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EFEITO DE HORMÔNIOS ESTEROIDAIS SOBRE A HIDRÓLISE DE NUCLEOTÍDEOS EXTRACELULARES EM TROFOZOÍTOS INTACTOS DE *Trichomonas vaginalis*

Porto Alegre, abril de 2009

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Dissertação apresentada como requisito para obtenção do grau de Mestre pelo Programa de Pós-Graduação em Biologia Celular e Molecular da Faculdade de Biociências da Pontifícia Universidade Católica do Rio Grande do Sul.

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RESUMO

O *Trichomonas vaginalis* é um protozoário flagelado causador da tricomonose, a doença sexualmente transmissível (DST), não-viral mais comum. Esteróides podem influenciar a patogênese do *Trichomonas vaginalis*. Estes hormônios podem influenciar o sistema imune e também a suscetibilidade para doenças causadas por parasitas. Estudos têm descrito que a modulação dos níveis de nucleotídeos pode ser essencial para a sobrevivência do parasito. A colonização por *T. Vaginalis* pode ser influenciada pela concentração de estrogênios na vagina. O papel dos estrogênios na patogênese do *T. vaginalis* é controverso, e estudos contraditórios são encontrados na literatura. Considerando que o *T. vaginalis* não realiza síntese *de novo* de purinas e pirimidinas, é importante avaliar as atividades enzimáticas envolvidas na produção de adenosina na presença de hormônios esteroidais. Nós investigamos o efeito do sulfato de deidroepiandrostenediona (S-DHEA) e do 17β-estradiol nas atividades da NTPDase e da ecto-5´-nucleotidase em um isolado clínico fresco (VP60) e em um isolado cultivado por longos períodos em laboratório (30236 ATCC), seguido pela análise transcricional dos genes. Os resultados demonstrram a influência do 17β-estradiol e do S-DHEA nas atividades da NTPDase e da ecto-5´-nucleotidase e padrões de expressão das NTPDases em trofozoítos intactos de *T. vaginalis*. A atividade da NTPDase no isolado 30236 foi inibida no ensaio *in vitro* e no tratamento na presença do hormônio S-DHEA por 12 h. No isolado VP60, a atividade da NTPDase foi inibida no tratamento com este hormônio por 2 e 12 h. A presença do hormônio esteróide S-DHEA por 12 h inibiu a expressão dos níveis de mRNA da NTPDaseA no isolado VP60. Em contraste, o 17β-estradiol ativou a atividade da NTPDase no isolado VP60 no ensaio *in vitro* e no tratamento por 12 h. A atividade da NTPDase no isolado 30236 foi ativada na presença do 17β-estradiol quando tratado por 12 h. Por outro lado, o tratamento com o hormônio 17β-estradiol por 2 h inibiu a atividade da NTPDase no isolado 30236. O tratamento na presença do S-DHEA por 2 h inibiu a hidrólise do AMP no isolado VP60 e no isolado 30236. O 17β-estradiol, no tratamento de 2 h, diminuiu a hidrólise do AMP no isolado 30236, mas não alterou a hidrólise do AMP no isolado VP60. O tratamento por 12 h na presença do hormônio S-DHEA inibiu a hidrólise do AMP. Considerando que a vagina é um ambiente constantemente afetado pelas mudanças hormonais e que os efeitos dos hormônios esteroidais são influenciados por receptores presentes no *T. vaginalis*, nossos resultados sugerem que a modulação dos níveis extracelulares de ATP, ADP e AMP durante a exposição aos hormônios esteroidais pode estar relacionada com a colonização do *T. vaginalis*.

Palavras-chave: sulfato de deidroepiandrosterona; 17β-estradiol; *Trichomonas vaginalis*; nucleosídeo trifosfato difosfoidrolase; nucleotidases; ecto-5´-nucleotidase.

ABSTRACT

Trichomonas vaginalis is a flagellate protozoan that causes trichomonosis, the most common, non-viral sexually transmitted disease (STD). Steroids may influence the pathogenesis of *Trichomonas vaginalis.* These hormones can influence the immune system and thus the susceptibility for diseases caused by protozoan parasites. Studies have described that the modulation of nucleotide levels seems to be essential for the survival of the parasite. *Trichomonas vaginalis* colonization may be influenced by the vaginal concentrations of estrogens. The role of estrogens in the pathogenesis of *T. vaginalis* has been controversial, and seemingly contradictory reports are found in the literature. Considering that *T. vaginalis* have not the ability to perform purine and pirimidine synthesis *de novo*, it becomes important to evaluate the enzyme activities involved in adenosine production in the presence of steroid hormones. We investigated the effect of dehydroepiandrosterone sulfate (DHEAS) and 17β-estradiol on NTPDase and ecto-5′-nucleotidase activities in fresh clinical (VP60) and long-term-grown isolates of *T. vaginalis* (30236 ATCC), followed by the NTPDase genes transcriptional analysis. The results presented demonstrate the influence of 17β-estradiol and DHEAS on nucleotidase activities and expression patterns in intact trophozoites of *T. vaginalis*. DHEAS significantly inhibited NTPDase activity. The NTPDase activity on 30236 isolate was inhibited i*n vitro* and in the treatment with DHEAS for 12 hours. NTPDase activity was inhibited in the treatment with the hormone for 2 and 12 hours in VP60 isolate. The presence of the steroid hormone DHEAS during 12 hours also inhibited the transcript levels of NTPDaseA in VP60 isolate. In contrast, 17β-estradiol activated the NTPDase activity *in vitro* and in the treatment of the hormone during 12 hours in the VP60 isolate. The NTPDase activity on 30236 isolate was also increased by the 17β-estradiol when treated by 12 hours. However,, the treatment with 17β-estradiol by 2 hours inhibited the NTPDase activity on 30236 isolate. Considering the ecto-5′-nucleotidase activity, the results demonstrated that the treatment with DHEAS by 2 h significantly inhibited the AMP hydrolysis in VP60 and 30236 isolates. 17β-estradiol by 2 h inhibited the AMP hydrolysis in the 30236 isolate whereas did not alter the AMP hydrolysis in the VP60 isolate. The treatment with DHEAS by 12 h inhibited AMP hydrolysis. Considering that the vaginal microenvironment is a mixture of hormones with constantly changing concentrations and the effect of steroid hormones on *T. vaginalis* is complex and is influenced by the presence of hormones receptors on *T. vaginalis*, our results suggest that the modulation of extracellular ATP, ADP, and AMP levels during exposure to steroid hormones may be related with their influence in *T. vaginalis* colonization.

Keywords: dehydroepiandrosterone sulfate (DHEAS); 17-beta estradiol; *Trichomonas vaginalis*, nucleoside triphosphate diphosphohydrolase; nucleotidases; ecto-5′-nucleotidase.

LISTA DE ABREVIATURAS

ACRs – regiões conservadas de apirase

ADP – adenosina 5'- monofosfato

AIDS – acquired immunedeficiency syndrome

AMP – adenosina 5'- monofosfato

ATCC – American Type Culture Collection

ATP – adenosina 5'- trifosfato

Ca^{2+} – íon cálcio

CDF – cell detaching factor

DHEA – deidroepiandrosterona

DST – doença sexualmente transmissível

E-NPP – ectonucleosídeo pirofosfatase/ fosfodiesterase

E-NTPDase – ectonucleosídeo trifosfato difosfoidrolase

FSH – hormônio folículo estimulante

GABA – ácido gama - aminobutírico

GMP – guanosina 5´-monofosfato

GPI – glicosilfosfatidilinositol

K^+ – íon potássio

K_M – constante de Michaelis

HIV – human immunedeficiency virus

Na^+ – íon sódio

NTPDase – nucleosídeo trifosfato difosfoidrolase

S-DHEA – sulfato de deidroepiandrostenediona

UDP – uridina 5´-difosfato

UMP – uridina 5´-monofosfato

UTP – uridina 5´-trifosfato

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1. CAPÍTULO 1 - INRODUÇÃO E OBJETIVOS

1.1 INTRODUÇÃO

1.1.1 *Trichomonas vaginalis*

O *Trichomonas vaginalis* apresenta um corpo celular muito plástico, adotando forma elipsóide, piriforme ou oval em preparações a fresco ou fixadas e coradas. Condições físico-químicas como pH, temperatura, tensão de oxigênio e força iônica afetam o aspecto dos organismos, que não possuem a forma cística, somente a trofozoítica. O *T. vaginalis* possui quatro flagelos anteriores, desiguais em tamanho, e uma membrana ondulante, que se adere ao corpo pela costa. O axóstilo é uma estrutura rígida e hialina, formada por microtúbulos, que se projeta através do centro do organismo, prolongando-se até a extremidade posterior. O núcleo é elipsóide, localizado próximo à extremidade anterior (HONIGBERG & BRUGEROLLE, 1990).

O *T. vaginalis* é um organismo eucariótico que difere da maioria das células eucarióticas em diversos aspectos significativos, principalmente nas exigências nutricionais e no metabolismo energético. O parasito depende de um grande número de metabólitos pré-formados como nutrientes, revelando a ausência das principais vias biossintéticas (MÜLLER, 1990).

O protozoário é anaeróbio facultativo e pode ser cultivado *in vitro* na ausência de oxigênio, na faixa de pH compreendida entre 5,0 e 7,5 e em temperaturas entre 20 e 40°C (DE CARLI, 2000). O flagelado utiliza carboidratos como a glicose, a maltose e a galactose como principais fontes de energia (READ, 1957), mas o glicogênio e o amido podem manter o crescimento. Sacarose e manose não são utilizadas como fontes de energia

(MÜLLER, 1990). O *T. vaginalis* é capaz de manter o glicogênio em reserva, o qual representa até 20% do peso seco do organismo (MICHAELS & TREICK, 1962). O protozoário também depende do fornecimento de aminoácidos ou proteínas digeridas, embora não exista informação disponível sobre a natureza essencial ou não essencial dos aminoácidos (MÜLLER, 1990). O *T. vaginalis* não apresenta a capacidade de realizar síntese *de novo* de purinas e pirimidinas, sendo dependente das vias de salvação para a obtenção desses compostos (HEYWORTH et al., 1982; 1984).

A principal característica destes protozoários e que os torna alvo de estudos bioquímicos e fisiológicos é a ausência de mitocôndrias. O *T. vaginalis* apresenta hidrogenossomos, os quais são grânulos densos distribuídos por todo o citoplasma e especialmente concentrados próximos ao axóstilo e à costa (MÜLLER, 1990; 1993; DE SOUZA, 2001). Os hidrogenossomos são portadores de uma piruvato: ferredoxina oxidorredutase capaz de transformar o piruvato em acetato pela oxidação fermentativa e de liberar ATP e hidrogênio molecular (KULDA, 1999). Além do papel exercido no metabolismo energético dos tricomonas, os hidrogenossomos são as organelas responsáveis pela ativação dos 5-nitroimidazóis, fármacos usados no tratamento da tricomonose, dentre os quais o mais utilizado é o metronidazol (KULDA, 1999).

1.1.2 Tricomonose

Trichomonas vaginalis é o protozoário flagelado causador da tricomonose (KASSAI et al., 1988), a doença sexualmente transmissível (DST) não viral mais comum no mundo. Apesar de o parasito ter sido descrito por Donné em 1836 (HONIGBERG, 1990) e do cultivo *in vitro* ter sido iniciado em 1920 (LINSTEAD, 1990), muitos aspectos biológicos do protozoário ainda são desconhecidos, o que revela a complexidade dos mecanismos

utilizados pelo patógeno para a sobrevivência. O *T. vaginalis* utiliza diferentes mecanismos patogênicos para estabelecer o parasitismo com sucesso: a) o estabelecimento do parasito na vagina, um ambiente constantemente modificado pelas variações de pH, hormônios, menstruação e fornecimento de nutrientes; b) a citoaderência às células vaginais epiteliais através de adesinas, proteínas multifuncionais que apresentam também funções enzimáticas metabólicas; c) o escape das respostas imunes do hospedeiro; d) a alternância da expressão dos genes de virulência em resposta a fatores ambientais como ferro e cálcio (ALDERETE et al., 2002).

As manifestações clínicas da tricomonose variam desde um estado assintomático até um estado de severa inflamação (vaginite) (PETRIN et al., 1998). Setenta por cento das mulheres com vaginite aguda causada por *T. vaginalis* freqüentemente têm corrimento devido à infiltração por leucócitos. O sintoma clássico de corrimento amarelo, abundante, espumoso e mucopurulento, no entanto, ocorre em somente 10-20% das mulheres (LEHKER & ALDERETE, 2000). Há também odor vaginal anormal e prurido vulvar. A vagina e a cérvice podem ser edematosas e eritematosas, com erosão e pontos hemorrágicos na parede cervical, conhecida como *colpitis macularis* ou cérvice com aspecto de morango. Embora essa aparência seja altamente específica para tricomonose, é vista somente em poucas mulheres (2-5%). Dor abdominal tem sido relatada entre muitas mulheres com tricomonose e pode ser indicativa de infecção do trato urogenital superior (REIN, 1990; PETRIN et al., 1998; LEHKER & ALDERETE, 2000). Diferente das mulheres, homens infectados podem ter somente uma infecção auto-limitada (KRIEGER et al. 1993; LEHKER & ALDERETE, 2000). A tricomonose em homens pode ser assintomática; aguda, caracterizado por uretrite purulenta abundante; doença sintomática leve, clinicamente indistingüível de outras causas de uretrite (PETRIN et al., 1998). No estado

sintomático, há corrimento escasso, disúria, prurido, ulceração peniana e sensação de queimação imediatamente após a relação sexual. As complicações são raras, mas podem incluir epididimite, infertilidade e prostatite (HOLMES et al., 1975; KRIEGER, 1984; 1990).

O *T. vaginalis* tem se destacado como um importante patógeno e está associado a sérias complicações de saúde. Estudos recentes mostraram que a tricomonose aumenta a susceptibilidade ao vírus da imunodeficiência humana (HIV, do inglês *human immunedeficiency virus*) e a síndrome da imunodeficiência adquirida (AIDS, do inglês *acquired immunedeficiency syndrome*) (LAGA et al., 1993; SORVILLO & KERNDT, 1998; SORVILLO et al., 2001) a tricomonose causa baixo peso de recém-nascidos, bem como nascimento prematuro (COTCH et al., 1991; 1997), predispõe mulheres à doença inflamatória pélvica atípica, câncer cervical e infertilidade (GRODSTEIN et al., 1993; ZHANG & BEGG 1994; VIIKKI et al., 2000). Entre outras seqüelas da tricomonose estão incluídas infecções do trato urinário e doença pulmonar crônica em recém-nascidos (HOFFMAN et al., 2003). Além disso, foi descrito um caso de co-infecção por *T. vaginalis* e *Pneumocystis* sp. em um paciente aidético (DUBOUGHER et al., 2003). Homens infectados com HIV e *T. vaginalis* apresentam altos números de partículas virais no sêmen, facilitando a transmissão do vírus (HOBBS et al., 1999). A tricomonose expande a porta de saída em pacientes infectados com HIV e a porta de entrada para pacientes não infectados pelo vírus (SORVILLO et al., 2001). Estima-se que 24% das infecções pelo HIV são diretamente atribuídas a tricomonose. Em estudo recente, Chesson et al. (2004) mostraram que a cada ano nos Estados Unidos, um número estimado de 746 novos casos de infecção pelo HIV entre mulheres pode ser atribuído aos efeitos facilitatórios da tricomonose na transmissão do vírus.

A interação *T. vaginalis*-hospedeiro é um processo complexo, no qual estão envolvidos componentes associados à superfície celular do parasito e às células epiteliais do hospedeiro e também componentes solúveis encontrados nas secreções vaginal e uretral. Esse patógeno atinge o parasitismo com sucesso através da aquisição de nutrientes por meio de receptores específicos: a vagina é um dos mais complexos sítios de infecção para um patógeno de mucosa como o *T. vaginalis*, por estar constantemente sob a influência do ciclo menstrual. A ausência de enzimas para a síntese ou conversão de lipídeos (BEACH et al., 1990) é compensada pela ligação mediada por receptores e captação de lipoproteínas encontradas no soro ou em secreções vaginais (PETERSON & ALDERETE, 1984) e por hemaglutinação específica (LEHKER et al., 1990), seguida de hemólise mediada por proteases (KRIEGER et al., 1983; DAILEY & ALDERETE, 1990). Além disso, o *T. vaginalis* requer grandes quantidades de ferro para crescimento e multiplicação (GORREL, 1985) e para a regulação dos genes de virulência (LEHKER et al., 1991; LEHKER & ALDERETE, 1992). Além da hemólise e da citotoxicidade estimularem o crescimento em ambientes ferro-restritivos, o parasito usa a lactoferrina, através de ligação a receptores específicos do patógeno (LEHKER & ALDERETE, 1992) e o grupamento heme da hemoglobina como fontes de ferro (ALDERETE et al. 2004).

Um dos mecanismos que também contribui para o parasitismo é a citoaderência mediada pelas adesinas, proteínas multifuncionais localizadas na superfície do *T. vaginalis* (AP23, AP33, AP51 e AP65) (ALDERETE & GARZA, 1988). Estas proteínas parecem interagir com as células do hospedeiro via interações do tipo receptor-ligante e são codificadas por famílias multi-gene (ENGBRING et al., 1996; ALDERETE et al., 1995; 1998; ENGBRING & ALDERETE, 1998). É importante salientar que a análise das seqüências de nucleotídeos e de aminoácidos revelou semelhança das adesinas com

enzimas metabólicas localizadas no hidrogenossomo (ENGBRING et al., 1996): a AP65 apresenta semelhança com a enzima málica e a AP51 e a AP33 com as subunidades α e β da succinil coenzima A sintetase (SCS), respectivamente (O'BRIEN et al., 1996; ENGBRING & ALDERETE, 1998; ALDERETE et al., 2001). A síntese dessas adesinas é regulada positivamente pela ligação das adesinas a células epiteliais e ao ferro (GARCIA et al., 2003). Imediatamente após o contato entre *T. vaginalis* e células vaginais epiteliais, os parasitos sofrem uma transformação morfológica dramática concomitante com a rápida síntese de adesinas e início da citoaderência (ARROYO et al., 1993).

Além disso, o escape da resposta imune do hospedeiro pode ocorrer devido ao revestimento da superfície dos parasitos com proteínas e outras macromoléculas do hospedeiro, evitando assim, o reconhecimento pelo sistema imune (PETERSON & ALDERETE, 1982). Também tem sido descrita a secreção de cisteína-proteinases que degradam imunoglobulinas e a porção C3 do complemento (PROVENZANO & ALDERETE, 1995). As proteinases dos tricomonas são capazes de degradar o inibidor de protease leucocitária secretória que é um fator de proteção das mucosas contra patógenos e que desempenham um papel importante na prevenção da transmissão do HIV (DRAPER et al., 1998). Outro aspecto importante é o fato de que o peróxido de hidrogênio neutraliza as cisteína-proteinases, mostrando o efeito protetor da flora normal formada por lactobacilos. Entretanto, os lactobacilos são removidos imediatamente na infecção por *T. vaginalis* através de fagocitose, assim o parasito consegue neutralizar mais uma resposta imune do hospedeiro (RENDÓN-MALDONADO et al., 1998).

A variação fenotípica promovida pela alternância da expressão de antígenos na superfície como a P270 (ALDERETE et al., 1986; ALDERETE, 1987; WANG et al.,

1987), bem como da expressão coordenada de genes de virulência em resposta a fatores ambientais, tais como concentrações de ferro e cálcio (CROUCH et al., 2001; ALDERETE et al., 2002; GARCIA et al., 2003) são mecanismos que podem estar relacionados com a interação *T.vaginalis* - hospedeiro.

Considerando o sério impacto desta DST na saúde pública, é importante estudar os aspectos bioquímicos deste parasito que pode contribuir na infecção do hospedeiro, bem como na sua patogênese.

1.1.3 Hormônios esteroidais

O colesterol é o precursor das cinco classes dos hormônios esteroidais: glicocorticóides, mineralocorticóides, andrógenos, estrógenos e progestinas. Estes hormônios são produzidos em tecidos específicos e sua secreção é desencadeada por outros hormônios.

Os hormônios esteroidais são transportados pelo sangue do seu sítio de síntese até os órgãos alvo, e devido a sua hidrofobicidade, atravessam a membrana e se ligam em receptores específicos no citoplasma ou no núcleo. (SIMPSON, 2003).

1.1.3.1 17β-Estradiol

O 17β-Estradiol é um importante hormônio do sistema nervoso central, o qual é produzido pelos ovários (SIMPSON, 2003). O estradiol na fase folicular está relacionado com o risco de desenvolvimento do câncer de mama (ELIASSEN et al., 2006). Este hormônio também está associado à menopausa, sendo que seus níveis diminuem, enquanto os níveis do hormônio FSH aumentam (BURGER et al., 1999).

No sistema nervoso central, pode atuar na maturação e plasticidade neural e exerce atividades neuroprotetoras cruciais contra vários danos ao sistema nervoso central (MCEWEN, 2001; MCCULLOUGH & HURN, 2003; GARCIA-SEGURA et al., 2003; KRETZ et al., 2004).

Com relação ao sistema cardiovascular, no período que precede a menopausa, as mulheres apresentam menor incidência de doença coronária comparando com homens da mesma idade. Com a terapia de reposição hormonal de estrogênios, há uma redução na mortalidade provocada por doenças coronárias em mulheres no período da menopausa (ETTINGER et al., 1996). O estrogênio se mostra um protetor do sistema cardiovascular e apresenta propriedades antioxidantes (SHWAERY et al., 1997; HUANG et al., 1999).

1.1.3.2 Sulfato de deidroepiandrostenediona (S-DHEA)

O S-DHEA é o hormônio esteroidal mais abundante no sistema circulatório, e é sintetizado nas adrenais e no fígado a partir do hormônio diidroepiandrosterona (DHEA) pela enzima DHEA sulfotranferase (LUU-THE et al., 1996). Os níveis plasmáticos de S-DHEA no homem e na mulher adultos são 100-500 vezes maior que o nível de testosterona e 1000-10000 vezes maior que o de estradiol. O S-DHEA serve como precursor de andrógenos e estrógenos. Altos níveis de S-DHEA em animais e humanos produzem uma variedade de atividades biológicas, incluindo aumento da memória (JOHNSON et al., 2000), diminuição da ansiedade (REDDY & KULKARNI, 1997), aumento da excitabilidade neuronal (STEFFENSEN, 1995), e ainda diminuição dos níveis de depressão e aumento da cognição (WOLKOWITZ et al., 1997; 1999).

A diminuição em 70 a 95% da síntese de DHEA e S-DHEA pelas adrenais durante o envelhecimento resulta em uma dramática redução da formação de andrógenos e estrógenos

nos tecidos periféricos glandulares, que pode estar envolvida em patogêneses relacionadas à idade, tais como resistência à insulina (COLEMAN et al., 1982; SCHRIOCK et al.; 1988) e obesidade (NESTLER et al.; 1988, MCEWEN & KURZMAN 1991; TCHERNOF et al., 1995). Baixos níveis circulantes de DHEA e S-DHEA também foram associados com câncer de mama (ZUMOFF et al., 1981) e o DHEA exerce atividade anti-oncogênica em uma série de modelos animais (LI et al., 1993).

1.1.4 Ações dos hormônios esteroidais em *Trichomonas vaginalis*

A interação de hormônios esteroidais, tais como estrogênios, com a patogênese do *T. vaginalis* é controversa. Um quadro de exacerbação da tricomonose geralmente ocorre próximo ao período da menstruação (GARBER et al., 1991). Também existe um aumento na incidência de tricomonose durante a gravidez, sugerindo que um alto nível de estrogênio pode aumentar a infectividade e/ou os sintomas (BROWN, 1972). Estudos demonstraram que a adesão do parasito a células epiteliais aumenta significativamente na presença de α-estradiol e β-estradiol (SILVA-FILHO & BONILHA, 1992). Martinotti & Savoia (1985) determinaram que concentrações de β-estradiol aumentam o crescimento do *T. vaginalis*, enquanto que a progesterona inibe o crescimento. Um dos fatores de virulência do *T. vaginalis,* o fator de descamação celular (*cell detaching factor* – CDF), tem a atividade significativamente reduzida na presença de estradiol, o que sugere que sintomas durante a infecção podem ser regulados pela concentração vaginal de estrogênios (GARBER et al., 1991).

1.1.5 Nucleotídeos extracelulares

Os nucleotídeos podem ser encontrados nos espaços extracelulares e são principalmente liberados pelas células em situações fisiológicas de ativo metabolismo ou situações de estresse, anóxia ou lesão. Essas moléculas desempenham um papel regulatório importante, influenciando funções em organismos unicelulares e em órgãos complexos e sistemas (CHOW et al., 1997). Essas funções incluem contração do músculo liso, neurotransmissão, resposta imune, inflamação, agregação plaquetária, dor, liberação de catecolaminas, exercícios físicos em demasia ou choque (FERREIRA et al., 1995; RALEVIC & BURNSTOCK; 1998; SNEDDON et al., 1999; CUNHA & RIBEIRO; 2000; DING et al., 2000; RALEVIC & BURNSTOCK, 2003; BURNSTOCK & KNIGHT, 2004).

O ATP pode ser armazenado e co-liberado juntamente com diversos outros neurotransmissores, tais como: acetilcolina, glutamato, noradrenalina, serotonina e ácido γ-amino butírico (GABA) (BURNSTOCK, 2004). Atualmente, se propõe que o ATP é uma molécula sinalizadora ubíqua, a qual foi retida como um cotransmissor em quase todos os tipos celulares, desempenhando importantes papéis tanto em estados fisiológicos quanto patológicos (CHOW et al., 1997; BURNSTOCK, 2004; 2006).

Estudos demonstraram que, em concentrações na faixa de milimolar, o ATP inibe a agregação plaquetária via mecanismos competitivos e não competitivos, bem como baixas concentrações de ATP podem ser estimulatórias (SOSLAU & YOUNGPRAPAKORN, 1997). Em relação ao ADP, sabe-se que ele induz alterações na forma e agregação das plaquetas. Diferentes estudos têm demonstrado o importante papel destes nucleotídeos nos processos de homeostase e na formação de trombos (COADE & PEARSON, 1989; PIEBER et al. 1991). Já o nucleosídeo adenosina, produzido pela degradação dos

nucleotídeos púricos, é uma estrutura hábil para atuar como vasodilatador e cardioprotetor (FRASSETTO et al, 1993; SOSLAU & YOUNGPRAPAKORN, 1997).

Os nucleotídeos e nucleosídeos extracelulares podem agir como compostos sinalizadores por se ligarem a receptores específicos, conhecidos como purinorreceptores. Os receptores de adenosina, ou receptores P1, são divididos em 4 subtipos, A_1, A_{2A}, A_{2B} e A_3, todos ligados a proteínas G. Os receptores P2 reconhecem ATP, ADP, UTP e UDP e são classificados em: P2X, que são receptores de canais iônicos e apresentam permeabilidade rápida e seletiva para cátions (Na^+, K^+ e Ca^{2+}), e está dividida em sete membros ($P2X_{1-7}$), distribuídos em neurônios, células gliais e no músculo liso (BURNSTOCK, 2004); P2Y, receptores associados a proteínas G e foram funcionalmente descritos oito membros ($P2Y_1$, $P2Y_2$, $P2Y_4$, $P2Y_6$, $P2Y_{11}$, $P2Y_{12}$, $P2Y_{13}$ e $P2Y_{14}$) (RALEVIC & BURNSTOCK, 1998; BURNSTOCK, 2006; 2007).

1.1.6 Nucleotidases

A sinalização mediada pelos nucleotídeos extracelulares pode ser inativada por hidrólise através da ação de ectonucleotidases. Um número considerável de enzimas localizadas na superfície celular pode estar envolvido na via de hidrólise extracelular de nucleotídeos. Estas enzimas incluem a família E-NTPDase (ectonucleosídeo trifosfato difosfoidrolase), a família E-NPP (ectonucleotídeo pirofosfatase/fosfodiesterase), ecto-5'-nucleotidase e fosfatases alcalinas (ZIMMERMANN, 2001).

Em mamíferos, oito membros da família das E-NTPDase foram clonados e caracterizados funcionalmente. Existe similaridade do gene destas enzimas com espécies de invertebrados, plantas, leveduras e protozoários (HANDA & GUIDOTTI, 1996; VASCONCELOS et al., 1996; SMITH et al., 1997). Membros dessa família enzimática

podem diferir consideravelmente apesar da identidade das seqüências. Entretanto, cinco seqüências de domínios altamente conservados ("regiões conservadas da apirase", ACRs) são compartilhadas por todas as seqüências e tornaram-se um marcador das E-NTPDases (HANDA & GUIDOTTI, 1996). Provavelmente, as ACRs têm grande importância no sítio catalítico destas enzimas (ZIMMERMANN, 2001).

A família E-NTPDase é classificada como NTPDases 1 a 3 e 8, as quais estão localizadas na membrana da célula e as NTPDases 4 a 7, situadas intracelularmente. Os sítios catalíticos dessas enzimas estão voltados para o meio extracelular ou para o lúmen de organelas intracelulares, tais como o complexo de Golgi, o retículo endoplasmático e lisossomos. A atividade máxima requer a presença de cátions divalentes, tais como cálcio ou magnésio e pH alcalino. As massas moleculares das enzimas glicosiladas estão na ordem de 70-80 KDa (ZIMMERMANN, 2001; SHI et al., 2001; BIGONNESSE et al., 2004).

A NTPDase 1 (ecto-ATP difosfoidrolase, ecto-apirase, CD39) é capaz de hidrolisar os substratos ATP e ADP na mesma proporção, em contraste com a NTPDase 2 (ecto-ATPase, CD39L1) que hidrolisa 30 vezes mais rápido o ATP do que o ADP (ZIMMERMANN, 2000; ZIMMERMANN, 1996). A NTPDse 3 e a NTPDase 8 tem maior preferência pelo ATP do que o ADP numa razão de hidrólise de aproximadamente 3:1 e 2:1, respectivamente (ZIMMERMANN, 2001).

As NTPDases 1 a 8 compartilham similaridades e diferenças nas propriedades bioquímicas. As principais características que distinguem essas enzimas são a localização celular e a preferência por substratos, bem como a razão de hidrólise ATP:ADP. Independente da localização, as NTPDases estão envolvidas na degradação de nucleosídeos 5'-di e 5'-trifosfatados, e as diferenças na razão de hidrólise ATP:ADP determinam a razão

nucleotídeos/nucleosídeos nos microambientes onde as enzimas estão localizadas, modulando a ativação dos purinorreceptores.

Vários estudos têm relatado a presença de ectonucleotidases na superfície de parasitos. A nucleosídeo trifosfato hidrolase em *Toxoplasma gondii* foi uma das primeiras enzimas a ser descrita (ASAI et al., 1983). Três ATPases foram identificadas em *T. vaginalis* (TURNER & LUSHBAUGH, 1991) e, posteriormente, foi realizada a caracterização de uma família de genes de ATPase do tipo-P (SHAH et al., 2002). A atividade da NTPDase1 foi caracterizada em *T. vaginalis* (MATOS et al., 2001), sendo capaz de hidrolisar nucleosídeos da purina e da pirimidina 5'-di e 5'-trifosfatados em uma faixa de pH ótimo de 6,0 a 8,0. Esta atividade enzimática é cálcio-dependente e não é inibida pelos inibidores clássicos das ATPases, tais como ouabaína, *N*-etilmaleimida, ortovanadato e azida sódica (5 mM). Porém, uma inibição significativa da hidrólise do ADP é observada na presença de azida sódica, um inibidor da NTPDase1, na concentração de 20 mM,. Levamisole, um inibidor específico de fosfatase alcalina, e P^1,P^5-diadenosina 5'-pentafosfato (Ap5A), inibidor específico da adenilato quinase, também não inibiram a atividade de hidrólise. Os valores de K_M (Constante de Michaelis) para Ca^{2+}-ATP e Ca^{2+}-ADP encontram-se na ordem de micromolar. O comportamento similar das atividades ATPásica e ADPásica e o gráfico de Chevillard (CHEVILLARD et al., 1993) sugerem que a hidrólise de ATP e ADP ocorre no mesmo sítio ativo. A presença de uma NTPDase1 em *T. vaginalis* pode ser importante para a modulação da razão nucleotídeos/nucleosídeos no meio extracelular, protegendo o parasito dos efeitos citolíticos dos nucleotídeos, principalmente ATP.

A ecto-5'-nucleotidase tem sido descrita em células bacterianas e vegetais e é vastamente distribuída nos tecidos de vertebrados (ZIMMERMANN, 1992). Trata-se de

uma enzima ancorada à membrana plasmática por glicosilfosfatidilinositol (GPI). A ecto-5'-nucleotidase encontra-se presente na maioria dos tecidos e sua principal função é a hidrólise de nucleotídeos monofosfatados extracelulares, tais como AMP, GMP ou UMP a seus respectivos nucleosídeos (ZIMMERMANN, 1996; ZIMMERMANN et al., 1998). Tasca et al. (2003) demonstraram a atividade de uma ecto-5'-nucleotidase na superfície de *T. vaginalis*.

Nosso laboratório tem investigado o sistema purinérgico de *T. vaginalis* através da hidrólise extracelular de nucleotídeos. A NTPDase1 e a ecto-5'-nucleotidase de *T. vaginalis* apresentam ecto-localização e a participação dessas enzimas na sinalização celular é sugerida, visto que elas modulam os níveis de nucleotídeos e nucleosídeos extracelulares, influenciando os efeitos induzidos pelos purinoceptores. Além disso, considerando o fato de que *T. vaginalis* não realiza síntese *de novo* de purinas e pirimidinas, as enzimas participam das vias de salvação fornecendo os nucleosídeos, principalmente adenosina (TASCA et al., 2004). Considerando experimentos realizados *in vitro*, o metronidazol e o tinidazol, medicamentos usados no tratamento da tricomonose, apresentam efeitos diversos na atividade da NTPDase1 de um isolado de *T. vaginalis* proveniente da ATCC cultivado por longo período no laboratório e de um isolado clínico fresco, sugerindo uma função da NTPDase1 como moduladora da concentração de nucleotídeos extracelulares na presença dos fármacos, uma condição adversa para o parasito (TASCA et al., 2003).

A modulação da concentração de nucleotídeos no espaço extracelular, a proteção dos efeitos citolíticos do ATP e a participação nas vias de salvação de nucleosídeos são alguns aspectos fisiológicos do *T. vaginalis* que envolvem a hidrólise do ATP. O *T. vaginalis,* assim como *Giardia lamblia, Entamoeba histolytica, Leishmania* spp. e *Trypanosoma* spp., não é capaz de realizar síntese *de novo* de purinas e pirimidinas e seu

crescimento depende das vias de salvação para gerar nucleotídeos (HEYWORTH et al., 1982; 1984; WANG 1990). Considerando os altos níveis de nucleotídeos púricos no sítio do *T. vaginalis*, a ausência de efeitos citolíticos nos parasitos e a ecto-localização das enzimas envolvidas na hidrólise de nucleotídeos, sugere-se que a NTPDase1 e a ecto-5'-nucleotidase modulam as concentrações extracelulares de ATP, ADP e AMP. O produto final dessas reações, o nucleosídeo adenosina, é recaptado pelas vias de salvação. Munagala & Wang (2003) mostraram que a adenosina é o precursor primário do *pool* de nucleotídeos púricos em *T. vaginalis*, e identificaram atividades de adenosina deaminase, IMP desidrogenase e GMP sintetase nos parasitos, sugerindo uma rota metabólica capaz de converter adenina a GMP via adenosina. Visto que a adenosina tem importância primordial na salvação de purinas em *T. vaginalis*, pode-se considerar a NTPDase1 e a ecto-5'-nucleotidase essenciais para as estratégias de sobrevivência do parasito durante a exposição a nucleotídeos extracelulares, principalmente ATP. Portanto, essas enzimas produzem adenosina e contribuem para a utilização do nucleosídeo através das vias de salvação e a ecto-localização das enzimas é fundamental para a realização dessas funções. Tasca et al. (2005) mostraram que a hidrólise de ATP, ADP e AMP em *T. vaginalis* é elevada em isolados clínicos frescos quando comparadas com isolados cultivados por longos períodos no laboratório que apresentam a hidrólise destes nucleotídeos mais baixa.

Estudos mostram efeitos de hormônios esteroidais na atividade das ectonucleotidases. Spychala et al. (2004) demostraram que a expressão e os níveis de mRNA da ecto-5'-nucleotidase são negativamente regulados pelo receptor de estrogênio, indicando uma correlação entre a presença da enzima e de adenosina e progressão de carcinoma mamário. Outros estudos envolvendo ratas ovariectomizadas mostraram um aumento na hidrólise de ATP, ADP e AMP no soro e uma diminuição da atividade de

hidrólise desses nucleotídeos pelas enzimas das plaquetas (POCHMANN et al., 2004; 2005). Rücker et al. (2004) mostraram que há um aumento na velocidade de hidrólise de AMP no córtex cerebral de ratas ovariectomizadas.

1.2. OBJETIVOS

1.2.1 Obetivo geral

Considerando o impacto da tricomonose na saúde pública com a geração de graves problemas como aumento da transmissão do HIV, do câncer cervical, de problemas relacionados à fertilidade e gestações, e que o estabelecimento do *T. vaginalis* na vagina é influenciado por diversos sintomas, dentre eles, os níveis de hormônios, é de fundamental importância para que se conheçam os aspectos biológicos do *T. vaginalis.* A variedade de efeitos causados pelos nucleotídeos e nucleosídeos extracelulares ocorre em inúmeros tecidos e órgãos, principalmente em sítios de infecção, onde há uma intensa liberação dessas moléculas em situações patológicas.

O objetivo geral deste projeto é investigar a influência de dois hormônios esteroidais, o 17β-estradiol e o S-DHEA sobre a hidrólise de nucleotídeos extracelulares em um isolado padrão de *T. vaginalis*, ATCC 30236, e em um isolado fresco, VP60, uma vez que estes dois isolados apresentam diferentes graus de virulência.

1.2.2 Objetivos específicos

a) Avaliar o efeito in vitro do17β-estradiol e do S-DHEA sobre a hidrólise de ATP, ADP e AMP em dois diferentes isolados de *T. vaginalis*, ATCC 30 236 e TVVP-60.

b) Investigar o efeito da adição de diferentes concentrações dos hormônios esteroidais no meio de cultura de *T. vaginalis* e verificar a sua influência sobre a hidrólise de ATP, ADP e AMP.

c) Avaliar as atividades nucleotidásicas após diferentes tempos de exposição aos hormônios esteroidais no meio de cultura: 2 e 12 horas.

d) Investigar a expressão gênica dos diferentes membros da família das NTPDases e 5´-nucleotidase nos dois isolados de *T. vaginalis* a serem estudados, bem como avaliar a influência dos hormônios esteroidais no nível de transcritos de mRNA dos membros destas famílias que foram identificados.

2. Capítulo 2

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

Caroline Rückert, Cristiane dos Santos Stuepp, Bárbara Gottardi, Jéssica Rosa, Júlia Cisilotto, Fernanda Pires Borges, Denis Broock Rosemberg, Mauricio Reis Bogo, Geraldo Attilio De Carli, Carla Denise Bonan

Artigo submetido ao periódico Molecular & Biochemical Parasitology

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

Caroline Rückert[1], Cristiane dos Santos Stuepp[2], Bárbara Gottardi[2], Jéssica Rosa[2], Júlia Cisilotto[2], Fernanda Pires Borges[2], Denis Broock Rosemberg[3], Maurício Reis Bogo[4], Tiana Tasca[5], Geraldo Attilio De Carli[2], Carla Denise Bonan[1,*]

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Running Head: Steroid hormones alter NTPDase in *T.vaginalis*

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Abstract

Steroids may influence the pathogenesis of *Trichomonas. vaginalis.* Studies have described that the modulation of nucleotide levels seems to be essential for the survival of the parasite. We investigated the effect of dehydroepiandrosterone sulfate (DHEAS) and 17β-estradiol on NTPDase activity in fresh clinical (VP60) and long-term-grown isolates of *T. vaginalis* (30236 ATCC), followed by the NTPDase genes transcriptional analysis. For *in vitro* studies, ATP hydrolysis was activated by 17β-estradiol (0.01-1.0 μM) in the VP60 isolate. In the same conditions, ADP hydrolysis was inhibited by 1.0-5.0 μM DHEAS in the ATCC isolate. The treatment of parasites in the presence of DHEAS (0.01-1.0 μM) for 2 h inhibited ATP and ADP hydrolysis in VP60 isolate whereas there were no significant changes on nucleotide hydrolysis in 30236 isolate. The treatment during 2 h with 17β-estradiol (0.01-1 μM) did not alter the nucleotide hydrolysis in VP60 isolate. However, there was a significant inhibition of ATP and ADP hydrolysis in 30236 isolate. During a 12 h-treatment with DHEAS, the nucleotide hydrolysis was inhibited in both isolates whereas it was activated by 17β-estradiol. Two NTPDase sequences were identified from phylogenetic analyses and named NTPDaseA and NTPDaseB. The treatment with DHEAS (0.1 μM) for 12 h was able to inhibit mRNA NTPDaseA transcript levels from VP60 isolate. Considering that 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 steroids may be related with their influence in *T. vaginalis* colonization.

Keywords: dehydroepiandrosterone sulfate (DHEAS); 17 beta estradiol; *Trichomonas vaginalis*, nucleoside triphosphate diphosphohydrolase; nucleotidases.

Introduction

Trichomonas vaginalis is a flagellate protozoan that causes trichomonosis, the most common, non-viral sexually transmitted disease (STD) [1]. The infection has been associated with serious health consequences including adverse pregnancy outcomes [2], infertility [3], predisposition to cervical cancer [4] and pelvic inflammatory disease [5]. Trichomonosis also impacts upon birth outcomes and it is a co-factor in human immunodeficiency virus (HIV) transmission and acquisition [6].

Adenine nucleotides (ATP, ADP, and AMP) and their nucleoside derivative adenosine are important signaling molecules that mediate diverse biological and pathological processes [7]. The biological effects of extracellular ATP are mediated by ionotropic P2X and metabotropic P2Y receptors [7,8]. 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 [9, 10]. 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 [7]. It has been described the presence of ecto-nucleotidases on the surface of parasites and previous studies from our laboratory have already characterized the NTPDase (ATP diphosphohydrolase, apyrase) activity in trophozoites of *T. vaginalis* [11].

In the past few years, evidence has accumulated that many hormones, especially the sex steroids, can influence the immune system and thus the susceptibility for diseases caused by protozoan parasites [12-14]. For example, estrogen, testosterone and progesterone treatment in insects stimulated the proliferation of their protozoan and helminth parasites infections [15]. In contrast, dehydroepiandrosterone (DHEA), DHEA-

sulfate (DHEA-S) and its analog 16α-bromoepiandrosterone have shown antimalarial activity against several strains of *Plasmodium falciparum in vitro* and *P. berghei in vivo* [16]. Interestingly, high circulating levels of DHEAS in Kenyan pubertal girls are correlated with lower *P. falciparum* parasitemia, which suggest a predictive role for this hormone in malaria [17]. Proliferation and survival of *Cryptosporidium parvum* in multiple animal models were also down-regulated by treatment with DHEA [18 - 20].

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. There is also an increased incidence of trichomonosis during pregnancy, suggesting that a high-estrogen state may enhance infectivity or symptoms [21]. Silva-Filho & Bonilha [22] showed that α-estradiol and 17β-estradiol increases the 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. This suggests that the symptoms of *T. vaginalis* infection may be influenced by the vaginal concentrations of estrogens [23].

Studies have demonstrated that ovariectomy on female rats promoted a significantly increase in ATP, ADP, and AMP hydrolysis in blood serum [24]. Considering the impact of trichomonosis on public health and that host-parasite interaction is influenced by hormone levels it becomes important to understand the effects promoted by these hormones on 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 a fresh clinical (VP60) and in a long-term-grown isolates (30236 ATCC) of *T. vaginalis*, followed by the NTPDase genes 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 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 the VP60, a fresh clinical isolate (EU816897) [25]. Both isolates were cultivated axenically in trypticase-yeast extract-maltose (TYM) medium [26] without agar (pH 6.0) supplemented with 10% (v/v) heat-inactivated cold 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 (NaCl 0.85%) (750 x *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 hours before washing with sterile saline solution (0.85% NaCl) (750 x *g* for 5 min) three times. The subsequent steps for incubation to measure the nucleotide hydrolysis were the same as described above.

2.4. 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, 5.0 μM) was added in the reaction media. The samples were preincubated for 5 min at 37ºC 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) [27]. 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 substrates. Specific activity was expressed as nmol Pi released/min/$1.5x10^6$ trophozoites/ml. All samples were run in triplicate, with similar results achieved in at least three different cell suspensions.

2.5. Protein determination

Protein was measured by the Coomassie blue method [28], using bovine serum albumin as standard.

2.6. Prediction of *T. vaginalis* NTPDase sequences

NTPDase sequences of *T. vaginalis* were obtained from the NCBI database using the systematic BLAST searches (www.ncbi.nlm.nih.gov/BLAST) [29]. The well-known amino acid sequences of human (**NP_001767**, **NP_982293**, **NM_001248**, **BC034477**, **NM_001249**, **BC025980**, **NM_020354,** and **AAR04374**) and mouse (**AAH11278**, **O55026**, **AY376710**, **BC043134**, **BC015247**, **BAE33807**, **NM_053103,** and **AAQ84519**) NTPDase members were used as queries. The protein blast was performed using the Swissprot protein sequences database and the algorithm blastp.

2.7. Phylogenetic and sequence analyses

The protein sequences alignment was performed using ClustalX program [30] and a phylogenetic tree was constructed according Neighbor-Joining method [31] using proportional (p) distance with MEGA 2.1 program [32] (Figure 1).

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

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

strategy adopted to construct the primers 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 (Table1), 0.2 mM dNTP, 2.0 mM $MgCl_2$ and 0.5 U Taq Platinum DNA polymerase (Invitrogen). 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 resolved by a 1.0% agarose gel containing ethydium bromide and visualized with ultraviolet light. The fragments length of PCR reactions was confirmed with Low DNA Mass Ladder (Invitrogen, USA) and the normalization was performed employing α-tubulin as a constitutive gene.

2.9. 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 have investigated the effects of the 17β-estradiol and DHEAS on the 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.

In order to evaluate a direct effect on ecto-nucleotidase activities we have tested *in vitro* 17β-estradiol or DHEAS concentrations varying from 0.01 to 5.0 μM in VP60 and 30236 ATCC isolates. Figure 2 shows the NTPDase activity measured in the presence of 17β-estradiol (Fig. 2A) and DHEAS (Fig. 2B) for the VP60 isolate. ATP hydrolysis was increased 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 did not present significantly changes 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 2D. 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 on ATP and ADP hydrolysis (Fig. 3). VP60 isolate in the presence of DHEAS for 2 h promoted a significant inhibition of 40%, 41%, and 25% ($P<0.05$) on ATP hydrolysis in all tested concentrations (0.01, 0.1, and 1.0 μM) whereas a 38% ($P<0.05$) decrease of 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. 4). The treatment of 30236 isolate in the presence of 17β-estradiol for 2 h

induced a significant decrease on 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. 4).

Figure 5 shows the effect of the treatment with DHEAS and 17β-estradiol for 12 h in the VP60 isolate on nucleotide hydrolysis. 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 of 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 during 12 h induced a significant inhibition on ADP hydrolysis (42 and 38% $P<0.05$) at 0.01 and 0.1 μM, respectively in 30236 isolate (Fig. 6). The treatment with 17β-estradiol for 12 h promoted an increase of 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. 6).

The effects promoted by the hormones 17β-estradiol or DHEAS could be a consequence of transcriptional control. From the eight-well characterized enzymes of mammals NTPDase family, four members, NTPDase 1-3 and 8, are tightly bound to plasma membrane with active site facing the extracellular milieu. A phylogenetic analysis was performed in order to verify possible orthologous genes in *T. vaginalis*.

Protein sequences of *Homo sapiens* and *Mus musculus* NTPDase members were retrieved from GenBank and used as query. NCBI Blast searches of GenBank yielded in two complete *T .vaginalis* NTPDase sequences from which **XM_001298945** corresponds to NTPDaseA and **XM_001579653** corresponds to NTPDaseB. No other related sequences were identified in *T. vaginalis* genome with the strategy adopted. 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. The ACR1 sequences are MVDAGSSGTRGFLY and MVDAGSSGTRAFVY, the ACR2 are ETPIYVYATAGMRLLS and STPMYVFATAGMRLLG, the ACR3 are VISGVEEGVYGWLSVNNLL and VINGVEEGVYGWLSVNLLL and the ACR4 are GSIDLGGASFQIALQVN and GAMDLGGASFQIAVEVN from NTPDase A and NTPDase B respectively. The ACR5 is identical in both sequences (DLSWAIGAM). Comparison of *T. vaginalis* NTPDase A to all proteins in GenBank shows that the best match with non trichomonads is to *Ornithorhynchus anatinus* which is similar to E-type ATPase (**XP_001509821**) with 27% of identity whereas the best match found to NTPDase B sequence is to *Caenorhabditis elegans* Nucleoside Triphosphatase family member ntp-1 (**NP_501289**) presenting 30% of identity.

The phylogenetic tree constructed used Neighbor-Joining method and proportional (*p*) distance (Figure 1). The phylogenetic tree grouped consistently (Mm) *Mus musculus* (Hs) *Homo sapiens* and *T. vaginalis* NTPDases sequences. 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 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 VP60 isolate with 0.1 μM DHEAS for 12 h promoted a decrease (42%) in NTPDaseA transcript levels (Fig. 5 A and B) whereas this treatment did

not alter the mRNA levels for both enzymes on 30236 isolate (Fig.5A and 5C). 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*. The treatment during 2 or 12 h with DHEAS and 17β-estradiol were able to modulate the 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 VP60 isolate after DHEAS treatment during 12 h.

Pochmann et al. [24] demonstrated that estradiol replacement therapy have a significant decrease of ATP, ADP, and AMP hydrolysis. Rats submitted to ovariectomy and with estradiol replacement showed a significant decrease in enzymatic 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 on parasites *S. mansoni* [33] and *Plasmodium* sp. [34]. Moreover, *in vivo* evidence on 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 the parasitemia [17]. DHEA has an

important effect in human malaria, in which the plasma levels of DHEA were associated with lower parasitemia and protection in hyperendemic areas [17, 35]. The mechanisms through which DHEA improves the immune response against the 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 [14, 36].

T. vaginalis has specific androgen and estrogen receptors, which suggests that steroid hormones could directly affect the parasite [37]. Animal models of *T. vaginalis* vaginitis require estrogenization to establish infection [38, 39]. It was also found that, in women volunteers, a high estrogenic state is required to establish a *T. vaginalis* vaginal infection [40]. Trichomonosis, as well as symptomatic genital gonococcal disease, are exacerbated during menstruation [41], characterized by additional estrogenic activity.

In addition, *T. vaginalis* is unable to synthesize the purines and pyrimidines de novo and its their growth and survival are dependent on the salvage pathway of these compounds to generate nucleotides [42, 43]. Moreover, studies have demonstrated that the extracellular ATP can act in citotoxic mechanisms [8, 44]. The concentration of purine nucleotides found in the vagina is around 10 mM [45]. The activation on NTPDase activity after the 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 participates on hydrolysis cascade of ATP is essential for the growth of the parasite in a hostile ambient under constant alterations, through the modulation of extracellular nucleotides concentrations. Furthermore, previous studies have shown that extracellular ATP, ADP, and AMP hydrolysis in *T. vaginalis* is higher in fresh clinical isolates, and indeed, higher

enzymatic activities were seen in fresh compared to representative long-term-grown isolates [46]. The differences promoted by DHEAS and 17B-estradiol in VP60 and 30236 ATCC isolates probably are due to the heterogeneity of these isolates, which results in different kinetic profile and sensitivity to hormones for NTPDase activity.

Transcriptional control could be responsible, at least in part, for the alterations promoted by the hormones on 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 have performed semiquantitative RT-PCR experiments. Interestingly, the results have demonstrated that NTPDaseA mRNA levels have been significantly inhibited 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 of NTPDase activity induced by this hormone can impair the growth and survival of *T. vaginalis* due to a possible reduction of adenosine levels, which is essential for parasite metabolism. Furthermore, a lower NTPDase activity might result in higher nucleotide levels, which present cytolytic effects [47].

Considering that 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 related with their influence in *T. vaginalis* colonization.

Acknowledgements

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Table1

PCR primer design

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

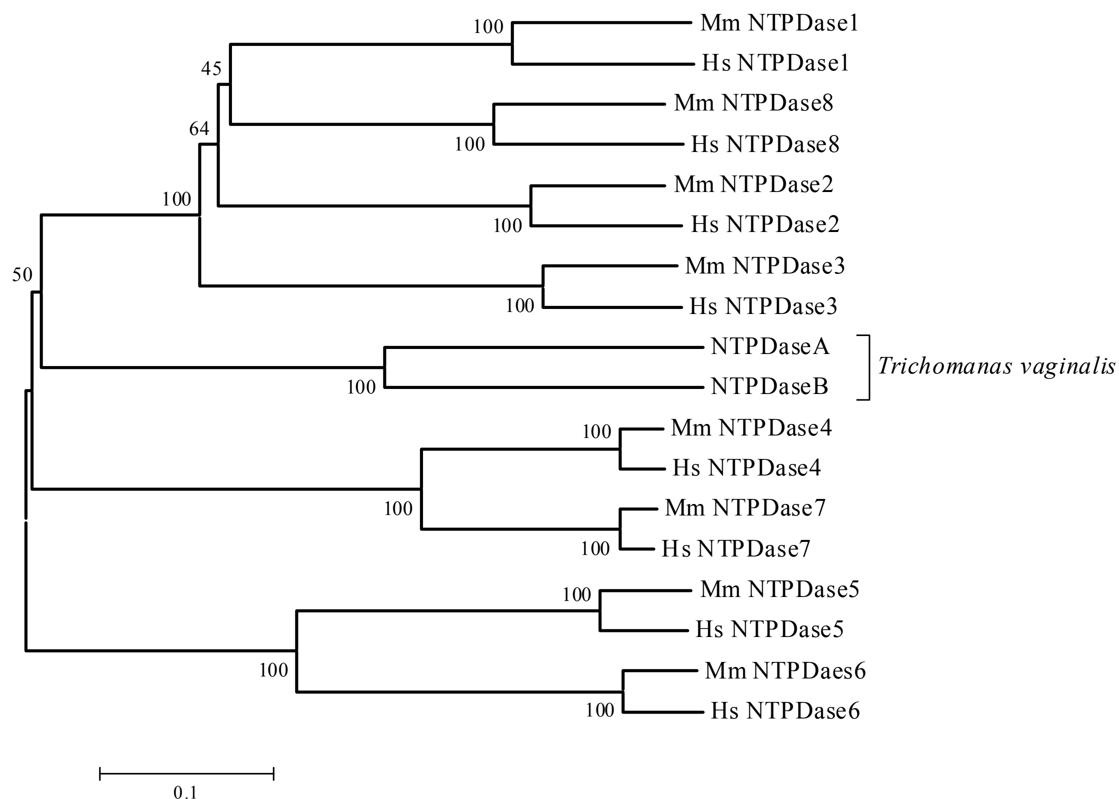

Figure 1: Phylogenetic analysis of NTPDase-related family members, demonstrating the existence of two distinct members in *T. vaginalis*: NTPDaseA (**XP_001298946**) and NTPDaseB (**XP_001579703**). The deduced amino acid sequences were aligned with ClustalX program and the phylogenetic tree was constructed using Neighbor-Joining method, using proportional (p) distance with MEGA 2.1 program. The phylogenetic tree grouped consistently (Mm) *Mus musculus* (Hs) *Homo sapiens* and *T. vaginalis* NTPDase sequences.

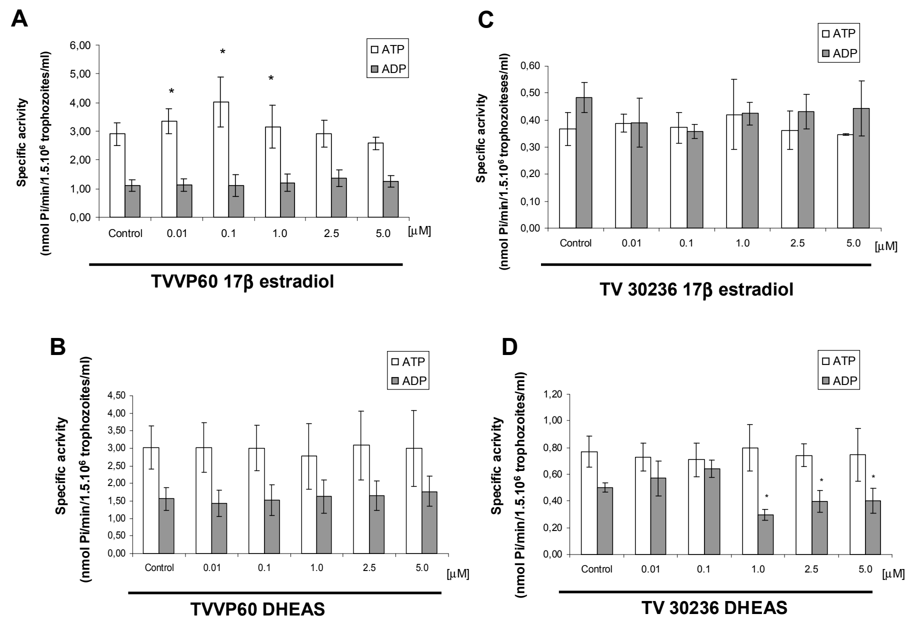

Figure 2: Effect of 17β-estradiol (A,C) and DHEAS (B,D) on ATP and ADP hydrolysis in VP60 and 30236 ATCC isolate. Bars represent the mean ± S.D. of at least four experiments (n=4) using different parasite suspensions, each in triplicate. * indicates significant difference from controls by one-way ANOVA,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

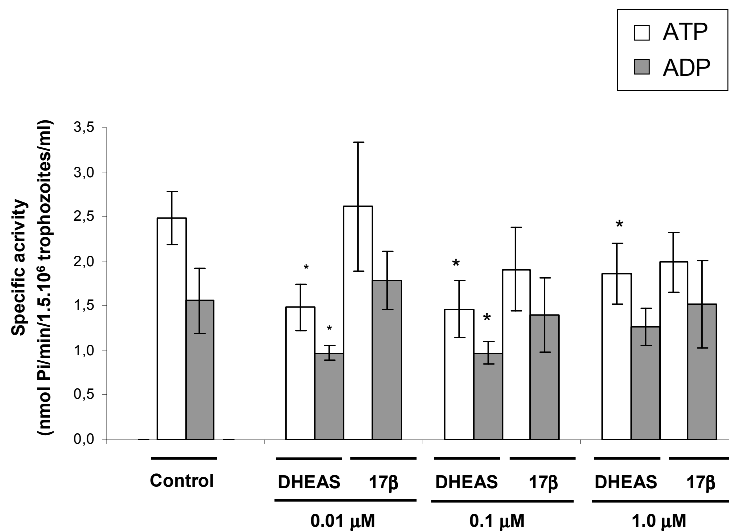

Figure 3: Effect of 17β-estradiol and DHEAS treatment during 2 h on ATP and ADP hydrolysis in VP60 isolate. Bars represent the mean ± S.D. of at least four experiments (n=4) using different parasite suspensions, each in triplicate. * indicates significant difference from controls by one-way ANOVA, followed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

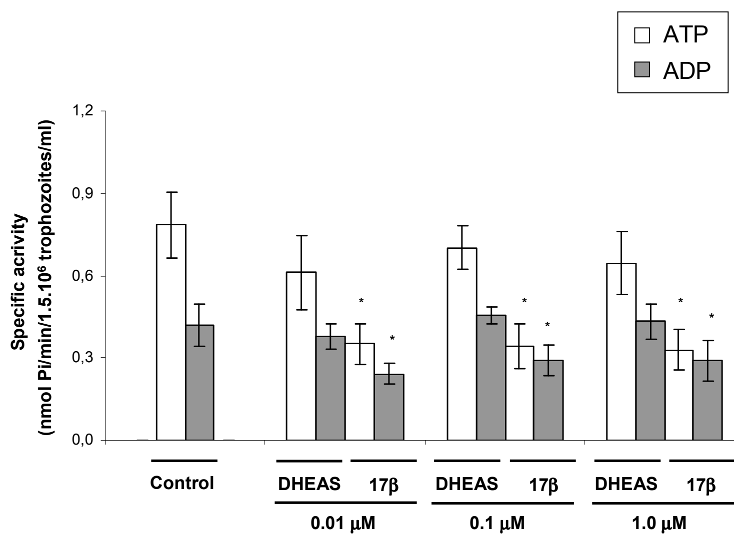

Figure 4: Effect of 17β-estradiol and DHEAS treatment during 2 h on ATP and ADP hydrolysis in 30236 ATCC isolate. Bars represent the mean ± S.D. of at least four experiments (n=4) using different parasite suspensions, each in triplicate. * indicates significant difference from controls by one-way ANOVA,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

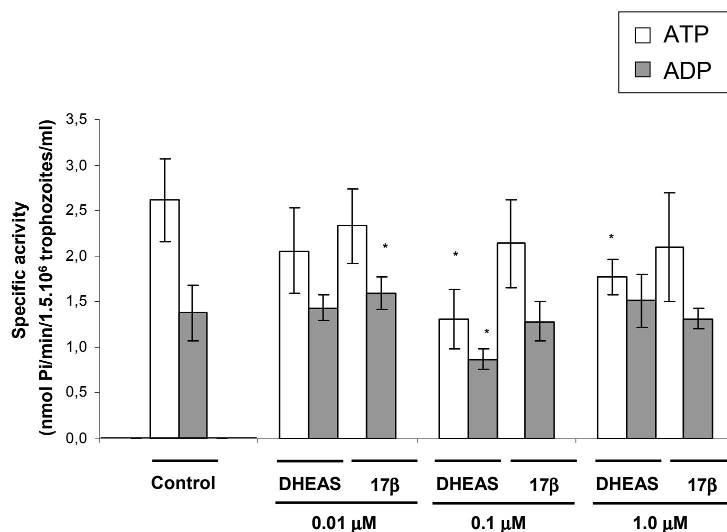

Figure 5: Effect of 17β-estradiol and DHEAS treatment during 12h on ATP and ADP hydrolysis in VP60 isolate. Bars represent the mean ± S.D. of at least four experiments (n=4) using different parasite suspensions, each in triplicate. * indicates significant difference from controls by one-way ANOVA, followed by Duncan test as a post-hoc (P<0.05).

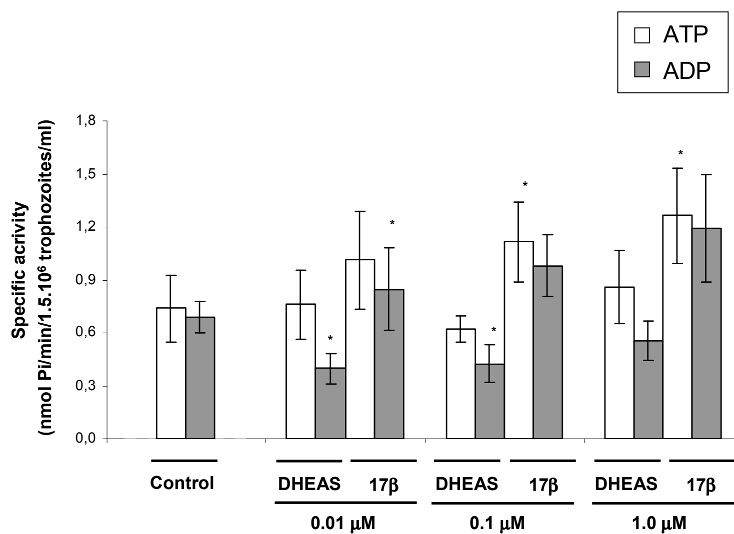

Figure 6: Effect of 17β-estradiol and DHEAS treatment during 12 h on ATP and ADP hydrolysis in 30236 isolate. Bars represent the mean ± S.D. of at least three experiments using different parasite suspensions, each in triplicate. * indicates significant difference from controls by one-way ANOVA, followed by Duncan test as a post-hoc ($P<0.05$).

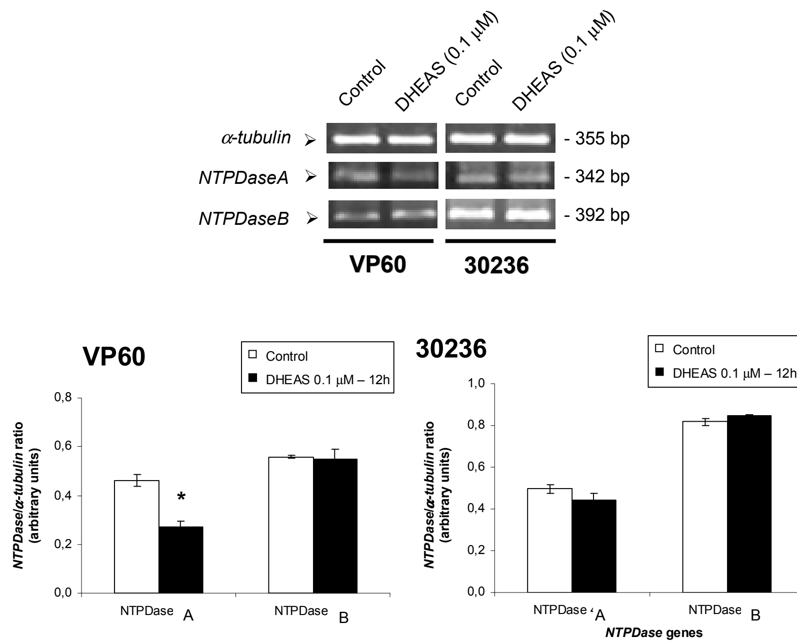

Figure 7: Gene expression patterns of NTPDase members after 12 h parasite treatment with DHEAS in VP60 and 30236 isolates. The α-tubulin gene was carried out as an internal standard and the optical densitometry analysis of the PCR products was made using Image J 1.37 for Windows. Three independent experiments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

3. Capítulo 3

Steroid hormones alter AMP hydrolysis in intact trophozoites of *Trichomonas vaginalis*

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Steroid hormones alter AMP hydrolysis in intact trophozoites of *Trichomonas vaginalis*

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Running Head: *Trichomonas vaginalis*: hydrolysis of AMP

Abstract

Steroid hormones can influence the immune system and thus susceptibility for diseases caused by protozoan parasites. *Trichomonas vaginalis* infection may be influenced by the vaginal concentrations of estrogens. We have investigated the effects of 17β-estradiol and dehydroepiandrosterone sulfate (DHEAS) on the ecto-5´-nucleotidase activity in a fresh clinical (VP60) and in a long-term-grown (30236 ATCC) isolates of *T. vaginalis.* For *in vitro* studies, AMP hydrolysis has not demonstrated difference between the investigated isolates. The treatment of parasites in the presence of DHEAS (0.01-1.0 μM) for 2 h inhibited AMP hydrolysis in VP60 isolate whereas there were no significant changes in the presence of 17β-estradiol on nucleotide hydrolysis. DHEAS and 17β-estradiol (0.01-1.0 μM) for 2 h inhibited AMP hydrolysis in 30236 isolate. The treatment during 12 h with DHEAS at 0.1 μM inhibited AMP hydrolysis in VP60 isolate, whilst 17β-estradiol did not alter the nucleotide hydrolysis in VP60 isolate. Our findings have shown that the complex effect of steroid hormones and their receptors on *T. vaginalis* promote changes in ecto-5´-nucleotidase activity during exposure to these hormones.

Keywords: dehydroepiandrosterone sulfate (DHEAS); 17 beta estradiol; *Trichomonas vaginalis*, ecto-5´-nucleotidase, steroid hormones.

1. Introduction

Trichomonas vaginalis is an amitochondriate parasitic flagellated protozoan that causes trichomonosis, the number one, non-viral sexually transmitted disease (STD) in the world. The parasite infects the urogenital tract of both women and men. Trichomonosis is associated with serious adverse health consequences to women that include infertility (Goldstein et al., 1993), atypical pelvic inflammatory disease (Moodley et al., 2002), preterm birth and low birth weight infants (Cotch et al., 1997), and predisposition to cervical neoplasia (Viikki et al., 2000). Trichomonosis among men can cause non-chlamydial, non-gonococcal urethritis (Bennett et al., 1989; Bakare et al., 1999) and, more recently, serum antibody to *T. vaginalis* was found to be related with prostate cancer (Sutcliffe et al., 2006). For both men and women, trichomonosis increases predisposition to HIV seroconversion (Guenthner et al., 2005; Mason et al., 2005; Rughooputh and Greenwell, 2005). The proteins and glycoproteins in the cell surface of trichomonads play a major role in cytoadhesion, host-parasite interaction, nutrient acquisition, and in the protection from the cytolytic effects (Petrin et al., 1998). Extracellular ATP may act as a signaling compound in cytolytic mechanisms (Filippini et al., 1990; Steinberg and Di Virgilio, 1991) and it is hydrolyzed to adenosine by a group of enzymes named NTPDases (nucleoside triphosphate diphosphohydrolase), which hydrolyze nucleoside di- and tri-phosphates, and the ecto-5′-nucleotidase (EC 3.1.3.5), which hydrolyzes nucleoside monophosphates (Sarkis et al., 1995; Zimmermann, 1996, 2001). Adenosine is a regulatory autocoid that is generated as a result of cellular injury or stress, and it interacts with specific G protein-coupled receptors on inflammatory and immune cells to regulate their function (Haskó and Cronstein, 2004).

5′-Nucleotidase activity has been described for bacteria and plant cells and the enzyme is also widely distributed in vertebrate tissues (Zimmermann, 1992). This enzyme acts on a variety of non-cyclic nucleoside monophosphates, such as AMP, CMP, UMP, IMP and GMP, inactivating them to the respective nucleosides and inorganic phosphate (Bianchi and Spychala, 2003). Although the 5'-nucleotidase has broad substrate specificity, AMP is considered to be the major physiological substrate with K_M values in the micromolar range (Zimmermann, 1992, 1996). The enzyme is variably expressed in a wide number of cells types under physiological and pathological conditions (Zimmermann, 1992). Several studies have reported the presence of ectonucleotidases on the surface of parasites. Recently, our laboratory has characterized activity of an ecto-5′-nucleotidase in trophozoites of *T. vaginalis* (Tasca et al., 2003) and *T.gallinae* (Borges et al., 2007). The presence of an enzyme that hydrolyzes AMP to adenosine provides the nucleoside required for parasite growth, due to the lack of *de novo* purine nucleotide synthesis among all trichomonad species (Heyworth et al., 1982, 1984; Wang, 1990; Munagala and Wang, 2003).

In the past few years, evidence has accumulated that many hormones, especially the sex steroids, can influence the immune system and thus susceptibility for diseases caused by protozoan parasites (Grossman, 1984; Roberts et al., 2001; Olsen et al., 2005). For example, estrogen, testosterone, and progesterone treatment on insects stimulated the proliferation of their parasitic infections (Lawrence, 1991). In contrast, dehydroepiandrosterone (DHEA), DHEA-sulfate (DHEA-S) and its analog 16α-bromoepiandrosterone have shown antimalarial activity against several strains of *Plasmodium falciparum in vitro* and *P. berghei in vivo* (Freilich et al., 2000). Interestingly,

high circulating levels of DHEA-S in Kenyan pubertal girls correlated with lower *P. falciparum* parasitemia suggest a predictive role for this hormone in malaria (Leenstra et al., 2003). Studies have demonstrated an increased incidence of trichomonosis during pregnancy, suggesting that a high-estrogen state may enhance infectivity or symptoms (Brown, M. T. 1972). Silva-Filho & Bonilha (1992) have shown that α-estradiol and 17β-estradiol increases the 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. This suggests that the symptoms of *T. vaginalis* infection may be influenced by the vaginal concentrations of estrogens (Garber 1991).Previous studies have shown that estrogen is associated with changes in 5′-nucleotidase activities (Murphy, 2001; Ramalingam, 1993). Rücker et al. (2004) demonstrate changes in the activity and expression of 5′-nucleotidase in synaptosomes from female rat cerebral cortex, following chronic steroid hormone deprivation induced by removal of ovaries. The results demonstrated an increase in AMP hydrolysis in the animals submitted to ovariectomy in cerebral cortex.

Considering that trichomonads have not the ability to perform purine and pirimidine synthesis *de novo*, it becomes important to evaluate the enzyme activity involved in adenosine production in the presence of steroid hormones. Therefore, in this study we investigated the effects of 17β-estradiol and dehydroepiandrosterone sulfate on the ecto-5′-nucleotidase activity in a fresh clinical (VP60) and in a long-term-grown (30236 ATCC) isolates of *T. vaginalis*.

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

2.2 Parasites and culture conditions

Two *Trichomonas vaginalis* isolates were used in this study: 30236, from the American Type Culture Collection (ATCC) and the 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) supplemented with 10% (v/v) heat-inactivated cold serum, penicillin (1,000 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 (NaCl 0.85%) (750 x *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 treatments

The trophozoites were kept in the absence (control) or in the presence of 17 β-estradiol or DHEAS (0.01, 0.1, and 1.0 μM) for 2 or 12 hours at 37°C (±0.5). During these pretreatments, the trophozoites were maintained in trypticase-yeast extract-maltose (TYM) medium (Diamond, 1957) without agar (pH 6.0) supplemented with 10% (v/v) heat-inactivated serum, penicillin (1,000 IU/ml), and streptomycin sulfate (1.0 mg/ml). After the pretreatments, the trophozoites were washed with sterile saline solution (0.85% NaCl) (750 x g for 5 min) three times. The subsequent steps for incubation to measure the nucleotide hydrolysis were the same as described above.

To evaluate the direct effect of hormones on the enzyme activity, 17β-estradiol or dehydroepiandrosterone sulfate (DHEAS), at the final concentrations of 0.01, 0.1, 1.0, 2.5 or 5.0 μM, were added directly in the reaction mixture and were maintained throughout the enzyme assays, as described below. For the control, the enzyme assays was performed in the absence of hormones.

2.4 Enzyme assays

Intact trophozoites of *T. vaginalis* were added to the reaction mixture containing 50 mM Tris buffer (pH 7.5) and 3.0 mM $MgCl_2$, for measuring ecto-5′-nucleotidase activity. The samples were preincubated for 5 min at 37ºC in 200 μl of the reaction mixture. The reaction was initiated by the addition of AMP to a final concentration of 3.0 mM and stopped, after 60 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 cellular 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 substrate. Specific activity is expressed as nmol Pi released/min/1.5x10^6 trophozoites/ml. All samples were run in triplicate, with similar results achieved in at least three different parasite suspensions.

2.5 Protein determination

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

2.6 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 have investigated the effects of the 17β-estradiol and DHEAS on the ecto-5´-nucleotidase activity in intact trophozoites of *T. vaginalis*. Cellular integrity and viability were assessed before and after the reactions by the motility of trophozoites and trypan blue dye exclusion. The integrity of the trophozoites was not affected by any of the conditions used in the assays.

In order to evaluate a direct effect on ecto-5´-nucleotidase activity we have tested *in vitro* 17β-estradiol or DHEAS concentrations varying from 0.01 to 5.0 μM in VP60 and 30236 isolates. AMP hydrolysis did not demonstrate difference between the investigated isolates (data not shown).

The treatment of VP60 isolate in the presence of DHEAS for 2 h promoted a significant inhibition of 43%, 52%, and 24% ($P<0.05$) for AMP hydrolysis in all tested concentrations (0.01, 0.1, and 1.0 μM), whereas 17β-estradiol did not significantly change AMP hydrolysis in VP60 isolate (Fig. 1).

Figure 2 shows the effect of the treatment with DHEAS and 17β-estradiol for 2 h in the 30236 isolate on ecto-5´-nucleotidase activity. AMP hydrolysis, at 0.01, 0.1, and 1.0 μM, was significantly inhibited in the presence of DHEAS (29%, 36%, and 31%, respectively; $P<0.05$). The treatment of 30236 isolate in the presence of 17β-estradiol promoted a significant inhibition of 25%, 22%, and 37% ($P<0.05$) for AMP hydrolysis in all tested concentrations (0.01, 0.1, and 1.0 μM).

The treatment with DHEAS during 12 h induced a significant inhibition in AMP hydrolysis (32%, $P<0.05$) at 0.1 μM in VP60 isolate (Fig. 3). In a similar treatment, 17β-estradiol did not significantly change AMP hydrolysis in the VP60 isolate (Fig. 3).

The treatment of 30236 isolate in the presence of DHEAS and 17β-estradiol for 12 h did not significantly change AMP hydrolysis (data not shown).

4. Discussion

The results presented demonstrate the influence of DHEAS and 17β-estradiol on ecto-5´-nucleotidase activity in intact trophozoites of *T. vaginalis.* The importance of testing two different isolates is explained by the fact that fresh clinical isolates have demonstrated higher extracellular nucleotide hydrolysis and different nucleotide hydrolysis ratios when compared to long-term growth isolates (Tasca et al., 2005). Due to these significant differences in nucleotide metabolism, we have investigated the effect of

hormones on these two isolates in order to observe if steroids could differentially modulate the adenosine production, mainly because this nucleoside is the primary precursor of the entire purine nucleotide pool in *T. vaginalis*, and adenine is converted to GMP via adenosine (Munagala & Wang, 2003). Our findings have shown that VP60, the fresh clinical isolate tested, is more sensitive to the effects induced by steroid hormones since the short-term treatment (2h) and a lower DHEAS concentration (0.1μM) were able to promote the most intense inhibition on AMP hydrolysis. Moreover, the 12-h treatment also promoted a decrease in AMP hydrolysis in the presence of 0.1 μM DHEAS, although the effects were less expressive probably due to adaptive mechanisms developed by the parasites to resist these environmental conditions. These results reinforce the idea that fresh clinical isolates are more susceptible to environmental changes induced by factors, such as hormones. In contrast, it is important to highlight that the ATCC 30236 isolate also presented a decrease in AMP hydrolysis during 2h-treatment whereas there was no significant response in the long-term treatment (12 h) for any concentrations of both hormones tested. The lower effects observed for this isolate are probably related to its long-term adaptation to *in vitro* cultivation and, therefore, to a lower responsiveness to the environmental conditions.

T. vaginalis has specific androgen and estrogen receptors, which suggests that steroid hormones could directly affect the parasite (Ford 1987). Furthermore, direct effects of hormones on growth and viability have been shown in several parasite organisms. Sugarman and Mummaw (1988) showed that estrogen decreases the growth of *T. vaginalis.* Another report showed that application of vaginal estradiol pellets appeared to ameliorate the clinical symptoms of vaginitis in 33 women tested (Lirosi and Guarascio, 1972). The adrenal hormone DHEA has been shown to mediate only inhibitory actions on parasites *S.*

mansoni (Morales-Montor, 2001) and *Plasmodium* sp. (Ayi, 2002). Carrero et al. (2006) presented evidence on the direct *in vitro* effect of hormone DHEA on growth and viability of *Entamoeba histolytica* trophozoites. Moreover, *in vivo* evidence on 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 the parasitemia (Leenstra 2003). The mechanisms through which DHEA improves the immune response against the 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, 2005; Bouyou-Akotet, 2004).

Contradictory reports are found in the literature considering the relationship between estrogen and 5´-nucleotidase. Some reports have demonstrated an increase in plasma 5´-nucleotidase activities in women with hyperemesis gravidarum, which is characterized by enhanced release of pregnancy-related hormones (Depue et al., 1987, Abell et al., 1992, Goodwin et al., 1992). An increase in plasma 5´-nucleotidase activities may be partly attributed to elevations of pregnancy-related hormones, suggesting changes in the purine metabolism in women with hyperemesis gravidarum (Yoneyama et al., 2002). Spychala et al. (2004) demonstrated that estradiol, acting through the estrogen receptor, strongly down-regulates the expression of ecto-5´-nucleotidase. Another report demonstrated an increase in AMP hydrolysis in the cortex of ovariectomyzed rats. Since 5´-nucleotidase activity is involved in the hydrolysis of AMP to adenosine in the synaptic cleft, ovariectomy may result in an increase in 5´-nucleotidase activity in cortical synaptossomes (Rücker et al., 2005).

In summary, the presence of enzymes that participates on hydrolysis cascade of ATP to adenosine may play an important role for the growth of the parasite in a hostile

ambient under constant alterations due to the lack of *de novo* purine nucleotide synthesis. Our findings have shown that the complex effect of steroid hormones and their receptors on *T. vaginalis* promotes changes in ecto-5´-nucleotidase activity during exposure to these hormones. Therefore, our results present an additional contribution for the complex picture of the hormone effects on nucleotide hydrolysis and their influence in pathophysiological conditions.

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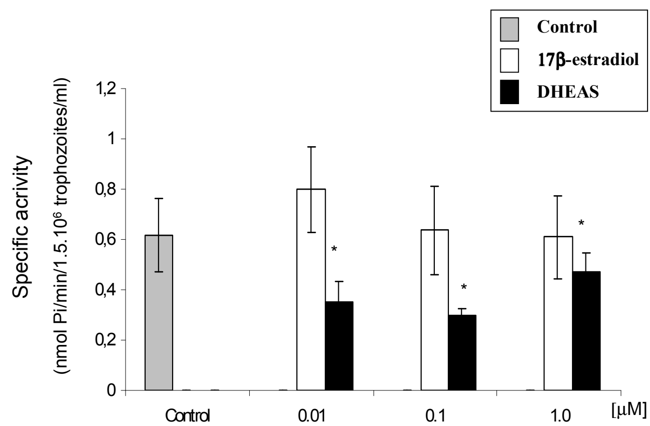

Figure1: Effect of 17β-estradiol and DHEAS treatment during 2 h on AMP hydrolysis in VP60 isolate. Bars represent the mean ± S.D. of at least four experiments (n=4) using different parasite suspensions, each in triplicate. * indicates significant difference from controls by one-way ANOVA, followed by Duncan test as a post-hoc (P<0.05).

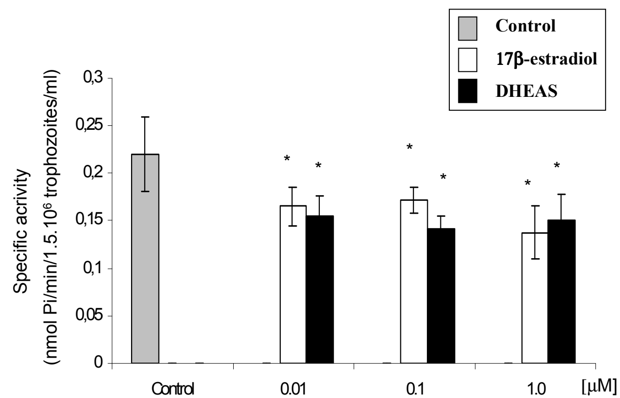

Figure 2: Effect of 17β-estradiol and DHEAS treatment during 2 h on AMP hydrolysis in 30236 ATCC isolate. Bars represent the mean ± S.D. of at least four experiments (n=4) using different parasite suspensions, each in triplicate. * indicates significant difference from controls by one-way ANOVA, followed by Duncan test as a post-hoc (P<0.05).

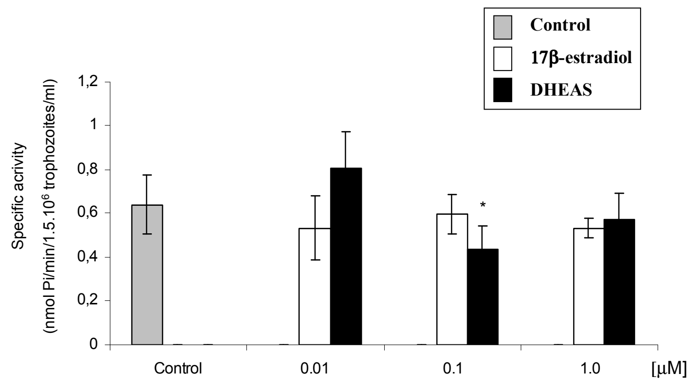

Figure 3: Effect of 17β-estradiol and DHEAS treatment during 12 h on AMP hydrolysis in VP60 isolate. Bars represent the mean ± S.D. of at least four experiments (n=4) using different parasite suspensions, each in triplicate. * indicates significant difference from controls by one-way ANOVA, followed by Duncan test as a post-hoc ($P<0.05$).

4. CONSIDERAÇÕES FINAIS

Os resultados apresentados mostram a influência de hormônios esteroidais, S-DHEA e 17β-estradiol, sobre as atividades das enzimas NTPDase e ecto-5´-nucleotidase em trofozoítos intactos de *T. vaginalis.*

Estudos têm mostrado que os hormônios esteroidais exercem efeitos sobre o crescimento e a viabilidade em diversos parasitos. Evidências apontam para a interferência dos níveis de estrogênios na incidência e desenvolvimento de doenças parasitárias (SYTRT & SUGARMAN, 1991). Por exemplo, 17β-estradiol pode modular a fase aguda de infecção do T. cruzi em camundongos (DE SOUZA, 2001) e administração de diidroepiandrosterona diminui a parasitemia em ratos (DOS SANTOS, 2005). O hormônio adrenal DHEA está envolvido em efeitos inibitórios nos parasitos *S. mansoni* (MORALES-MONTOR, 2001) e *Plasmodium sp*. (AYI, 2002). Carrero et al. (2006) demonstraram evidências do efeito *in vitro* do hormônio DHEA no crescimento e viabilidade de trofozoítos de *Entamoeba histolytica,* mostrando que o DHEA é um potente inibidor da proliferação dos trofozoítos.

A terapia de reposição de estradiol inibiu a hidrólise de ATP, ADP e AMP. Ratas ovariectomizadas submetidas à reposição hormonal com estradiol apresentaram uma inibição significativa da atividade das enzimas, sugerindo uma relação entre o sistema hormonal e enzimas que hidrolisam nucleotídeos de adenina no soro de ratas (POCHMAN et al., 2004).

O capítulo 2 mostra os efeitos dos hormônios esteroidais S-DHEA e 17β-estradiol sobre a atividade da NTPDase. O hormônio S-DHEA mostrou ser um potente inibidor da hidrólise de ATP e ADP. No isolado 30236, a atividade da NTPDase foi inibida no ensaio *in vitro* e no tratamento por 12 h, e no isolado VP60 a atividade da enzima foi inibida no

tratamento com o hormônio por 2 e 12 h. A presença do hormônio por 12 h foi capaz de inibir os níveis de mRNA da NTPDaseA no isolado VP60. Em contraste, o hormônio 17β-estradiol ativou a atividade da enzima NTPDase no isolado VP60 no ensaio *in vitro* e no tratamento por 12 h. Entretanto, na presença de 17β-estradiol por 2 h, a atividade da enzima foi inibida no isolado 30236. A ativação da enzima NTPDase após o tratamento com 17β-estradiol foi provavelmente devida a uma tentativa do parasito de modular a concentração dos níveis de nucleotídeos extracelulares. A presença de enzimas que participam da cascada de hidrólise do ATP é essencial para o crescimento do parasito em um ambiente hostil, sob constantes alterações, e pode contribuir para os mecanismos de escape do parasito da resposta imune do hospedeiro através da degradação do ATP, protegendo o parasito dos efeitos citolíticos dos nucleotídeos, principalmente o ATP.

O controle transcricional pode ser responsável por alterações promovidas pelos hormônios na atividade das NTPDases. Para verificar se o gene da NTPDase pode ser modulado quando trofozoítos intactos de *T. vaginalis* são expostos aos hormônios foi realizada a análise semiquantitativa de RT-PCR. Os resultados demonstram que os níveis de mRNA da NTPDaseA foram significativamente inibidos no isolado clínico fresco, na presença do hormônio S-DHEA por 12 h, sugerindo que a inibição da atividade neste tratamento pode estar diretamente relacionada com um baixo nível de expressão da NTPDaseA. Estudos prévios demonstram que a hidrólise extracelular de ATP, ADP e AMP em *T. vaginalis* é mais alta em isolados clínicos frescos comparada à hidrólise destes nucleotídeos em isolados mantidos em laboratório por longos períodos (Tasca et al., 2005). As diferenças promovidas pelo S-DHEA e pelo 17β-estradiol nos isolados VP60 e 30236

são provavelmente devido a heterogeneidade destes isolados, que resulta em diferente perfil cinético e sensibilidade aos hormônios para a atividade da NTPDase.

O capítulo 3 mostra os efeitos dos hormônios esteroidais sobre a atividade da ecto-5´-nucleotidase em trofozoítos de *T. vaginalis*. O tratamento com S-DHEA por 2 h inibiu significativamente a hidrólise de AMP nos isolados VP60 e 30236. 17β-estradiol por 2 h diminui significativamente a hidrólise de AMP no isolado 30236, enquanto no isolado VP60 a hidrólise de AMP não foi alterada. O tratamento por 12 h na presença do hormônio S-DHEA inibiu a hidrólise de AMP. Estudos contraditórios sobre a interação de hormônios esteroidais e a enzima ecto-5´-nucleotidase são encontrados na literatura. Alguns estudos demonstram um aumento na atividade da 5´-nucleotidase em mulheres com hiperemíase na gravidez, que é caracterizada por um aumento nos níveis dos hormônios relacionados com a gravidez (DEPUE et al., 1987; ABELL et al., 1992; GOODWIN et al., 1992). O aumento na atividade da 5´-nucleotidase pode ser atribuído a elevação dos hormônios durante a gravidez, sugerindo mudanças ocorridas no metabolismo purinérgico em mulheres com hiperemíase na gravidez. Spychala et al. (2004) demonstraram que o estradiol, agindo através do receptor de estrogênio, inibe a expressão da ecto-5´-nucleotidase. Outro estudo mostra um aumento na hidrólise do AMP em córtex de ratas ovariectomizadas (RÜCKER et al., 2005). Considerando que a atividade da 5´-nucleotidase está envolvida na hidrólise de AMP até adenosina na fenda sináptica, a ovariectomia pode resultar em um aumento na atividade da 5´-nucleotidase em sinaptossomas de córtex (RÜCKER et al., 2005).

T. vaginalis tem receptores específicos para androgênio e estrgênios, o que sugere que os hormônios esteroidais podem afetar diretamente os parasitos (FORD, 1987). Modelos animais para vaginite por *T. vaginalis* requerem estrogenização para estabelecer a

colonização (CAPPUCCINELLI, 1974; MAESTRONE, 1967). Azuma (1968) descreveu que em mulheres são precisos altos níveis de estrogênios para estabelecer a infecção por *T. vaginalis.* Os sintomas da tricomonose, assim como os sintomas por infecção por gonococos, são exacerbados durante a menstruação, que é caracterizada por atividade estrogênica adicional (PENZA, 1973).

A presença das enzimas que participam da hidrólise do ATP em *T. vaginalis* pode ser importante para a modulação da concentração de nucleotídeos no espaço extracelular. Protegendo o parasito dos efeitos citolíticos dos nucleotídeos, através da degradação do ATP e para a produção de adenosina necessária para o crescimento do parasito. Além disso, *T. vaginalis* é incapaz de sintetizar purinas e pirimidinas de novo e seu crescimento e sobrevivência depende das vias de salvação para gerar nucleotídeos (Heyworth et al., 1982; 1984).

Considerando que a vagina é um ambiente constatemente afetado pelas mudanças hormonais e que os efeitos dos hormônios esteroidais são influenciados por receptores presentes no *T. vaginalis* e pela concentração de hormônios na vagina, nossos resultados sugerem que a modulação dos níveis extracelulares de ATP, ADP e AMP durante a exposição aos hormônios esteroidais pode estar relacionada com a colonização do *T. vaginalis.*

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