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Trichomonas vaginalis: Dehydroepiandrosterone sulfate and 17β-estradiol alter NTPDase activity and gene expression

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ABSTRACT

We investigated the effect of dehydroepiandrosterone sulfate (DHEAS) and 17β-estradiol on NTPDase activity in fresh clinical (VP60) and long-term-grown (30236 ATCC) isolates of *Trichomonas vaginalis* followed by NTPDase gene transcriptional analysis. ATP hydrolysis was activated *in vitro* by 17β-estradiol (0.01–1.0 μM) in the VP60 isolate. Treatment for 2 h with 17β-estradiol (0.01–1 μM) promoted an inhibition in nucleotide hydrolysis in the 30236 isolate whereas the 12 h-treatment promoted an activation of nucleotide hydrolysis in both isolates. ADP hydrolysis was inhibited *in vitro* by 1.0–5.0 μM DHEAS in the ATCC isolate. The treatment with DHEAS (0.01–1.0 μM) for 2 h inhibited ATP and ADP hydrolysis in VP60; however, during a 12 h-treatment with DHEAS, nucleotide hydrolysis was inhibited in both isolates. Two NTPDase orthologous (NTPDaseA and NTPDaseB) were identified and the treatment with DHEAS for 12 h was able to inhibit mRNA NTPDaseA transcript levels from the VP60. These findings demonstrate that NTPDase activity and gene expression pattern are modulated by exposure to steroids in *T. vaginalis*.

1. Introduction

Trichomonas vaginalis is a flagellate protozoan that causes trichomonosis, the most common, non-viral sexually transmitted disease (STD) (WHO, 2007). The infection has been associated with serious health consequences, including adverse pregnancy outcomes (Cotch et al., 1997), infertility (Goldstein et al., 1993), predisposition to cervical cancer (Viikki et al., 2000), and pelvic inflammatory disease (Cherpes et al., 2006). Trichomonosis also affects birth outcomes and is a co-factor in human immunodeficiency virus (HIV) transmission and acquisition (Van der Pol et al., 2008).

Adenine nucleotides (ATP, ADP, and AMP) and their nucleoside derivative, adenosine, are important signaling molecules that mediate diverse biological and pathological processes (Burnstock, 2007). The biological effects of extracellular ATP are mediated by ionotropic P2X and metabotropic P2Y receptors (Burnstock, 2007; Ralevic and Burnstock, 1998). The signaling effects induced by extracellular ATP are directly correlated to the action of ecto-nucleotidases, since these enzymes trigger enzymatic conversion of ATP to adenosine (Zimmermann, 2001; Robson et al., 2006). Ecto-nucleotidases comprise a group of ecto-enzymes involved in the control of nucleotide and nucleoside levels. These enzymes include the NTPDase (nucleoside triphosphate diphosphohydrolase) family, which is able to hydrolyze nucleoside 5′ -tri and diphosphates (Burnstock, 2007). The presence of ecto-nucleotidases has been related on the surface of parasites and previous studies from our laboratory have already characterized this enzyme activity (NTPDase, ATP diphosphohydrolase, or apyrase) in trophozoites of *T. vaginalis* (Matos et al., 2001).

In the past few years, accumulating evidence has suggested that many hormones, especially the sex steroids, can influence the im-

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Table 1
Primer sequences and PCR conditions.

Enzymes	Primer sequences (5′ → 3′)	Annealing temperature (°C)	PCR product (bp)	GenBank accession number (mRNA)
NTPDase A	F-TGAAGAAGAGTTGAAGGGCAAAG R-AATTCTTCGACAGGAGGCATTG	53	342	XM_001298945
NTPDase B	F-CGACTACA0054CATCTCTTGCCGATC R-GACTCTCTTATGTATCTTTGGGCAG	53	397	XM_001579653
α-Tubulin	F-GCCAACATGATGGTTAAGTGCGATCCAC R-CAGCTTCTTCCATACCCTCACCGACG	61	355	XM_001330630

mune system and, thus, the susceptibility to diseases caused by protozoan parasites (Grossman, 1984; Roberts et al., 2001; Olsen and Kovacs, 2005). For example, estrogen, testosterone, and progesterone treatments in insects stimulated the proliferation of their protozoan and helminth parasite infections (Lawrence, 1991). In contrast, dehydroepiandrosterone (DHEA), DHEA-sulfate (DHEA-S) and its analog, 16α-bromoepiandrosterone, have shown antimalarial activities against several strains of *Plasmodium falciparum in vitro*, and *Plasmodium berghei in vivo* (Freilich et al., 2000). Interestingly, high circulating levels of DHEAS in Kenyan pubertal girls are correlated with lower *P. falciparum* parasitemia, which suggests a predictive role for this hormone in malaria (Leenstra et al., 2003). Proliferation and survival of *Cryptosporidium parvum* in multiple animal models were also down-regulated by treatment with DHEA (Rasmussen et al., 1991, 1995; Rasmussen and Healey, 1992).

The role of estrogens in the pathogenesis of *T. vaginalis* has been controversial, and seemingly contradictory reports are found in the literature. Exacerbation of trichomonosis often occurs around the time of menses (Petrin et al., 1998). Silva-Filho and Bonilha (1992) showed that α-estradiol and 17β-estradiol increase parasite adhesion. However, the activity of cell-detaching factor (CDF), a virulence factor of *T. vaginalis*, in the presence of 17β-estradiol, was significant diminished. These data suggest that the symptoms of *T. vaginalis* infection may be influenced by the vaginal concentrations of estrogens (Garber et al., 1991).

Studies have demonstrated that ovariectomy in female rats promotes a significant increase in ATP, ADP, and AMP hydrolysis in blood serum (Pochmann et al., 2004). Considering the impact of trichomonosis on public health and the fact that host–parasite interaction is influenced by hormone levels, it is important to understand the effects promoted by these hormones on the nucleotidase pathway in *T. vaginalis*. Therefore, the aim of this study was to evaluate the effects of 17β-estradiol and DHEAS on NTPDase activity in fresh clinical isolates (VP60) and in long-term-grown isolates (30236 ATCC) of *T. vaginalis* followed by NTPDase gene transcriptional analysis after hormone treatment.

2. Materials and methods

2.1. Chemicals

Trizma Base, malachite green, ammonium molybdate, polyvinyl alcohol, nucleotides, Coomassie Blue G, bovine serum albumin, calcium and magnesium chloride, 17β-estradiol (cyclodextrin-encapsulated 17β-estradiol), dehydroepiandrosterone sulfate (5-androsten-3β-ol-17-one sulfate), and trypan blue dye were

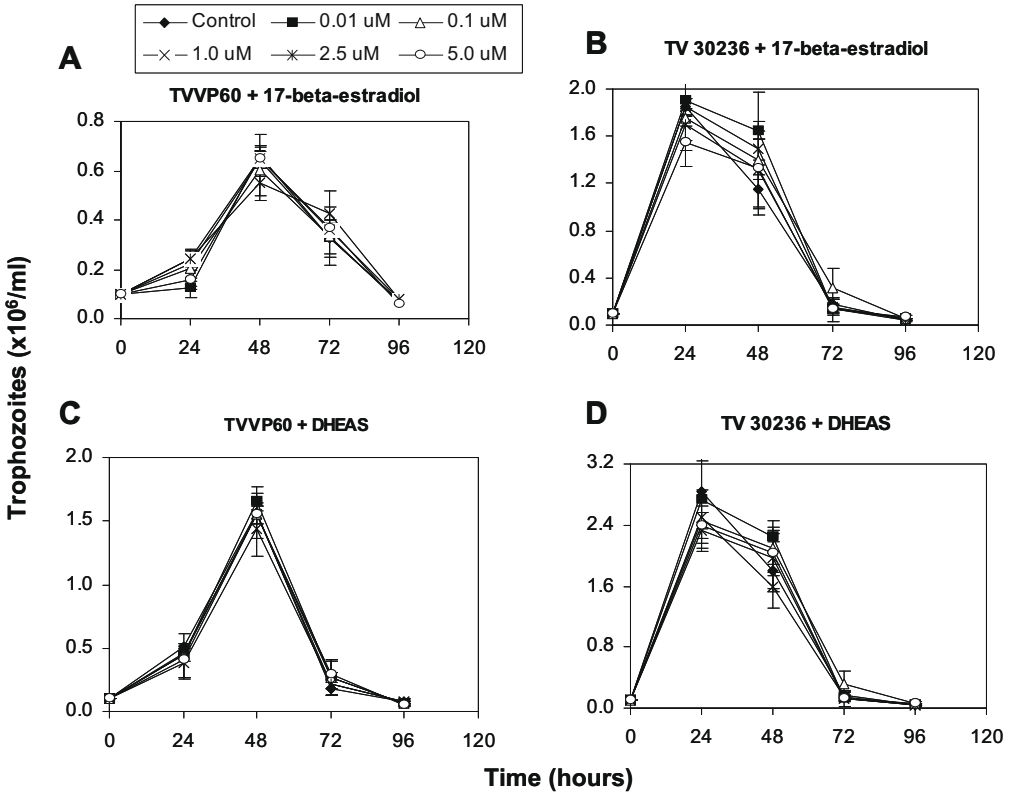

Fig. 1. Kinetic growth curve of VP60 and 30236 ATCC isolates in presence of 17β-estradiol (A and B) and DHEAS (C and D). Lines represent the means ± S.D. of three experiments ($n = 3$) using different parasite suspensions.

purchased from Sigma (St. Louis, MO, USA). All other reagents used were of analytical grade.

2.2. Parasites and culture conditions

Two *T. vaginalis* isolates were used in this study: 30236, from the American Type Culture Collection (ATCC) and VP60, a fresh clinical isolate (EU816897) (Michel et al., 2006). Both isolates were cultivated axenically in trypticase-yeast extract-maltose (TYM) medium (Diamond, 1957) without agar (pH 6.0) and supplemented with 10% (v/v) heat-inactivated serum, penicillin (1000 IU/ml), and streptomycin sulfate (1.0 mg/ml) in aerobiosis at 37 °C (±0.5). Isolates were subcultured every 48 h in TYM medium. Trichomonads in the logarithmic phase of growth and within 48 h of subculture (exhibiting more than 95% mobility and normal morphology) were harvested and washed with sterile saline solution (0.85% NaCl) (750 × g for 5 min) three times. Parasites were resuspended in saline solution and counted with a haemocytometer and adjusted to a concentration of 1.5×10^6 trophozoites/ml, corresponding to 0.3–0.7 mg/ml protein. All experiments were performed using intact organisms and the cellular viability was assessed, before and after incubations, by motility and trypan blue dye exclusion. The viability was not affected by incubation conditions.

2.3. Parasite treatment

The trophozoites were kept in the absence (control) or in the presence of 17β-estradiol or DHEAS (0.01, 0.1, and 1.0 μM) for 2 or 12 h before washing with sterile saline solution (0.85% NaCl) (750 × g for 5 min) three times. The subsequent steps for incubation to measure the nucleotide hydrolysis were the same as those described above.

2.4. Kinetic growth curve in the presence of hormones

In order to investigate the influence of the hormones on *T. vaginalis* growth, experiments on the kinetic growth curve were performed with both isolates, with an initial innoculum of 1.0×10^5 trophozoites/ml, in presence and absence of 17β-estradiol or DHEAS (0.01, 0.1, 1.0, 2.5, and 5.0 μM), in TYM medium. The results were expressed as the percentage of living organisms compared to parasite control (absence of hormones) counted each 24 h of kinetic growth curve.

2.5. Enzyme assays

Intact trophozoites of *T. vaginalis* were added to the reaction mixture containing 50 mM Tris buffer (pH 7.2) and 5.0 mM $CaCl_2$ to determine NTPDase activity. For *in vitro* assays, hormone 17β-estradiol or dehydroepiandrosterone sulfate (DHEAS) (0.01, 0.1, 1.0, 2.5, and 5.0 μM) was added to the reaction media. The samples were preincubated for 5 min at 37 °C in 200 μl of the reaction mixture. The reaction was initiated by the addition of ATP or ADP to a final concentration of 1.0 mM and stopped, after 40 min, by adding 200 μl 10% trichloroacetic acid. The samples were chilled on ice for 10 min before assaying for the release of inorganic phosphate (Pi) (Chan et al., 1986). Incubation times and parasite density were chosen in order to ensure the linearity of the reactions. Controls with the addition of the intact trophozoites, after mixing trichloroacetic acid, were used to correct non-enzymatic hydrolysis of sub-

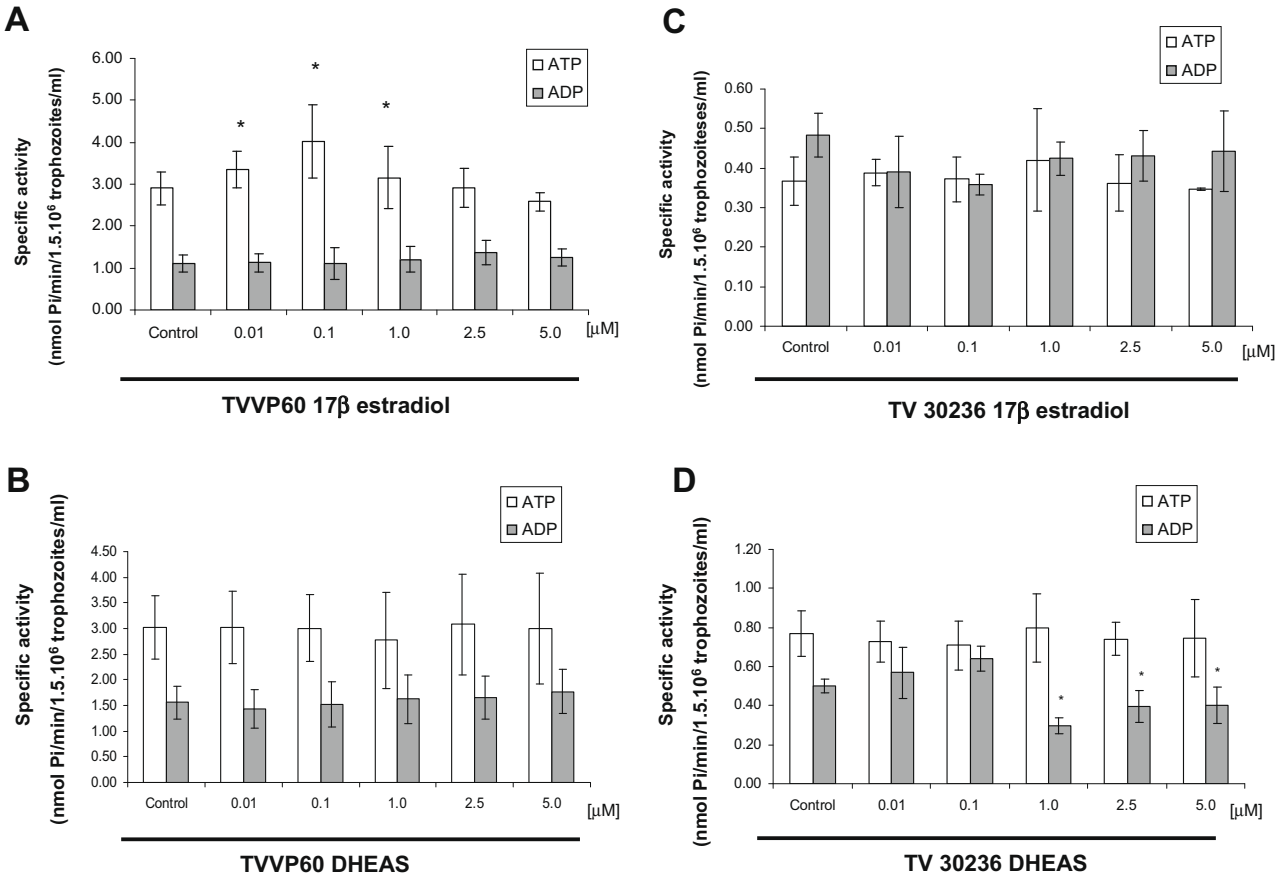

Fig. 2. Effect *in vitro* of 17β-estradiol (A and C) and DHEAS (B and D) on ATP and ADP hydrolysis in VP60 and 30236 ATCC isolate. The steroid hormones were added in the reaction medium and maintained throughout the enzyme assay, as described in Materials and methods (Subsection 2.5). Bars represent the means ± S.D. of at least four experiments ($n = 4$) using different parasite suspensions, each in triplicate. *Indicates significant difference from controls by one-way ANOVA, followed by Duncan test as a post hoc ($P < 0.05$).

strates. Specific activity was expressed as nmol Pi released/min/ 1.5×10^6 trophozoites/ml. All samples were run in triplicate, with similar results achieved in at least three different cell suspensions.

2.6. Protein determination

Protein was measured by the Coomassie blue method (Bradford, 1976), using bovine serum albumin as a protein standard.

2.7. Prediction of T. vaginalis NTPDase sequences

NTPDase sequences of *T. vaginalis* were obtained from the NCBI database and UniProtKB using the systematic BLAST searches (Altschul et al., 1990). The well-known amino acid sequences of human (NP_001767, NP_982293, NM_001248, BC034477, NM_001 249, BC025980, NM_020354, and AAR04374) and mouse (AAH 11278, O55026, AY376710, BC043134, BC015247, BAE33807, NM_053103, and AAQ84519) NTPDase members were used as queries. The protein blast was performed using the non-redundant protein sequences (nr), Reference proteins (refseq_protein) and Swissprot protein sequences (swissprot) databases and the algorithm blastp.

2.8. Phylogenetic and sequence analyses

NTPDases orthologous sequences from related parasites *Toxoplasma gondii* (AAC80187 and AAC80188), *Trypanosoma cruzi* (AAS75599), *Trypanosoma brucei* (AAZ13145), *Schistosoma mansoni* (XP_002579239), *Schistosoma japonicum* (AAW26231), *P. falciparum* (AAN36910), *Leishmania major* (DAA04950), and *Entamoeba histolytica* (XP_648646, XP_652062, XP_654244, and XP_656157) were retrieved from databases and included in the phylogenetic analysis.

Protein sequences alignment was performed using the ClustalX program (Thompson et al., 1997) and a phylogenetic tree was constructed according to the Neighbor-Joining method (Saitou and Nei, 1987) using a proportional (p) distance with the MEGA 4.0 program.

2.9. Reverse transcription-polymerase chain reaction (RT-PCR)

The search for specific NTPDase primers was performed using regions with low scores of similarity among the sequences, which were designed using the Oligos 9.6 program. In order to confirm primer specificity, each primer was compared with the *T. vaginalis* genome and it was able to recognize only its specific target sequence. Therefore, the strategy adopted to construct the primers

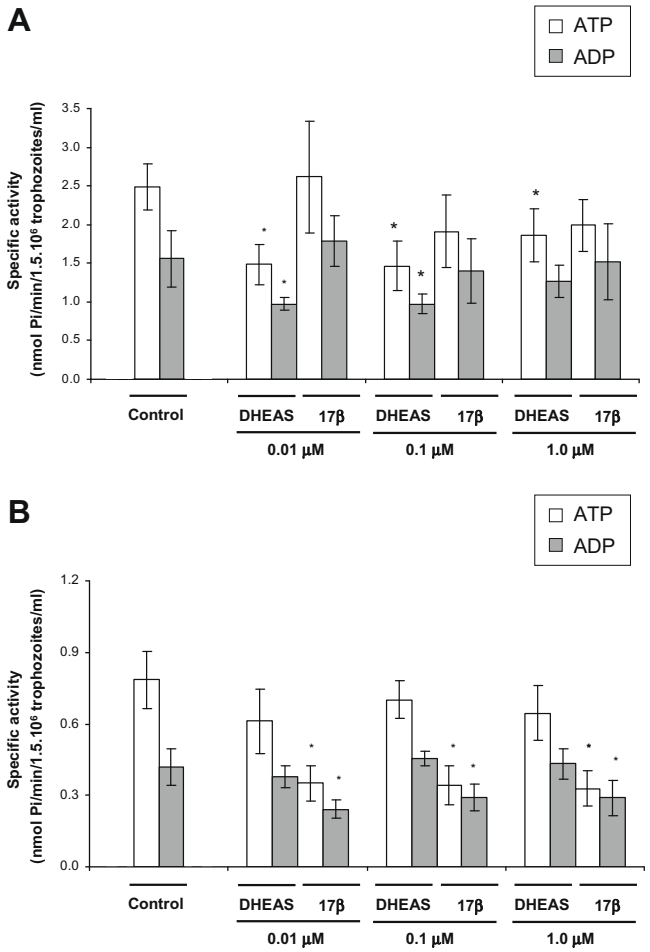

Fig. 3. Effect of 17β-estradiol and DHEAS treatment for 2 h on ATP and ADP hydrolysis in the VP60 isolate (A) and the 30236 ATCC isolate (B). Bars represent the means ± S.D. of at least four experiments ($n = 4$) using different parasite suspensions, each in triplicate. *Indicates significant difference from controls by one-way ANOVA, followed by Duncan test as a post hoc ($P < 0.05$).

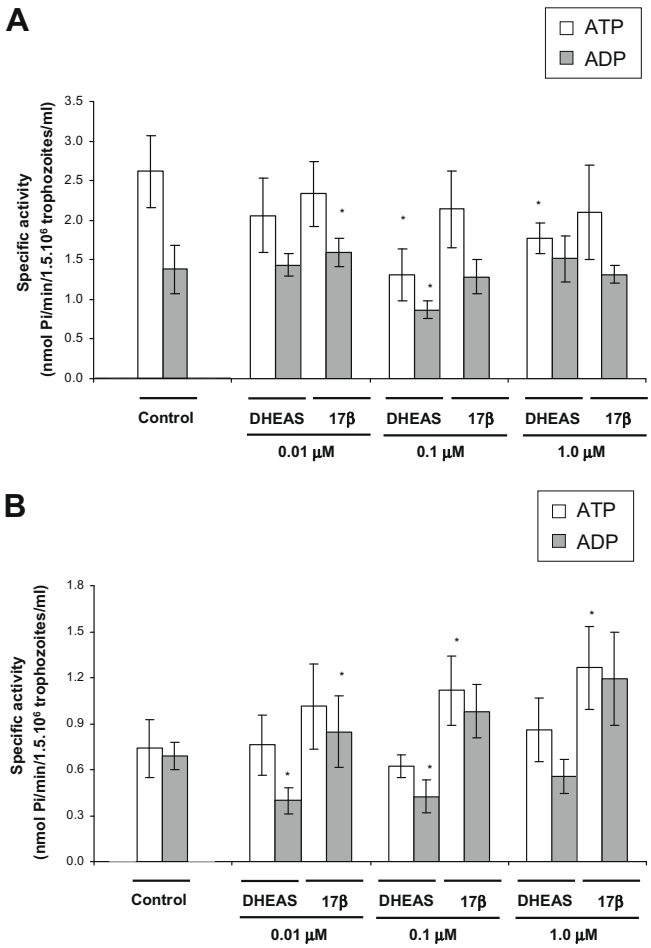

Fig. 4. Effect of 17β-estradiol and DHEAS treatment during 12 h on ATP and ADP hydrolysis in VP60 isolate (A) and 30236 ATCC isolate (B). Bars represent the means ± S.D. of at least four experiments ($n = 4$) using different parasite suspensions, each in triplicate. *Indicates significant difference from controls by one-way ANOVA, followed by Duncan test as a post hoc ($P < 0.05$).

did not allow cross-amplification. The α-tubulin primers were designed and the optimal PCR conditions were determined (Table 1).

RT-PCR conditions were optimized in order to determine the number of cycles that would allow product detection within the linear phase of mRNA transcripts amplification. NTPDaseA, NTPDaseB, and α-tubulin PCR assays were performed using 20 μl, 0.1 mM primers (Table 1), 0.2 mM dNTP, 2.0 mM $MgCl_2$, and 0.5 U Taq Platinum DNA polymerase (Invitrogen, Carlsbad, CA, USA). PCR assays were carried out using 2.0 μl cDNA as template. The following conditions were used for PCR reactions: 1 min at 94 °C, 1 min for annealing temperature (see Table 1), 1 min at 72 °C for 25 cycles. A post-extension cycle at 72 °C was performed for 10 min. For each set of PCR reactions, a negative control was included. PCR products were separated by electrophoresis with 1.0% agarose gel containing ethidium bromide and visualized with ultraviolet light. The fragment lengths of PCR reactions were

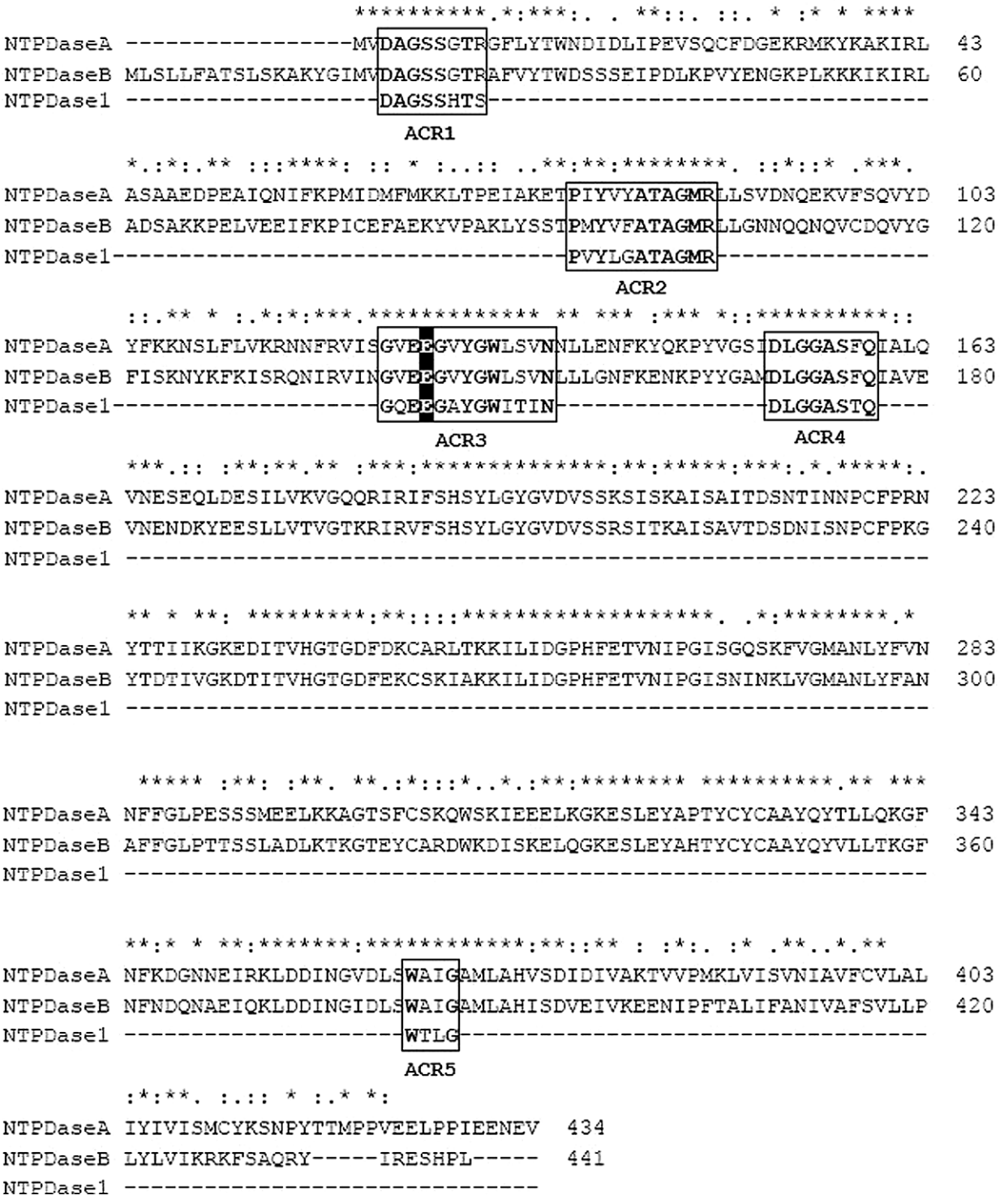

Fig. 5. Deduced amino acid sequences alignment of *T. vaginalis* NTPDaseA and NTPDaseB sequences. The conserved apyrase domains (ACRs) are aligned with human NTPDase1. The Glu residue essential for enzyme activity is marked. The asterisks (*) indicate similarities among *T. vaginalis* NTPDase sequences after ClustalX alignment.

confirmed with a Low DNA Mass Ladder (Invitrogen, USA) and the normalization was performed, employing α-tubulin as a constitutive gene.

2.10. Statistical analysis

Statistical analysis was conducted by one-way ANOVA (analysis of variance), followed by Duncan test as a post hoc, considering a level of significance of 5%.

3. Results

In the present study, we investigated the effects of 17β-estradiol and DHEAS on NTPDase activity in intact trophozoites of *T. vaginalis*. Parasite integrity and viability were assessed before and after the reactions by the mobility of trophozoites and trypan blue dye exclusion. The integrity of trophozoites was not affected by any of the conditions used in the assays. Furthermore, in order to investigate the effects of 17β-estradiol and DHEAS on *T. vaginalis* growth and morphology, kinetic growth experiments with parasites in presence of different concentrations of both hormones were performed. Fig. 1 shows that the *in vitro* growth of two isolates, TVVP60 and 30236 ATCC, was not affected by these hormones. In addition, no morphology alterations were observed in the trophozoites treated with the hormones when compared to controls.

In order to evaluate a direct effect on ecto-nucleotidase activities, we tested *in vitro* several 17β-estradiol or DHEAS concentrations varying from 0.01 to 5.0 μM on ATP and ADP hydrolysis in VP60 and 30236 ATCC isolates. Fig. 2 shows the NTPDase activities assayed in the presence of 17β-estradiol (Fig. 2A) and DHEAS (Fig. 2B) for the VP60 isolate. ATP hydrolysis was increased by 15%, 38%, and 9% in the presence of 0.01, 0.1, and 1 μM 17β-estradiol, respectively ($P < 0.05$). On the other hand, ADP hydrolysis was not significantly changed in the presence of 17β-estradiol (Fig. 2A). DHEAS did not significantly alter the ATP and ADP hydrolysis at the concentrations tested, when compared to the control (no hormone added) for the VP60 isolate (Fig. 2B). Likewise, the enzyme activities were tested in the presence of the steroid hormones in the 30236 isolate, as shown in Fig. 2C and D. 17β-Estradiol did not significantly change the ATP and ADP hydrolysis (Fig. 2C) and DHEAS did not alter the ATP hydrolysis (Fig. 2D) in the 30236 isolate. In contrast, ADP hydrolysis of the this isolate was affected by DHEAS 1.0, 2.5, and 5.0 μM, revealing an inhibition of 40%, 20%, and 20%, respectively ($P < 0.05$) (Fig. 2D).

The treatment of VP60 isolate in the presence of 17β-estradiol for 2 h did not induce significant differences in ATP and ADP hydrolysis (Fig. 3A). VP60 isolate in the presence of DHEAS for 2 h promoted a significant inhibition of 40%, 41%, and 25% ($P < 0.05$) in ATP hydrolysis at 0.01, 0.1, and 1.0 μM, respectively, whereas a 38% ($P < 0.05$) decrease in ADP hydrolysis was observed at 0.01 and 0.1 μM. In a similar treatment, DHEAS did not significantly change ATP and ADP hydrolysis in the 30236 isolate (Fig. 3B). The treatment of 30236 isolate in the presence of 17β-estradiol for 2 h induced a significant decrease in ATP (55%, 56%, and 58%) and ADP (43%, 31%, and 31%) hydrolysis at 0.01, 0.1, and 1.0 μM, respectively ($P < 0.05$) (Fig. 3B).

Fig. 4A shows the effect of the treatment with DHEAS and 17β-estradiol for 12 h on nucleotide hydrolysis in the VP60 isolate. ATP hydrolysis, at 0.1 and 1.0 μM, was significantly inhibited in the presence of DHEAS (50% and 32%, respectively; $P < 0.05$) and a decrease in ADP hydrolysis was observed only at 0.1 μM DHEAS (37% $P < 0.05$). In contrast, 17β-estradiol increased ADP hydrolysis (16% $P < 0.05$) at 0.01 μM.

The treatment with DHEAS for 12 h induced a significant inhibition in ADP hydrolysis (42% and 38% $P < 0.05$) at 0.01 and 0.1 μM, respectively, in the 30236 isolate (Fig. 4B). The treatment with 17β-estradiol for 12 h promoted an increase in ATP hydrolysis (50% and 32% $P < 0.05$) at 0.1 and 1.0 μM, respectively. This treatment also increased ADP hydrolysis (74% $P < 0.05$) at 1.0 μM (Fig. 4B).

The effects promoted by the hormones 17β-estradiol or DHEAS could be a consequence of transcriptional control. From the eight-well characterized enzymes of the mammal NTPDase family, four members (NTPDase1–3 and 8) are tightly bound to the plasma membrane with the active site facing the extracellular milieu. A phylogenetic analysis was performed in order to verify possible orthologous genes in *T. vaginalis* genome. Protein sequences of *Homo sapiens* and *Mus musculus* NTPDase members were retrieved from GenBank and used as a query. NCBI Blast searches of GenBank and Blast Search in UniProtKB yielded two complete *T .vaginalis* NTPDase sequences from which XM_001298945 corresponds to NTPDaseA and XM_001579653 corresponds to NTPDaseB. The NTPDase A (434 amino acids) and B (441 amino acids) sequences share 61% of identity and 76% of similarity. Both sequences present the five apyrase conserved regions (ACR1–5), which are essential for enzyme activity. Differences in ACR1–4 sequences were found when a comparison was performed (Fig. 5). No other related sequences were identified in the *T. vaginalis* genome with the strategy adopted. Table 2 shows the percentage of amino acid identity and similarity between the parasite proteins.

The NTPDase sequences from mammals and orthologues sequences from parasites were aligned and a phylogenetic tree was constructed according Neighbor-Joining method using proportional (p) distance with MEGA 4.0 program (Fig. 6). The phylogeny constructed resulted in 13 well-resolved terminal clades supported by high bootstrap values. *Mus musculus* (Mm) and *Homo sapiens* (Hs) sequences were grouped consistently in eight clades corresponding to NTPDase1–8 proteins respectively. The ninth clade was formed by *T .vaginalis* sequences. The 10th clade grouped *T. cruzi*, *T. brucei* and *L. major* sequences whereas the 11th clade was formed by *S. mansoni* and *S. japonicum*. The 12th clade grouped consistently the *T. gondii* sequences. The four *E. histolytica* sequences were the most divergent and formed the 13th clade. *P. falciparum* sequence was placed independently between the mentioned 13 clades. The tree topology strongly suggests homologous functions on *T vaginalis* genome. The sequences obtained were used to construct specific primers (Table 1).

The semiquantitative RT-PCR analyses were performed when kinetic alterations had occurred. The treatment in the presence of

Table 2
Relatedness between *T. vaginalis* (Tv) NTPDase proteins and their *E. histolytica* (Eh), *L. major* (Lm), *P. falciparum* (Pf), *S. japonicum* (Sj), *S. mansoni* (Sm), *T. brucei* (Tb), *T. gondii* (Tg), and *T. cruzi* (Tc) orthologues.

	Tv NTPDaseA		Tv NTPDaseB	
	Identity	Similarity	Identity	Similarity
Tv NTPDaseA	–	–	56.8	73.5
Tv NTPDaseB	56.8	73.5	–	–
Eh (XP_648646)	14.2	31.1	16.6	32.2
Eh (XP_652062)	17.0	33.2	15.7	31.5
Eh (XP_654244)	16.0	32.5	18.9	34.9
Eh (XP_656157)	16.6	33.9	17.1	33.1
Lm (DAA04950)	19.4	41.2	19.1	39.5
Pf (AAN36910)	15.2	24.7	16.1	25.7
Sj (AAW26231)	16.9	30.8	19.2	32.2
Sm (XP_002579239)	17.2	32.1	19.4	33.7
Tb (AAZ13145)	14.5	30.3	16.9	32.0
Tg (AAC80188)	15.8	30.3	19.4	33.3
Tg (AAC80187)	16.0	30.4	20.0	33.8
Tc (AAS75599)	15.3	29.6	17.1	31.1

Note: The percentage of amino acid identity and similarity between the proteins was determined by scoring matrix BLOSUM62 using MatGAT 2.01 software.

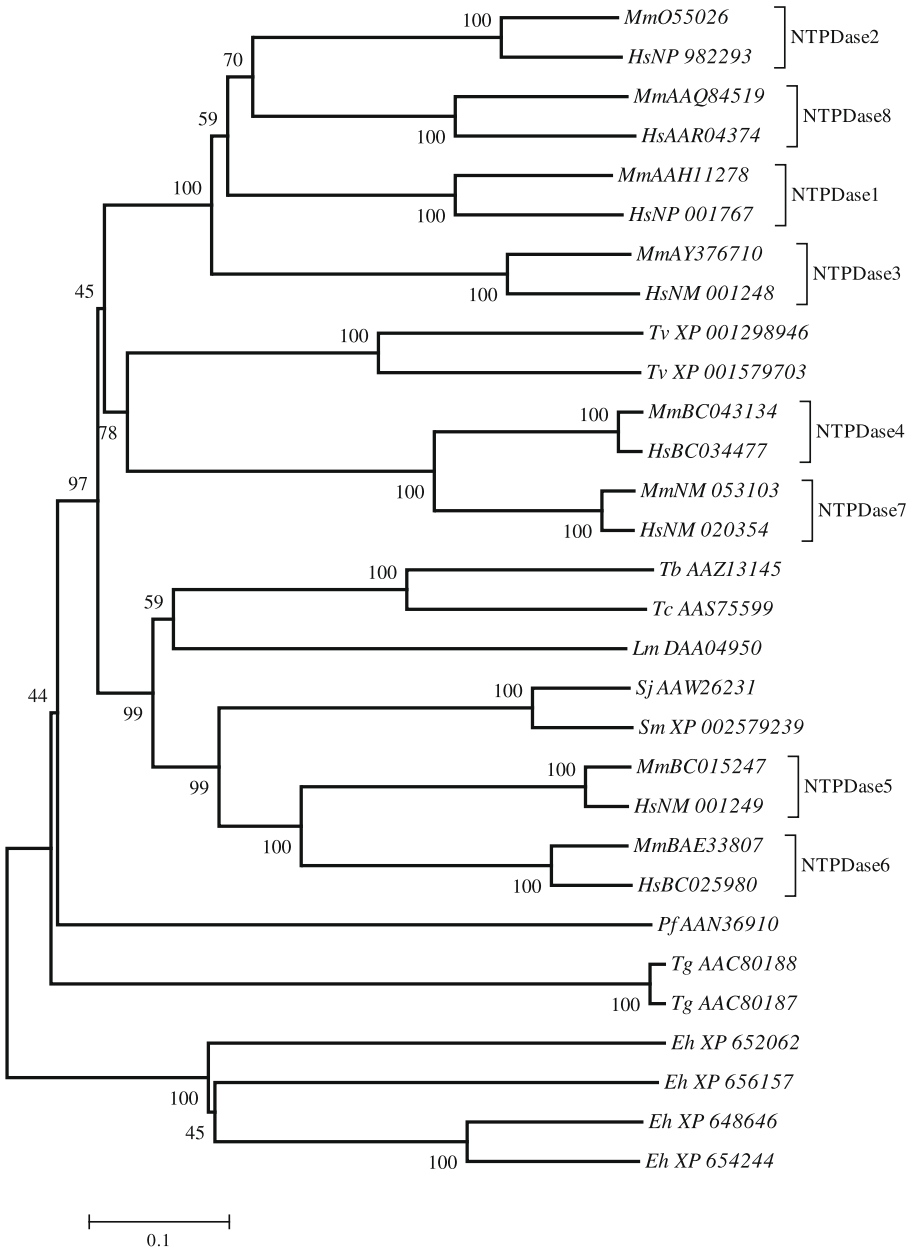

Fig. 6. Phylogenetic analysis of NTPDase-related family members, demonstrating the existence of two distinct members in *T. vaginalis*: NTPDaseA (XP_001298946) and NTPDaseB (XP_001579703). The deduced amino acid sequences were aligned with the ClustalX program and the phylogenetic tree was constructed using the Neighbor-Joining method, using proportional (*p*) distance by the MEGA 2.1 program. The phylogenetic tree grouped consistently (Mm) *Mus musculus* (Hs) *Homo sapiens* and *T. vaginalis* NTPDase sequences.

17β-estradiol or DHEAS for 2 h did not alter the transcript levels in both isolates and for both enzymes, NTPDaseA and NTPDaseB (data not shown). The treatment of the VP60 isolate with 0.1 μM DHEAS for 12 h promoted a decrease (42%) in NTPDaseA transcript levels (Fig. 7A and C) whereas this treatment did not alter the mRNA levels for both enzymes in the 30236 isolate (Fig. 7A and C). The treatment with 17β-estradiol for 12 h did not alter the mRNA levels for NTPDase A and NTPDase B for both isolates (data not shown).

4. Discussion

These findings demonstrated the influence of 17β-estradiol and DHEAS on NTPDase activity and gene expression patterns in intact trophozoites of *T. vaginalis*. Treatments for 2 or 12 h with DHEAS and 17β-estradiol were able to modulate nucleotide hydrolysis. Two NTPDase sequences were identified from phylogenetic analyses and named NTPDaseA and NTPDaseB. However, changes in gene expression pattern were only observed for NTPDaseA of the VP60 isolate after DHEAS treatment during 12 h.

Pochmann et al. (2004) demonstrated that estradiol replacement therapy causes a significant decrease in ATP, ADP, and AMP hydrolysis. Rats submitted to ovariectomy plus estradiol replacement showed a significant decrease in enzyme activities, suggesting a relationship between the hormonal system and the enzymes that hydrolyze adenine nucleotides in rat blood serum.

Direct effects of hormones on growth and viability have been shown in several parasite organisms. Sexual hormones exert diverse actions in a variety of parasites. The adrenal hormone, DHEA, has been shown to mediate only inhibitory actions in parasites *S. Mansoni* (Morales-Montor et al., 2001) and *Plasmodium* sp. (Ayi

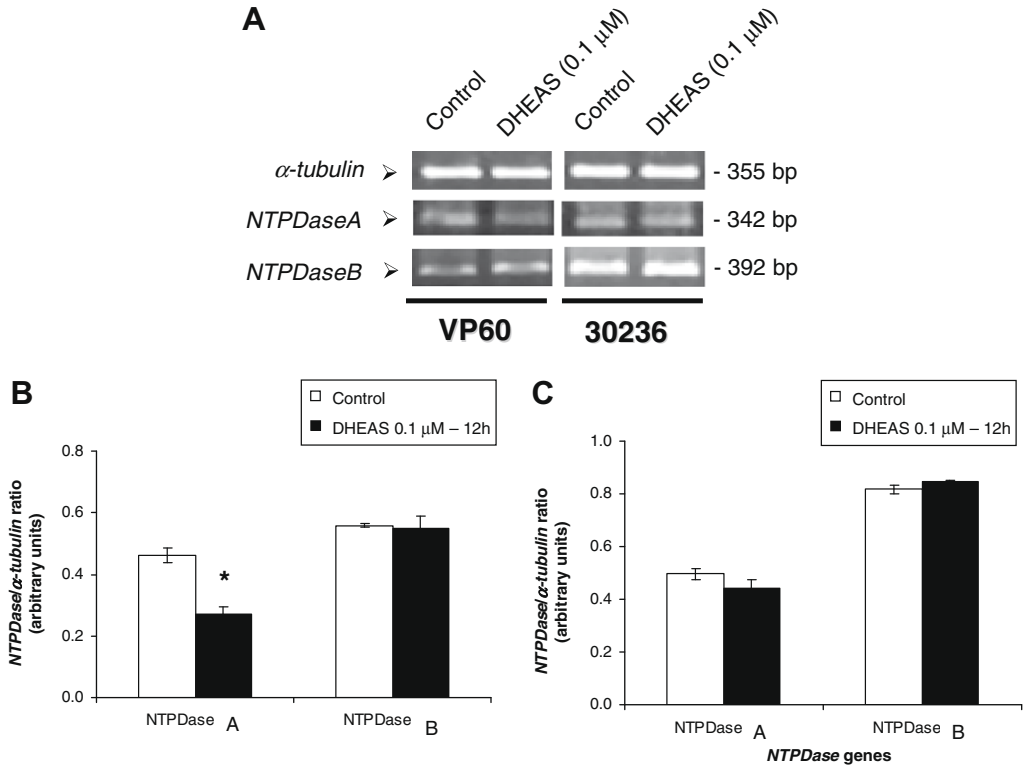

Fig. 7. Gene expression patterns (A) of NTPDase members after 12 h parasite treatment with DHEAS in VP60 (B) and 30236 isolates (C). The α-tubulin gene was carried out as an internal standard and the optical densitometry analysis of the PCR products was made using Image J 1.37 for Windows. Three independent experiments using different isolate suspensions were performed, with entirely consistent results.

et al., 2002). Moreover, *in vivo* evidence of the anti-malarial effect of DHEA came from the inverse correlation, found in young women from Kenya, between the levels of blood-circulating DHEA and parasitemia (Leenstra et al., 2003). DHEA has an important effect on human malaria and plasma levels of DHEA have been associated with lower parasitemia and protection in hyperendemic areas (Leenstra et al., 2003; Kurtis et al., 2001). The mechanisms through which DHEA improves the immune response against this parasite are not well defined, but some reports have demonstrated a role for androgens in the regulation and modulation of the activity of certain immune cell types, such as T cells, natural killer, and B cells (Olsen and Kovacs, 2005; Bouyou-Akotet et al., 2004).

T. vaginalis presents specific androgen and estrogen receptors, which suggests that steroid hormones could directly affect the parasite (Ford et al., 1987). Animal models of *T. vaginalis* vaginitis require estrogenization to establish infection (Cappuccinelli et al., 1974; Maestrone and Semar, 1967). Trichomonosis, as well as symptomatic genital gonococcal disease, are exacerbated during menstruation (Penza, 1973) and characterized by additional estrogenic activity. The menstrual flow provides nutrients to the parasite, as well as a supply of iron, which is a key factor in gene regulation of *T. vaginalis.* Iron upregulates various adhesins, immunogens, and proteinases that are essential for the ability of the parasite to deal with stress in the ever-changing vaginal environment (Petrin et al., 1998).

In addition, *T. vaginalis* is unable to de novo synthesize purines and pyrimidines and its growth and survival are dependent on the salvage pathway of these compounds to generate nucleotides (Heyworth et al., 1982, 1984). Moreover, studies have demonstrated that extracellular ATP can act in cytotoxic mechanisms (Ralevic and Burnstock, 1998; Ding et al., 2000). The concentration of purine nucleotides found in the vagina is around 10 mM (Munagala and Wang, 2003). The activation of NTPDase activity after treatment with 17β-estradiol was probably due to an attempt by the parasite to modulate the nucleotide concentration in the extracellular space. The presence of enzymes that participate in the hydrolysis cascade of ATP is essential for the growth of the parasite in a hostile ambient, under constant alterations, through the modulation of extracellular nucleotide concentrations.

Previous studies have shown that extracellular ATP, ADP, and AMP hydrolysis in *T. vaginalis* is higher in fresh clinical isolates and indeed higher enzyme activities were seen in fresh compared to representative long-term-grown isolates (Tasca et al., 2005). Considering ATP and ADP hydrolysis, both isolates tested were responsive to hormones, with some variation in concentration, hormone type, and time of treatment. The differences promoted by DHEAS and 17β-estradiol in VP60 and 30236 ATCC isolates are probably due to the heterogeneity of these isolates, which results in different kinetic profiles and sensitivities to hormones to NTPDase activity. The adaptability of *T. vaginalis* to challenging environmental pressures may be explained by the fact that trichomonads possess signal transduction pathways that link changes in the environment with appropriate changes in transcriptional and post-transcriptional regulatory mechanisms (Lehker and Alderete, 2000). Transcriptional control could be responsible, at least in part, for the alterations promoted by the hormones in NTPDase activity. In order to verify whether the NTPDase gene could be modulated when intact trophozoites of *T. vaginalis* were exposed to DHEAS or 17β-estradiol after 2 h and 12 h, we performed semiquantitative RT-PCR experiments. Interestingly, results demonstrated that NTPDaseA mRNA levels were significantly decreased in the fresh clinical isolate after DHEAS exposure, suggesting that the inhibition of NTPDase activity, observed in this treatment, may be directly related to a lower NTPDaseA expression level. A decrease in the NTPDase activity induced by this hormone can impair the growth and survival of *T. vaginalis* due to a possible reduction in adenosine levels, which are essential for parasite metabolism. Furthermore, a lower NTPDase activity might result in higher

nucleotide levels, which present cytolytic efficiency (Filippini et al., 1990). Only VP60 isolate presented the reduction on expression probably because it is a fresh clinical isolate and more responsive to challenging environmental pressures through appropriate changes in transcriptional and post-transcriptional regulatory mechanisms.

As the vaginal microenvironment is a mixture of hormones with constantly changing concentrations, our results suggest that the modulation of extracellular ATP and ADP levels during exposure to steroid hormones may be associated with their influence on *T. vaginalis* colonization.

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