibody recognized a protein of 139 KDa in trophozoites, corresponding in size to the *G. intestinalis* putative PFOR (figure 2, lane T). The recognition was specific, because this protein was not detected by the pre-immune serum. The anti-ADHE antibody specifically detected a *G. intestinalis* 97 kDa protein (the expected molecular mass for ADHE). Identical results were obtained with $1x10^6$ whole parasites per lane (figure 3). Figure 2 shows other smaller non-specific bands that were also detected by the pre-immune serum. PFOR and ADHE proteins were not detected in *in vitro*-derived cyst extracts under the conditions described (figure 2, lane C), i.e., they are not found in the cyst stage or they are present at a concentration below the method's sensitivity threshold. The same results were obtained when complete *in vitro* and *in vivo*-derived cysts ($1x10^6$ per lane) were used (figure 3).

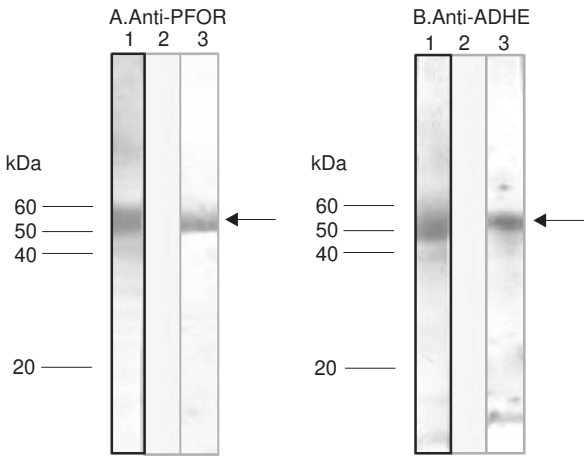

Figure 1. Immunodetection of PFOR and ADHE recombinant proteins. 1. Coomassie blue blue stain of SDS-PAGE gel. 2. Immunodetection using preimmune serum as primary antibody. 3. Immunodetection using purified IgGs.

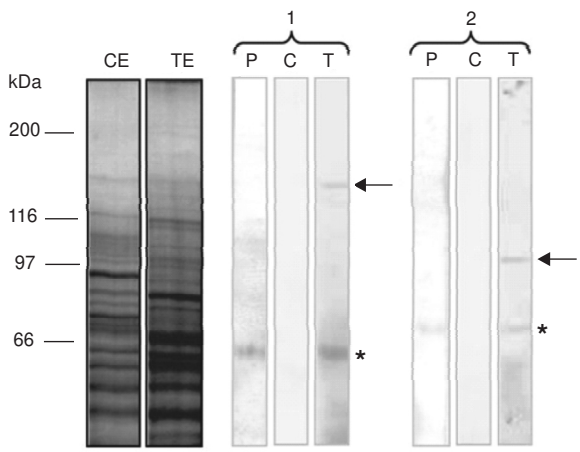

Figure 2. Immunodetection of PFOR and ADHE on *Giardia intestinalis* trophozoite- and *in vitro* derived-cyst extracts. Lane CE: 50 μg SDS-PAGE gel on of *in vitro*-derived cyst extract on SDS-PAGE gel, Coomassie blue stain. Lane TE: 50 μg SDS-PAGE gel on of trophozoite extract on SDS-PAGE gel, Coomassie blue stain. Lane P: Inmunodetection on 50 μg trophozoite extract using preimmune serum as primary antibody. Lane C: Immunodetection on 50 μg *in vitro*-derived cyst extract. Lane T: Immunodetection on 50 μg trophozoite extract. Panel 1: Immunodetection using anti-PFOR antibody 1:100 as primary antibody. Panel 2: Immunodetection using anti-ADHE antibody 1:500 as primary antibody. Arrows indicate 139 kDa PFOR and 97 kDa ADHE immunodetected proteins. Asterisks indicate signals detected with both, preimmune serum and anti-PFOR or anti-ADHE antibodies.

Immunodetection of PFOR and ADHE proteins during the excystation process

Panel A in figure 4 shows the levels of PFOR using the anti-PFOR antibody. The protein was not detected in fecal or in *in vitro*-derived cysts (Q). No signal was observed in the induction phase (I), but the protein was detected by the antibody after the excystation was finished (II). This meant that PFOR was synthesized during excystation and that its level increased sufficiently to be detected at the end of the process. Panel B shows that ADHE was not detected in cysts (Q) but that a signal was observed in cysts after the induction phase (I).

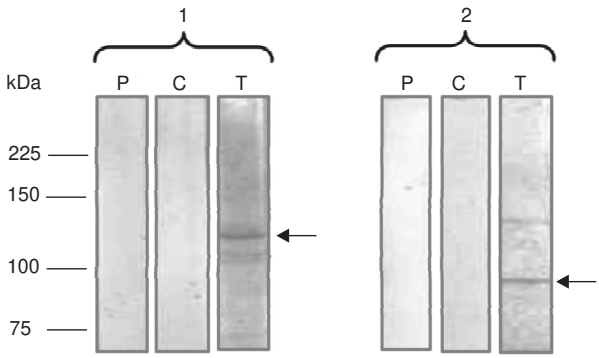

Figure 3. Immunodetection of PFOR and ADHE on in *Giardia intestinalis* trophozoites and *in vitro*-derived complete cysts. Lane P: Immunodetection using preimmune serum. Lane C: 1x10^6 complete cysts. Lane T: 1x10^6 complete trophozoites. Panel 1: Immunodetection using anti-PFOR antibody 1:100 as primary antibody. Panel 2: Immunodetection using anti-ADHE antibody 1:500 as primary antibody. Arrows indicate 139 kDa PFOR and 97 kDa ADHE proteins.

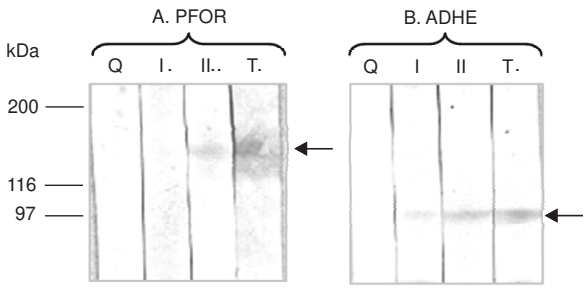

Figure 4. Immunodetection of PFOR and ADHE proteins during the *Giardia intestinalis* excystation process. Panel A: Immunodetection using anti-PFOR 1:100 as primary antibody. Panel B: Immunodetection using anti-ADHE 1:500 as primary antibody. Lane Q: Immunodetection on 2 x 10^6 purified cysts from clinical samples. Lane I: Immunodetection on 2 x 10^6 cysts subjected to Bingham's acid induction phase I. Lane II: Immunodetection on 2 x 10^6 cysts subjected to the complete excystation process. Lane T: Positive control, immunodetection on 2 x 10^6 trophozoites. Arrows indicate 139 kDa PFOR and 97 KDa ADHE proteins.

Once the excystation ended (II), the signal was more intense. Clearly, ADHE was synthesized early during excystation and that its level increased gradually throughout the process.

Enzymatic activity of ADHE and GDH in G. intestinalis *trophozoites and cysts*

ADHE enzymatic activity was detected in trophozoite extracts, but not in cysts. The activity values of ADHE and GDH in trophozoite protein extracts, expressed in mU/mg, were 56.7 ± 3.9 and 231.2 ± 9.4 respectively. In cyst extracts no ADHE activity was detected whereas GDH had an activity value of 128.8 ± 11.6 mU/mg. The detection of ADHE activity in trophozoites and not in cysts agreed with the Western blot results that indicated the presence of ADHE protein in trophozoites but not in cysts.

Discussion

Giardia intestinalis excystation is a cellular differentiation process that results in the generation of four metabolically active trophozoites capable of vegetative growth in a human host from an apparently non active cyst. This process can be reproduced *in vitro* in about 1 h. Because it is a process that occurs in one of the earliest divergent eukaryotes, it an interesting target to study molecular changes involved in cellular differentiation.

The analysis of PFOR and ADHE protein expression in *G. intestinalis* trophozoites and cysts showed significant differences in the two stages. PFOR and ADHE proteins, as well as their mRNAs (4), were present in the trophozoite vegetative stage, but not in the cyst stage. The same behavior was observed both *in vitro*-derived cysts and in those purified from fecal samples. This finding is relevant as it demonstrated expression differences in PFOR and ADHE, two specific and metabolic important proteins in the *G. intestinalis* life cycle. It also showed that the excystation process involved changes in the genetic expression and induction of the synthesis of new proteins, whereas the encystation process involved their degradation. If the electrophoretic pattern of the *in vitro* derived cyst is compared with that of the trophozoite, considerable differences are apparent and suggest a significant change in the proteome during the differentiation processes (figure 2).

These observations also indicate that PFOR and ADHE proteins are synthesized during the excystation process, but not in the cyst stage. This may be a general mechanism for proteins not present in cysts but that are required for trophozoite survival. Additionally, the observations confirm and complement previous results (4) and support the idea that the very short excystation period is not only a process of mechanical rearrangement of cell components, but also the beginning of a complex pattern of regulated gene expression. Clearly ADHE is already detectable at the end of the acid induction (phase I), whereas PFOR is detectable only after total excystation. This may indicate that protein synthesis is initiated as soon as cysts receive the initial stimulus, but that some proteins are synthesized at different times or with different rates.

ADHE enzymatic activity was detected in trophozoites but not in cysts, whereas glutamate dehydrogenase (GDH) activity was detected both in trophozoites and cysts. The value of GDH specific activity in trophozoites, about twice the activity in cysts, agrees with the previous report (4) where GDH mRNA levels in trophozoites were about twice the levels present in cysts. The presence of GDH activity also confirmed that the quality of the cyst protein extract is similar to that of the trophozoite protein extract, and that the absence of ADHE activity in cysts was not due to extraction problems.

ADHE detection and its increase after induction suggest that the Bingham induction solution directly generates a stimulus linked to gene expression. Further assays are required to better establish which of the solution components play a role in this regulation, be it through pH-mediated processes or ion fluxes (Na^+, K^+, Ca^{+2}).

Conflict of interest

The authors declare that they did not incur in any conflict of interest during the present study.

Financial support

The current study was funded by the Universidad Nacional de Colombia (Research Division, Bogotá, D.C., División Investigación sede Bogotá (DIB) and by the Instituto Colombiano para el Desarrollo de la Ciencia Francisco José de Caldas-Colciencias, project 1101-05-14649.

References

1. **Adam R.** Biology of *Giardia lamblia.* Clin Microbiol Rev. 2001;14:447-75.
2. **Hernandez P, Wasserman M.** Do genes from the cholesterol synthesis pathway exist and express in *Giardia intestinalis*? Parasitol Res. 2006;98:194-9.
3. **Hetsko L, McCaffery J, Svärd S, Meng T, Que X, Gillin F.** Cellular and transcriptional changes during excystation of *Giardia lamblia in vitro*. Exp Parasitol. 1998;88:172-83.
4. **Niño C, Wasserman M.** Transcription of metabolic enzyme genes during the excystation of *Giardia lamblia*. Parasitol Int. 2003;52:291-8.
5. **Luján H, Mowatt M, Byrd L, Nash T.** Cholesterol starvation induces differentation of the intestinal parasite *Giardia lamblia*. Proc Natl Acad Sci USA. 1996;93:7628-33.
6. **Gillin F, Reiner D, McCaferry J.** Cell Biology of the primitive eukaryote *Giardia lamblia*. Annu Rev Microbiol. 1996; 50:679-705.
7. **Thompson R, Reynoldson J, Mendis A.** *Giardia* and giardiasis. Adv Parasitol. 1993;32:71-160.
8. **Paget T, Macechko P, Jarroll E.** Metabolic changes in *Giardia intestinalis* during differentiation. J Parasitol. 1998;84:222-6.
9. **Lindmark D.** Energy metabolism of the anaerobic protozoon *Giardia lamblia*. Mol Biochem Parasitol. 1980;1:1-12.
10. **Müller M.** Energy metabolism of protozoa without mitochodria. Annu Rev Microbiol. 1998;42:465-88.
11. **Townson S, Upcroft J, Upcroft P.** Characterisation and purification of pyruvate: ferredoxin oxidoreductase from *Giardia lamblia*. Mol Biochem Parasitol. 1996;79:183-93.
12. **Sanchez L.** Aldehyde dehydrogenase (CoA-acetylating) and the mechanism of ethanol formation in the amitochondriate protist *Giardia lamblia*. Arch Biochem Biophys. 1998; 354:57-64.
13. **Keister D.** Axenic culture of *Giardia lamblia* in TYI-S-33 medium supplemented with bile. Trans R Soc Trop Med Hyg. 1983;77:487-8.
14. **Kane A, Waed H, Keusch G, Pereira M.** *In-vitro* encystations of *Giardia lamblia*: large-scale production of *in-vitro* encystations efficiency. J Parasitol. 1991;177:974-81.
15. **Bingham A, Meyer E.** *Giardia* excystation can be induced *in vitro* in acidic solutions. Nature. 1979;227:301-2.
16. **Coligan J, Dunn B, Ploegh H, Speicher D, Wingfield P.** Current protocols in protein science. New York: John Wiley and Sons, Inc.; 1997.
17. **Harlow E, Lane D.** Antibodies a laboratory manual. New York: Cold Spring Harbour Laboratory; 1998.
18. **Park J, Schofield P, Edwards M.** *Giardia intestinalis*: Characterization of a NADP-dependent glutamate dehydrogenase. Exp Parasitol. 1998;88:131-8.