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PERFIL BIOQUÍMICO E MOLECULAR DAS ECTONUCLEOTIDASES NO SISTEMA CARDIOVASCULAR DE RATOS DIABÉTICOS POR ESTREPTOZOTOCINA

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PARTE I

RESUMO

Ectonucleotidases constituem uma cascata enzimática altamente organizada para regulação da sinalização mediada por nucleotídeos, controlando a quantidade e a taxa de degradação destes, além da formação do respectivo nucleosídeo. Nucleotídeos/ nucleosídeos extracelulares são conhecidos por regularem diversas respostas fisiológicas, incluindo o tônus vascular, função cardíaca e homeostasia. Neste estudo nós analisamos a presença dos membros das famílias das E-NTPDases, E-NPPs e ecto-5'-nucleotidase em tecido de ventrículo esquerdo (VE) de ratos. A análise por PCR mostrou a expressão da NTPDase1, 2, 3,5,6, ecto-5'-NT/CD73, NPP2 e NPP3, excluindo a presença da NPP1 e NTPDase8. Através da análise por PCR em tempo real, encontramos o maior nível de expressão de mRNA para a NTPDase2 no ventrículo esquerdo. Também caracterizamos as propriedades bioquímicas e atividades enzimáticas em sinaptossomas de terminal nervoso (sinaptossoma cardíaco) em VE. No estudo das propriedades das E-NTPDases e ecto-5'-NT, observamos dependência de cátions divalentes, pH ótimo de 8.0 para as hidrólises de ATP e ADP e 9.5 para a hidrólise de AMP. Os valores de K_M aparente são 40 µM, 90 µM, 39 µM e os valores de V_{max} aparente são 537, 219 and 111 nmol Pi liberado/min/mg de proteína para as hidrólises de ATP, ADP e AMP, respectivamente. Oligomicina e azida, ambos inibidores de ATPases mitocondriais, inibiram somente a hidrólise de ATP. A hidrólise de AMP não foi afetada por levamisole e tetramisole, enquanto molibdato de amônio praticamente aboliu a atividade da ecto-5'-nucleotidase. Utilizando o *p*-nitrofenil-5'-timidina monofosfato (*p*-Nph-5'-TMP) como substrato marcador para E-NPPs na preparação de sinaptossoma cardíaco, observamos um pH alcalino, dependência de cátions divalentes e valores de K_M de 91.42 ± 13.97 µM e V_{max} calculado de 63.79 ± 3.59 nmol *p*-nitrofenol liberado/min/mg de proteína. Não houve alteração na hidrólise do substrato com adição de levamisole no pH de 8.9 (pH ótimo caracterizado), porém o suramin reduziu fortemente a hidrólise de *p*-Nph-5'-TMP. Para estudarmos se estas enzimas poderiam participar na patofisiologia das doenças diabéticas cardiovasculares, usamos um modelo de diabetes experimental por estreptozotocina (STZ) 65mg/kg. A injeção de STZ causou aumento da glicemia, perda de peso corporal, diminuição dos batimentos cardíacos e pressão sangüínea. Seis dias de tratamento com insulina foram capazes de reverter as alterações encontradas no diabetes. Houve uma inibição das hidrólises de ATP e ADP, mas não de AMP e *p*-Nph-5'-TMP em sinaptossoma cardíaco. O tratamento com insulina reverteu as hidrólises para os níveis do controle (citrato). A análise do metabolismo extracelular de ATP em sinaptossoma cardíaco por HPLC mostrou que o ATP é hidrolisado mais lentamente no grupo diabético. Dados preliminares de análise molecular sugerem a participação das NTPDases 1 e 2 nas hidrólises de nucleotídeos em sinaptossoma cardíaco.

Com o intuito de analisar um material biológico de fácil obtenção, avaliamos as atividades das ectonucleotidases e o perfil extracelular de hidrólise de ATP em soro de ratos diabéticos e também a influência da glicemia nestas atividades. Trinta dias após a injeção de STZ, observamos um aumento nas hidrólises de ATP, ADP, AMP e *p*-Nph-5'-TMP, quando comparados ao grupo citrato e este aumento foi revertido com 6 dias de tratamento com insulina. Glicose (20 mM), *in vitro*, não afetou a atividade enzimática em soro de ratos controle, após 24 h de pré-incubação. A análise por HPLC mostrou uma rápida hidrólise de ATP em soro de animais diabéticos, diminuindo os níveis de ATP e ADP mais rapidamente do que os grupos controle e insulina.

Mostramos uma alteração na hidrólise de nucleotídeos em sinaptossoma cardíaco e em soro de ratos diabéticos. A alteração encontrada em coração parece fazer parte da etiologia da doença cardiovascular diabética. No entando, a alteração encontrada no soro dos ratos diabéticos, diminuição de ADP (pró-agregante plaquetário) e aumento de adenosina (antiagregante plaquetário) parece participar de um sistema de proteção a possíveis danos vasculares decorrentes do diabetes. .

ABSTRACT

Ectonucleotidases constitute a highly organized enzymatic cascade in the regulation of nucleotide-mediated signaling, controlling the rate, amount and timing of nucleotide degradation and ultimately, the nucleoside formation. Extracellular nucleotides/nucleosides are known to regulate several physiological responses, including vascular tone, cardiac function and haemostasis. In this study, we have analyzed the presence of the E-NTPDase family members, ecto-5'-nucleotidase/CD73 and E-NPPs in rat heart left ventricle. RT-PCR analysis from left ventricle tissue demonstrated different levels of expression of NTPDase1, 2, 3, 5, 6, 5'-NT/CD73, NPP2 and NPP3, but excluded the presence of NPP1 and NTPDase8. By quantitative real-time PCR we identified the NTPDase2 as the enzyme with the highest mRNA expression in rat left ventricle. Moreover, we characterized the biochemical properties and enzyme activities from synaptosomes of the nerve terminal endings (cardiac synaptosomes) of heart left ventricle. In the E-NTPDases study, we observed divalent cation-dependent enzymes that presented optimum pH of 8.0 for ATP and ADP hydrolysis, and 9.5 for AMP hydrolysis. The apparent K_M values are 40 μM, 90 μM and 39 μM and apparent V_{max} values are 537, 219 and 111 nmol Pi released/min/mg of protein for ATP, ADP and AMP hydrolysis, respectively. Oligomycin and sodium azide, both mitochondrial ATPase inhibitors, inhibited only the ATP hydrolysis. AMP hydrolysis was not affected by levamisole and tetramisole, whereas ammonium molybdate practically abolished the ecto-5'-nucleotidase activity. Using *p*-nitrophenyl-5'-thymidine monophosphate (*p*-Nph-5'-TMP) as substrate for E-NPPs in rat cardiac synaptosomes, we observed an alkaline pH dependence, divalent cation dependence and the K_M value corresponded to 91.42 ± 13.97 μM and V_{max} value calculated was 63,79 ± 3,59 nmol *p*-nitrophenol released/min/mg of protein. Levamisole was ineffective as inhibitor of *p*-Nph-5'-TMP hydrolysis in pH 8.9 (optimum pH for the enzyme characterized). Suramin strongly reduced the hydrolysis of *p*-Nph-5'-TMP.

In order to study if these enzymes could participate in the pathophysiology of diabetic cardiovascular diseases, we induced experimental diabetes with an injection of streptozotocin 65mg/kg to study diabetic complications. The STZ injection caused a rise in glucose levels, loss of body weight, lower heart rate (HR) and systolic blood pressure (SBP) when compared to citrate group (control). The 6 days insulin treatment was able to reverse these parameters. There was an inhibition in ATP and ADP, but not in AMP and *p*-Nph-5'-TMP hydrolysis in cardiac synaptosomes and the insulin treatment returned the hydrolysis to the citrate group levels. The analysis of extracellular ATP metabolism in cardiac synaptosomes by HPLC shows that ATP was hydrolyzed slower in diabetes group. Preliminar data from molecular analysis suggest the participação of NTPDases 1 and 2 on nucleotide hydrolysis in cardiac synaptosomes. In order to analyse a readily available biological material, we evaluated the ectonucleotidases activities and the profile of extracellular ATP metabolism in blood serum of streptozotocin-diabetic rats and the influence of glycemic status. The 30 days of STZ injection was associated with a raise in ATP, ADP, AMP, and 5'-TMP hydrolysis in blood serum, when compared to the citrate group. I*n vitro,* 20 mM glucose added did not affect ectonucleotidases activities in normal blood serum of rats with 24 h of pre-incubation. HPLC analysis showed a rapid hydrolysis of extracellular ATP by diabetic animals, decreasing the ATP and ADP levels faster than in serum of control and insulin groups, leading to the formation of high levels of adenosine when compared with citrate and insulin groups.

We showed alterations in the nucleotides hydrolysis in the cardiac synaptosomes and in serum from diabetic rats. The cardiac alteration seems to be related to the ethiology of cardiac problems. However, the serum hydrolysis alterations seems to be related to a vascular protection, with the diminution of ADP (pro-agregant) and an increase in adenosine (antiagregant), found in diabetic serum rats.

LISTA DE ABREVIATURAS

ADA – Associação Americana de Diabetes

ADK - adenosina quinase

Ado - adenosina

AVC - acidente vascular cerebral

ACR – regiões conservadas da apirase

CD39 - antígeno de ativação celular linfóide

CD73 - ecto-5'-nucleotidase

DAC - doença arterial coronariana

DAP - doença arterial periférica

DM - diabetes *mellitus*

EBV - vírus Epstein Barr

Ecto-ADA - ecto-adenosina deaminase

Ecto-ATPDase - ecto-ATP difosfoidrolase

E-NPP - ecto-nucleotídeo pirofosfatase/fosfodiesterase

E-NTPDase - ecto-nucleosídeo trifosfato difosfoidrolase

GPI - glicosil-fosfatidilinositol

HB6 - ATPase tipo E clonada de cérebro humano

IM - infarto do miocárdio

IRC - insuficiência renal crônica

mRNA - RNA mensageiro

NAC - neuropatia autonômica cardiovascular

NAD - nicotinamida adenina dinucleotídeo

NE - norepinefrina

NTPDase – nucleosídeo trifosfato difosfoidrolase

OMS - Organização Mundial da Saúde

P2X - receptor purinérgico ionotrópico

P2Y - receptor purinérgico metabotrópico

p-nitrophenyl-5'-TMP - *p*-nitrofenil-5'-timidina monofosfato

RT-PCR – reção em cadeia da polimerase – transcriptase reversa

SBD - Sociedade Brasileira de Diabetes

SOD - superóxido dismutase

STZ - estreptozotocina

VE - ventrículo esquerdo

WHO – Organização Mundial da Saúde

1. INTRODUÇÃO

1.1 Sistema Purinérgico

1.1.1 Nucleotídeos Extracelulares e seus receptores

ATP e outros nucleotídeos e nucleosídeos são encontrados em todos os sistemas orgânicos animais, onde são capazes de produzir efeitos tanto por mecanismos intracelulares, quanto extracelulares. O ATP intracelular é primordialmente utilizado como molécula energética, em processos ativos como transporte, motilidade celular e biossíntese, enquanto o ATP extracelular tem sido considerado uma importante molécula sinalizadora (Yegutkin, 2008).

O conceito de neurotransmissão purinérgica foi primeiramente proposto por G. Burnstock há mais de três décadas (Burnstock, 1972) e desde então os nucleotídeos extracelulares purínicos (ATP, ADP e o nucleosídeo adenosina) e pirimidínicos (UTP e UDP) são considerados moléculas sinalizadoras importantes. Estas moléculas modulam uma multiplicidade de funções teciduais, incluindo neurotransmissão no sistema nervoso central, contração não-colinégica e não-adrenérgica em músculo liso e interações neurônio-glia (Ralevic & Burnstock, 1998), efeitos inotrópicos, cronotrópicos e arritmogênicos no miocárdio (Vassort, 2001), funções gastrointestinais e hepáticas (Roman & Fitz, 1999), regulação da resposta de células epiteliais (Schwiebert & Zsembery, 2003), regulação do fluxo sangüíneo (Gonzalez-Alonso et al., 2002), resposta imune e inflamação (Bours et al., 2006) e agregação plaquetária em sítios de injúria vascular (Marcus et al., 2003; Gachet, 2006). Em adição à sinalização de eventos agudos, tem sido postulado que as purinas e pirimidinas também apresentam papéis tróficos na

proliferação e crescimento celular (Burnstock, 2006), indução de apoptose e atividade anticâncer (Bours et al., 2006; White & Burnstock, 2006), formação da placa aterosclerótica (Di Virgilio & Soloni, 2002), cicatrização, formação e reabsorção óssea (Hoebertz et al., 2003) e alterações vasculares no diabetes (Solini et al., 2004).

Os efeitos dos nucleotídeos extracelulares são mediados por receptores presentes na superfície das membranas celulares (Figura 1), chamados receptores purinérgicos ou purinoceptores. Os receptores purinérgicos foram definidos pela primeira vez em 1976 (Burnstock, 1976) e dois anos mais tarde foram distinguidos dois tipos de purinoceptores identificados como P1 ou de adenosina e P2, ativados por ATP, ADP, UTP e UDP (Burnstock, 1978; Erlinge & Burnstock, 2008).

Os receptores P1 se subdividem em A_1, A_{2A}, A_{2B} e A_3 e são ativados por adenosina, com o potencial agonista na ordem adenosina > AMP > ADP> ATP. Enquanto os receptores A_1 e A_3 são acoplados a proteína G_i e inibem a adenilato ciclase, ambos os receptores A_{2A} e A_{2B} são acoplados à proteína G_s e estimulam a adenilato ciclase (Ralevic & Burnstock, 1998; Czajkowski & Baranska, 2002).

A classificação dos receptores P2 foi inicialmente baseada em critérios farmacológicos (Burnstock & Kennedy, 1985), e posteriormente reforçada através de técnicas de clonagem e expressão em sistemas heterólogos (Evans et al., 1995). Estes receptores podem ser classificados em duas famílias: receptores P2X e P2Y. Os receptores P2X atuam como canais ionotrópicos ativados por ATP e estão divididos em sete subtipos ($P2X_{1-7}$). Os receptores metabotrópicos P2Y são acoplados a proteínas G e estão divididos nos subtipos $P2Y_1$, $P2Y_2$, $P2Y_4$,

$P2Y_6$, $P2Y_{11}$, $P2Y_{12}$, $P2Y_{13}$ e $P2Y_{14}$ (Ralevic & Burnstock, 1998; Communi et al., 2001; Hollopeter et al., 2001; Erlinge & Burnstock, 2008). Todas as células do sistema cardiovascular expressam um ou mais subtipos de receptores de purinas e os efeitos extracelulares das mesmas, em geral, estão relacionados à cardioproteção (Ralevick & Burnstock, 2003).

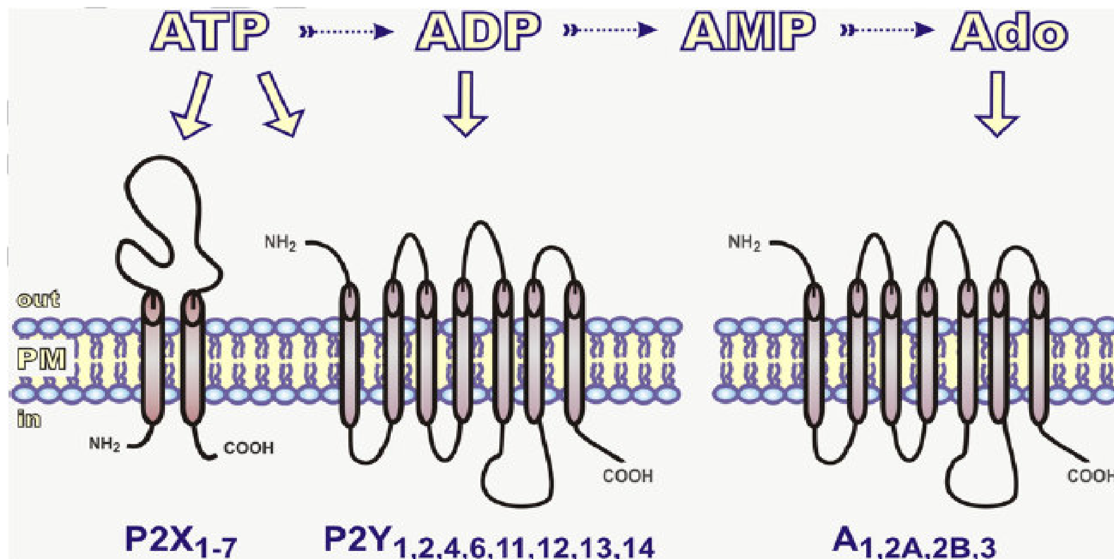

Figura 1. Vias de sinalização purinérgica. Sinalização mediada por nucleotídeos através de receptores ionotrópicos P2X e metabotrópicos P2Y. O nucleosídeo gerado, adenosina, age em quatro receptores próprios acoplados à proteína G. *Yegutkin G. (2008).*

1.1.2 Os nucleotídeos ATP e ADP no sistema cardiovascular

Os nucleotídeos extracelulares, ATP e ADP, têm sido implicados em um grande número de funções fisiológicas (Kunapuli & Daniel, 1998; Ralevick & Burnstock, 1998; Yegutkin, 2008). ATP e ADP podem ser liberados de pelo menos cinco diferentes fontes corporais e subseqüentemente, alcançar órgãos-alvo: 1) o ATP é liberado juntamente com a norepinefrina em neurônios simpáticos; 2) ATP e ADP são estocados nos grânulos densos das plaquetas e são liberados na corrente

sangüínea sob ativação; 3) as células da medula adrenal também liberam nucleotídeos; 4) tanto as células musculares lisas quanto as células endotelias transportam nucleotídeos do citosol através da membrana; e finalmente, 5) o rompimento de células libera nucleotídeos celulares que têm vários efeitos sobre as plaquetas, células endoteliais, leucócitos e células vasculares musculares lisas durante uma injúria vascular (Kunapuli & Daniel, 1998). A liberação de nucleotídeos endógenos representa um componente crítico para o início da cascata de sinalização (Yegutkin, 2008).

Em 1929, Drury and Szent-Györgi descreveram pela primeira vez a ação de nucleotídeos e nucleosídeos de purinas em coração e vasos sangüíneos (Drury & Szent-Györgi, 1929). Desde então, evidências têm sugerido um importante papel do sistema purinérgico na regulação cardiovascular (Menezes de Oliveira et al., 1997; Marcus et al., 2003; Carneiro-Ramos et al., 2004; Oses et al., 2004; Fürstenau et al., 2006; Barreto-Chaves et al., 2006; Bours et al., 2006; Gachet et al., 2006; Tamajusuku et al., 2006; Erlinge & Burnstock, 2008; Pochmann et al., 2008) e consequentemente seu envolvimento em diferentes condições clínicas cardiovasculares (Di Virgílio & Solini, 2002; Rossato et al., 2003; Hoebertz et al., 2003; Erlinge & Burnstock, 2008; Fürstenau et al., 2008).

A demonstração da liberação de ATP em nervos sensoriais, em 1954, foi a primeira evidência da função neurotransmissora do ATP (Holton & Holton, 1954). Posteriormente, Burnstock (1972) demonstrou que além da transmissão colinérgica e noradrenérgica até então conhecidas, existe uma transmissão purinérgica em sistema nervoso autônomo, onde o ATP atua como o principal neurotransmissor (Burnstock, 1972). O ATP liberado como co-transmissor de nervos simpáticos é

capaz de contrair a musculatura vascular lisa via receptores P2X, enquanto o ATP liberado dos nervos motores sensoriais durante a atividade do "arco-reflexo" pode dilatar os vasos via receptores P2Y. Além disso, o ATP liberado das células endoteliais durante mudanças do fluxo sangüíneo (shear stress) ou durante uma hipóxia, é capaz de agir em receptores P2Y nestas células e liberar óxido nítrico (NO), resultando em relaxamento (Burnstock, 2002). O ATP também pode exercer influência sobre o sistema vascular por interferir no processo de agregação plaquetária e promover proliferação de células musculares lisas e células endoteliais (Ralevic & Burnstock, 2003).

Em adição aos seus efeitos pós-sinápticos, o ATP é capaz de afetar a transmissão adrenérgica pela ação nos purinoceptores em terminais nervosos através de modulação pré-sináptica (Burnstock, 1999) (Figura 2). Foi demonstrado que, quando o ATP liga em receptores ionotrópicos P2X em culturas primárias de neurônios ganglionares, ocorre um aumento da exocitose de norepinefrina (NE), enquanto isso quando o ATP se liga a receptores metabotrópicos P2Y, esta liberação é atenuada. Isso sugere que o ATP endógeno age por um mecanismo de "feedback" autócrino em terminais nervosos simpáticos cardíacos a partir do qual ele é liberado (Sesti et al., 2002). Evidências recentes demonstram a ação do ATP como co-transmissor com NE em terminais nervosos simpáticos cardíacos, aumentando a liberação de NE através de um mecanismo de "feedback" positivo (Sesti et al., 2003). Por outro lado, a inibição pré-sináptica da liberação de norepinefrina em nervos simpáticos por ATP e adenosina já foi demonstrada em diversos vasos sangüíneos (Ralevic & Burnstock, 1991).

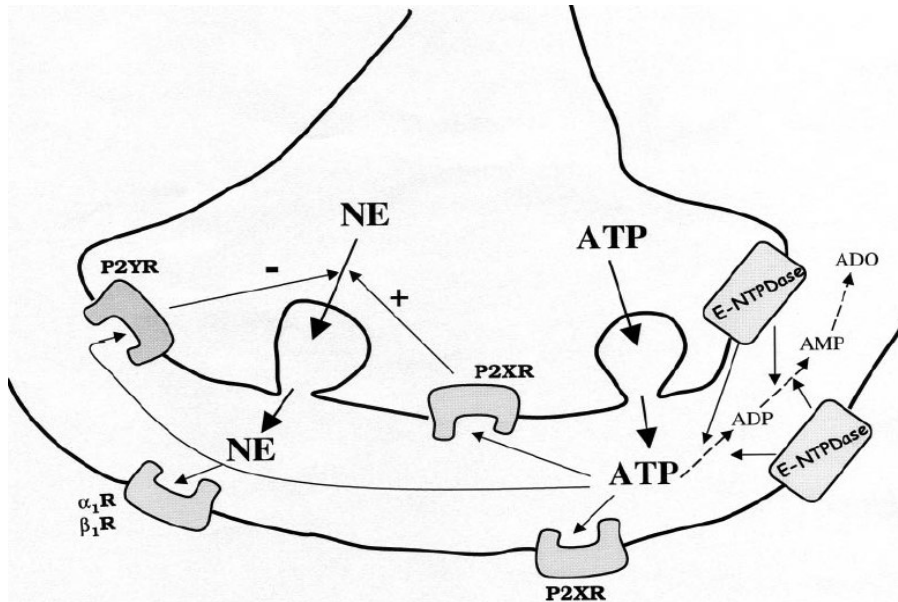

Figura 2. Representação esquemática do papel do sistema purinérgico na modulação da liberação de NE em terminais nervosos simpáticos cardíacos. *Sesti et al., (2002).*

Além dos efeitos na vasculatura cardiovascular e na modulação pré-sináptica da liberação de norepinefrina em terminais nervosos, o ATP apresenta efeitos cronotrópicos, dromotrópicos e inotrópicos em preparações de coração isolado (Rongen et al., 1997). O ATP geralmente é responsável por efeitos inotrópicos positivos e sua rápida aplicação em células induz várias formas de arritmia. Outros possíveis papéis do ATP no coração incluem a hipertrofia, precondicionamento e apoptose (Vassort, 2001).

O principal efeito funcional do ADP é a estimulação da agregação das plaquetas, uma parte importante do papel hemostático (Boarder & Hourani, 1998), sendo o controle dos níveis extracelulares de ADP fundamental para a regulação dos processos trombóticos e/ou hemorrágicos. Em adição, o ADP pode atuar nos receptores $P2Y_1$ das células endoteliais e musculares lisas, causando dilatação

(Ralevick e Burnstock, 2003), sendo que esta também pode ocorrer pela liberação de NO que o ADP é capaz de estimular (Kunapuli & Daniel, 1998).

1.1.3 O nucleosídeo adenosina

A adenosina (Ado), um precursor ou um metabólito dos nucleotídeos da adenina, é uma molécula presente em todas as células do sistema biológico (Hori & Kitakase, 1991). Desde o clássico trabalho de Drury e Szent-Györgi em 1929, no qual as propriedades antiarrítmicas e vasodilatadoras deste nucleosídeo foram pela primeira vez demonstradas, tem se tornado claro que a Ado desempenha não somente um importante papel no metabolismo celular, mas também um importante papel fisiológico no sistema cardiovascular (Mubagwa, et al., 1996; Balas, 2002).

A formação de Ado intracelular pode se dar por duas vias principais que são: a clivagem da S-adenosil-homocisteína pela enzima S-adenosil-homocisteína hidrolase, e pela degradação de AMP por ação de uma 5'-nucleotidase citosólica (Patel & Tudball, 1986). Depois de formada, a Ado pode passar através da membrana plasmática por difusão facilitada, através de transportadores de nucleosídeos. Estes transportadores são bidirecionais e equilibram os níveis intracelulares e extracelulares de Ado (Dunwiddie & Masino, 2001). Além de ser liberada como tal para o meio extracelular, a Ado também pode ser formada no espaço extracelular, através da hidrólise do AMP extracelular por ação de uma ecto-5'-nucleotidase (Zimmermann, 1992; Dunwiddie & Masino, 2001).

Após interagir com receptores específicos, a ação da Ado pode ser finalizada através da enzima ecto-adenosina deaminase (ecto-ADA), ou ainda

através de fosforilação até 5'-AMP catalisada pela enzima adenosina quinase (ADK) (Dunwiddie & Masino, 2001; Yegutkin, 2008).

O coração e os vasos sangüíneos apresentam diversos mecanismos para proteção a estímulos capazes de causar algum tipo de injúria, dentre eles o efeito da Ado via receptores adenosinérgicos ou receptores P1 (A_1, A_2, A_{2B} e A_3) (Willems et al., 2005). Estudos descreveram a vasodilatação coronária mediada por este nucleosídeo durante hipóxia, como uma tentativa de aumentar o fluxo sangüíneo e restaurar o suprimento de oxigênio ao tecido cardíaco (Ralevic & Burnstock, 2003). Sabe-se que a vasodilatação induzida pela Ado é resultado da ativação dos receptores A_2, que são expressos em praticamente todo o sistema vascular de mamíferos (Ralevic & Burnstock, 2003). Recentes trabalhos mostram a diferença entre os efeitos agudos da Ado (Peart & Headrick, 2003; Flood et al., 2003) verso efeitos de pré-condicionamento adenosinérgico (Peart & Headrick, 2003), mostrando múltiplas vias de proteção deste nucleosídeo (Willems et al, 2005). Os efeitos da Ado também envolvem a atenuação pré-sináptica da liberação de norepinefrina em terminais nervosos simpáticos em adição aos seus efeitos pós sinápticos (Richardt et al, 1987). A Ado é capaz de antagonizar os efeitos inotrópicos positivos e arritmogênicos dos agonistas β-adrenérgicos no miocárdio ventricular (Mubagwa et al., 1996). Em termos de alvos celulares, a Ado parece proteger diretamente cardiomiócitos ou tecido miocárdico (provavelmente via receptores A_1 e A_3) (Roscoe et al., 2000) e, além disso, protege por limitar a inflamação em tecido miocárdico e vascular (Vinten-Johansen et al.,1999). A Ado também é capaz de atuar como um inibidor da agregação plaquetária, sendo por

este motivo, também conhecida como uma molécula antitrombogênica (Kitakaze et al., 1991; Kawashima et al., 2000).

Tendo em vista a evidente participação das purinas em diferentes processos envolvidos na funcionalidade cardiovascular normal (Figura 3), deve-se atentar para o fato de que distúrbios ou prejuízos na sinalização purinérgica podem estar relacionados ao desenvolvimento de patologias vasculares. Desta maneira, a manutenção da sinalização purinérgica normal, incluindo desde as moléculas sinalizadoras, até seus receptores e suas enzimas de degradação, têm se mostrado importantes alvos para o tratamento de doenças cardiovasculares (Ralevick & Burnstock, 2003; Erlinge & Burnstock, 2008).

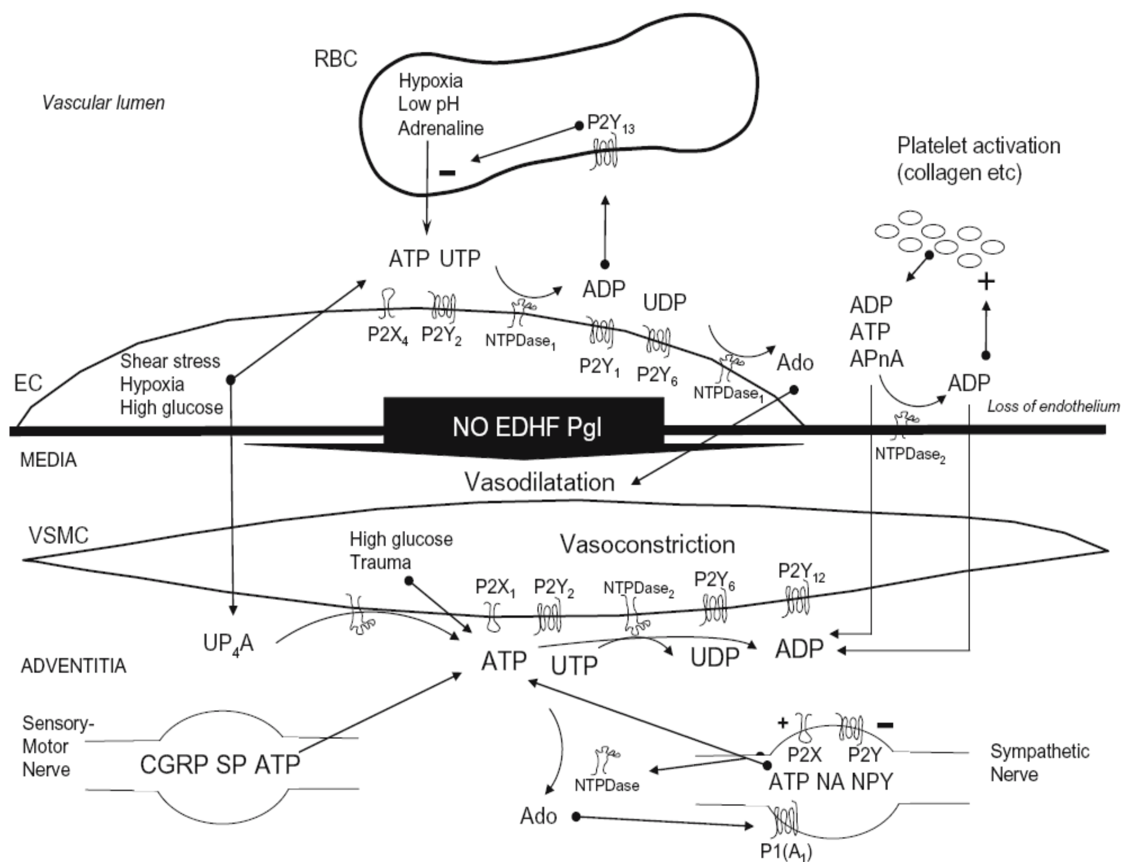

Figura 3. Regulação do sistema purinérgico na circulação. *Erlinge & Burnstock, 2008.*

1.2 A família das Ectonucleotidases

Após a liberação no meio extracelular, os nucleotídeos da adenina exercem seus efeitos através da interação com receptores específicos localizados na membrana celular e posteriormente são metabolizados através da ação de ecto-enzimas que fazem a conversão destes nucleotídeos até Ado. O sítio catalítico destas ecto-enzimas está voltado para o meio extracelular e elas são, em geral, proteínas ligadas à membrana, embora isoformas extracelulares clivadas e solúveis também existam. A atividade catalítica máxima é adaptada ao meio extracelular e requer a presença de cátions divalentes, tais como cálcio ou magnésio e um pH levemente alcalino. Além disso, na maioria dos casos, os valores de K_M estão na faixa de micromolar (Zimmermann, 2001).

Um grande número de trabalhos tem demonstrado que a degradação do ATP extracelular envolve um conjunto de enzimas que constituem a “via das ectonucleotidases”. Estas enzimas revelam não somente uma sobreposição de distribuição tecidual, mas também de especificidades por substratos. Desta forma, os nucleosídeos di e trifosfatados podem ser hidrolisados principalmente por membros das famílias E-NTPDase (ecto-nucleosídeo trifosfato difosfoidrolase), E-NPP (ecto-nucleotídeo pirofosfatase/fosfodiesterase) e fosfatases alcalinas. Os nucleosídeos monofosfatados estão sujeitos à hidrólise pela ecto-5’-nucleotidase e pelas fosfatases alcalinas (Zimmermann, 2001; Robson et al., 2006). Esta via pode resultar na inativação da sinalização mediada pelo ATP via receptores P2 e contribuir para a sinalização mediada pela Ado, através dos receptores P1 (Richardson et al., 1987; Sebastião et al., 1999). Desta forma, as ectonucleotidases constituem um eficiente

mecanismo de controle dos níveis de nucleotídeos e nucleosídeos no espaço extracelular (Zimmermann, 1996; Zimmermann, 2001).

1.2.1 Ecto-nucleosídeo trifosfato difosfoidrolases (E-NTPDases)

A presença de enzimas que hidrolisam especificamente nucleotídeos na superfície de muitos tipos celulares tem sido intensamente reportada. Na literatura mais antiga, informações sobre estas enzimas podem ser encontradas sob diferentes nomes, incluindo ecto-ATPase, ATP difosfoidrolase, apirase, ATPDase, nucleosídeo difosfatase, etc (Plesner, 1995; Zimmermann, 1996). Atualmente, baseado em sua estrutura e propriedades catalíticas, particularmente na relação de hidrólise ATP/ADP, a família das NTPDases em mamíferos está constituída de oito membros clonados e funcionalmente caracterizados: NTPDase 1 (CD39), NTPDase 2 (CD39L1), NTPDase 3 (CD39L3, HB6), NTPDase 4 (UDPase), NTPDase 5 (CD39L4) e NTPDase 6 (CD39L2), NTPDase 7 e NTPDase 8 (Figura 4).

Como características comuns, estas enzimas são capazes de hidrolisar nucleosídeos tri e ou difosfatos, mas não os monofosfatos, necessitam concentrações milimolares de cátions como Ca^{2+} e Mg^{2+} para atingirem a atividade máxima e são insensíveis a inibidores específicos de ATPases do tipo P (ex. $Na^{+}K^{+}$ATPase), tipo V (ex. bomba de prótons vacuolar) e tipo F (ex. ATPase mitocondrial) (Zimmermann et al., 1998;Yegutkin, 2008). Além disso, após a análise das sequências de diversas ecto-ATPDases e ecto-ATPases, foi demonstrado que estas enzimas compartilham cinco regiões altamente conservadas chamadas de “regiões conservadas da apirase”, ACR (ACR 1 - 5 apyrase conserved regions) (Handa & Guidotti, 1996; Schulte et al., 1999). A

existência desses sítios conservados pode estar relacionada com a formação do sítio catalítico das enzimas e/ou com a integridade estrutural das E-NTPDases (Grinthal & Guidotti, 2000).

Dentre os oito tipos de genes clonados e caracterizados, as NTPDases 1, 2, 3 e 8 estão localizadas na superfície celular com sítios catalíticos voltados para o meio extracelular, as NTPDases 5 e 6 apresentam localização intracelular e sofrem secreção depois de expressão heteróloga. Já as NTPDases 4 e 7 têm localização totalmente intracelular com o sítio ativo voltado para o lúmem das organelas intracelulares (Zimmermann, 2001; Robson et al., 2006)(Figura 4).

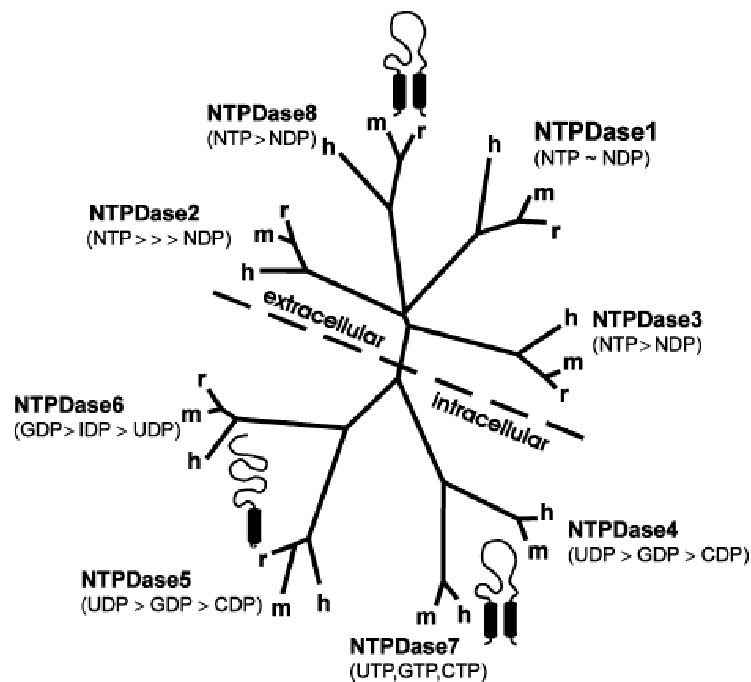

Figura 4: Árvore filogenética hipotética para os membros da família das NTPDases (NTPDase 1 a 8) de rato (r), humano (h) e camundongo (m). O comprimento das linhas indica as diferenças entre as seqüências de aminoácidos. O gráfico representa a separação entre NTPDases localizadas na superfície (superior) e intracelular (inferior). A preferência por substrato para cada subtipo e a topografia na membrana para cada grupo de enzimas está também representada (um ou dois domínios transmembrana, indicados por cilindros). *Robson et al. 2006.*

Os subtipos de NTPDases, além de diferirem na localização celular, apresentam diferentes propriedades funcionais. Enquanto todos os membros da família catalisam a hidrólise de ambos nucleosídeos trifosfato (NTP) e nucleosídeos difosfatos (NDP), as razões de hidrólise (NTP:NDP) variam significativamente para essas reações, resultando em enzimas que hidrolisam preferencialmente NTPs (NTPDase2), preferencialmente NDPs (NTPDase 5 e 6) ou ambos nucleotídeos (NTPDase 1, 3 e 8) (Zimmermann, 2001; Grinthal & Guidotti, 2002; Robson et al., 2006) (Figura 4). Diferenças na seqüência, mas também nas estruturas secundárias, terciárias e quaternárias podem estar relacionadas às diferenças nas propriedades catalíticas encontradas entre os diferentes tipos de NTPDases (Heine, 2001; Grinthal e Guidotti, 2004).

A NTPDase1, que apresenta a mesma preferência pela hidrólise do ATP e do ADP (NTP/NDP 1:1), tem sido a mais estudada dos membros da família da E-NTPDases. O nome ATP difosfoidrolase ou apirase (EC 3.6.1.5), foi primeiramente proposto por Meyerhof em 1945, como uma designação geral para enzimas que hidrolisam todos os di- e trifosfonucleosídeos até seus respectivos nucleosídeos monofosfatos e fosfato inorgânico (Pi), liberando 2 mol de Pi por mol de nucleosídeo trifosfatado e 1 mol de Pi por nucleosídeo difosfatado. Então, a partir de 1945, mas principalmente nas duas últimas décadas, ATP difosfoidrolases foram descritas em diferentes fontes. Apirases foram descritas em tecidos vegetais (Krishnan, 1949; Valenzuela et al., 1989), insetos (Ribeiro et al., 1989; Sarkis et al, 1996) aves (Carl e Kirley, 1997) e tecidos de mamíferos, como preparações em sistema nervoso central (SNC) e periférico (Battastini et al., 1991; Sarkis & Saltò, 1991), aorta bovina (Cote et al, 1992), secreções seminais

(Rosenberg et al, 1988), vasos umbilicais humanos (Yagi et al., 1992), pulmão bovino (Picher et al., 1993), plaquetas e soro de ratos (Frasseto et al, 1993; Oses et al., 2004), plaquetas humanas (Pilla et al., 1996), células neoplásicas humanas (Dzhandzhugazyan et al., 1998), células de sertoli de ratos (Casali et al., 2001), linfócitos humanos (Leal et al., 2005), glândulas submandibulares de ratos (Henz et al., 2006), células de tumor de Walker 256 (Buffon et al., 2007), dentre outros.

Em 1994, Malisweski e colaboradores identificaram molecularmente o primeiro membro da família E-NTPDase. Um protótipo da enzima foi clonado, seqüenciado e identificado como um antígeno de ativação celular, CD39, principalmente expressa em linfócitos ativados (Malisweski et al., 1994) e inicialmente descrita como proteína marcadora de células B transformadas pelo vírus Epstein Barr (EBV) (Wang e Guidotti, 1996). Experimentos subseqüentes com apirases solúveis e clonadas de batata (Handa e Guidotti, 1996) e de diferentes tecidos de mamíferos (Kaczmarek et al., 1996) confirmaram a homologia desta enzima ao CD39 humano.

A NTPDase 1 tem sido extensivamente estudada em células endoteliais e em plaquetas, possuindo um papel bem descrito na regulação do fluxo sanguíneo e trombogênese. A enzima em associação com a ecto-5’-nucleotidase presente na superfície das células endoteliais e plaquetas converte o ADP, pró-agregante plaquetário, em adenosina, anti-agregante, limitando a extensão da agregação plaquetária intravascular (Frassetto et al., 1993; Pilla et al., 1996; Kaczmarek et al., 1996; Marcus et al., 1997; Imai et al., 1999; Koziak et al., 1999). Em concordância, NTPDase1 solúvel recombinante, bloqueia a agregação plaquetária induzida *in vitro* por ADP (Gayle et al, 1998).

Estudos prévios demonstraram que ratos deficientes de CD39/ATP difosfoidrolase apresentam problemas relacionados com hemostasia e trombogênese (Enjyoji et al., 1999). Além disso, nos terminais nervosos simpáticos, onde NE e ATP são co-liberados, NTPDase1 tem sido associada à degradação do ATP até adenosina, posssuindo um possível papel na modulação da exocitose de NE, via receptores P2X ou P2Y. A liberação excessiva de NE é a principal causa de arritmias e disfunção vascular coronariana na isquemia miocárdica (Benedict et al., 1996; Machida et al., 2005). Assim, formas solúveis e ligadas à membrana da NTPDase1/CD39 são potenciais agentes terapêuticos para a inibição de processos trombogênicos (Gayle et al., 1998) e arrítmicos (Machida et al, 2005) e podem representar uma nova geração de moléculas cardioprotetoras (Marcus et al., 2005).

Diferentemente da NTPDase1 e dos outros dois membros relacionados, NTPDases3 e 8, a característica que mais distingue a NTPDase2 é a sua clara preferência pelos nucleosídeos trifosfatados. A enzima hidrolisa os nucleosídeos difosfatados apenas marginalmente, tendo uma preferência de até 30 vezes pelo ATP em relação ao ADP como substrato (Kegel et al., 1997).

A NTPDase2 é particularmente associada com eventos na superfície das adventícias de vasos da musculatura onde, em contraste à NTPDase1, a NTPDase2 vascular pode promover a agregação plaquetária pela presença de ADP (Sevigny et al., 2002). Também é descrita em cultura de astrócitos, células de Schwan e outras células glias do sistema nervoso central e periférico (Wink et al., 2006; Langer et al., 2007). As propriedades bioquímicas e a localização específica da NTPDase2 sugerem que a enzima possa regular funções cardíacas

como a regulação da ativação de receptores P2X ou P2Y, sendo capaz de promover a degradação do ATP, que exerce efeitos inotrópicos e cronotrópicos, além de poder participar da modulação da liberação de NE (Sevigny et al., 2002, Marcus et al., 2005).

A NTPDase3 (CD39L3) é considerada um intermediário funcional entre a NTPDase1 e a NTPDase2, pois hidrolisa o ATP e o ADP em uma razão de 3:1. Muito do conhecimento sobre a NTPDase3 está relacionado a sua estrutura, entretanto muito pouco é sabido a respeito do seu papel fisiológico, bem como sua distribuição na natureza. A expressão da NTPDase3 tem sido principalmente associada com estruturas neuronais no cérebro, onde pode agir como regulador dos níveis de ATP na pré-sinapse, além de coordenar muitos eventos homeostáticos (Belcher et al, 2006). A sua exata contribuição e relação com NTPDase 1, 2 e 8 permanece a ser elucidada nos diversos tecidos onde há sobreposição de suas funções.

As NTPDases4 (UDPases), embora possuam a mesma estrutura geral das NTPDases1 a 3, diferem principalmente em relação à localização celular. NTPDases4 de humanos estão localizadas no complexo de Golgi (NTPDase4β) ou em vacúolos lisossomais (NTPDase4α) (Wang & Guidotti, 1998). Ambas enzimas hidrolisam nucleosídeos di e trifosfatados, mas possuem uma baixa preferência por ATP e ADP. Elas diferem em sua preferência por nucleotídeos e também na dependência de cátions divalentes. A NTPDase4α tem alta preferência por UTP e TTP, enquanto que CTP e UDP são os melhores substratos da NTPDase 4β.

Já as NTPDases5 (CD39L4) e NTPDases6 (CD39L2) encontram-se ancoradas na membrana celular somente pela porção NH_2 terminal e possuem uma larga região COOH terminal extracelular. Ambas enzimas são ativadas por cátions divalentes e apresentam uma maior preferência por nucleotídeos difosfatados. A NTPDase5 tem maior preferência para hidrolisar principalmente GDP e UDP, enquanto a NTPDase6 hidrolisa preferencialmente GDP e IDP. Acredita-se que estas enzimas sejam liberadas da membrana e então secretadas para o meio extracelular, indicando tratar-se de enzimas na forma solúvel.

A NTPDase5 está localizada no retículo endoplasmático, enquanto a NTPDase6, encontra-se no complexo de Golgi (Zimmermann, 2001). A identificação e caracterização bioquímica da NTPDase5 (Mulero et al., 2000) não permitiu ainda um completo entendimento da função fisiológica da CD39L4. Devido a sua clara preferência por nucleosídeos difosfatados, acredita-se que o seu papel fisiológico seja reduzir os níveis circulantes do ADP e não do ATP.

Já a NTPDase6, embora a expressão tenha sido demonstrada em tecidos de diversos órgãos (cérebro, fígado, rim, pulmão, placenta, músculo esquelético, pâncreas, etc) (Chadwick & Frischauf, 1998), é consenso que a sua maior expressão é em coração, principalmente em células do músculo cardíaco e em células endoteliais capilares (Hicks-Berger et al., 2000; Braun et al., 2000, Yeung et al., 2000).

As NTPDases7 e 8 preferem como substratos nucleosídeos trifosfatados. Entretanto, a NTPDase7 está localizada em vesículas intracelulares, enquanto que a NTPDase8 foi a última a ser clonada, caracterizada e descrita como uma ecto-enzima de membrana expressa em fígado, rins e intestino de camundongos com

uma razão de hidrólise de aproximadamente 2:1 (Bigonnesse et al., 2004; Zimmermann, 2001). Recentemente, uma NTPDase8 também foi clonada e caracterizada em fígado humano, e uma forma solúvel desta enzima foi gerada por expressão de seu domínio extracelular em células embrionárias de rim humano (HEK293) (Knowles & Li, 2006). A expressão desta enzima também foi encontrada em canalículos hepáticos, sendo sugerido um papel regulatório na secreção biliar e/ou no salvamento de nucleosídeos (Fausther et al., 2007). Assim, os dados encontrados na literatura, até então, sugerem um forte papel desta enzima principalmente em eventos relacionados ao sistema hepático.

1.2.2 Ecto-nucleotídeo pirofosfatases/fosfodiesterases (E-NPPs)

A família das E-NPPs consiste em sete membros estruturalmente relacionados (NPP1 a NPP7) que foram numerados de acordo com a sua ordem de descobrimento. Os membros desta família multigênica possuem uma ampla especificidade de substratos e são capazes de hidrolisar ligações pirofosfato e fosfodiester em (di) nucleotídeos, ácidos nucléicos, nucleotídeos açúcares, assim como ésteres de fosfato de colina e lisofosfolipídeos (Stefan et al., 2005; Yegutkin, 2008). O *p*-nitrofenil-5'-timidina-monofosfato (*p*-nitrophenyl-5'-TMP) tem sido usado como um substrato artificial, específico para as E-NPPs (Sakura et al., 1998).

Os membros da família E-NPPs possuem uma ampla distribuição tecidual e incluem a NPP1(PC-1), NPP2 (PD-Iα, autotaxina), NPP3 (PD-Iβ, B10, gp130^{RB13-6}), NPP4, NPP5, NPP6 e NPP7 (esfingomielinase alcalina). Exceto para a NPP2,

que é secretada no meio extracelular, todos os demais membros são ligados à membrana por um único domíno transmembrana N-terminal e apresentam um domínio para clivagem proteolítica, sugerindo que possam ocorrer como enzimas solúveis (Zimmermann, 2001; Stefan et al., 2005; Stefan et al., 2006). No entanto, somente as NPP1-NPP3, que possuem um ancestral comum, têm sido responsabilizadas pela hidrólise de nucleotídeos (Stefan et al., 2006).

NPP1-NPP3, sozinhas ou combinadas, têm sido expressas em vários tipos celulares estudados (Bollen et al., 2000), apesar de isoformas individuais estarem confinadas a subestruturas e/ou tipos celulares específicos (Narita et al., 1994; Harahap & Goding, 1988; Johnson et al., 2001; Blass-Kampmann et al., 1997; Goding et al., 2003). NPP1 foi originalmente descoberta na superfície de linfócitos B de camundongo como um antígeno de diferenciação celular plasmático (PC-1) (Takahashi et al., 1970). Em humanos é altamente expressa em ossos e cartilagens, tendo expressão intermediária em coração, fígado, placenta e testículos (Goding et al., 2003). Camundongos deficientes em NPP1 revelaram uma produção excessiva de tecido ósseo, sugerindo que esta enzima desempenha um papel essencial no controle da mineralização óssea, pela produção de PP_i (Stefan et al., 2005; Stefan et al., 2006). Além disso, esta enzima também está presente nos rins, ductos de glândulas salivares, cérebro e epidídimo (Harahap et al., 1988; Yano et al., 1985). Stefan e colaboradores, em 1999, verificaram a presença de um híbrido NPPγ-cDna em tecidos de ratos, que estavam principalmente presentes em fígado, rins e coração. Em muitos estudos, a NPP1 também tem sido

correlacionada com a sinalização da insulina e na etiologia da resistência à insulina (Gijsbers et al., 2003; Goding et al., 2003; Stefan et al., 2005).

A NPP2 foi descoberta como um fator de motilidade autócrino (autotaxina, NPP2α) (Stracke et al., 1992) e é expressa em diversos tipos celulares (Bollen et al., 2000; Bächner et al., 1999), o que indica que esta enzima possui capacidades multifuncionais (Stefan et al., 2005). A NPP2 é uma proteína secretada que se acumula em fluidos corporais, tais como plasma e fluido cerebroespinhal. Esta enzima tem sido correlacionada com estímulo da proliferação, contração e migração celular (Stefan et al., 2005; Stefan et al., 2006), além de participar da formação de vasos sanguíneos e progressão do câncer (Koike et al., 2006). Alguns estudos demonstram a expressão desta enzima em cérebro, pulmão, duodeno e glândulas adrenais (Stefan et al., 1999) e existem evidências para a sua presença também em coração humano (Bollen et al., 2000).

NPP3 ($gp130^{RB13-6}$ or B10) foi inicialmente reconhecida pelo anticorpo monoclonal RB13-6 como uma glicoproteína de células precurssoras gliais de cérebro de ratos (Deissler et al., 1995; Deissler et al., 1999). A NPP3 possui um papel importante em alergias, sendo definida como um marcador de ambos basófilos e mastócitos (Stefan et al., 2005). Foram encontrados poucos transcritos de NPPβ (NPP3) em cérebro e glândulas adrenais de ratos, mas abundantes em fígado, rins e coração (Stefan et al., 1999).

Muitas vezes se torna difícil a distinção entre os membros das famílias das E-NPPs e E-NTPDases , devido sua co-expressão em tecidos de mamíferos e por possuírem similaridades nas suas especificidades por substratos. O que se sabe é

que muitas vezes estas enzimas trabalham em conjunto ou consecutivamente, como já foi proposto em trabalhos do nosso grupo (Fürstenau et al., 2006; Henz et al., 2007; Cognato et al., 2008) (Figura 5).

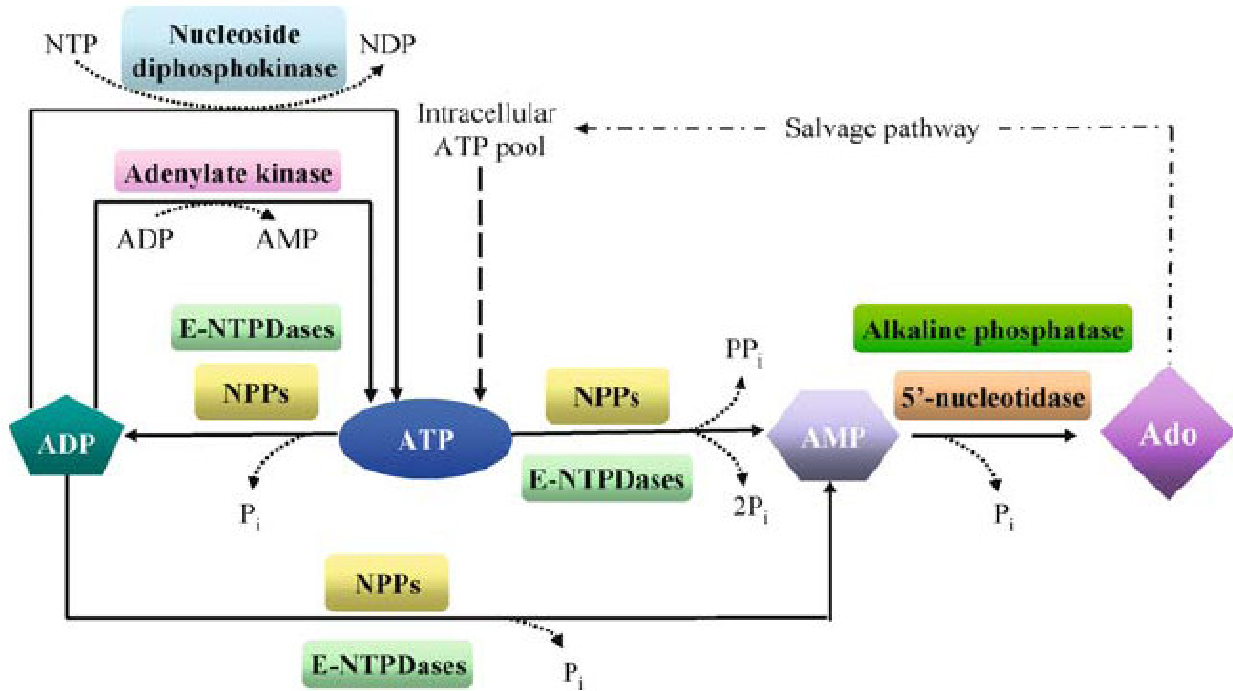

Figura 5: E-NPPs participando da rede de metabolismo de nucleotídeos extracelulares. A concentração de nucleotídeos no meio extracelular é resultado da liberação de nucleotídeos a partir de células, síntese por nucleosídeos difosfocinases e adenilato cinases e hidrólise por ectonucleotidases. *Stefan et al., 2006.*

1.2.3 Ecto- 5'-nucleotidase

A ecto-5'-nucleotidase ("lymphocyte surface protein CD73") também participa do metabolismo dos nucleotídeos da adenina, atuando em conjunto com as E-NTPDases ou E-NPPs (Figura 5). A ecto-5'-nucleotidase é uma enzima ancorada à membrana plasmática por glicosil-fosfatidilinositol (GPI), que representa um marcador de maturação para os linfócitos T e B, sendo ausente nas

células imaturas (Airas et al., 1997). O ancoramento da enzima pode ser clivado por uma fosfolipase C específica para GPI, dando origem às formas solúveis da enzima (Zimmermann, 1992). A ecto-5'-nucleotidase pode exercer uma ampla variedade de funções dependendo de sua expressão tecidual e celular. Ela encontra-se presente na maioria dos tecidos e sua principal função é a hidrólise de nucleosídeos monofosfatados extracelulares, tais como AMP, GMP ou UMP, a seus respectivos nucleosídeos (Sträter, 2006). Sua presença tem sido descrita em cólon, rins, cérebro, fígado, pulmão e coração (Zimmermann, 1992; Zimmermann, 1996; Moriwaki et al., 1999). Na vasculatura, a ecto-5'-nucleotidase está predominantemente associada com o endotélio vascular de grandes vasos como a aorta, carótida e artéria coronária (Koszalka et al., 2004). O principal papel fisiológico atribuído a ecto-5'-nucleotidase, é a formação de Ado a partir do AMP extracelular e a subsequente ativação dos receptores P1, que em sistema nervoso, resulta principalmente na inibição da liberação de neurotransmissores excitatórios (Brundege & Dunwidie, 1997), enquanto que em sistema vascular, resulta em vasodilatação e na inibição da agregação plaquetária (Kawashima et al., 2000).

1.3 Diabetes *mellitus*

1.3.1 Conceito e epidemiologia

Diabetes *mellitus* (DM) representa um grupo de doenças de etiologia heterogênea, caracterizada por uma hiperglicemia crônica e outras anormalidades

metabólicas, em decorrência a uma deficiente ação da insulina. Esta deficiência, a base comum do diabetes, leva a anormalidades características no metabolismo de carboidratos, lipídeos, proteínas e outros (Kuzuya et al., 2002). Em longo prazo, esta desorganização metabólica pode evoluir para complicações micro e macrovasculares e também neuropáticas (Kuzuya et al., 2002; Schmid et al., 2003; De Lorenzo et al., 2006).

A classificação etiológica do diabetes e desordens na glicemia incluem diabetes tipo 1, diabetes tipo 2, desordens em mecanismos específicos ou relacionados à doenças e diabetes gestacional (Kuzuya et al., 2002). Os mais comuns são o DM tipo 1 e DM tipo 2. O DM tipo 1 se aplica à doença caracterizada por destruição das células β do pâncreas, com deficiência grave na secreção de insulina, sendo 95 % dos casos causados por auto-imunidade e 5 % idiopáticos. Os pacientes geralmente são propensos à cetoacidose e requerem tratamento com insulina. O DM tipo 2 é a forma mais prevalente (90 %) e é uma doença heterogênea, relacionando-se mais frequentemente a defeitos na ação da insulina e secundariamente, à disfunção das células β (Schmid, 2003).

Segundo a Organização Mundial de Saúde (OMS), o diabetes é uma doença que está devastando a humanidade e que tem impactos sociais e econômicos. Hoje mais de 250 milhões de pessoas no mundo todo convivem com a doença e a cada ano mais 7 milhões de pessoas desenvolvem o diabetes *mellitus*. A previsão da OMS é de que em 2030, aproximadamente 370 milhões de pessoas no mundo apresentem a doença (Figura 6).

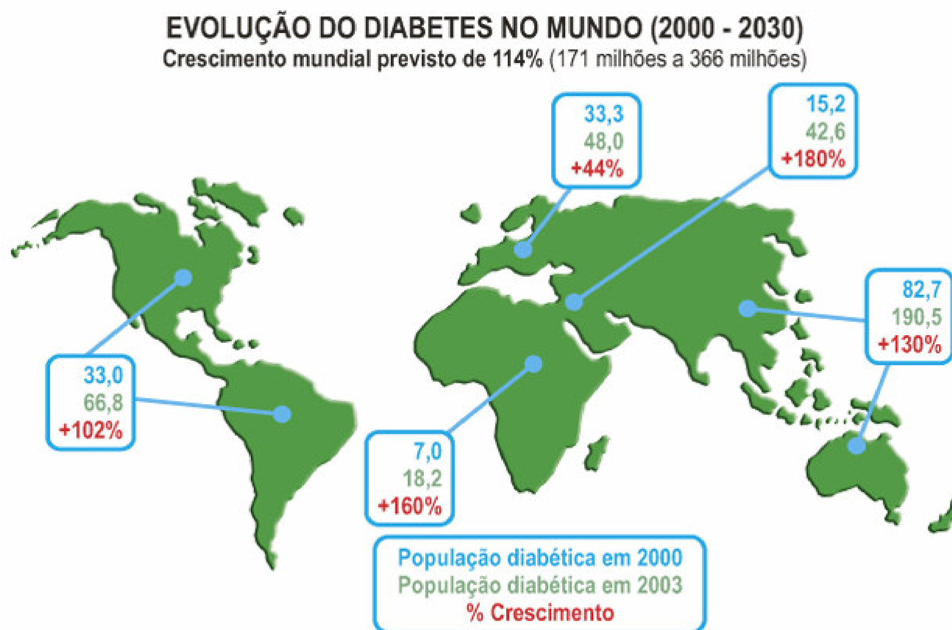

Figura 6: Estimativa da evolução do número de portadores de diabetes no período de 2000 a 2030, em nível mundial e regional. O crescimento previsto é de 114% em 2030, em relação à população diabética em 2000 (OMS). *http://www.diabetesebook.org.br/capitulo/aspectos-epidemiologicos-do-diabetes-mellitus-e-seu-impacto-no-individuo-e-na-sociedade/, 2008.*

A prevalência da doença em indivíduos brasileiros entre 30 e 64 anos de idade é de 7,0% e 8,9%, em homens e mulheres, respectivamente. No Brasil atualmente existem mais de 4,5 milhões de pacientes diabéticos e estima-se que em 30 anos este número chegue a mais de 11 milhões de pacientes (WHO Database, 2008).

As complicações crônicas micro e macrovasculares que podem acompanhar o DM estão associadas a elevada morbidade e mortalidade. As doenças cardiovasculares (DCV) são responsáveis por mais de 50 % da morte de pacientes diabéticos e também por 30 % das internações em centros de tratamento intensivo. A nefropatia diabética acomete mais de um terço dos pacientes e é a causa mais

comum da doença renal terminal e por ingressos em programas de hemodiálise (Sociedade Brasileira de Diabetes, 1999). Mais de 70 % de todas as amputações de pés e membros inferiores estão relacionadas ao diabetes *mellitus* e em algumas regiões, estes níveis podem chegar a 90 % (Schmid et al., 2003). O tratamento destas complicações consome parte significativa do orçamento destinado à assistência médica (Silvestre, 1997). Além disso, estima-se 4 milhões de óbitos anuais mundiais relacionados a presença desta doença, com importante contribuição de complicações cardiovasculares (Diretrizes Sociedade Brasileira de Diabetes, 2007).

1.3.3 Diabetes *mellitus* e alterações cardiovasculares

Embora o diabetes seja um distúrbio metabólico, atualmente também é considerado um distúrbio vascular. Trata-se, portanto, de uma síndrome dismetabólica cardiovascular (Fagan & Deedwania, 1998).

A doeça arterial coronariana (DAC) é uma importante causa de óbito nos EUA e o diabetes ocupa lugar de destaque, visto que em 25 % dos pacientes diabéticos, a primeira manifestação da DAC é o infarto agudo do miocárdio (IM) ou a morte súbita. Quando ocorre o primeiro infarto, a sobrevida de pacientes diabéticos é reduzida (Mazzone, 2004). Indivíduos com DM tipo 1 ou 2 têm risco aumentado, de duas a quatro vezes, para DAC, acidente vascular cerebral (AVC) e doença arterial periférica (DAP) (Colwell & Nesto, 2003). Os vários sistemas que mantêm a homeostase, decorrente de um equilíbrio normal, assegurando uma delicada estabilidade entre fatores pró-trombóticos e mecanismos fibrinolíticos, rompem-se no

diabetes, atingindo a integridade e funcionalidade dos vasos, favorecendo um acentuado estado pró-trombótico e levando a trombose vascular (Mudaliar, 2004).

Uma outra importante complicação crônica do diabetes são as chamadas neuropatias, dentre elas a neuropatia autonômica cardiovascular (NAC). A NAC ocorre quando há lesão das fibras autonômicas periféricas (simpáticas e parassimpáticas), relacionadas ao sistema cardiovascular, resultando em distúrbios na regulação neuro-humoral (Rolim et al., 2008). Essa complicação, com prevalência média de 30 % dos pacientes, pode levar a taquicardia de repouso, hipotensão ortostática grave, síncope, intolerância ao exercício físico (por bloqueio das respostas cronotrópicas e inotrópicas), isquemia e infarto do miocárdio assintomáticos, disfunção sistólica e diastólica do ventrículo esquerdo (VE) e riscos aumentados de insuficiência renal crônica (IRC), AVC e morte súbita de origem cardíaca (Schmid, 2007; Rolim et al., 2008).

1.4 Diabetes *mellitus* e o sistema purinérgico

O entendimento da patogênese e a prevenção e/ou retardamento das complicações associadas ao diabetes, tem sido o maior objetivo das pesquisas em DM nas últimas décadas. Na literatura atual existem alguns trabalhos correlacionando o diabetes com o sistema purinérgico, desde o seu desenvolvimento, até as patologias relacionadas, dentre elas as alterações a nível cardiovascular.

Em 2000, foi descrito que alterações nos níveis de Ado poderiam estar envolvidas em complicações renais, cardíacas e hepáticas em ratos diabéticos por

estreptozotocina, devido a diminuição da expressão da Ado quinase nestes tecidos (Pawelczyk et al, 2000). O mesmo grupo, dois anos depois, demonstrou que o tratamento com insulina nestes ratos foi capaz de restaurar a expressão da enzima nos mesmos tecidos estudados (Sakowicz & Pawelczyk, 2002). Este grupo também foi o responsável por demonstrar que existe uma alteração na expressão dos receptores de Ado no coração de ratos diabéticos e que esta alteração poderia estar envolvida na patogênese do desenvolvimento da cardiomiopatia diabética (Grden et al., 2005).

Já Lunkes e colaboradores (2003) demonstraram que existe uma alteração na hidrólise de nucleotídeos em plaquetas de pacientes com DM tipo 2 e um ano depois, o mesmo grupo demonstrou que estas alterações também ocorriam em ratos diabéticos por aloxano, tanto em plaquetas quanto em sinaptossoma de sistema nervoso central (Lunkes et al., 2004). Recentemente foi demonstrado que o aumento na atividade de hidrólise de ATP e ADP encontrado em plaquetas de pacientes diabéticos seria em função de uma maior expressão da CD39 (NTPDase1) (Lunkes et al., 2008; Schetinger et al., 2008).

Também em fibroblastos, elementos estruturais chaves das paredes arteriais, foi demonstrado um aumento da atividade de receptores $P2X_7$ em pacientes com DM tipo 2 e este seria um possível mecanismo de dano vascular causado pelo diabetes (Solini et al., 2004).

Tem sido reportado que alterações em fosfodiesterases occorem em doenças cardiovasculares associadas ao diabetes (Nagaoka et al., 1998; Goding et al., 2003). Nagaoka e colaboradores (1998) mostraram um aumento na atividade da NPP3 em aorta de ratos resistentes à insulina propensos à

aterosclerose e esse aumento se relacionou positivamente ao aumento dos níveis de mRNA da NPP3. Outros estudos têm demonstrado a correlação entre o aumento da expressão da NPP1 e a resistência à insulina. Foi visto que pacientes portadores de DM tipo 2 tiveram elevação tanto nos níveis de proteína, quanto de atividade enzimática para a NPP1 (Maddux et al., 1995; Maddux et al., 2000). No entanto, alguns trabalhos publicados na literatura, mostram o contrário. Whitehead e colaboradores (1997) demonstraram que pacientes com lesões genéticas nos receptores de insulina apresentaram significante redução na expressão da NPP1 em fibroblastos.

Tendo em vista essa diversidade de trabalhos que sugerem uma possível participação do sistema purinérgico na patofisiologia do diabetes, torna-se extremamente interessante elucidar as possíveis relações entre o sistema purinérgico e as patologias associadas ao diabetes, como as decorrentes do sistema cardiovascular, tão freqüentes nos pacientes portadores desta doença. Assim, o uso do modelo de estreptozotocina tem sido utilizado por diversos grupos para estudar os mais diferentes efeitos do diabetes (De Angelis et al., 2002; Schaan et al., 2004; Howarth et al., 2005; Howarth et al., 2006; Lin et al., 2008). A estreptozotocina (STZ), que atualmente é chamada de estreptozocina nos Estados Unidos, é uma nitrosurea isolada, derivada do *Streptomyces griseus,* que, ao ser administrada em animais sadios, causa grave deficiência na liberação da insulina pancreática. Pode ser aplicada numa dose única, variando de 50 a 100 mg/Kg de peso corporal ou pode ser administrada em múltiplas pequenas doses (Yamamoto et al., 1981).

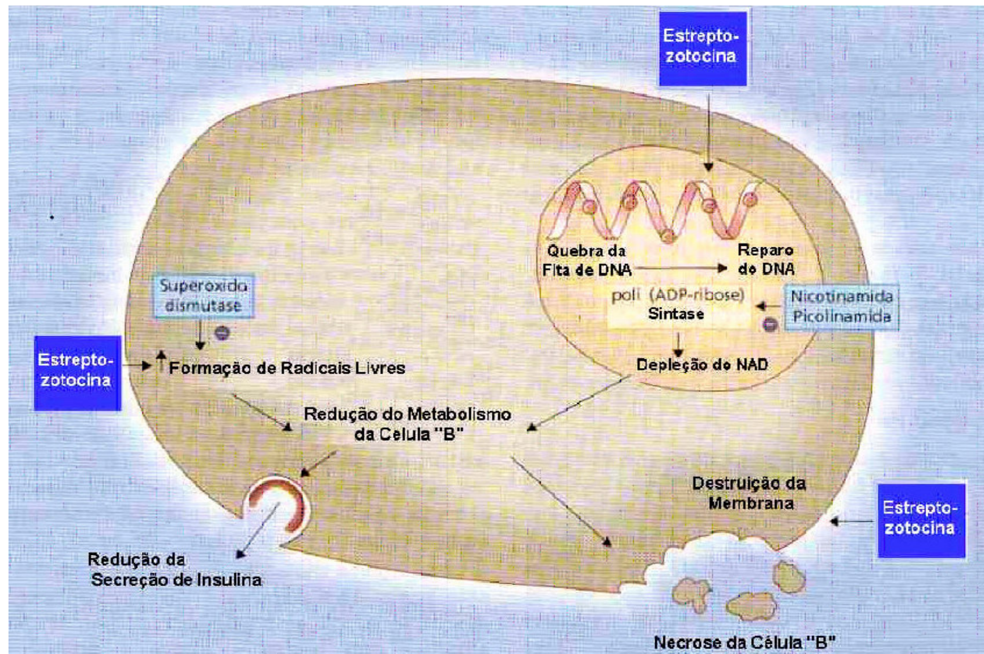

Figura 7: Mecanismo de ação da estreptozotocina no desenvolvimento do diabetes. *Pickup & Williams, 1997.*

A injeção da STZ em ratos leva ao desenvolvimento de uma síndrome clínica caracterizada por hiperglicemia, poliúria e perda de peso, o que é similar ao diabetes humano tipo I (Schaan et al., 2004). Além disso, este modelo desenvolve complicações crônicas microvasculares, como nefropatia, além de causar neuropatia autonômica e periférica, também semelhante ao que ocorre em humanos (De Angelis et al., 2002; Schaan et al., 2004).

2. OBJETIVOS

Este trabalho está apresentado na forma de capítulos constituídos por artigos científicos publicados e/ou em preparação, que visaram cumprir os seguintes objetivos:

Capítulo I – Identificar molecularmente a presença dos membros das famílias das E-NTPDases e ecto-5'-nucleotidase em ventrículo esquerdo de ratos adultos, além de caracterizar as propriedades bioquímicas destas enzimas em sinaptossomas de terminal nervoso cardíaco, para um melhor entendimento do controle dos níveis cardíacos de nucleotídeos extracelulares.

Capítulo II - Identificar molecularmente a presença dos membros da família das E-NPPs em ventrículo esquerdo de ratos adultos, além de caracterizar as propriedades bioquímicas destas enzimas em sinaptossomas de terminal nervoso cardíaco, com a proposta de avaliar a contribuição desta família como parte integrante de um sistema enzimático, responsável pelo metabolismo dos nucleotídeos da adenina e controle dos níveis dos nucleotídeos extracelulares no coração de ratos adultos.

Capítulo III – Investigar o sistema purinérgico no sistema cardíaco de ratos diabéticos, utilizando o modelo da estreptozotocina, e tratados com insulina, visto que ainda não foram elucidados todos os mecanismos responsáveis

pelas alterações cardiovasculares encontradas em grande parte dos pacientes portadores dessa doença.

Capítulo IV – Avaliar a atividade das ectonucleotidases em sistema vascular de ratos diabéticos por estreptozotocina e tratados com insulina, através da avaliação da hidrólise de nucleotídeos no soro destes animais, além de verificar uma posssível influência da glicemia na atividade destas enzimas, visto que pacientes diabéticos apresentam frequentemente doença vascular e que os níveis de nucleotídeos/nucleosídeos na circulação são importantes por participarem do controle de eventos homeostáticos.

PARTE II

CAPÍTULO I

E-NTPDases and ecto-5'-nucleotidase expression profile in rat heart left ventricle and the extracellular nucleotide hydrolysis by their nerve terminal endings

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E-NTPDases and ecto-5′-nucleotidase expression profile in rat heart left ventricle and the extracellular nucleotide hydrolysis by their nerve terminal endings

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Abstract

In this study, we have identified the E-NTPDase family members and ecto-5′-nucleotidase/CD73 in rat heart left ventricle. Moreover, we characterize the biochemical properties and enzyme activities from synaptosomes of the nerve terminal endings of heart left ventricle. We observe divalent cation-dependent enzymes that presented optimum pH of 8.0 for ATP and ADP hydrolysis, and 9.5 for AMP hydrolysis. The apparent K_M values are 40 μM, 90 μM and 39 μM and apparent V_{max} values are 537, 219 and 111 nmol Pi released/min/mg of protein for ATP, ADP and AMP hydrolysis, respectively. Ouabain, orthovanadate, NEM, lanthanum and levamisole do not affect ATP and ADP hydrolysis in rat cardiac synaptosomes. Oligomycin (2 μg/mL) and sodium azide (0.1 mM), both mitochondrial ATPase inhibitors, inhibit only the ATP hydrolysis. High concentrations of sodium azide and gadolinium chloride show an inhibition on both, ATP and ADP hydrolysis. Suramin inhibit more strongly ATP hydrolysis than ADP hydrolysis whereas Evans blue almost abolish both hydrolysis. AMP hydrolysis is not affected by levamisole and tetramisole, whereas 0.1 mM ammonium molybdate practically abolish the ecto-5′-nucleotidase activity. RT-PCR analysis from left ventricle tissue demonstrate different levels of expression of *Entpd1* (*Cd39*), *Entpd2* (*Cd39L1*), *Entpd3* (*Cd39L3*), *Entpd5* (*Cd39L4*) *Entpd6*, (*Cd39L2*) and 5′-NT/CD73. By quantitative real-time PCR we identify the *Entpd2* as the enzyme with the highest expression in rat left ventricle. Our results contribute to the understanding about the control of the extracellular nucleotide levels in and cardiac system.
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Keywords: E-NTPDases; Ecto-5′-nucleotidase/CD73; Left ventricle; Cardiac synaptosomes

Introduction

Extracellular nucleotides can be hydrolyzed by a variety of enzymes that are located on the cell surface or may also be soluble in the interstitial medium or within body fluids (Zimmermann, 2001). Signaling events induced by extra cellular adenine nucleotides are controlled by the action of ectonucleotidases, including members of E-NTPDase (ecto-nucleoside triphosphate diphosphohydrolase) family, E-NPP (ecto-nucleotide pyrophosphatases/phosphodiesterase) family, alkaline phosphatases as well as the ecto-5′-nucleotidase/CD73 (Zimmermann, 2001; Wink et al., 2006).

E-NTPDase encompass a family of mammalian enzymes that catalyze the hydrolysis of γ- and β-phosphate residues of nucleotides, albeit with different abilities (Zimmermann, 2001). This family, so far, is constituted by eight enzymes (Robson et al., 2006) that are tightly bound to the plasma membrane and/or to intracellular organelles by one or two transmembrane domains (Bigonnesse et al., 2004). NTPDase1-3 and 8 are expressed in the cell surface and their catalytic site faces the extracellular milieu (Vollmayer et al., 2001; Bigonnesse et al., 2004; Belcher et al., 2006; Wink et al., 2006). NTPDase4-7 have an intracellular

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localization for instance the Golgi apparatus and endoplasmic reticulum (Wang and Guidotti, 1998; Biederbick et al., 2000). NTPDase5 and 6 may also be found on the plasma membrane and could be secreted following proteolytic cleavage (Mulero et al., 1999). The ecto-5′-nucleotidase (EC 3.1.3.5), also known as CD73, is a GPI-anchored enzyme that represents the major enzyme responsible for the formation of extracellular adenosine from 5′-AMP and the subsequent activation of P1 adenosine receptors (Vollmayer et al., 2001; Zimmermann, 2001). This enzyme has a broad tissue distribution and its soluble and cleaved forms have also been described (Zimmermann, 2001).

These ectoenzymes constitute a highly organized enzymatic cascade in the regulation of nucleotide-mediated signaling, controlling the rate, amount and timing of nucleotide (e.g. ATP) degradation and ultimately, the nucleoside (e.g. adenosine) formation. Extracellular nucleotides/nucleosides are known to regulate several physiological responses, including vascular tone, cardiac function and haemostasis (Burnstock, 1990; Kunapuli and Daniel, 1998; Ralevic and Burnstock, 2003).

Extracellular ATP influences cardiac function both, indirectly, via blood vessels and nerves, and directly via myocites (Kunapuli and Daniel, 1998). ATP is found in sympathetic neurons where it plays a significant role as a co-transmitter and acts on vascular smooth muscle to cause vasoconstriction (Kunapuli and Daniel, 1998; Burnstock, 2002). ATP normally elicits positive ionotropic effects, and upon rapid application to cell induces various forms of arrhythmia (Vassort, 2001). Additional roles for ATP in the heart might include hypertrophy, preconditioning and apoptosis (Ralevic and Burnstock, 2003). These effects are mediated by cell-surface P2 receptors expressed in the heart (Kunapuli and Daniel, 1998; Burnstock, 2002; Gendron et al., 2002). Moreover, the breakdown of ATP can produce adenosine, a metabolite of adenine nucleotide, capable of regulating a variety of physiological functions by the subsequent activation of P1 adenosine receptors. Adenosine (Ado) is an important endogenous mediator of the cardiovascular system (Balas et al., 2002) and is believed to be an important cardioprotective molecule (Obata, 2002).

Numerous studies indicate the involvement of ectonucleotidases on nucleotide and nucleoside signaling in the cardiac nervous system. The relative contribution of each enzyme isoforms is, however, poorly understood and needs to be further defined. In the present study, we identify the molecular levels of an ecto-5′-nucleotidase and E-NTPDase family members in the rat heart left ventricle. Furthermore, we characterize the biochemical properties of these enzyme activities, specifically in the rat heart synaptosomes from the nerve terminal endings in order to better understand the control of the extracellular nucleotide levels in cardiac system.

Materials and methods

Chemicals

Nucleotides, oligomycin, sodium azide, ouabain, orthovanadate, NEM, lanthanum, levamisole, tetramisole, suramin, Evans blue, gadolinium chloride, HEPES, Trizma Base and EDTA were obtained from Sigma Chemical CO. (ST. Louis, MO, USA). Collagenase type II was obtained from Worthington Biochemical Corporation (Lakewood, NJ, USA). The LDH Kit was purchased from Dolles Reagents (Goiânia, Goiás, Brazil). Ringer-solution was purchased from Basa Ltda (Caxias do Sul, RS, Brazil). M-MLV RT and dNTPs were purchased from Promega (Madison, WI, USA) and Taq polymerase from CenBiot-UFRGS (Porto Alegre, RS, Brazil). Oligonucleotides and Trizol LS reagent were obtained from Invitrogen (Carlsbad, CA, USA). All others reagents were also of analytical grade.

Animals

Male Wistar rats (age 60–80 days; weighing about 230–280 g) from our breeding stock were used in the study. Animals were maintained on 12 h light/dark cycle at a constant temperature of 23±2 °C, with free access to food and water. Procedures for the care and use of animals were adopted according to the regulations of Colégio Brasileiro de Experimentação Animal (COBEA), based on the Guide for the Care and Use of Laboratory Animals (National Research Council).

Preparation of crude synaptosomal-mitochondrial fraction from rat heart

The crude synaptosomal-mithocondrial fraction was prepared as previously described by Aloyo et al., 1991. Briefly, the rat was killed by decapitation and immediately after the sacrifice the heart was removed and reperfused by 10 min with a Ringer-Lactate solution. This procedure ensured that blood did not remain in the coronary circulation. After this, the left ventricle was separated and minced in ice-cold solution 0.32 M sucrose containing 1.0 mM EGTA, pH 7.5. The mince was transferred to a HEPES buffered Krebs-Ringer (KRH) solution (50 mM HEPES, 144 mM NaCl, 1.2 mM $MgCl_2$, 1.2 mM $CaCl_2$, 5.0 KCl mM, 10 mM glucose, 1.0 mM ascorbic acid, pH 7.4) containing 12 U of collagenase type II per mg of tissue. This suspension was then incubated at 37 °C for 40 min with continuous bubbling with oxygen. After low speed centrifugation (10 min, 120× g, 4 °C), the resulting pellet was suspended in 10 volumes of 0.32 M sucrose and homogenized with a Teflon/glass homogenizer. Cellular debris, nuclei and large tissues shards were removed by centrifugation (10 min, 650× g, 4 °C). The resulting supernatant was centrifuged (20 min, 21,000× g, 4 °C). The pellet was resuspended in oxygenated, ice-cold KRH and recentrifuged twice (20 min, 22,000× g, 4 °C). The pellet containing cardiac synaptosomes was resuspended in KRH, to a final protein concentration of approximately 0.5–0.7 mg/mL.

Cardiac synaptosomes integrity

The integrity of cardiac synaptosomes was evaluated by measuring the lactate dehydrogenase (LDH) activity. The ratio of this enzyme activity measured in intact and disrupted synaptosomes can be regarded as a measure of damage particles (Frasseto et al., 1993). The protocol was carried out according to the manufacture′s instructions. Triton X-100 (1.0%, final concentration) was used to disrupt the cardiac synaptosomes

preparation. The measurement of LDH activity showed that most synaptosomes (approximately 85%±2%, $n=3$) were intact after the isolation procedure (data not shown).

Protein determination

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

Enzyme assays

ATP hydrolysis was assayed in a reaction medium containing 2.0 mM $CaCl_2$, 5.0 mM KCl, 10 mM glucose, 225 mM sucrose, 2.0 µg/mL oligomycin, 0.1 mM sodium azide, 50 mM Tris–HCl buffer, pH 8.0, and 6.0 µg protein in a final volume of 200 µL. To assay ADP hydrolysis, the reaction medium containing 2.0 mM $CaCl_2$, 5.0 mM KCl, 10 mM glucose, 225 mM sucrose, 50 mM Tris–HCl buffer, pH 8.0, and 6.0 µg of protein in a final volume of 200 µL. The reaction to assay AMP hydrolysis was made using 2.0 mM $MgCl_2$, 150 mM sucrose and 50 mM glycine, pH 9.5, and 10 µg of protein, in a final volume of 200 µL. The synaptosomal fractions were added to the reaction mixture, preincubated for 10 min and incubated (for 6 min for ATP or ADP and 10 min for AMP hydrolysis) at 37 °C. The reaction was initiated by the addition of substrate to a final concentration of 1.0 mM and stopped by the addition of 200 µL 10% trichloroacetic acid. The samples were chilled on ice for 10 min and the released inorganic phosphate (Pi) was measured (Chan et al., 1986). Incubation times and protein concentration were chosen in order to ensure the linearity of the reactions. Controls, with the addition of the enzyme preparation, after addition of trichloroacetic acid were used to correct nonenzymatic hydrolysis of the substrates. All assays were done in duplicate or triplicate. Enzymes activities were expressed as nmol of phosphate released per min per mg of protein. Oligomycin were added from a concentrated ethanol solution, in a final concentration of 1.0% in the ATP incubation medium. The addition of ethanol did not arrest the enzyme activity in the absence of inhibitor.

Divalent cation dependence

In order to identify the divalent cation dependence for ATP hydrolysis, Mg^{2+} or Ca^{2+}, in the range of 0.5–6.0 mM or EDTA, in the range 0.05–0.5 mM were added to the reaction mixture containing 5.0 mM KCl, 10 mM glucose, 225 mM sucrose, 2.0 µg/mL oligomycin, sodium azide 0.1 mM, 50 mM Tris–HCl buffer, pH 8.0. For ADP hydrolysis, the same reaction medium without inhibitors was used and for AMP hydrolysis the same conditions were tested in the medium, containing 150 mM sucrose and 50 mM glycine, pH 9.5. For control groups, neither divalent cations nor EDTA were added to the reaction medium.

pH dependence

For analysis of ATP from pH 5.5 to 9.0, a buffer solution reaction mixture containing 50 mM Tris–HCl buffer, 50 mM HEPES buffer, 2 mM $CaCl_2$, 5.0 mM KCl, 10 mM glucose, 225 mM sucrose, 2.0 µg/mL oligomycin, sodium azide 0.1 mM, was used. For ADP hydrolysis, the same conditions were tested without inhibitors in the reaction mixture. For AMP hydrolysis, the pH tested ranged form 7.0 to 10.5, in a reaction mixture containing 50 mM Tris–HCl and 50 mM glycine buffers, 2.0 mM $MgCl_2$, 150 mM sucrose.

Differential effects of some compounds on ATP, ADP and AMP hydrolysis

The effects on ATP and ADP hydrolysis of the following compounds were analyzed: 1.0 mM ouabain, 0.1 mM orthovanadate, 1.0 mM NEM (N-ethylmaleimide), 0.1 mM lanthanum, 1.0 mM levamisole, 0.1 mM suramin, 0.1 mM Evans blue, 2.0 µg/mL oligomycin, 0.1, 5.0, 10 and 20 mM sodium azide, 0.3 and 0.5 mM gadolinium chloride. Incubation times, protein and substrate concentrations were used as described above. Synaptosomes were preincubated in the presence of each inhibitor for 10 min at 37 °C followed by the addition of substrate. Results are expressed as percentage of control enzyme activity and data were analyzed by one-way ANOVA, followed by Student–Neuman–Keuls *post hoc* test ($P<0.05$; $P<0.01$).

RT-PCR analysis

RNA was isolated from left ventricle using the Trizol Reagent. The cDNA species were synthesized from 5.0 µg of total RNA in a total volume of 25 µL with both oligo (dT) primer and random hexamers in accordance with the manufacturer's instructions. One microliter of the RT reaction mix was used as a template for PCR, except for *Entpd1* and *Entpd6* (2.0 µL) in a total volume of 20 µL using a concentration of 0.25 µM of each primer indicated below and 0.5 units *Taq*DNA polymerase. The PCR was run at the following conditions: 1 min at 95 °C denaturation, followed by 35 cycles of 1 min at 94 °C, 1 min at 60 °C, 1 min at 72 °C and a final 10 min extension at 72 °C. Ten microliters of the PCR reaction were analyzed on a 1.5% agarose gel. The primer sets used for rat *Entpd1* (*Cd39*), *Entpd2* (*Cd39L1*), *Entpd3* (*Cd39L3*), *Entpd5* (*Cd39L4*), *Entpd6* (*Cd39L2*) were described by Vollmayer et al., 2001; for rat 5′-NT/CD73 and β-actin RT-PCR were described by Wink et al., 2003a. For NTPDase8 the sequences were 5′-AGG TGC CTT TGG TTG GAT C-3′ 5′-GGT AGC TGT GAG TGT AGA C-3′ (amplification product 227 bp). Negative controls were performed with water as template and positive controls were plasmids with cDNA sequences for mouse *Entpd1*, rat *Entpd2* (Kegel et al., 1997; Sévigny et al., 2002) and human *ENTPD3* (Smith and Kirley, 1998) *ENTPD5* (Mulero et al., 1999) and *ENTPD6* (Yeung et al., 2000), ecto-5′-nucleotidase/CD73 rat astrocytes cultures (Wink et al., 2003b) and NTPDase8 rat liver (Fausther et al., 2006).

Real time PCR

Total RNA and cDNA were generated as described in RT-PCR analysis. SYRB Green I-based real-time PCR was carried out on MJ Research DNA Engine OpticonTM Continuous

Fluorescence Detection System (MJ Research Inc., Walthan, MA) as described (Zerbini et al., 2003). All PCR mixtures contained: PCR buffer (final concentration 10 mM Tris–HCl (pH 9.0), 50 mM KCl, 2.0 mM $MgCl_2$, and 0.1% Triton X-100), 250 μM deoxy-NTP (Roche), 0.5 μM of each PCR primer, 0.5xSYBR Green I (Molecular Probes), 5.0% DMSO, and 1 U taq DNA polymerase (Promega, Madison, WI) with 2.0 μL cDNA in a 25 μL final volume reaction mix. The samples were loaded into wells of Low Profile 96-well microplates. The PCR was run at the following conditions: 1 min at 95 °C denaturation, followed by 35 cycles of 1 min at 94 °C, 1 min at 60 °C, 1 min at 72 °C and a final 10 min extension at 72 °C. The fluorescence signal was measured right after incubation for 5 s at 79 °C following the extension step, which eliminates possible primer dimer detection. At the end of the PCR cycles, a melting curve was generated to identify specificity of the PCR product. For each run, serial dilutions of human GAPDH plasmids were used as standards for quantitative measurement of the amount of amplified DNA. For normalization of each sample, β-actin primers were used to measure the amount of β-actin cDNA. All samples were run in triplicate and the data were presented as ratio of enzymes/β-actin. The primers used for real time PCR are described in RT-PCR analysis.

Results

Biochemical properties of ATP, ADP and AMP hydrolysis

The optimum pH for the ATP, ADP and AMP hydrolysis were determined (Fig. 1). The biochemical analysis of synaptosomal preparation showed that the optimum pH for ATP and ADP hydrolysis was 8.0, which is in accordance with those previously described for E-NTPDases (Zimmermann, 2001; Knowles et al., 2002; Leal et al., 2005). For AMP hydrolysis, the optimum pH was 9.5 and this alkaline pH was previously described in rat heart (Naito and Lowenstein, 1981).

We also tested the hydrolysis rate for ATP, ADP and AMP in the presence and in the absence of divalent cations, or EDTA, as indicated in Materials and methods. As shown in Fig. 2, in the presence of 0.05 mM EDTA the hydrolysis of all nucleotides was lower than the control groups. Control groups were considered the ones that did not receive additional cations in the reaction medium during the incubation. The remaining activity seen in the absence of added ions (Fig. 2), can be explained by the cations present in the Krebs Ringer buffer used in the synaptosomes preparation or additionally, released from cardiac tissue during the homogenate preparation. Ectonucleotidases are generally inhibited by chelators of divalent cations (Zimmermann, 2000; Casali et al., 2001) and it was confirmed by the decrease of the enzyme activity in presence of EDTA. On the other hand, ATP and ADP hydrolysis were greatly stimulated by addition in the reaction medium of Ca^{2+} whereas for AMP hydrolysis, Mg^{2+} was responsible for the increase of the enzyme activity. The ecto-5′-nucleotidase is usually considered to be Mg^{2+}-dependent (Zimmermann, 1992), which is in accordance with our results. Increasing concentrations of divalent cations added to the reaction mixture containing EDTA 0.5 mM partially

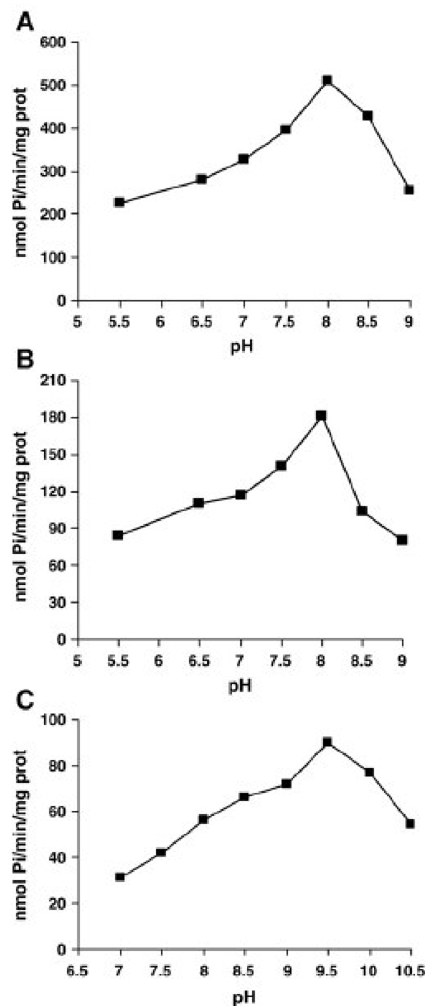

Fig. 1. Effect of pH variation on ATP (A), ADP (B) and AMP (C) hydrolysis from rat cardiac synaptosomes. Buffers used were 50 mM Tris–HCl and 50 mM HEPES for ATP and ADP hydrolysis and 50 mM Tris–HCl and 50 mM glycine for AMP hydrolysis. Conditions are described in Materials and methods. Data are representative of four independent experiments. The activity was expressed as nmol Pi/min/mg of protein.

recovered E-NTPDases and 5′-nucleotidase activities. Thus, considering that the enzymes responsible for ATP, ADP, and AMP hydrolysis in rat cardiac synaptosomes are cation-dependent, we established 2.0 mM Ca^{2+}/1.0 mM ATP, 2.0 mM Ca^{2+}/1.0 mM ADP and 2.0 mM Mg^{2+}/1.0 mM AMP as optimal conditions for measuring the ectonucleotidases activities.

Michaelis constant (K_M) and maximal velocity (V_{max}) values were calculated from the Eadie–Hofstee plot (Fig. 3) with ATP, ADP and AMP as substrates. Substrate concentrations tested ranged from 75 to 2000 μM (figures insets), indicating that the

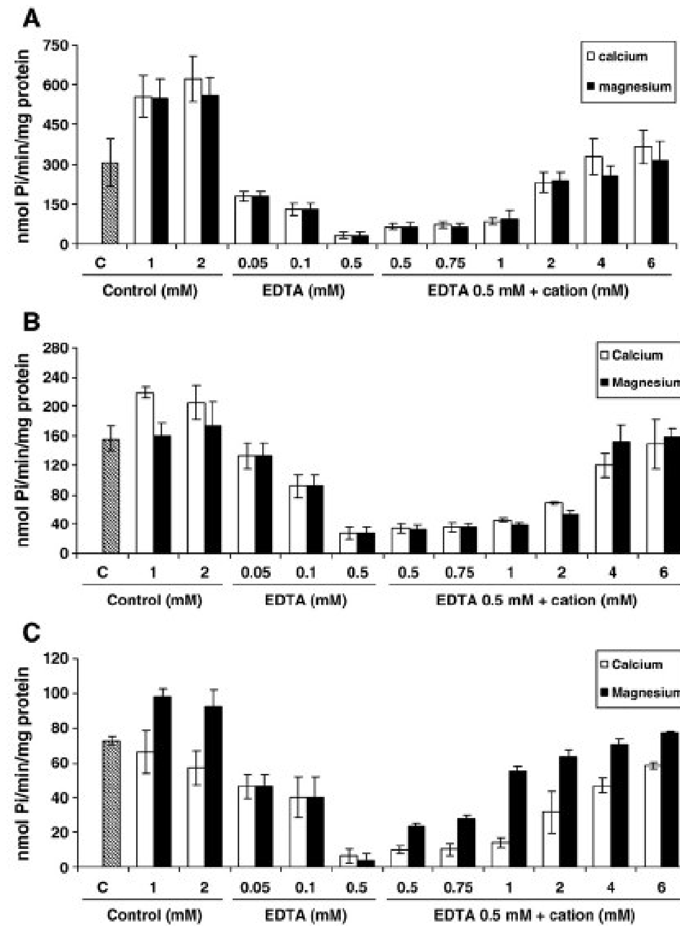

Fig. 2. Divalent cations dependence on ATP (A), ADP (B) and AMP (C) hydrolysis. Hydrolysis of ATP, ADP and AMP by rat cardiac synaptosomes was analyzed without addition of cations (c), in the presence of 1.0 and 2.0 mM Ca^{2+} or Mg^{2+}. EDTA, in a range from 0.05 to 0.5 mM, was used as a cation chelator. The activity was determined in the presence of 0.5 mM EDTA plus cation concentration (0.5–6.0 mM calcium or magnesium) as shown in the figure. Bars represent means ± SD for three independent experiments. Results are expressed as nmol Pi/min/mg of protein.

enzyme activities increased with rising nucleotide concentrations under saturation. The K_M and the V_{max} values calculated for ATP, ADP, and AMP hydrolysis were 140.4±13 μM and 536.9±35 nmol Pi released/min/mg of protein (mean ± SD, n=5), 90.0±15 μM and 219.5±36 nmol Pi released/min/mg of protein (mean ± SD, n=5) and 38.7±7 μM and 110.5±10 nmol Pi released/min/mg of protein (mean ± SD, n=3), respectively.

Effects of some compounds

The classical inhibitors of Na^+, K^+-ATPase, ouabain and orthovanadate; inhibitors of Ca^{2+}, Mg^{2+}-ATPase, NEM and lanthanum; alkaline phosphatase inhibitor, levamisole (Plesner, 1995; Oliveira et al., 1997; Buffon et al., 2007) were ineffective as inhibitors of ATP and ADP hydrolysis in rat cardiac synaptosomes (Table 1). Oligomycin and 0.1 mM sodium azide, both mitochondrial ATPase inhibitors (Knowles and Nagy, 1999; Sasaki et al., 2001), inhibited ATP hydrolysis by 66% and 42%, respectively. This result is not surprising, since the presence of mitochondrial ATPase in heart is well documented (Sasaki et al., 2001; Grover et al., 2004) and the fraction analyzed is a "crude synaptosomal-mitochondrial fraction" (Aloyo et al., 1991). However, it is important to note that the mitochondria fraction present in the preparation was not released from the synaptosomes, but they were purified together with the synaptosomes. For this reason, all incubations using ATP as substrate were done in the presence of the inhibitors to exclude the involvement of this enzyme in our assay.

High concentrations of sodium azide (5.0, 10 and 20 mM) and gadolinium chloride have been demonstrated to inhibit E-NTPDases from several sources (Knowles and Nagy, 1999; Oses et al., 2004; Escalada et al., 2004; Fürstenau et al., 2006;

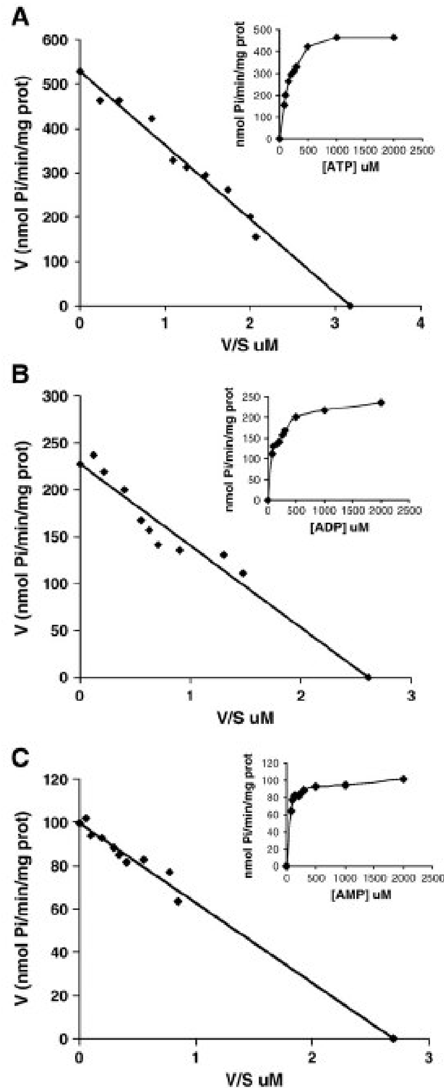

Fig. 3. Eadie–Hofstee plot for extracellular hydrolysis of ATP (A), ADP (B) and AMP (C). The substrate concentrations tested ranged from 75 to 1000 μM (insets figures). The K_M and the maximal velocity (V_{max}) values calculated for ATP, ADP and AMP hydrolysis were 140.45±13.55 mM and 536.93±35.02 nmol Pi released/min/mg of protein (mean ± SD, n=5), 90.06±14.70 mM and 219.48±36.46 nmol Pi released/min/mg of protein (mean ± SD, n=5) and 38.69±6.65 mM and 110.53±10.02 nmol Pi released/min/mg of protein (mean ± SD, n=3), respectively. Data represents a typical experiment.

Buffon et al., 2007). Our results showed that 5.0, 10 and 20 mM of sodium azide inhibited ATP (65%, 75% and 78%, respectively) and ADP hydrolysis (16%, 31% and 45%, respectively). Gadolinium chloride inhibited ATP hydrolysis by 30% and ADP hydrolysis by 20% when tested at a final concentration of 0.3 mM. When it was tested at a final concentration of 0.5 mM, ATP hydrolysis was inhibited by 39% and ADP hydrolysis by 29%. The antagonists of P2 receptors, suramin and Evans blue, that have been used as ectonucleotidases inhibitors (Heine et al., 1999; Fürstenau et al., 2006; Wink et al., 2006) cause inhibition on ATP and ADP hydrolysis in rat cardiac synaptosomes. Suramin inhibited ATP hydrolysis by 83% and ADP hydrolysis by 36%. Evans blue was the most effective inhibitor since almost abolished ATP hydrolysis (97% of inhibition) and strongly inhibited ADP hydrolysis (86% of inhibition).

Considering that the ideal pH for ecto-5′-nucleotidase assay was 9.5, as demonstrated by pH curve (Fig. 1C), we tested the alkaline phosphatase classical inhibitors, levamisole and tetramisole (Borges et al., 2007) to discard its participation in the AMPase activity. As seen in the Table 2, these inhibitors did not decrease the AMPase activity. Thus, ammoniun molybtade,

Table 1
Effect of inhibitors on ecto-ATPase and ecto-ADPase activities in rat cardiac synaptosomes

Inhibitors	Concentration (mM)	% of control enzyme activity	
		ATP	ADP
Ouabain	1.0	100.8±9.4	99.9±8.9
Orthovanadate	0.1	96.5±6.4	99.1±2.6
NEM	1.0	93.6±6.6	95.5±5.8
Lanthanum	0.1	93.2±6.9	98.3±8.1
Levamisole	1.0	94.9±12	101.3±4.5
Suramin	0.1	12.9±2.7**	64.2±6.7**
Evans blue	0.1	2.8±0.3**	14.1±2.0**
Oligomycin	2 μg/mL	43.6±2.9**	94.7±2.4
Sodium azide	0.1	58.3±3.8**	97.6±6.2
	5.0	35.4±4.0**	84.5±7.9*
	10.0	25.9±2.8**	69.1±8.7**
	20.0	22.4±3.2**	55.8±9.5**
Gadolinium chloride	0.3	70.4±8.7**	80.1±8.0*
	0.5	61.1±8.0**	70.8±6.0**

ATPase and ADPase activities were assayed as described in Materials and methods and expressed as percent of control obtained in the presence of inhibitor. The average control values (100%) from different experiments were 1188.11±68.04 nmol Pi/min/mg protein for ATP hydrolysis and 177.22±5.13 nmol Pi/min/mg protein for ADP hydrolysis. Results are the mean ± SD (n=5). Data was analyzed statistically by one-way analysis of variance (ANOVA) followed by Student–Neuwman–Keuls test.

* Represents difference from control enzyme activity (100%) (P<0.05).

** Represents difference from control enzyme activity (100%) (P<0.001).

Table 2
Effect of inhibitors on AMP hydrolysis from rat cardiac synaptosomes

Inhibitors	Concentration (mM)	% of control enzyme activity
Levamisole	1.0	98.9±6.2
	5.0	93.9±7.8
Tetramisole	1.0	108.8±9.0
Molybdate	0.1	17.6±5.7**

AMPase activity was assayed as described in Materials and methods and expressed as percent of control obtained in the presence of inhibitor. The average control values (100%) from different experiments were 104±4.9 nmol Pi/min/mg protein. Results are the mean ± SD (n=3). Data was analyzed statistically by one-way analysis of variance (ANOVA) followed by Student–Neuwman–Keuls test.

** Represent difference from control enzyme activity (100%) (P<0.01).

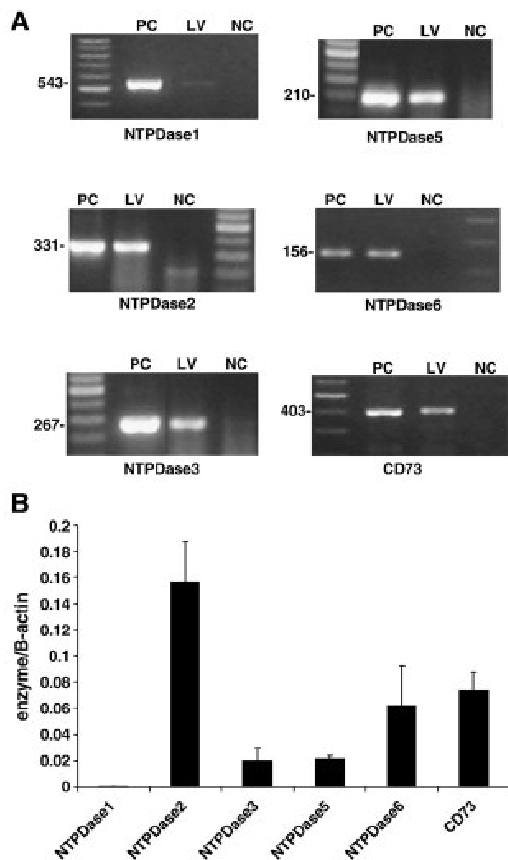

Fig. 4. RT-PCR and quantitative real-time PCR analysis of *Entpds* and 5′-nucleotidase gene expression rat left ventricle. (A) Total RNA was isolated from left ventricles and the cDNA was analyzed by PCR with primers for *Entpds* and 5′-nucleotidase as described in Materials and methods. (PC) positive control; (LV) left ventricles; and (NC) negative control. The length (bp) of the PCR products obtained with each pair of primers is given in the figure. (B) The expression of NTPDases and 5′-nucleotidase/CD73 in left ventricles was quantitatively analyzed by real-time PCR as described in Materials and methods. The results are presented as ratio of cDNA enzyme/β-actin. Bars represent mean ± SD for two experiments.

which are known an ecto-5′-nucleotidase inhibitor, dramatically inhibited the AMPase activity (82% of inhibition).

Ectonucleotidases mRNAs expression in rat cardiac left ventricle

As ATP, ADP and AMP can be metabolized by different members of the ectonucleotidases family (Zimmermann, 2001) we investigated the mRNA expression of *Entpd* 1, 2, 3, 5, 6 and 8 and 5′-NT/CD73 in rat left ventricle. Using specific primers, oligonucleotide fragments were amplified and analyzed on agarose gels. Among the mRNAs analyzed, all members of ectonucleotidases were detected at different intensities in rat left ventricle with exception of *Entpd8*, (Fig. 4A). The length of the oligonucleotide fragments obtained in the samples was comparable to the positive controls and corresponded to the expected sizes. In order to analyze and compare the different levels of mRNA expression of *Entpd* family and 5′-NT/CD73 in rat left ventricle, we performed quantitative real-time RT-PCR analysis (Fig. 4B). The results showed that from *Entpds* genes, the *Entpd2* is the highest expressed from all genes investigated, followed by 5′-NT/CD73 and *Entpd6*.

Discussion

Signaling via nucleotides is widespread in the peripheral nervous system (Zimmermann, 1996; Sesti et al., 2002). It is clear that specific ectonucleotidases expressed by cells regulate the metabolism of extracellular nucleotides in their microenvironment. In accordance with their importance in cell signaling, the extracellular concentration of nucleotides is tightly regulated. We report here the NTPDases and ecto-5′-nucleotidase expression profile in rat heart left ventricle and the extracellular ATP metabolism by their nerve terminal endings. RT-PCR analysis shows that the left ventricle express multiple ectonucleotidases with the potential to hydrolyze nucleotides to their respective nucleosides. By quantitative real-time PCR analysis, we demonstrate that among the NTPDases members that preferentially hydrolyses tri-phosphonucleosides, the *Entpd2* (*Cd39L1*) is the major gene expressed.

Ecto-ATPase activity has been observed in synaptosomal fractions from various sources, implying ectonucleotidases activity of nerve cells (Robson et al., 2006). This ATPase activity can be observed in our results and the high expression of the NTPDase2 in heart suggests the possible presence of this enzyme in cardiac synaptosomes. Furthermore, it has been suggested this activity may be regulated by the release of ATP from nerve endings. Noradrenaline (NE) and ATP are co-released from sympathetic nerve terminals of the guinea pig heart. It has been demonstrated that ATP enhances the noradrenaline release by a mechanism controlled by ectonucleotidases, described as NTPDase1, but the presence of other NTPDases was not investigated in this study (Machida et al., 2005). Our results demonstrated a low expression of the NTPDase1 in left ventricle and this may suggest that other enzymes could participate in nucleotide hydrolysis in rat cardiac synaptosomes. Biochemical analyses of murine and porcine heart tissues have demonstrated ATPase/ADPase ratios of 10, suggesting expression of enzymes with preferential ATPase activity (Lemmens et al., 2000; Sévigny et al., 2002). Concurring with these findings, it was showed the presence of NTPDase2 in heart by Northern blotting (Chadwick and Frischauf, 1998) and an ectoenzyme that preferentially hydrolyze NTPs was already demonstrated in sarcolemmal membranes from rat heart (Oliveira et al., 1997). Given the levels of NTPDase2 mRNA expression in murine and human hearts, this enzyme would be a likely candidate responsible for such activity (Chadwick and Frischauf, 1997; Kegel et al., 1997; Chadwick and Frischauf, 1998).

The soluble NTPDases 5 and 6 are also identified in the rat left ventricle. These enzymes present both soluble and membrane-bound forms (Hicks-Berger et al., 2000) and exhibit higher affinity for nucleoside diphosphates than for nucleoside

triphosphates (Mulero et al., 1999). After the NTPDase2, the NTPDase6 is the most NTPDase expressed in the left ventricle. This enzyme is related to be highly expressed in heart tissues and the preferred ADP hydrolysis suggests that this enzyme may participate in regulating haemostasis (Yeung et al., 2000). In cardiac synaptosomes, NTPDase6 could be one of the most important enzymes responsible for the ADP hydrolysis. The NTPDase5 shows lower expression and could also contribute for the ADP degradation, once that release of soluble nucleotidases with undefined molecular structure from nerve endings has previously been reported (Todorov et al., 1997).

The pattern of biochemical parameters obtained here is in accordance with the expression of these enzymes, revealing a rapid hydrolysis of extracellular ATP with specific activity of 520 ± 22 and 181 ± 8 nmol Pi/min/mg for ADP, resulting in a hydrolysis ratio (ATPase/ADPase) of 2.9. The ratio indicates the participation of NTPDase3 in rat cardiac synaptosomes. It has been suggested that NTPDase3 may act as a presynaptic regulator of extracellular ATP levels (Belcher et al., 2006). However, considering the low expression of NTPDase3 and high expression of NTPDase2, it is most likely that the 2.9 ratio is a consequence of the participation of the enzymes capable of hydrolyze ADP. Then, ATP could be hydrolyzed mainly by NTPDases 2 and also by NTPDase3 and the ADP by the NTPDases 3, 5 and 6.

In the sense to investigate the different enzymes activities present in our preparation, some enzyme inhibitors were tested in rat cardiac synaptosomes. However, considering the lack of specific inhibitors able to distinguish among the several ectoenzymes already described, the antagonists of P2 receptors, suramin and Evans blue, have been used as ectonucleotidases inhibitors (Heine et al., 1999; Fürstenau et al., 2006; Wink et al., 2006). Both of them cause inhibition on ATP and ADP hydrolysis in rat cardiac synaptosomes. It is interesting to note, that suramin, that has been shown to preferentially inhibit ecto-ATPase (Heine et al., 1999; Wink et al., 2006), reduce strongly ATP hydrolysis than ADP hydrolysis in cardiac synaptosomes, while Evans blue, that has been demonstrated to be more efficient to inhibit apyrase activity, practically abolish the activities. This pattern of inhibition reinforces the participation of more than one member of the E-NTPDase family in cardiac synaptosomes. Besides the E-NTPDase, the E-NPP family could also contribute in the control of nucleotide hydrolysis in synaptosomes (Rucker et al., 2007), but there are no specific inhibitors to distinguish these enzymes. However, in the conditions tested, we possibly are working principally in the presence of an E-NTPDase.

It is known that stimulated sympathetic nerves of guinea pig vas deferens release not only ATP and NE, but also enzyme activity that degrades ATP to adenosine. The latter exhibits similarities to E-NTPDases and ecto-5′-nucleotidase, but their molecular identity has not been defined (Mihaylova-Todorova et al., 2002). Our results also demonstrate an ecto-5′-nucleotidase activity and its molecular expression. This is the second enzyme more expressed on left ventricle. Ecto-5′-nucleotidase is the major enzyme responsible for the formation of extracellular adenosine from released adenine nucleotides (Zimmermann, 2000). Then, the co-existence of the E-NTPDases members and ecto-5′-nucleotidase is very important for their participation in an "enzymatic chain" for the complete hydrolysis of ATP to adenosine and for the control of the nucleotide/nucleoside ratio in nerve terminal endings.

It was demonstrated that ATP and NE during sympathetic neurotransmission are released together. Following release, ATP is metabolized to AMP by E-NTPDases, either by direct action or through transient formation of ADP. The AMP formed is further converted to adenosine by the action of ecto-5′-nucleotidase (Marcus et al., 2005). Enhanced adrenergic activity and NE release are etiologic factors in clinical cardiac dysfunction, including arrhythmias and sudden cardiac death during myocardial ischemia (Braunwald and Sobel, 1988; Benedict et al., 1996). It was reported that ATP increases NE released via positive feedback mechanism. Besides, P2X receptor is responsible for the vasoconstrictor responses produced as a consequence of the release of ATP from sympathetic perivascular nerves (Ralevic and Burnstock, 1991). Then E-NTPDases could exert cardioprotection by the breakdown of the neurotransmitter ATP and, consequently, reducing the release of NE. The effects of adenosine also involve a presynaptic attenuation of the released of NE from sympathetic nerve endings (through presynaptic P1 receptors) (Burnstock, 2002), in addition to its postsynaptic effects (Richardt et al., 1987). The inhibition of NE release may contribute to a reduction of cardiac work during ischemia and reperfusion (Shryock and Belardinelli, 1997).

In this study we demonstrate the presence of the E-NTPDase family and ecto-5′-nucleotidase only in rat left ventricle. Our results are crucial since in some pathological situations, such as a congestive heart failure caused by myocardial infarction, exist differential changes in the status of sympathetic nerves in the left and right ventricles (Sethi et al., 1997). Then, the study of the different pattern of the expression and activities of ectonucleotidases could be important in normal and pathological conditions, and these enzymes may contribute to the normal cardiovascular function and disturbances in purinergic signaling involved in cardiovascular diseases (Burnstock, 1990). In conclusion, these findings may contribute to understand the contribution of the different members of ectonucleotidases family in the control of the extracellular nucleotide levels in cardiac system.

Acknowledgements

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CAPÍTULO II

Biochemical characterization of ecto-nucleotide pyrophosphatase/phosphodiesterase (E-NPP, E.C. 3.1.4.1) from rat heart left ventricle

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Biochemical characterization of ecto-nucleotide pyrophosphatase/phosphodiesterase (E-NPP, E.C. 3.1.4.1) from rat heart left ventricle

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Abstract In the present study we investigate the biochemical properties of the members of NPP family in synaptosomes prepared from rat heart left ventricles. Using *p*-nitrophenyl-5′-thymidine monophosphate (*p*-Nph-5′-TMP) as substrate for E-NPPs in rat cardiac synaptosomes, we observed an alkaline pH dependence, divalent cation dependence and the K_M value corresponded to 91.42 ± 13.97 µM and the maximal velocity (V_{max}) value calculated was 63.79 ± 3.59 nmol *p*-nitrophenol released/min/mg of protein (mean ± SD, $n = 4$). Levamisole (1 mM), was ineffective as inhibitor of *p*-Nph-5′-TMP hydrolysis in pH 8.9 (optimum pH for the enzyme characterized). Suramin (0.25 mM) strongly reduced the hydrolysis of *p*-Nph-5′-TMP by about 46%. Sodium azide (10 and 20 mM) and gadolinium chloride (0.3 and 0.5 mM), E-NTPases inhibitors, had no effects on *p*-Nph-5′-TMP hydrolysis. RT-PCR analysis of left ventricle demonstrated the expression of NPP2 and NPP3 enzymes, but excluded the presence of NPP1 member. By quantitative real-time PCR we identified the NPP3 as the enzyme with the highest expression in rat left ventricle. The demonstration of the presence of the E-NPP family in cardiac system, suggest that these enzymes could contribute with the fine-tuning control of the nucleotide levels at the nerve terminal endings of left ventricles that are involved in several cardiac pathologies.

Keywords E-NPPs · Left ventricle · Cardiac synaptosomes

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Introduction

Nucleotides, a ubiquitous class of intracellular molecules, have the capacity to regulate pathophysiological functions in the extracellular environment. Extracellular ATP has long been recognized as an agonist that mediates a wide variety of biological responses in the central and peripheral nervous system, chiefly by binding to the G protein-coupled receptor (P2Y) or a ligand-gated ionotropic receptors (P2X) [1]. The interaction between ATP/ADP and their P2 receptors elicits vasoconstriction/vasodilatation responses, platelet aggregation, and cellular proliferation [2].

There are important regulatory mechanisms that control extracellular concentration of nucleotides and hence regulate P2 -mediated effects. Members of several families of ectonucleotidases are able to hydrolyze extracellular nucleotides. These include ecto-nucleoside triphosphate diphosphohydrolase (E-NTPDases) family, ecto-nucleotide pyrophosphatases/phosphodiesterases (E-NPPs) family, alkaline phosphatases and ecto-5′-nucleotidase/CD73 [3, 4].

The family of E-NPPs consists of seven structurally related enzymes that are located at the cell surface, either expressed as transmembrane proteins or as secreted enzymes [5]. Only NPP1-3, which have a common ancestor, have been implicated in the hydrolysis of nucleotides [6–9], while NPP6-7 are only known to hydrolyze

phosphodiester bonds in lysophospholipids or other choline phosphodiesters [9, 10].

NPP1-3 has been detected in almost all tissues [7], although individual isoforms are usually confined to specific substructures and/or cell types [11–14]. In addition, NPP members have been localized in different cellular compartments and are differentially targeting to plasma membranes of polarized cells, suggesting specific physiological functions of NPPs in cells and tissues [15].

NPP1 was originally discovered on the surface of mouse B-lymphocytes as the plasma cell differentiation antigen (PC-1) [16] and has been described to be involved in bone and matrix mineralization, soft tissue calcification [15, 17, 18], insulin signalling and in the etiology of insulin resistance [18]. NPP2 was discovered as an autocrine motility factor (autotoxin, NPP2α) [19] and has been implicated in cell growth, motility, blood vessel formation and cancer progression [20]. NPP3 (gp130^{RB13-6} or B10) was initially recognized by the monoclonal antibody RB13-6 as a glycoprotein of rat brain glial precursor cells [21, 22].

Data concerning the presence of ecto-nucleotide pyrophosphatase/phophodiesterase enzymes in the mammalian heart are very limited, although it is known that adenine nucleotides are continually present in quite variable amounts in the extracellular space of the heart tissue. ATP can elicit positive inotropic effects, and upon rapid application to cells induces various forms of arrhythmia [23], since ATP and norepinephrine (NE) are thought to be stored together in cytoplasmic vesicles of adrenergic nerve cells. During sympathetic neurotransmission, they are released together and ATP can promote adrenergic transmission by action on presynaptic purinoceptors [24]. ATP plays a significant cotransmitter role in sympathetic nerves supplying hypertensive blood vessels. Further therapeutic targets for P2 receptor agonists and antagonists include congestive heart failure, hypertension, stroke, and angina [25]. Adenosine, generated by enzymatic degradation of ATP released by cells, has modulatory properties in the sympathetic nerve endings and is considered a cardioprotective molecule because of its vasodilatatory effects [2].

Thus, in the present study, we have identified and characterized in the rat heart left ventricle the E-NPP family members, in order to contribute with the understanding about the control of the extracellular nucleotide levels in cardiac system.

Materials and methods

Chemicals

p-Nitrophenyl-5′-thymidine monophosphate (*p*-Nph-5′-TMP), EDTA, Trizma Base, sodium azide, levamisole, suramin, and gadolinium chloride were obtained from Sigma Chemical CO. (ST. Louis, MO, USA). Collagenase type ll was obtained from Worthington Biochemical Corporation (Lakewood, NJ, USA). The LDH Kit was purchased from Dolles Reagents (Goiânia, Goiás, Brazil). Ringer-solution was purchased from Basa Ltd (Caxias do Sul, RS, Brazil). M-MLV RT and dNTPs were purchased from Promega. Trizol LS reagent (Life Technologies), Taq polymerase (CenBiot-UFRGS) and oligonucleotides were obtained from Invitrogen. All others reagents were also of analytical grade.

Animals

Male Wistar rats (age 60–80 days; weighing about 250–300 g) from our breeding stock were used in the study. Animals were maintained on 12 h light/dark cycle at a constant temperature of 23 ± 2°C, with free access to food and water. Procedures for the care and use of animals were adopted according to the regulations of Colégio Brasileiro de Experimentação Animal (COBEA), based on the Guide for the Care and Use of Laboratory Animals (National Research Council).

Preparation of crude synaptosomal fraction from rat heart

The crude heart synaptosomal fraction was prepared as described previously by Aloyo et al. (1991) [26]. Briefly, the rat was killed by decapitation and immediately following it the heart was removed and reperfused by 10 min with a Ringer-Lactate solution. This procedure ensured that blood did not remain in the coronary circulation. After this, the left ventricle was separated and minced in ice-cold 0.32 M sucrose containing 1 mM EGTA, pH 7.5. The mince was transferred to a HEPES buffered Krebs–Ringer (KRH) solution (HEPES 50 mM, NaCl 144 mM, $MgCl_2$ 1.2 mM, $CaCl_2$ 1.2 mM, KCl 5 mM, glucose 10 mM, ascorbic acid 1 mM, pH 7.4) containing 12 units of collagenase type ll per mg of tissue. This suspension was then incubated at 37°C for 40 min with continuous bubbling with oxygen. After this, the suspension was centrifuged (10 min, 120 × *g*, 4°C) and the resulting pellet was suspended in 10 volumes of 0.32 M sucrose and homogenized with a Teflon/glass homogenizer. Cellular debris, nuclei and large tissues shards were removed by centrifugation (10 min, 650 × *g*, 4°C). The resulting supernatant was centrifuged (20 min, 21,000 × *g*, 4°C). The pellet was resuspended in oxygenated, ice-cold KRH and recentrifuged twice (20 min, 22,000 × *g*, 4°C). The pellet containing cardiac synaptosomes was resuspended in KRH, to a final protein concentration of approximately 0.5–0.7 mg/ml.

Protein determination

Protein was measured by the Coomassie Blue method using bovine serum albumin as standard [27].

Assay of ecto-nucleotide pyrophosphatase/phosphodiesterase activity

The phosphodiesterase activity was assessed using *p*-Nph-5′-TMP as substrate as previously outlined [28]. The reaction medium containing 50 mM Tris–HCl buffer, 5 mM KCl, 10 mM glucose, 225 mM sucrose, pH 8.9, was preincubated approximately with 10 μg of synaptosomal protein per tube at 37°C in a final volume of 200 μl. The enzyme reaction was started by the addition of *p*-Nph-5′-TMP to a final concentration of 0.5 mM. After 30 min of incubation, 200 μl NaOH 0.2 N was added to the medium to stop the reaction. An incubation time of 30 min was chosen, in order to ensure the linearity of the reaction. The amount of *p*-nitrophenol released from the substrate was measured at 400 nm using a molar extinction coefficient of 18.8×10^{-3}/M/cm. Controls to correct for non-enzymatic substrate hydrolysis were performed by adding synaptosomal preparation after the reaction had been stopped with NaOH as described above. All samples were performed in duplicate. Enzyme activities were generally expressed as nmol *p*-nitrophenol released per minute per milligram of protein.

Divalent cations dependence, pH dependence and inhibitors

In order to investigate the possibility of divalent cations dependency for cardiac synaptosomes enzymes, we tested the hydrolysis rate for the *p*-Nph-5′-TMP in the presence or absence of EDTA or divalent cations (EDTA in the range of 0.1–0.3 mM and Mg^{2+} or Ca^{+} in the range of 2–8 mM). EDTA or cations were added to the reaction mixture containing 50 mM Tris–HCl buffer, 5 mM KCl, 10 mM glucose, 225 mM sucrose, and pH 8.9. For control groups, neither EDTA either divalent cations were added to the reaction medium.

For analysis in the pH range from 6 to 10, a buffer solution reaction mixture containing 50 mM HEPES buffer, 50 mM Tris–HCl buffer, 50 mM glycine, 5.0 mM KCl, 10 mM glucose, and 225 mM sucrose was used.

The inhibitory effect on *p*-Nph-5′-TMP hydrolysis of the following compounds was analyzed: 1 mM levamisole, 0.25 mM suramin, 10 and 20 mM sodium azide, 0.3 and 0.5 mM gadolinium chloride. Enzyme was preincubated with the inhibitor for 10 min at 37°C followed by the addition of substrate.

RT- PCR analysis

RNA was isolated from left ventricle using the Trizol Reagent (Invitrogen). The cDNA species were synthesized from 5 μg of total RNA in a total volume of 25 μl with both oligo (dT) primer and random hexamers in accordance with the manufacturer's instructions. One microliter of the RT reaction mix was used as a template for PCR in a total volume of 20 μl using a concentration of 0.25 μM of each primer indicated below and 0.5 units *Taq*DNA polymerase. The PCR was run at the following conditions: 1 min at 95°C denaturation, followed by 35 cycles of 1 min at 94°C, 1 min at 60°C, 1 min at 72°C and a final 10 min extension at 72°C. Ten ml of the PCR reaction was analyzed on a 1.5% agarose gel. The primers used for rat NPP2 were 5′ GAAAATGCCTGTCACTGCTC 3′ and 5′ GCTGTAATCCATAGCGGTTG 3′ (amplification product 449 bp). For NPP1 and NPP3 were as described by Vollmayer et al. (2001) [29]. Negative controls were performed with water as template and positive controls were C6 rat glioma cells (NPP1), Walker cell (NPP2) and astrocytes (NPP3).

Real time PCR

Total RNA and cDNA were generated as described in RT-PCR analysis. SYRB Green I-based real-time PCR was carried out on MJ Research DNA Engine OpticonTM Continuous Fluorescence Detection System (MJ Research Inc.,Walthan, MA) as described [30]. All PCR mixtures contained: PCR buffer (final concentration 10 mM Tris–HCl (pH 9.0), 50 mM KCl, 2 mM $MgCl_2$, and 0.1% Triton X-100), 250 μM deoxy-NTP (Roche), 0.5 μM of each PCR primer, 0.5× SYBR Green I (Molecular Probes), 5% DMSO, and 1 U taq DNA polymerase (Promega, Madison, WI) with 2 μl cDNA in a 25 μl final volume reaction mix. The samples were loaded into wells of Low Profile 96-well microplates. After an initial denaturation step for 1 min at 94°C, conditions for cycling were 35 cycles of 30 s at 94°C, 30 s at 56°C, 1 min at 72°C. The fluorescence signal was measured right after incubation for 5 s at 79°C following the extension step, which eliminates possible primer dimer detection. At the end of the PCR cycles, a melting curve was generated to identify specificity of the PCR product. For each run, serial dilutions of human GAPDH plasmids were used as standards for quantitative measurement of the amount of amplified DNA. Also, for normalization of each sample, *β*-actin primers were used to

measure the amount of β-actin cDNA. All samples were run in triplicate and the data were presented as ratio of enzymes/β-actin. The primers used for real time PCR are described in RT-PCR analysis.

Results

Cardiac synaptosomes integrity

The integrity of cardiac synaptosomal fraction was evaluated by measuring the lactate dehydrogenase (LDH) activity. The ratio of this enzyme activity measured in intact and disrupted synaptosomes can be regarded as a measure of damaged particles [31]. The protocol was carried out according to the manufacture's instructions. Triton X-100 (1%, final concentration) was used to disrupt the cardiac synaptosomes preparation. The measurement of LDH activity showed that most of cardiac synaptosomes (approximately 86% ± 3%, mean ± SD, n = 3) were intact after the isolation procedure (data not shown).

Biochemical properties of p-Nph-5′-TMP hydrolysis by E-NPPs

The apparent optimum pH for the p-Nph-5′-TMP hydrolysis was determined in a medium with 50 mM HEPES, 50 mM Tris–HCl and 50 mM glycine buffering from pH 6–10, since E-NPPs are described as enzymes that act at alkaline pH [7]. We could observe that at lower pHs (6–7.5) the enzyme activity was reduced and the apparent optimum pH was obtained in the range 8.9–9.3 for p-Nph-5′-TMP hydrolysis (Fig. 1). Then, the pH 8.9 was chosen for experimental procedures.

The results of the experiments done to investigate the divalent cation dependence were represented in Fig. 2. As

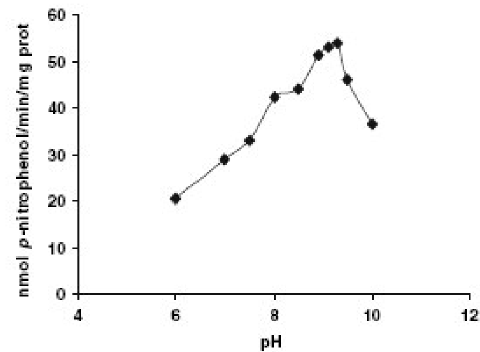

Fig. 1 Effect of pH variation on the E-NPP activity from rat cardiac synaptosomes, with p-Nph-5′-TMP as substrate. Conditions are described in Material and methods. Data are representative of three independent experiments. The activity was expressed as nmol p-nitrophenol/min/mg of protein

indicated in Material and methods, calcium and magnesium concentrations were tested in the range of 2–8 mM and 0.5 mM of substrate was used. As expected, increasing concentrations of EDTA (0.1, 0.2 and 0.3 mM) greatly reduced the catalytic activity, indicating that we are working with a cation-dependent enzyme. However, the p-Nph-5′-TMP hydrolysis was not further stimulated by the addition of the cations, when compared to the control levels. The catalytic rates were similar in the presence of Ca^{2+} added, when compared to the control group without cation addition and was a slightly increased by Mg^{2+} in a non-significant manner.

Michaelis constant (K_M) and V_{max} values were calculated from an Eadie–Hofstee plot (Fig. 3) with p-Nph-5′-TMP as substrate. Substrate concentrations tested ranged from 50 to 1,000 µM. The K_M value corresponded to 91.42 ± 13.97 µM (mean ± SD, n = 4) and the maximal velocity (V_{max}) value calculated was 63.79 ± 3.59 nmol p-nitrophenol released/min/mg of protein (mean ± SD, n = 4).

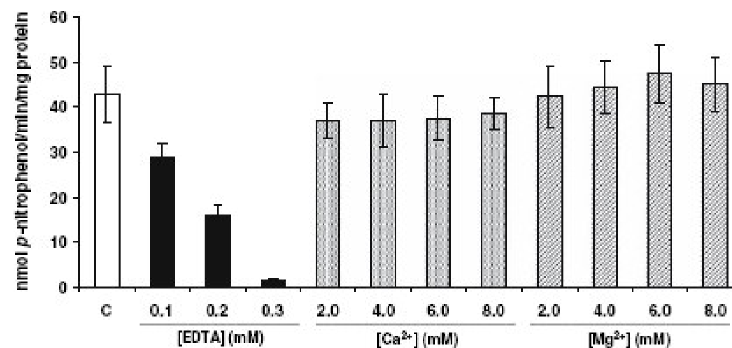

Fig. 2 Divalent cations dependence on p-Nph-5′-TMP hydrolysis. Hydrolysis of p-Nph-5′-TMP by rat cardiac synaptosomes was analyzed in the absence of cations (C), in the presence of 2–8 mM Ca^{2+} or 2–8 mM Mg^{2+}. EDTA in a range from 0.1 to 0.3 mM was used as a cation chelator. Bars represent means ± SD for three independent experiments. Results are expressed as nmol p-nitrophenol/min/mg of protein

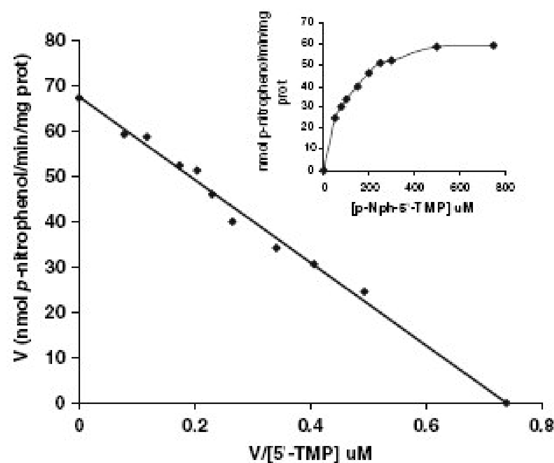

Fig. 3 Eadie–Hofstee plot for *p*-Nph-5′-TMP hydrolysis. The substrate concentrations tested ranged from 50 to 1000 μM (inset figure). The *KM* value corresponded to 91.42 ± 13.97 μM and the maximal velocity (V_{max}) value calculated was 63.79 ± 3.59 nmol *p*-nitrophenol released/min/mg of protein (mean ± SD). Data are representative of four different experiments

Action of inhibitors

Intracellular ATPases and possible enzymatic associations were eliminated by using enzyme specific inhibitors. As shown in Table 1, the classical alkaline phosphatase inhibitor, levamisole (1 mM) [32], was ineffective as inhibitor of *p*-Nph-5′-TMP hydrolysis in pH 8.9. On the other hand, suramin (0.25 mM), a P2 receptor antagonist and an inhibitor of E-NTPDase [33] and E-NPP activities [34], strongly reduced the hydrolysis of *p*-Nph-5′-TMP by approximately 46%. Sodium azide (10 and 20 mM) that is known to inhibit E-NTPDases at high-concentration, had no effects on *p*-Nph-5′-TMP hydrolysis. Gadolinium chloride (0.3 and 0.5 mM), a lanthanide that interacts with different pathways of intracellular and extracellular ATP action and that has been considered the most potent inhibitor for both soluble and membrane-bound NTPDases [35, 36] had no effects on *p*-Nph-5′-TMP hydrolysis.

Table 1 Effects of inhibitors on E-NPPs activities in cardiac synaptosomes

Inhibitors	Concentration (mM)	% of control enzyme activity
Suramin	0.25	54.22 ± 0.03*
Levamisole	1.0	91.60 ± 0.09
Azide	10.0	101.00 ± 0.04
	20.0	100.74 ± 0.04
Gadolinium chloride	0.30	94.13 ± 0.04
	0.50	96.17 ± 0.01

E-NPPs activities were assayed as described in Materials and methods and expressed as percent of control obtained in the absence of inhibitor. The average control values (100%) from different experiments were 58.75 ± 6.48 nmol *p*-nitrophenol/min/mg of protein. Results are mean ± SD of five experiments. Statistics significance was analyzed with the Student's *t*-test (*$P < 0.01$)

mRNA expression and quantitative analysis of E-NPPs in rat left ventricle

As *p*-Nph-5′-TMP can be metabolized by three different members of the ecto-nucleotide pyrophosphatases/phosphodiesterases (E-NPPs) family, we investigated the mRNA expression in rat left ventricle. Using specific primers, mRNAs for NPP2 and NPP3 were detected, while NPP1 signal in rat left ventricle was undetectable (Fig. 4A). The length of the oligonucleotide fragments obtained for the samples was compared to that obtained for the positive controls and corresponded to the expected sizes. In order to analyze e compare the different levels of mRNA expression of E-NPPs on rat left ventricle, we performed quantitative real-time RT-PCR analysis (Fig. 4

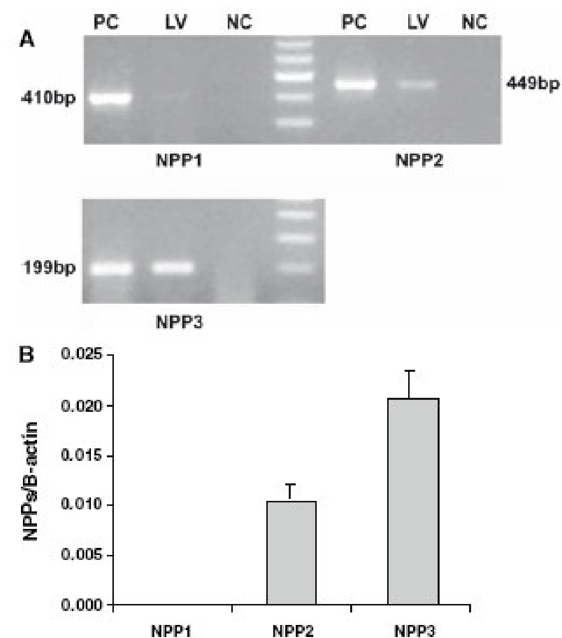

Fig. 4 RT-PCR and quantitative real-time PCR analysis of E-NPPs genes expressed by rat left ventricle. (A) Total RNA was isolated from left ventricles and the cDNA was analyzed by PCR with primers for NPP1, NPP2, NPP3 as described in Material and methods. (PC) positive control; (LV) left ventricles; and (NC) negative control. The length (bp) of the PCR products obtained with each pair of primers is given in the figure. (B) The expression of E-NPPS in left ventricles was quantitatively analyzed by real-time PCR as described in Material and methods. The results are presented as ratio of cDNA NPPs/β-actin. Bars represent mean ± SD for two experiments

B). The results showed that from E-NPPs, the NPP3 is the highest expressed from all genes are investigated.

Discussion

Extracellular nucleotides/nucleosides are known to regulate several physiological responses, including vascular tone, cardiac function, and hemostasis [37–39]. The hydrolysis ATP, ADP and AMP by ecto-enzymes (ecto-ATPase, NTPDases and 5′-nucleotidase) was thought to be a very simple process during a long time. Nowadays it is well known that this is a complex process involving several families of ectonucleotidases [15]. The relative contribution of the distinct ectonucleotidases species to the modulation of purinergic signaling may depend on differential tissue and cell distribution, regulation of expression, targeting to specific membrane domains, but also on substrate availability and substrate preference [9]. One of the four structurally unrelated families of ectonucleotidases is represented by E-NPPs.

In this study the enzyme activity obtained from intact cardiac synaptosomes shares the major biochemical properties already described for E-NPPs. The pH curve showed a maximal enzymatic activity at alkaline pH in the range 8.9–9.3. These pH values are in accordance with those previously described for E-NPPs [7]. The hydrolysis of *p*-Nph-5′-TMP by NPPs was determined in the presence of divalent cations (Ca^{2+} or Mg^{2+}) and the results indicates that the enzyme is cation-dependent, considering that increasing concentrations of EDTA greatly reduced the catalytic activity. It has been demonstrated that E-NPPs are metalloenzymes as their activity is blocked by metal chelators [7]. The activity was not further stimulated, when compared to the control levels, by the addition of the cations, since the catalytic rates were similar in the presence of Ca^{2+} or Mg^{2+}. We believe that the amounts of divalent cations present in the buffer used in the synaptosomes preparation could be sufficient for the enzyme activity.

Members of E-NPP family generally have wider substrate specificity than intracellular pyrophosphatases and phosphodiesterases. The *p*-nitrophenyl ester of TMP is used routinely for the in vitro assay of E-NPPs. Then, Michaelis constant (K_M) and V_{max} values were calculated from an Eadie–Hofstee plot with *p*-Nph-5′-TMP as substrate. The K_M value corresponded to 91.42 ± 13.97 μM (mean ± SD, $n = 4$) and the maximal velocity (V_{max}) value calculated was 63.79 ± 3.59 nmol *p*-nitrophenol released/min/mg of protein (mean ± SD, $n = 4$). This is in accordance with the NPP reaction that is characterized by a K_M of 50 to 500 μM and a V_{max} of 5–300 μmol/min/mg enzyme [8, 40, 41]. In the sense to eliminate the possible participation of other enzymes, we tested compounds like levamisole, suramin, sodium azide, and gadolinium chloride. The results could suggest that in the conditions tested we worked with a predominant E-NPP activity in our system incubation, since only suramin, an inhibitor of E-NPP activities [34, 35], strongly reduced the hydrolysis of *p*-Nph-5′-TMP.

NPP1-3, alone or in combination, are expressed in every cell type that has been analyzed for their presence [42, 43]. Our data demonstrated that left ventricle express two of these three members of the E-NPP family. Others researchers have been demonstrated the presence of the E-NPP family in heart, however, without analyze specifically the enzymes present in the left ventricle [7, 44]. It is known that the expression pattern of the E-NPPs isozymes is remarkably species dependent [7]. Human NPP1 is highly expressed in bone and cartilage cells, with intermediate expressed in heart, liver, placenta and testis [15]. In addition, NPP1 is also presented in kidney, salivary gland ducts, brain capillary endothelium, and the epydidymis [12, 42, 43]. Stefan et al. [44] verified the presence of NPPγ-cDna hybridized (NPP1) in rat tissues with two transcripts that were mainly present in liver, heart, and kidney. No expression of NPP1 has been detected in neurons and glial cells, although it has been detected in rat C6 glioma cells [45]. Our RT-PCR results demonstrated no expression of NPP1 in rat left ventricle. These results show some difference in the expression of this enzyme considering the total heart or only in left ventricle. NPP2 is abundantly expressed in embryonic tissues and various cancer cells [20]. There are some results showing the expression of NPP2 in rat brain, lung, duodenum, and adrenals [44] and, as showed by Bollen et al. [7], there are evidences for the presence of a NPP2 in human heart. Our results demonstrate an NPP2 in rat left ventricle. However, as we can observe, there is a small expression of this enzyme in this tissue, when compared with the NPP3 expression. It was found that NPPβ (NPP3) transcript was scarce in rat brain and adrenals, but abundant in liver, heart and kidney [44]. Our results are in agreement with the previous work and show the NPP3 as the most expressed of the E-NPP family in rat left ventricle.

The action of E-NPP 1-3 (in) directly interfere in the termination of nucleotide signaling, promoting the salvage of nucleotides and/or the generation of new messengers like ADP, adenosine or pyrophosphate [9]. It is clear that specific ectonucleotidases expressed by the cells regulates the ecto-nucleotides metabolism in their microenvironment. In accordance with their importance in cell signaling, the extracellular concentration of nucleotides is tightly regulated. In neurons, ATP is stored in synaptic vesicles in millimolar concentrations and is released into the synaptic cleft upon excitation [15]. Both pre- and post-synaptic

neuromodulation by ATP and its degradation product adenosine have been demonstrated, and are mediated by the activation of P2 and P1 purinoceptors respectively [15, 46, 47]. Recent works has been demonstrated that noradrenaline and ATP are co-released from sympathetic nerve terminals of the guinea pig heart whereby ATP enhances noradrenalin releases by a mechanism controlled by ectonucleotidases, possibly NTPDase1 [2, 24, 48]. Previous studies have reported that cells and tissues can co-express distinct ectonucleotidases that share common characteristics [35, 49, 50]. Then, we could propose a multiple system with the participation of an E-NPP activity for extracellular nucleotide hydrolysis control by sympathetic nerve terminals. It is important, since enhanced adrenergic activity and NE released are known causes of clinical cardiac dysfunction, arrhythmias, and sudden cardiac death during myocardial ischemia [24]. Besides, it is important that the study of differences in ventricles, since in some pathological situations, like a congestive heart failure caused by myocardial infarction, exists differential changes in the status of sympathetic nerves in the left and right ventricles. There is an increased sympathetic activity only in the left ventricle [51]. Then, the nucleotide hydrolysis control could exert a cardioprotective action by reducing ATP-mediated NE released with the maintenance of normal physiological process.

There is evidence that ecto-nucleotides contribute to a number of processes involved in normal cardiovascular function and that disturbances in purinergic signaling are involved in some cardiovascular diseases [37]. The role of E-NPPs in the cardiovascular system remains to be clarified, but this work presents, for the first time, this enzyme activity in synaptosomes from cardiac left ventricle. In this way, our results will open up new avenues for research into the physiological roles of this family enzymes heart tissue and their possible therapeutic potential to cardiovascular system.

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CAPÍTULO III

Streptozotocin-induced diabetes alters ATP and ADP hydrolysis in rat heart left ventricle

Artigo em preparação para ser submetido ao periódico *Life Sciences*

Streptozotocin-induced diabetes alters ATP and ADP hydrolysis in rat heart left ventricle

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ABSTRACT

Aims: Streptozotocin (STZ) has long been used to induce experimental diabetes *mellitus* in order to study diabetic complications. There are many controversies regarding cardiac alterations in diabetes and evidence is accumulating suggesting an important role for the purinergic system in cardiovascular regulation. In the present study we investigated if the STZ treatment is capable to cause any alteration on nucleotide metabolism in rat heart left ventricle.

Main methods: Glucose levels, body weight (BW), heart rate (HR), and systolic blood pressure (SBP) were evaluated after 30 days of a single intraperitoneal STZ injection to confirm the diabetes induction and also after 6 days of insulin treatment. Nucleotide hydrolyses were evaluated in cardiac synaptosomes.

Key findings: STZ injection caused a rise in glucose levels, a loss of body weight, and a decrease in HR and SBP when compared to control group (citrate-treated). Besides, the insulin treatment was able to reverse all these parameters. There was an inhibition in ATP and ADP, but not in AMP and 5'-TMP hydrolysis in cardiac synaptosomes, which were completely recovered with insulin treatment. The analysis of extracellular ATP metabolism in cardiac synaptosomes by HPLC showed that ATP was slowly hydrolyzed in diabetes group. Significance: In summary, we showed here a possible involvement of the purinergic system in the cardiac alterations due to diabetes pathology.

Keywords: diabetes, ectonucleotidases, cardiac synaptosomes, STZ, insulin

INTRODUCTION

Diabetes has been currently considered as an important public health problem due its increasing prevalence all over the world (Garcia et al. 2007). This disease is characterized by chronic hyperglycemia secondary to a reduction in the functional efficacy and/or a deficiency of insulin (Liu et al. 2008). Cardiovascular complications are the leading cause of diabetes-related morbidity and mortality (Goede et al. 2008). A common and severe complication of diabetes mellitus is the peripheral neuropathy that can leads to dysfunction in the cardiovascular system (Giudice et al. 2002; Howarth et al. 2006; Malone et al. 2007). The reduced ability to finely regulate heart rate (HR) in long-term diabetes is attributed to profound disturbances in autonomic function (McLeod 1992). Previous studies have demonstrated that an unbalanced sympathetic/parasympathetic tone, with a prevalence of sympathetic activity, is associated with higher cardiovascular mortality in diabetic patients (Kleiger et al. 1987; Tsuji H. 1994; Manzella and Paliosso 2005). Such unbalanced sympathetic/parasympathetic tone can be responsible for many cases of sudden death in diabetic patients, despite the absence of documented pre-existing heart disease (Tsuji H. 1994).

Evidence has been accumulated suggesting an important role for the purinergic system in cardiovascular regulation (Burnstock and Kennedy 1986; Burnstock 2002; Di Virgilio and Solini 2002; Erlinge and Burnstock 2008). In this sense, it is already known that purines are involved in different processes such as vasoconstriction and vasodilatation, growth of vascular muscle cells and endothelial cells, angiogenesis, vascular remodeling, platelet aggregation,

coagulation, inflammation and several aspects of cardiac function (Erlinge and Burnstock 2008).

It is well established that norepinephrine (NE) and ATP function as co-transmitters at peripheral adrenergic neuroeffector junctions (Bursntock 1999; Machida 2005). The release of NE from sympathetic nerves in the heart is regulated by several presynaptic receptors (Boehm and Kubista 2002) that can be either inhibitories (like adenosine A1 and P2YR) or facilitatories (P2XR). Unlike NE, whose actions are terminated predominantly by re-uptake into the nerve endings by a specific transporter, ATP, once released, is extracellularly metabolized by E-NTPDases via sequential conversion to ADP and AMP, which is converted to adenosine by 5'-nucleotidase (Zimmermann and Braun 1999). ATP has been implicated with inotropic and chronotropic effects in myocardium (Vassort 2001) and the release into extracellular space under pathophysiological conditions could be arrythmogenic (Kuzmin et al. 1998). Ectonucleotidases are, therefore, a key element in purinergic system because they modulate the ultimate effects of released nucleotides (Sesti et al. 2002).

The presence of ectonucleotidases in rat heart was demonstrated by different groups (Zinchuck 1999; Sesti et al. 2001). Recently, we reported the expression of E-NTPDases, E-NPPs and ecto-5'-nucleotidase in rat heart left ventricle and the profile of extracellular nucleotide hydrolysis in cardiac synaptosomes (Rucker et al. 2007; Rucker et al. 2008). Besides, it has been demonstrated that the purinergic system could be altered in diabetes *mellitus* (Pawelczyk et al. 2000; Grden et al. 2005; Lunkes et al. 2003; Lunkes et al. 2004;

Lunkes et al. 2008; Schetinger et al. 2008); however there are no reports regarding the involvement of purinergic signalling in diabetes cardiac problems.

In the present study, we investigated the activities of E-NTPDase, E-NPP and ecto-5'-nucleotidase family members in the heart left ventricle of STZ-diabetic rats, treated or not with insulin.

MATERIALS AND METHODS

Chemicals

Streptozotocin, nucleotides, *p*-nitrophenyl thymidine 5'-monophosphate, oligomicyn, sodium azide and Trizma Base were obtained from Sigma-Aldrich (St. Louis, MO, USA). Tetrabutylammonium chloride was purchased from Merck (Darmstadt, Germany) and anesthetic sodium thiopental from Cristália (São Paulo, SP, Brazil). NPH insulin (Novolin NPH) was from Novo Nordisk (Araucária, Paraná, Brazil). Collagenase type II was obtained from Worthington Biochemical Corporation (Lakewood, NJ, USA). Ringer-solution was purchased from Basa Ltda (Caxias do Sul, RS, Brazil). All others reagents were also of analytical grade.

Animals

Male *Wistar* rats (± 60 days, weighing between 170-220g) from our breeding stock were used in the study. Animals were maintained on 12 h light/dark cycle at a constant temperature of 23 ± 2 °C, with free access to food and water. Procedures for the care and use of animals were adopted according to the regulations of

Colégio Brasileiro de Experimentação Animal (COBEA), based on the Guide for the Care and Use of Laboratory Animals (National Research Council).

Experimental protocols

Animals were fasted overnight and rendered diabetic by a single intraperitoneal injection of STZ (65 mg/kg body weight) dissolved in 0.1 M citrate buffer, pH 4.5. Control rats (hereafter referred as citrate) were injected with citrate instead of STZ. On the day 7, blood glucose levels were measured from tail blood and only rats with values of glucose levels higher than 300 mg/dL were selected for further studies. Glucose levels and BW were evaluated on the days 0, 7, 30 and 36 and are summarized on Table1. Two different protocols were conducted: *Protocol 1 –* to investigate the *effect of diabetes:* the animals were divided in 2 groups: citrate and diabetes that were killed 30 days after the citrate or STZ injection. *Protocol 2 – to determine the effect of short-term insulin treatment:* Thirty-day diabetic rats were evaluated after 6 days of treatment with NPH insulin (subcutaneous) 2 U in the morning (8:30 a.m.), and NPH insulin 4U in the afternoon (5:30 p.m.) (insulin group) or saline in the same volume (saline group). This insulin regimen was previously described by Freitas et al. 2005.

Hemodynamic parameters determination

In all rats, heart rate (HR) and systolic blood pressure (SBP) were measured in awake animals, at approximately the same time of the day by tail-cuff plethysmography (Kent Scientific; RTBP1001 Rat Tail Blood Pressure System for

rats and mice, Litchfield, USA). Rats were conditioned with the apparatus before measurements were taken. In protocol 1, SBP was recorded at day 0 (before the beginning of the treatment), and at the day 30 (the end of the treatment). In protocol 2, SBP was recorded at day 0 (before the beginning of the treatment), and at the day 30 (before the beginning of the insulin treatment) and at the day 36 (the end of the insulin treatment). The heart rate values were derived from the pulsations detected by SBP.

Preparation of synaptosomal fraction from rat heart

The synaptosomal fraction was prepared as previously described by Aloyo et al. 1991. Briefly, the rat was killed by decapitation and immediately after the sacrifice the heart was removed and reperfused for 10 min with a Ringer-Lactate solution. This procedure ensured that blood did not remain in the coronary circulation. After this, the left ventricle was separated and minced in ice-cold solution 0.32 M sucrose containing 1.0 mM EGTA, pH 7.5. The mince was transferred to a HEPES buffered Krebs-Ringer (KRH) solution (50 mM HEPES, 144 mM NaCl, 1.2 mM $MgCl_2$, 1.2 mM $CaCl_2$, 5.0 KCl mM, 10 mM glucose, 1.0 mM ascorbic acid, pH 7.4) containing 12 U of collagenase type II per mg of tissue. This suspension was then incubated at 37 °C for 40 min and continuously bubbled with oxygen. After low speed centrifugation (10 min, 120 x *g*, 4 °C), the resulting pellet was suspended in 10 volumes of 0.32 M sucrose and homogenized with a Teflon/glass homogenizer. Cellular debris, nuclei and large tissues shards were removed by centrifugation (10 min, 650 x *g*, 4 °C). The resulting supernatant was centrifuged (20 min, 21,000 x *g*, 4 °C). The pellet was resuspended in oxygenated, ice-cold KRH and recentrifuged

twice (20 min, 22,000 x *g*, 4 °C). The pellet containing cardiac synaptosomes was resuspended in KRH to a final protein concentration of approximately 0.5-0.7 mg/mL.

Protein determination

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

E-NTPDases and 5'-nucleotidase assays

ATP hydrolysis was assayed in a reaction medium containing 2.0 mM $CaCl_2$, 5.0 mM KCl, 10 mM glucose, 225 mM sucrose, 2.0 μg/mL olygomicin, 0.1 mM sodium azide, 50 mM Tris-HCl buffer, pH 8.0, and 6.0 μg protein. To assay ADP hydrolysis, the reaction medium containing 2.0 mM $CaCl_2$, 5.0 mM KCl, 10 mM glucose, 225 mM sucrose, 50 mM Tris-HCl buffer, pH 8.0, and 6.0 μg of protein . The reaction mixture to assay AMP hydrolysis contained 2.0 mM $MgCl_2$, 150 mM sucrose and 50 mM glycine, pH 9.5, and 10 μg of protein. All enzyme reactions were performed in a final volume of 200 μL. The synaptosomal fractions were added to the reaction mixture, preincubated for 10 minutes and incubated (6 min for ATP and ADP, and 10 min for AMP hydrolysis) at 37 °C. The reaction was initiated by the addition of substrate to a final concentration of 1.0 mM and stopped by the addition of 200 μL 10% trichloroacetic acid. The samples were chilled on ice for 10 minutes and the inorganic phosphate (Pi) released was measured (Chan et al. 1986). Incubation times and protein concentration were chosen in order to

ensure the linearity of the reactions. Controls, with the addition of the enzyme preparation after the addition of trichloroacetic acid were used to correct for the non-enzymatic hydrolysis of substrates. All assays were done in duplicate or triplicate. Enzyme activities are expressed as nmol of phosphate released per min per mg of protein. Oligomycin was added from a concentrated ethanol solution in a final concentration of 1.0 % in the ATP incubation medium. The addition of ethanol did not arrest the enzyme activity in the absence of the inhibitor.

Ecto-nucleotide pyrophosphatase/phosphodiesterase (E-NPPs) assay

The phosphodiesterase activity was assessed using *p*-Nph-5'-TMP as substrate as previously outlined (Sakura 1998). The reaction medium containing 50 mM Tris-HCl buffer, 5 mM KCl, 10 mM glucose, 225 mM sucrose, pH 8.9, was preincubated with approximately 10 μg of synaptosomal protein per tube at 37 ^{0}C in a final volume of 200 μL. The enzyme reaction was started by the addition of *p*-Nph-5'-TMP to a final concentration of 0.5 mM. After 30 min of incubation, 200 μL NaOH 0.2 N was added to the medium to stop the reaction. An incubation time of 30 min was chosen in order to ensure the linearity of the reaction. The amount of *p*-nitrophenol released from the substrate was measured at 400 nm using a molar extinction coefficient of 18.8 x 10^{-3}/M/cm. Controls to correct for non-enzymatic substrate hydrolysis were performed by adding synaptosomal preparation after the reaction had been stopped with NaOH . All samples were performed in duplicate. Enzyme activities are expressed as nmol *p*-nitrophenol released per minute per milligram of protein.

Analysis of extracellular ATP metabolism by High Pressure Liquid Chromatography (HPLC)

Synaptosomal samples were obtained as described before. The same reaction mixture as well as the protein concentration were used to analyze ATP metabolism. However, to start the reactions, ATP was added to the medium in a final concentration of 0.1 mM at 37ºC. Aliquots of the sample were collected at different incubation times (0, 5, 10, 20, 30, 60 and 90 min), with the reaction being stopped on ice. All samples were centrifuged 14,000 X *g* for 15 minutes. Aliquots of 40 µL were applied to a reversed-phase HPLC system using a 25 cm C_{18} Shimadzu column (Shimadzu, Japan) at 260 nm with a mobile phase containing 60 mM KH_2PO_4, 5 mM tetrabutylammonium chloride, pH 6.0, in 30% methanol according to a method previously described (Voelter 1980). The peaks of purines (ATP, ADP, AMP, and adenosine) were identified by their retention times and quantified by comparison with standards. The results are expressed as nmoles of the different compounds per mg of protein for each different incubation time. All incubations were carried out in triplicate and the controls to correct non-enzymatic hydrolysis of nucleotides were performed by measuring the peaks present into the same reaction medium without synaptosomes. The control for intrinsic synaptosomal purines was performed by incubation of the preparation without the substrate under the same conditions.

Data Analysis

Results are expressed as mean ± standard deviation (S.D.). The comparison among groups was analyzed by Student's *t* test for independent samples or one-way ANOVA followed by Student-Newman-Keuls *post hoc* test ($P<0.05$; $P<0.01$).

RESULTS

Diabetes model validation

In the present study, a single intraperitoneal injection of STZ in rats lead to the development of a clinical syndrome characterized by hyperglycemia, excessive osmotic diuresis and loss of body weight, which resembles the human diabetes (Schaan et al. 2004). Moreover, STZ-diabetic rat develops the usual chronic microvascular complications (nephropathy, peripheral and autonomic neuropathy) as observed in diabetic patients (Jensen et al. 1981; Schmid and Plural 1986; Schaan et al. 2004).

As shown in Table 1, the injection of STZ was associated with a significant rise in glucose levels from 106 ± 4 mg/dL on day 0 to 493 ± 45 on day 30, while in the citrate group the glucose levels were 111 ± 11 on day 0 and 104 ± 7 mg/dL on day 30. The saline group presented the similar rise 107 ± 14 mg/dL on day 0 and 486 ± 70 mg/dL on day 36. On the other hand, insulin group presented glucose levels similar to those of diabetes and saline group (109 ± 9 mg/dL on day 0, 481 ± 72 mg/dL on day 30) but after six days of insulin treatment, glucose levels returned to the levels of day 0 (114 ± 42 mg/dL) and were similar to the citrate group (107 ± 8 mg/dL) at the end of the experiment (36 days).

With regard to the body weight, we observed a normal increase in citrate group, which was not present in the other groups. Six days of insulin treatment, when the glucose levels were controlled, were enough to bring the body weight at the levels of insulin group, although it remained minor comparing to the citrate group (Table 1).

We observed that rats presented lower heart rate (HR) 30 days after STZ induction in diabetes (316±15 bpm), saline (321±14 bpm) and insulin (328±15) groups when compared to citrate group (369±14). However, with 6 days of insulin treatment, this parameter returned to the control value and saline group was still altered, as shown in Table 1. Besides, all groups 30 days after STZ injections showed hypotension (Table1) that disappeared in the insulin group.

Effect of STZ injection and insulin treatment on ectonucleotidase activities in cardiac synaptosomes

STZ injection was associated with an inhibition in ATP (Fig. 1A) and ADP (Fig. 1B) but not in AMP (Fig. 1C) and 5'-TMP (Fig. 1D) hydrolysis in cardiac synaptosomes when compared to the citrate group. The hydrolysis was inhibited 42% and 31% for ATP and ADP, respectively. The saline group (STZ injection +6 days of saline) presented the same pattern of substrates hydrolysis of diabetes group, however, in the insulin group (STZ injection + 6 days of insulin), the ATP and ADP hydrolysis returned to the citrate group levels.

Metabolism of extracellular ATP in cardiac synaptosomes of diabetic rats and/or treated with insulin

We also followed the pattern of extracellular ATP metabolism in cardiac synaptosomes of diabetic rats and diabetic rats treated with insulin by HPLC analysis during 90 min. As shown in Figure 2, with minor differences, the pattern of citrate group (Fig. 2A) is similar to the insulin group (Fig. 2D), while the pattern of diabetes group (Fig. 2B) is similar to the saline group (Fig. 2C). The diabetes group and saline group hydrolyzed slowly extracellular ATP and ADP, when compared with the citrate and insulin groups, however, the adenosine formation remains unchanged in all groups.

DISCUSSION

The role of ATP as an extracellular signalling molecule is now well established and evidence is accumulating that ATP and other nucleotides (like ADP) play important roles in cardiovascular physiology and pathophysiology acting via P2X (ion channel) and P2Y (G proteincoupled) receptors (Erlinge and Burnstock 2008). Then, considering the importance of cardiovascular disease as the most common complication in diabetes *mellitus* (Giudice et al. 2002) and that the consequences of diabetes induced by streptozotocin on sympathetic neurotransmission (Ralevic et al. 1995) are still unknown, this investigation reports the ectonucleotidases activities and extracellular ATP metabolism in synaptosomes of heart left ventricle from STZ-diabetic rats, treated or not with insulin.

It is well known that experimental diabetes induced by STZ has been used by several investigators to study disorders of the autonomic control of the

cardiovascular system (Angelis et al. 2002; Schaan et al. 2004; Howarth et al. 2005; Howarth et al. 2006; Lin et al. 2008). Since the autonomic nervous system modulates beat-to-beat fluctuations in heart rate (HR), methods to quantify HR and blood pressure have been evaluated as indicators of sympathetic and parasympathetic modulation of the cardiovascular functions in humans (Eur Soc Cardiol, 1996) and in experimental models (Angelis et al. 2002; Schaan et al. 2004). Our results showed that after 30 days of STZ injection, the diabetic animals developed a bradycardia when compared to the control group (Table 1). Besides, the same animals presented SBP reduced after the same period. These results are in accordance with other investigations (Grimm et al. 2002; Schaan et al. 2004; Howarth et al. 2005; Malone et al. 2007), however, the exactly mechanism responsible for these alterations remain poorly understood.

Many authors have been attributed the cardiac alterations in diabetes to disturbances in the sympathetic/parasympathetic tone (Kleiger et al. 1987; Tsuji 1994; Manzella and Paliosso 2005). It is well established that ATP acts as an extracellular signal in many tissues and is involved in a variety of regulatory processes including the control of vascular tone, muscle contraction, pain, or neuronal communication (Burnstock 1997). ATP serves as a neurotransmitter or co-transmitter in central, as well as in peripheral neurons (Bean 1992; Burnstock 1997). For instance, ATP is co-released with noradrenaline or acetylcholine from sympathetic or parasympathetic nerve endings, respectively, and even from neuromuscular synapses (Burnstock 1997). Besides, ectonucleotidases have been proposed to be co-released with ATP from nerve terminals (Todorov et al. 1997).

We could observe that there were alterations in ATP and ADP hydrolysis and these observations could reveal the possible participation of the purinergic system in the cardiac pathophysiology of diabetes. Drury and Szent-György, in 1929, reported a negative chronotropic effect of purines. Further studies suggested that ATP has dose-dependent effects; small doses producing tachycardia while relatively larger doses of ATP slow the heart rate and induce atrioventricular nodal conduction block (Hollander et al. 1957; Stoner et al. 1948). Since we observed a diminution in ATP and ADP hydrolysis, it is probably that these nucleotides may be accumulating and these alterations may participate with other altered mechanisms in the changing HR and SBP in STZ-induced diabetes. However, we can not exclude the participation of adenosine in this process. There are observations indicating that negative chronotropic action is in part due to P1-purinergic activation by ATP degradation to adenosine (Xu et al. 1994, Vassort 2001). We did not observed alterations in AMP hydrolysis, indicating that the effects observed probably are due to the alterations only in ATP and ADP content and the activation of P2 receptors. It is known that in addition to its postsynaptic effects, ATP affects adrenergic transmission by acting on purinoceptors at sympathetic nerve endings (Burnstock 1999). In primary cultures of dissociated rat superior ganglions neurons, ATP-gated ionotropic P2X purinoceptors (P2XR) are known to enhance NE exocytosis whereas metabotropic G-protein-coupled P2Y purinoceptors (P2YR) may attenuate it. It has been suggested that endogenous ATP acts by an autocrine feedback mechanism on cardiac sympathetic terminals from which is released (Sesti et al. 2002) and then, it could participate in the control of the NE release.

With our results, we have some difficult to explain what is the exactly mechanism that is causing the diminution of the ATP and ADP hydrolysis. It could be a down regulation of ectonucleotidases activity, probably E-NTPDases, since the hydrolysis of 5'-TMP, an artificial marker used to test the presence of E-NPPs, did not change. Besides, the expression of these enzymes could be altered and molecular techniques are necessary to clarify these points.

Other possibility is that the P2 receptors could be altered in the terminal endings of diabetic individuals, presenting differential sensitivity to these ligands. Since we observed that the glycemic control returned the ATP and ADP hydrolysis to the control levels, we could suggest that the hyperglycemia may participate in this process. Hyperglycemia has been considered as an important cause of diabetic cardiomyopathy, as a result of abnormal cellular metabolism and altered gene expression (Li et al. 2007)

Taken together, our results demonstrate that the cardiac synaptosomes from STZ-diabetic rats had an altered ATP and ADP hydrolysis. It is, therefore, tempting to suggest a possible involvement of the purinergic system in cardiac diabetes problems.

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Figure Legends

Figure 1: Effect of diabetes on ectonucleotidases activities from cardiac synaptosomes from citrate, diabetes, saline (30 days diabetes + 6 days of saline) and insulin (30 days diabetes + 6 days of insulin) treatment. ATP (A), ADP (B), AMP (C) and 5'-TMP (D) hydrolysis were evaluated in synaptosomes of 30 days diabetic rats. ATP and ADP hydrolysis were significantly inhibited and 6 days of insulin treatment restored the enzyme activities. Conditions are described in Material and Methods. Results are expressed as means ± SD (at least n=10 for each substrate tested). The comparison among groups was analyzed statistically by one-way analysis of variance (ANOVA) followed by Student-Newman-Keuls test. * Represents significant statistical difference comparing to the respective citrate group, considering $P \leq 0.05$. # Represents significant statistical difference comparing to the diabetes and saline groups, considering $P \leq 0.05$.

Figure 2: ATP metabolism and product formation in rat cardiac synaptosomes of diabetic rats. The synaptosomes were incubated with 100 μM ATP. Citrate (A), diabetes (B), saline (C), and insulin (D) groups. Data represents a typical result of three independent experiments.

Table 1

Table 1: Characterization of diabetic and control rats 0, 30 and 36 days after STZ injection.

	0 day				30 days				36 days		
	C (n=8)	D (n=8)	S (n=8)	I (n=8)	C (n=8)	D (n=7)	S (n=6)	I (n=6)	C (n=8)	S (n=5)	I (n=5)
BW (g)	192±17	194±17	195±21	183±18	270±13	202±25*	211±18*	208±11*	295±15	208±14*	226±14*
Glucose (mg/dL)	111±11	106±4	107±14	109±9	104±7	493±45*	480±55*	481±72*	107±8	486±70*	$114 \pm 42^{\#}$
HR (bpm)	373±16	372±10	378±16	372±19	369±14	316±15*	321±14*	328±15*	364±10	310±11*	$370 \pm 14^{\#}$
SBP (mmHg)	112±6	114±8	112±4	115±6	114±6	100±4*	101±5*	102±4*	112±4	101±5*	$110 \pm 5^{\#}$

BW: body weight in grams (g); HR: heart rate in beats per minute (bpm); SBP: systolic blood pressure in mmHg; from citrate (C), diabetes (D), saline (S: 30 days diabetes + 6 days of saline) and insulin (**I**: 30 days diabetes + 6 days of insulin) treatment. Results are expressed as mean ± S.D. The number of animals per group is indicated in the parenthesis. The symbol * represents statistical difference from citrate group (P<0.01) and the symbol # represents statistical difference from saline group (P<0.01).

Figure 1

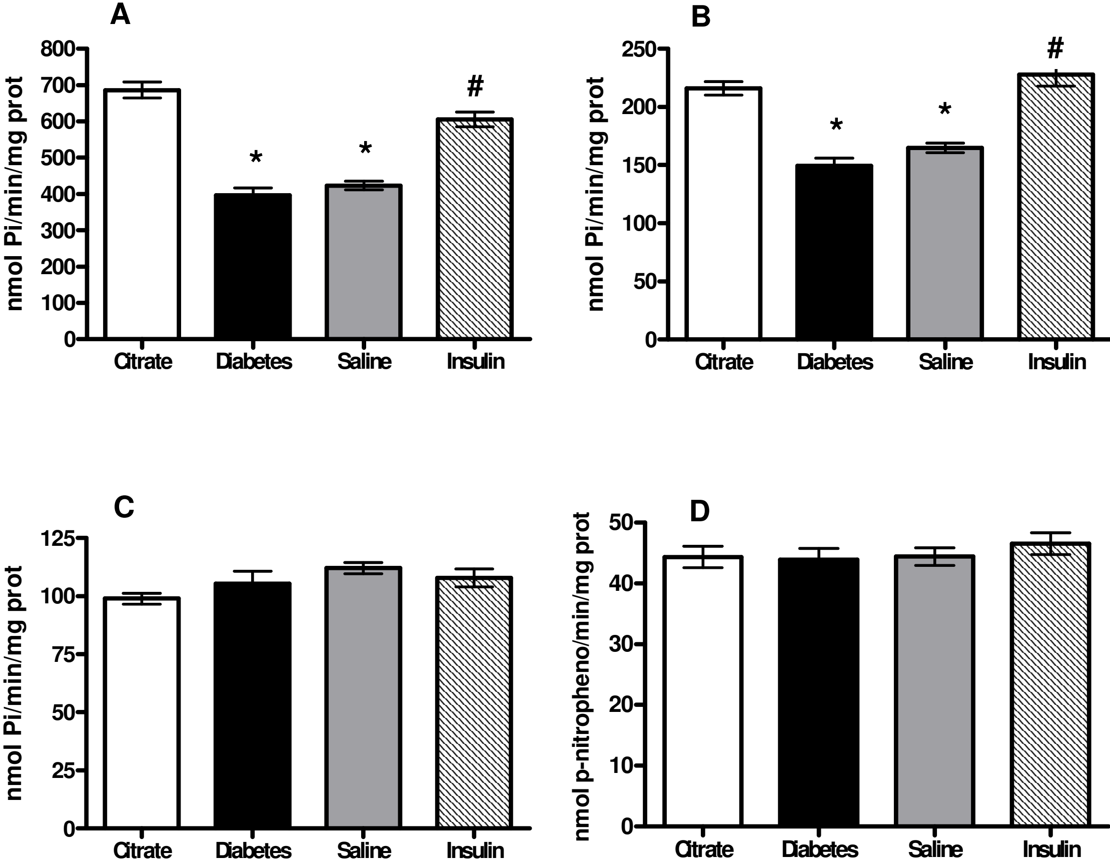
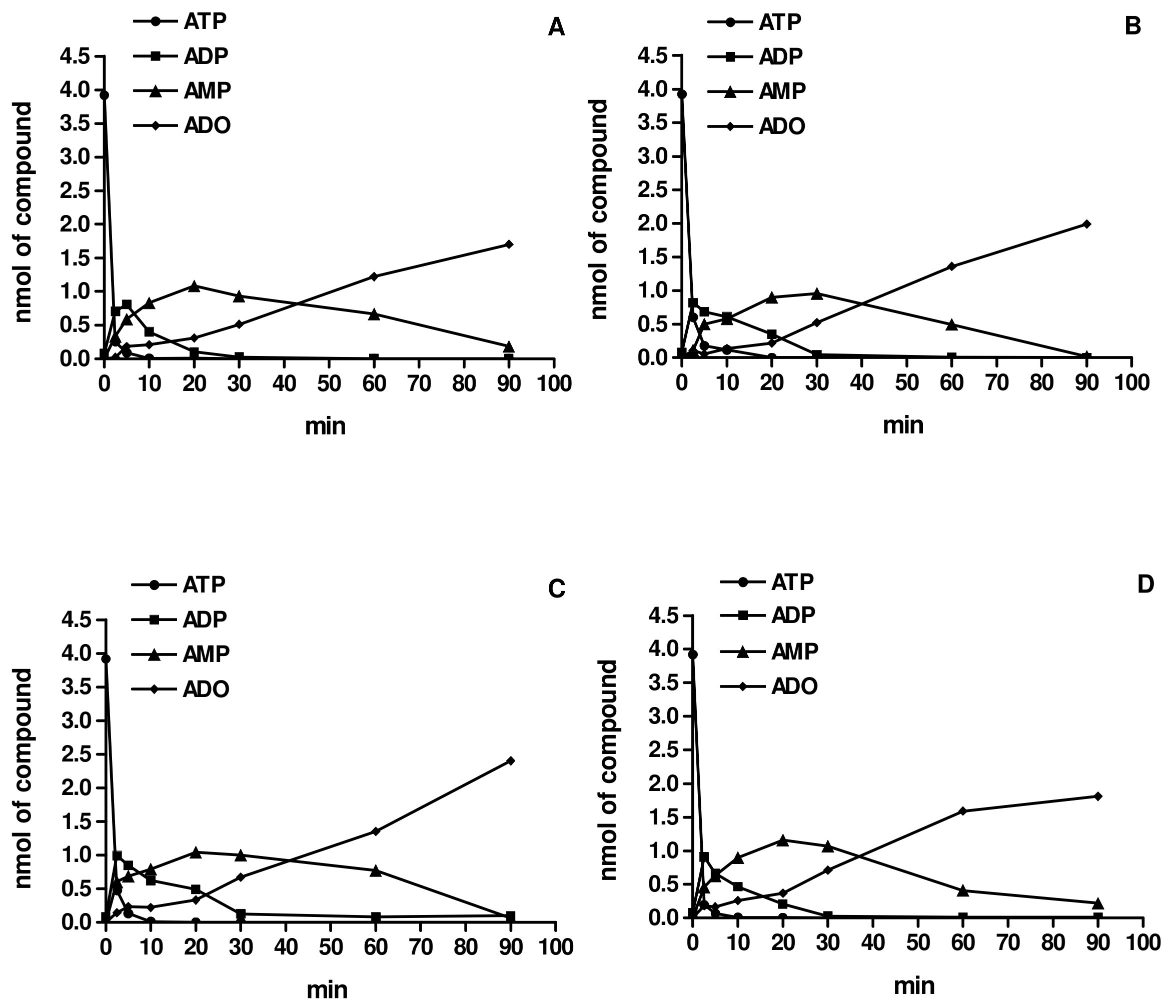


Figure 2



CAPÍTULO IV

The nucleotide hydrolysis is altered in blood serum of streptozotocin-induced diabetic rats

Artigo a ser submetido ao periódico *Molecular and Celullar Biochemistry*

The nucleotide hydrolysis is altered in blood serum of streptozotocin-induced diabetic rats

Shortened title: Nucleotide hydrolysis in blood serum of diabetic rats

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ABSTRACT

Ectonucleotidases and the nucleotide metabolism have been implicated as important regulators of various tissue functions in diabetes disease. In this study, we evaluated the ectonucleotidase activities and the profile of extracellular ATP metabolism in blood serum of streptozotocin (STZ) - induced diabetic rats and the influence of glycemic status. The 30 days of STZ injection was associated with a raise in ATP, ADP, AMP, and 5'-TMP hydrolysis in blood serum rats, when compared to the citrate group (vehicle control of STZ). The saline group (30 days of STZ injection, treated 6 days with saline) presented the same pattern of substrate hydrolysis of the STZ group (30 days of STZ injection). However, in the insulin group (36 days of STZ injection, treated 6 days with insulin), ATP, ADP, AMP, and 5'-TMP hydrolysis returned significantly to the citrate group levels. *In vitro,* 20 mM of glucose added did not affect ectonucleotidase activities in normal blood serum rats with 24 h of preincubation time. Extracellular ATP metabolism by rat serum was estimated by HPLC analysis. A rapid hydrolysis of extracellular ATP by diabetic and saline animals was observed, decreasing the ATP and ADP levels faster than in serum of citrate and insulin groups, leading to the formation of high levels of adenosine when compared with citrate and insulin groups. Since in diabetes the vascular disease is frequently present, the alterations observed are important, because these enzymes control the nucleotides/nucleosides ratio in the circulation and thus the events related to haemostais.

Keywords: E-NTPDases, ecto-5'-nucleotidase/CD73, E-NPPs, rat blood serum, diabetes

INTRODUCTION

Diabetes mellitus is a group of disease characterized by chronic hyperglycemia due to deficiency of insulin secretion, insulin action or both [1], and it is associated with the multiple defects in various tissues [2]. With long duration of diabetic metabolism, diabetes-specific complications, chiefly involving small vessels (retinopathy, nephropathy, and neuropathy), may ensue, and lead to serious outcomes such as visual disturbance, renal failure and gangrene [3]. Accelerated atherosclerosis and microvascular complications are perhaps the leading cause of coronary heart disease, blindness and renal failure, which could account for disabilities and high mortality rates in patients with diabetes. Several thrombogenic abnormalities have been shown to play a central role in the pathogenesis of these devastating complications [4].

Circulating nucleotides are released as signaling substances or during pathological events [5,6]. Extracellular purines, such as adenosine, adenosine diphosphate (ADP) and adenosine triphosphate (ATP), are important signalling molecules that mediate diverse biological effects in many tissues, including the vascular system [7]. ATP acts as vasoconstrictor and may be a citotoxic structure [8], while could be a potent vasodilator in most vascular beds [9], depending on its concentration and receptor ligand [10]. ADP acts upon platelets, modifying their shape and regulating their aggregation [11] and adenosine, the nucleoside produced by the nucleotide catabolism, opposes platelet aggregation, prevents microthrombosis and is capable of acting as vasodilator in general [12].

There are important regulatory mechanisms that control the external concentration of nucleotides and hence regulate P2-receptor mediated effects. The

actions induced by extracellular ATP and adenosine are directly correlated to the activity of a variety of ectoenzymes that are either, located on the cell surface, or in a soluble form in the interstitial medium, or within body fluids [13]. These ectoenzymes constitute a highly organized enzymatic cascade for the regulation of nucleotide-mediated signaling, controlling the rate, amount and timing of nucleotide (e.g. ATP) degradation and ultimately nucleoside (e.g. adenosine) formation [14]. The most important ectoenzymes involved in adenine extracellular nucleotide hydrolysis are the ectonucleotidases, including members of ecto-nucleotide pyrophosphatases/phosphodiesterases (E-NPPs) and ecto-nucleoside triphosphate diphosphohydrolases (E-NTPDases) families as well as the ecto-5'-nucleotidase/CD73 [13,15,16].

Some studies have been indicated that diabetes is capable to alter the metabolism of nucleotides/nucleosides. It has been demonstrated that nucleotide hydrolysis are altered in platelets and synaptosomes from CNS [17], cerebrospinal fluid [18] and the ENTPDase1 was considered a vascular protective factor in diabetic nephropathy that modulates glomerular inflammation and thromboregulation [19].

Since the modulation of extracellular nucleotide metabolism is an important factor in several acute and subacute models of vascular injury, like diabetes, the aim of this study was evaluate the ectonucleotidases activities and the pattern of extracellular ATP metabolism in blood serum of diabetic rats induced by streptozotocin model.

MATERIALS AND METHODS

Chemicals

Streptozotocin, nucleotides, *p*-nitrophenyl thymidine 5'-monophosphate and Trizma Base were obtained from Sigma-Aldrich (St. Louis, MO, USA). Tetrabutylammonium chloride was purchased from Merck (Darmstadt, Germany) and anesthetic sodium thiopental from Cristália (São Paulo, SP, Brazil). NPH insulin (Novolin NPH) was from Novo Nordisk (Araucária, Paraná, Brazil). All others reagents were also of analytical grade.

Animals

Male *Wistar* rats (± 60 days, weighing about 170-220g) from our breeding stock were used in the study. Animals were maintained on 12 h light/dark cycle at a constant temperature of 23 ± 2 °C, with free access to food and water. Procedures for the care and use of animals were adopted according to the regulations of Colégio Brasileiro de Experimentação Animal (COBEA), based on the Guide for the Care and Use of Laboratory Animals (National Research Council).

Experimental protocols

The animals were fasted overnight and rendered diabetic by a single intraperitoneal injection of STZ (65 mg/kg body weight). STZ was dissolved in 0.1 M citrate buffer, pH 4.5. Control rats (hereafter referred as citrate) were injected with citrate instead of STZ. On the day 7 blood glucose levels were measured from tail blood and only rats with glucose levels of >300 mg/dL were selected for further

studies. Glucose levels and body weight were evaluated on the days 0, 7, 30 and 36 and are summarized on table1. Two protocols of study were conducted. *Protocol 1 – effects of diabetes:* the animals were divided in 2 groups: citrate and diabetes that were killed 30 days after the citrate or STZ injection. *Protocol 2 – effects of short-term insulin treatment:* Thirty-day diabetic rats were evaluated after 6 days of treatment with NPH insulin (subcutaneous), 2 U in the morning (8:30 a.m.) and NPH insulin, 4U in the afternoon (5:30 p.m.) or saline in the same volume. This insulin regimen was previously described by Freitas et al, 2005 [20].

Isolation of Blood Serum

For enzymatic assays, the blood samples were obtained as previously described by Yegutkin (1997) [21], with minor modifications [22], in 30th day (citrate group and diabetes group) and 36th day [diabetes + saline group (referred as saline group) and diabetes + insulin group (referred as insulin group)]. After the induction of the model, blood was drawn after decapitation of male *Wistar* rats and was allowed to clot at room temperature for 30 minutes. Blood was centrifuged in glass tubes at 5,000 X g for 5 minutes at room temperature. The resultant serum samples were kept on ice and immediately used for enzymatic assays.

Assays of Ectonucleoside triphosphate diphosphohydrolase (E-NTPDase) and Ecto-5'-nucleotidase Activities

For the incubation of E-NTPDase and Ecto-5'-nucleotidase from blood serum samples, the reaction mixtures containing 112.5 mM Tris-HCl, pH 8.0 was used. The content of protein was 1.0 mg per tube. To start the reactions, substrates were

added to the medium in a final concentration of 3.0 mM. TCA 5% (final concentration) was used to stop the enzymatic reactions after 40 minutes of incubation. Incubation times and protein concentrations were chosen to ensure the linearity of the enzymatic reactions. The amount of inorganic phosphate (Pi) released was carried out using a colorimetric method as previously outlined [23]. Controls were performed to correct the non-enzymatic substrate hydrolysis by adding blood serum after the reactions had been stopped with TCA. All samples were performed in triplicate. Enzyme activities were generally expressed as nmol Pi released per minute per milligram of protein.

Assay of Ecto-nucleotide pyrophosphatase/phosphodiesterase (E-NPP) Activity

The phosphodiesterase activity was assessed using *p*-nitrophenyl 5'-thymidine monophosphate (*p*-Nph-5'-TMP – an artificial marker substrate that is used routinely for the *in vitro* assay of this activity) as previously outlined [24]. For assay of E-NPP activity in blood serum samples, the reaction medium containing 100 mM Tris-HCl, pH 8.9, was preincubated with approximately 1.0 mg of serum protein for 10 minutes at 37ºC. The enzyme reactions were started by the addition of *p*-Nph-5'-TMP to a final concentration of 0.5 mM. After 8 minutes of incubation, 200 µL NaOH 0.2 N was added to the medium to stop the reactions. Incubation times and protein concentrations were chosen in order to ensure the linearity of the reaction. The amount of *p*-nitrophenol released from the substrate was measured at 410 nm using a molar extinction coefficient of 18.8 X 10^{-3}/M/cm. Controls to correct the non-enzymatic substrate hydrolysis were performed by adding blood serum after the reaction had been stopped with NaOH. All samples were

performed in triplicate. Enzyme activities were generally expressed as nmol *p*-nitrophenol released per minute per milligram of protein.

Analysis of extracellular ATP metabolism by High Pressure Liquid Chromatography (HPLC)

The serum samples were obtained as described above. The reaction was started by adding ATP at final concentration of 100 µM to the same incubation medium and conditions as described above for E-NTPDase. After the incubation time (0, 5, 10, 20, 30, 60 and 90 min), the reaction was stopped on ice. Further, the proteins were denaturated by the addition of 0.6 mol/L of perchloric acid. Then, all samples were centrifuged (14,000 X *g* for 10 minutes) and the obtained supernatants were neutralized with 4N KOH and were clarified with a second centrifugation (14,000 X *g* for 15 minutes). Aliquots of 40 µL were applied to a reversed-phase HPLC system using a 25 cm C_{18} Shimadzu column (Shimadzu, Japan) at 260 nm with a mobile phase containing 60 mM KH_2PO_4, 5 mM tetrabutylammonium chloride, pH 6.0, in 30% methanol according to a method previously described [25]. The peaks of purines (ATP, ADP, AMP, and adenosine) were identified by their retention times and quantified by comparison with standards. The results are expressed as nmoles of the different compounds per mg of protein in the incubation time. All incubations were carried out in triplicate and the controls to correct non-enzymatic hydrolysis of nucleotides were performed by measuring the peaks present into the same reaction medium incubation without

serum. The control for serum purines was performed by incubation the serum without the substrate under the same conditions described above.

Glucose interference in vitro assay

To evaluate some interference of glucose in ectonucleotidases activities *in vitro,* experiments were performed with 20 mM glucose added in blood serum from control rats (normal rats), as described previously by Lunkes et al. (2008)[26]. Pre-incubation of 24 h were used and the blood serum was incubated as described previously. As control of our assay, we incubated blood serum of diabetic (30 days STZ injection) and normal rats in the same conditions, without glucose added.

Protein Determination

Protein was measured by the Coomassie Blue method using bovine serum albumin as standard [27].

Data Analysis

Results are expressed as mean ± standard deviation (S.D.). The comparison among groups was analyzed by one-way ANOVA followed by Student-Newman-Keuls *post hoc* test (P<0.05; P<0.01).

RESULTS

Model Validation

Table 1 shows the weights and glucose levels of the fours groups of rats. In the present study, a single intraperitoneal injection of STZ was associated with a significant rise in glucose levels from 108 ± 6 mg/dL on day 0 to 481 ± 56 on day 30 after injection on diabetes group, while the citrate group the glucose levels were 111 ± 12 on day 0 and 105 ± 24 mg/dL on day 30. The similar rise we observed in the group treated with saline that presented 107 ± 13 mg/dL on day 0 and 443±76 mg/dL on day 36. On the other side, insulin group presented glucose levels similar to the diabetes and saline group until 30 days (480 ± 73 mg/dL, 481 ± 53 and 473 ± 54, insulin, diabetes and saline groups, respectively), but after six days of insulin treatment, glucose levels returned to the levels on day 0 (114 ± 42 mg/dL) and were similar to the citrate group (110 ± 16 mg/dL) on the end of the experiment (36 days after STZ injection).

With regard to the body weight, we could observed a normal increase in citrate group; however in the other groups this normal rise did not occurred. Even with six days of insulin treatment, when the glucose levels were controlled, the body weight raised of insulin group was significantly minor than compared with citrate group (Table 1), but we can observe that there is an rapid body weight gain in the insulin group when compared to the saline group in this period.

Effect of STZ injection and insulin treatment on ectonucleotidase activities in serum

After 30 days of STZ injection there was an increase in ATP (Fig. 1A), ADP (Fig. 1B), AMP (Fig. 1C) and 5'-TMP (Fig. 1D) hydrolysis in blood serum, when compared to the citrate group. The hydrolysis raise was of 85%, 96%, 132% and

44% (ATP, ADP, AMP and 5'-TMP, respectively). The saline group (36 days of STZ injection, treated 6 days with saline) presented the same pattern of substrates hydrolysis of the diabetes group (30 days of STZ injection), however, in the insulin group (36 days of STZ injection, treated 6 days with insulin), the ATP, ADP, AMP and 5'-TMP hydrolysis returned significantly to the citrate group levels.

Metabolism of extracellular ATP in serum of diabetic rats treated with insulin

We also investigated the pattern of extracellular ATP metabolism in serum of diabetic rats treated or not with insulin, by HPLC analysis throughout a period of 90 min. As shown in Fig. 2, with minor differences, the pattern of citrate group (Fig. 2A) is similar to the insulin group (Fig. 2D), while the pattern of diabetes group (Fig. 2B) is similar to the saline group (Fig. 2C). The diabetes and saline groups quickly hydrolyzed extracellular ATP and ADP, when compared with the citrate and insulin groups. After 60 min, ATP was completely hydrolyzed in diabetes and saline groups, while in citrate and insulin groups this not occurred. In this same time, the ADP is more hydrolyzed in these groups. The end of extracellular ATP cascade produces high quantities of adenosine in the diabetes and saline groups (Fig. 2B and 2C).

The data presented above is in agreement with the enzymatic assays, where we observed an increase in the ectonucleotidases activities in the diabetes and saline groups. On the other side, the insulin was able to return these enzyme activities to the control levels. This could be observed in the nucleotide hydrolysis profile, when the pattern of insulin group was similar to citrate group.

Glucose interference in vitro assay

The effect of glucose on ectonucleotidases *in vitro* is shown in figure 3. As we can observe, 20 mM of glucose added *in vitro* did not affect ectonucleotidases activities in normal blood serum rats with 24 h of pre-incubation time. On the other hand, diabetic rats demonstrated the same rise in ectonucleotidases activities previously demonstrated.

DISCUSSION

Diabetes accelerates and exacerbates the occurrence of arteriosclerosis, increasing the risk of myocardial infarction, cerebral infarction and occlusive artery disease of the lower extremities [3]. Also, the hypercoagulable state of patients with diabetes arises as a substantial factor accelerating atherosclerosis development and the incidence of arterial thrombotic pathogenesis [28].

Many authors have reported the use of STZ to induced experimental diabetes due to its specific toxicity on pancreatic β-cells [29,30]. This model is used to study the different complications related to the disease. Within days after STZ injection, animals develop severe hyperglycemia and diuresis coupled with weight loss [1, 31], as observed in diabetic patients. In accordance with literature, our data validate the model of streptozotocin (table 1).

Extracellular nucleotides can be released into blood by vascular cells e.g. erythrocytes, platelets, leucocytes and endothelium [32,33]. At sites of inflammatory, vascular mechanical or oxidative stress, there are marked increases in nucleotides (and nucleosides) released from locall cells [33] generating an

autocrine and paracrine signalization. The release of these mediators is followed by purinergic/adenosinergic signaling through well-defined cell surface nucleotide or adenosine receptors. It is well established that a multienzimatic system for nucleotide hydrolysis, constituted at least by the enzymes E-NTPDase, E-NPP and ecto-5'-nucleotidase, is present in serum [24,34] and may have an important role in maintaining normal physiology [33] by the control of nucleotide circulating levels.

Our results demonstrate an increased in ATP, ADP, AMP and 5'-TMP hydrolysis in serum of diabetic rats as demonstrated in figure 1. Also, in diabetes and saline groups (figure 2), we observed that the ATP was hydrolyzed faster than in citrate and insulin groups. These results could indicate the participation of the ectonucleotidases in vascular system of diabetic animals for nucleotides/nucleosides levels control. It has been demonstrated that high levels of extracellular glucose causes an ATP and/or UTP release in endothelial and pancreatic β cells [35,36], and also, a marked increased in the proatherogenic nuclear factor of activated T cells signaling pathway in vascular smooth muscle cells (VSMC) [37]. This effect is mediated via glucose-induced release of ATP and UTP, which subsequently activate P2 receptors. Thus, nucleotide release is a potential metabolic sensor for the arterial VSMC response to high glucose levels [38]. By the other hand, high glucose induced release of extracellular nucleotides, acting on P2Y receptors to stimulate VSMC growth, via NFAT (nuclear factor of activated T cell) activation [37]. Lunkes et al. [39] demonstrated an increase in NTPDase and 5'-nucleotidase activities of platelet, from patients with type 2 diabetes. The authors suggested an involvement of physiological responses in

diabetes and other important mechanisms of thromboregulation. The same group demonstrated that high glucose levels added *in vitro* in human platelet could increase ATP, ADP and AMP hydrolysis, suggesting a direct effect of glucose in the enzymes activities [26], however, in blood serum, we did not observed this effect (figure 3). Since high glucose concentrations induce a series of metabolic changes that ultimately lead to the genesis of both microvascular complications and macrovascular damage [40], we believe that high glucose *in vivo* could induced local release of extracellular nucleotides and this rise of nucleotides could induce the rise in ectonucleotidases activities. Another possibility is an increase in enzymes molecules release that could be an adjuvant system to prevent injury or alterations in hemostatic mechanisms.

To evaluate in a different manner the interference of high glucose levels in serum ectonucleotidases of diabetic rats, we observed a modification in ectonucleotidases activities in diabetic rats treated with insulin. As observed in figures 1 and 2, the control of glucose levels with 6 days of insulin treatment may restore the ectonucleotidases activities. However, Sakowick and Pawelczyk [2] demonstrated that insulin could restore expression of an adenosine kinase in streptozotocin-induced diabetes mellitus rats in liver, kidney and heart, but this effect depends on the insulin and is not related to the glucose levels. In this sense, the insulin effects in blood serum of diabetic rats should be investigated with more details.

Taken together, the results cited may provide a link between purinergic system and diabetic vascular disease. It is important to consider that the enzymes present in blood serum, in platelets [41,42] and in vascular wall [43] could work

together to avoid spontaneous platelet aggregation and thrombus formation [34]. Blood serum enzymes, like NTPDases and NPPs, may contribute to remove ATP and ADP, and, together with 5'-nucleotidase, may increased the concentration of adenosine, a potent vasodilator [44]. Also, NPPs might act to as "guard dogs" to prevent subversion of the cell by destroying incoming DNA or RNA liberated upon tissue injury and cell death [34].

The data presented in this study suggests that soluble nucleotidases could represent an important auxiliary effector system for local inactivation of acutely elevated nucleotides. In this way, our results could contribute to the understood of the pathophysiology of the vascular complications related to diabetes and the involvement of ectonucleotidases family in this system.

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TABLE 1

Table 1: Weigth and blood glucose levels.

	Weight (g)				*Blood glucose (mg/dL)*			
Days / ***Groups***	*0*	*7*	*30*	*36*	*0*	*7*	*30*	*36*
Citrate	205±16	235±15	287±22	300±13	111±12	118±20	105±24	110±16
Diabetes	196±14	193±22*	196±24*	-	108±6	479±80*	481±53*	-
Saline	195±20	194±22*	212±17*	221±21*	107±13	457±87*	473±54*	443±76*
Insulin	187±18	190±17*	203±17*	232±20*	109±9	470±78*	480±73*	114±42#

Weight (g) and blood glucose (mg/dL) from citrate (control), diabetes, saline (diabetes + 6 days of saline) and insulin (diabetes + 6 days of insulin). Data are reported as mean ± SD (at least *n*=10 for each group).

***** represents difference from citrate group (*P*<0.01).

represents difference from saline group (*P*<0.01).

Figure Legends

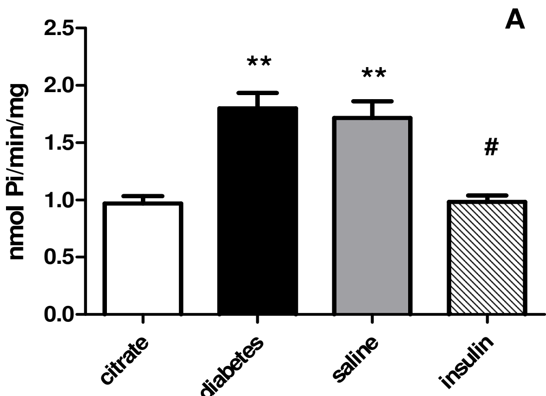
Figure 1: Effects of diabetes on nucleotides hydrolysis from citrate, diabetes, saline (30 days diabetes + 6 days of saline) and insulin (30 days diabetes + 6 days of insulin) treatment. ATP (A), ADP (B), AMP (C) and 5'-TMP (D) degradation was increased in serum of 30 day diabetic rats. The treatment of 6 days with insulin restored the enzyme activities to the level of citrate group (control). Results are expressed as means ± SD (at least n=10 for each substrate tested). The comparison among groups was analyzed statistically by one-way analysis of variance (ANOVA) followed by Student-Newman-Keuls test. ** Represents significant statistical difference to the respective citrate group, considering $P \leq 0.05$. [#] Represents significant statistical difference to the groups diabetes and saline between insulin groups, considering $P \leq 0.05$.

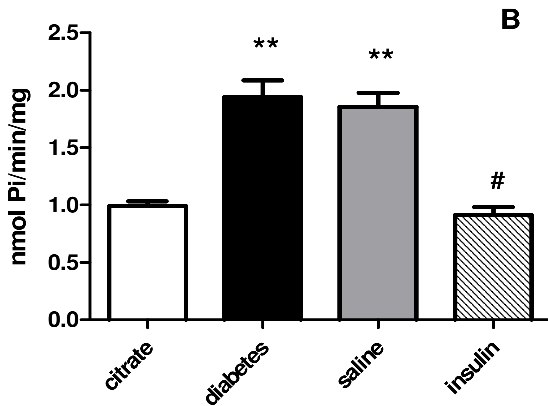
Figure 2: ATP metabolism and product formation in rat blood of diabetic rats. The blood serum was incubated, as described in Materials and Methods, containing 100 μM ATP and nucleotides were analyzed by HPLC. Citrate (A), diabetes (B) saline (C) and insulin (D) groups. Data represents a typical experiment of tree different experiments.

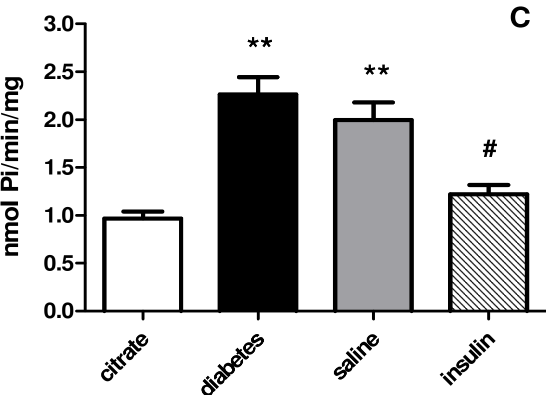
Figure 3: *In vitro* effect of 24 h of preincubation time with 20 mM glucose on ectonucleotidase activities in blood serum rats. The incubation conditions are described in Material and Methods (control = no glucose added, diabetes = serum

of 30 days diabetic rats and 20 mM glucose = glucose added). Results are expressed as means ± SD (n=3). The comparison among groups was analyzed statistically by one-way analysis of variance (ANOVA) followed by Student-Newman-Keuls test. * Represents significant statistical difference to the respective control (no glucose added) group, considering $P \leq 0.05$.

Figure 1







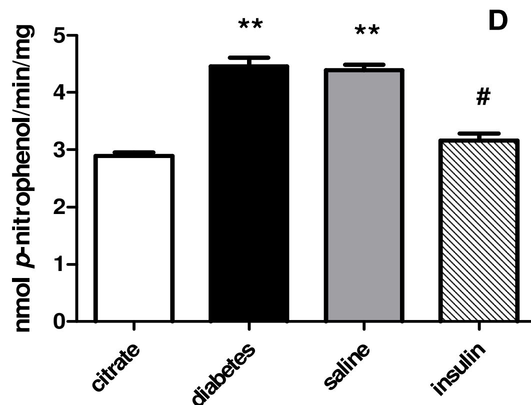


Figure 2

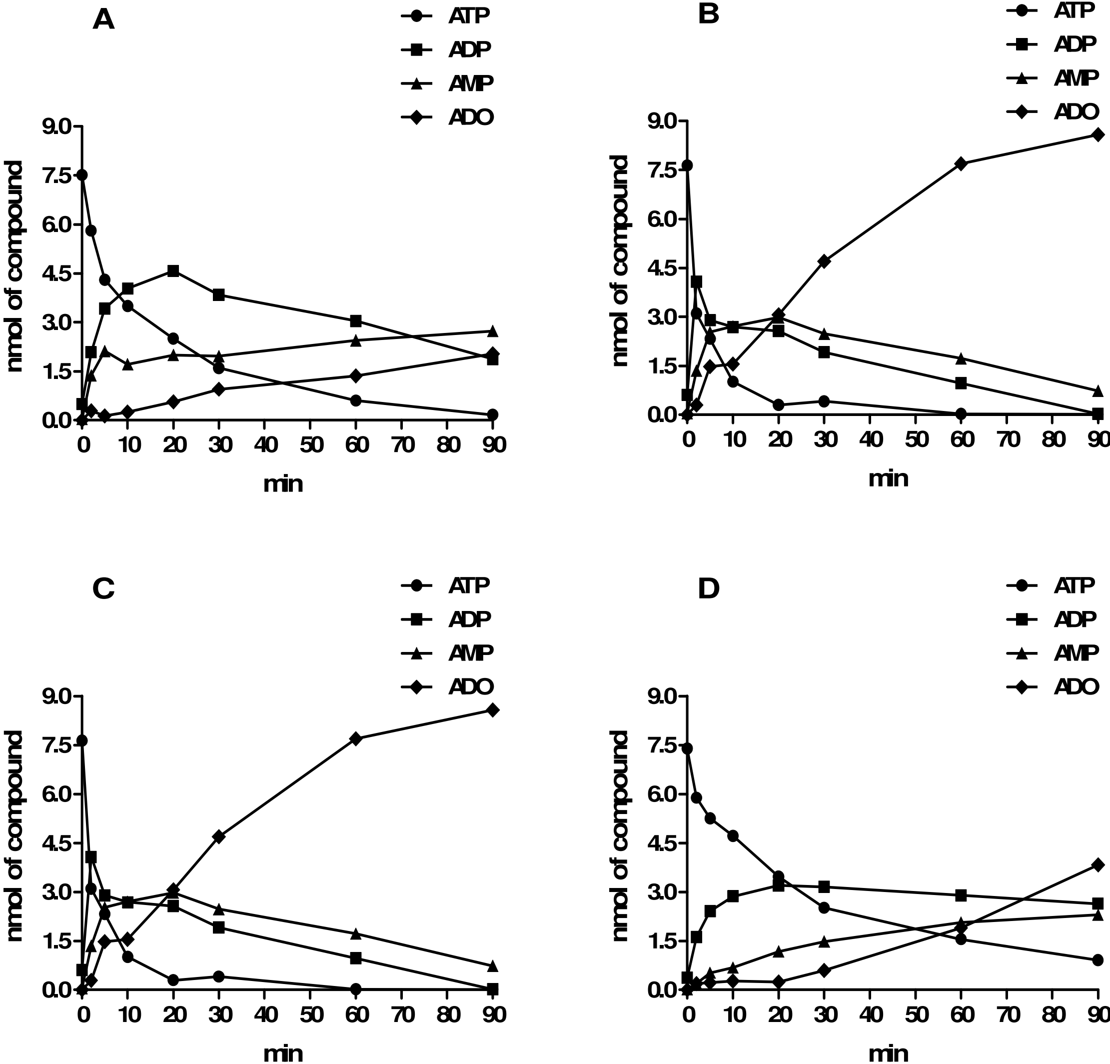
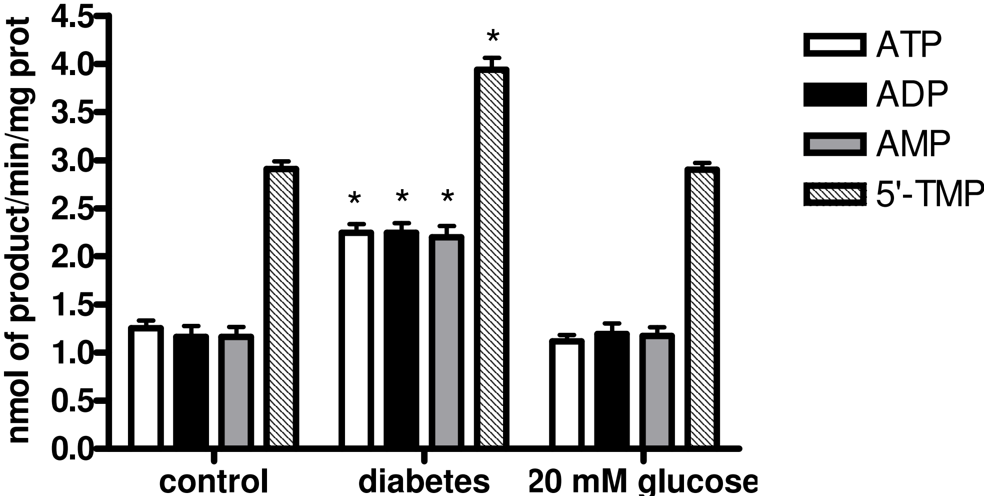


Figure 3



ANEXO I

Resultados preliminares que serão incluídos no artigo referente ao capítulo 3.

Materials and Methods

Real time PCR from heart left ventricle

Total RNA and cDNA were generated as described in RT-PCR analysis. SYRB Green I-based real-time PCR was carried out on MJ Research DNA Engine OpticonTM Continuous Fluorescence Detection System (MJ Research Inc.,Walthan, MA) as described (Zerbini et al., 2003). All PCR mixtures contained: PCR buffer (final concentration 10 mM Tris-HCl (pH 9.0), 50 mM KCl, 2.0 mM $MgCl_2$, and 0.1% Triton X-100), 250 µM deoxy-NTP (Roche), 0.5 µM of each PCR primer, 0.5 x SYBR Green I (Molecular Probes), 5.0 % DMSO, and 1 U taq DNA polymerase (Promega, Madison, WI) with 2.0 µL cDNA in a 25 µL final volume reaction mix. The samples were loaded into wells of Low Profile 96-well microplates. The PCR was run at the following conditions: 1 min at 95 °C denaturation, followed by 35 cycles of 1 min at 94 °C, 1 min at 60 °C, 1 min at 72 °C and a final 10 min extension at 72 °C. The fluorescence signal was measured right after incubation for 5 sec at 79 °C following the extension step, which eliminates possible primer dimer detection. At the end of the PCR cycles, a melting curve was generated to identify specificity of the PCR product. For each run, serial dilutions of human GAPDH plasmids were used as standards for quantitative measurement of the amount of amplified DNA. For normalization of each sample, β-actin primers were used to measure the amount of β-actin cDNA. All samples were run in triplicate and the data were presented as ratio of enzymes/β-actin. The primer sets used for rat *Entpd1* (*Cd39*), *Entpd2* (*Cd39L1*), *Entpd3* (*Cd39L3*),

Entpd5 (*Cd39L4*) *Entpd6,* (*Cd39L2*) were described by Vollmayer *et al*., 2001; for rat 5`-NT/CD73 and β-actin RT-PCR were described by Wink *et al.,* 2003. The primers used for rat NPP2 were 5'GAAAATGCCTGTCACTGCTC3' and 5'GCTGTAATCCATAGCGGTTG3' (amplification product 449 bp). For NPP1 and NPP3 were as described by Vollmayer *et al*. (2001).

Antibodies

The following rabbit polyclonal antibodies were used to identify the E-NTPDase family members in synaptosomal fraction from heart left ventricle : antibody C10F, raised against mouse NTPDase1 (Enjyoji et al., 1999); BZ3-4F, raised against rat NTPDase2 (Sevigny et al., 2002; Dranoff et al., 2002; Vlajkovic et al., 2004); respectively, obtained from published sequences of the N-terminal region of these NTPDases.

Western blotting

Synaptosomes prepared as described were lysed in lysis buffer (100 mM NaCl, 1% Nonidet P40, 1 mM sodium orthovanadate, 100 mM sodium fluoride, 0.5 ug/ml aprotinin, 1 ug/ml leupeptin and 1 mM phenylmethylsulfonyl fluoride, 20 mM Tris, pH 7.5). Protein content was determined by using Bicinchoninic acid assay using bovine serum albumin (BSA) as standard (Pierce, São Paulo/Brazil). SDS–polyacrylamide gel electrophoresis (SDS-PAGE) was performed loading a total of 40 ug of protein on a 4–12% polyacrylamide gel (40 ul/well) under reducing conditions followed by transfer to PVDF membrane (Immobilon P, Millipore,

Bedford, MA, USA) by electroblotting. After blocking with 5% milk in tris–saline buffer containing 0.1% Tween 20, membranes were probed with an appropriate primary antibody (NTPDases 1 and 2 1:1,000) overnight, at 4 ℃. After, membranes were washed and incubated with alkaline phosphatase-conjugated secondary antibodies for 2 h at room temperature and developed with ECL (Amersham, São Paulo/Brazil).

Legend to figures

Figure 1: The expression of ectonucleotidases in left ventricle of citrate, diabetes and insulin groups were quantitatively analyzed by real-time PCR as described in Material and Methods. The results are presented as ratio of cDNA enzyme/β-actin. Bars represent mean ± SD for two experiments.

Figure 2: Representative Western blotting from cardiac synaptosomes to NTPDase1 and NTPDase2 in left ventricle of citrate (C), diabetes (STZ) and insulin (I) groups. Positions of molecular size markers are shown in kDa.

Figure 1

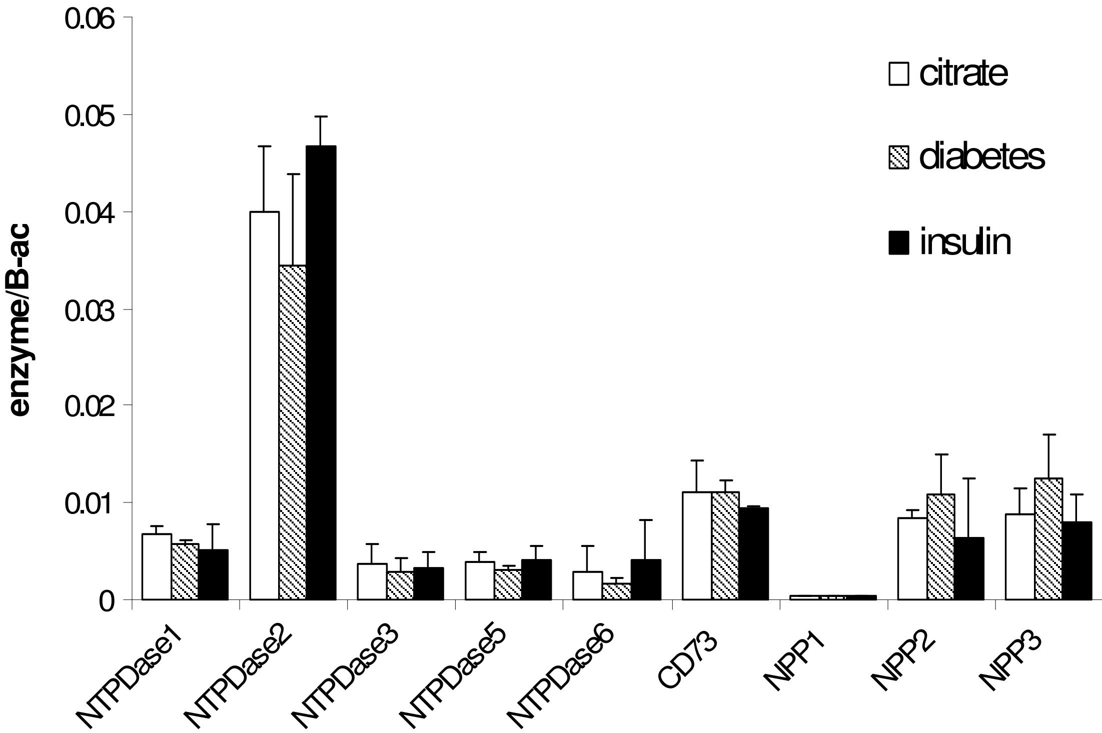
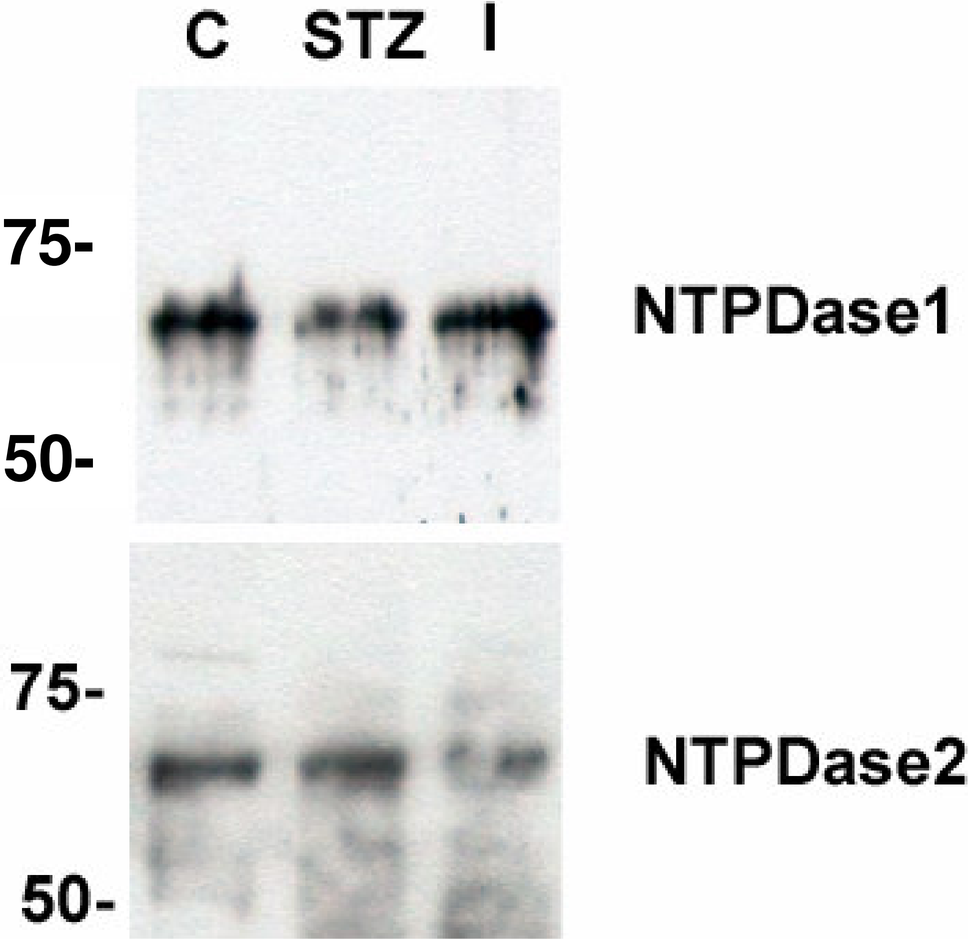


Figure 2



PARTE III

1. DISCUSSÃO

1.1 Ectonucleotidases presentes no ventrículo esquerdo do coração de ratos adultos

Durante muito tempo se pensou que a hidrólise de nucleotídeos como ATP e ADP por ectoenzimas fosse um processo simples, realizado por uma apirase e uma ecto-ATPase (Plesner, 1995). Hoje está bem estabelecido de que se trata de um processo complexo, onde estão envolvidos uma série de membros da família das ectonucleotidases, com sobreposição de atividades, cujas funções fisiológicas ainda não são totalmente compreendidas. A contribuição dos diferentes membros desta família na modulação da sinalização purinérgica pode diferir nos tecidos, compartimentalização celular, regulação da expressão, disponibilidade e preferência por substratos (Stefan et al., 2006). Há muito tempo é sabido que enzimas capazes de degradar nucleotídeos participam de enventos cardíacos, tanto fisiológicos quanto patológicos, entretanto a participação destas enzimas nestes diferentes processos necessita ser melhor esclarecida.

Os dois primeiros capítulos desta tese demonstraram a presença da família das ectonucleotidases, mais especificamente as ENTPDases, E-NPPs e a ecto-5'-nucleotidase, em VE de ratos adultos. Além disso, observamos a hidrólise de nucleotídeos em uma preparação de terminal cardíaco de ratos adultos, chamada sinaptossoma cardíaco. Esta fração sinaptossomal é uma preparação que tem sido utilizada por outros grupos para estudo da hidrólise de nucleotídeos, porém careciam dados cinéticos e bioquímicos sobre quais as enzimas estariam

presentes nessa preparação. Assim, também caracterizamos cinética e bioquimicamente estas enzimas em condições fisiológicas para que posteriormente esta preparação pudesse ser usada para o estudo de condições patológicas cardiovasculares, como aquelas observadas no diabetes.

Outro ponto importante desta tese que deve ser ressaltado é o fato de termos usado apenas o VE para estudo das ectonucleotidases. Como o coração possui diferentes compartimentos e cada um desempenha uma função distinta, fez-se necessário focar o estudo numa região específica. O VE foi o escolhido pelo fato de existirem relatos demonstrando alterações patológicas que ocorrem apenas nesse ventrículo, como alterações que podem ocorrer nas perfomances de sístole e diástole no diabetes (Pfeffer et al., 1979; Cosson & Kevorkian, 2003; Nemoto et al., 2006).

1.1.1 Hidrólise de nucleotídeos em sinaptossoma cardíaco e perfil da expressão das E-NTPDases e ecto-5'-nucleotidase no ventrículo esquerdo de ratos adultos

O estudo das propriedades bioquímicas demonstrou que a preparação de sinaptossoma cardíaco hidrolisa ATP, ADP e AMP em um pH ótimo de 8.0 para ATP e ADP e 9.5 para AMP. O elevado pH para a hidrólise do AMP encontrado já havia sido descrito em tecido cardíaco (Naito & Lowenstein, 1981). Além disso, a preparação necessitou de cátions divalentes para a sua atividade de hidrólise máxima, com uma preferência para o Ca^{2+} para hidrólise de ATP e ADP e Mg^{2+} para a hidrólise de AMP. A preferência de Mg^{2+} para a hidrólise de AMP também está bem descrita na literatura (Zimmerman, 1992). Além disso, estudamos a

hidrólise dos nucleotídeos em presença de diferentes compostos considerados inibidores de diferentes enzimas. Sendo a preparação usada considerada uma fração sinaptossomal-mitocondrial crua, primeiramente estudamos o efeito de 2 ug/mL de oligomicina e 0.1 mM de azida sódica, inibidores de ATPases mitocondriais. Como é sabido que o tecido cardíaco possui elevadas concentrações de ATPases mitocondriais (Sasaki et al., 2001; Grover et al., 2004), não foi surpresa encontrarmos uma alta inibição na hidrólise de ATP, mas não de ADP, na presença destes compostos. Isto foi importante, pois padronizamos o uso destes compostos na incubação da fração sinaptossomal para excluir a atividade destas enzimas na tentativa de estudar apenas a participação das NTPDases no processo de hidrólise de ATP. Já na presença de altas concentrações de azida sódica, considerada um inibidor de ATP difosfoidrolases de várias fontes (Plesner, 1995; Knowles & Nagy, 1999) e de cloreto de gadolínio, inibidor de E-NTPDases solúveis e ligadas à membrana (Escalada et al., 2004), uma significativa inibição foi observada na hidrólise do ATP e ADP (Capítulo 1, Tabela 1). Os antagonistas de receptores P2, Suramin e Evans Blue, também considerados inibidores de ectonucleotidases, causaram inibição das hidrólises de ATP e ADP. Observamos neste caso, que o suramin, que tem sido associado a uma maior inibição da ecto-ATPase (NTPDase2) (Heine et al., 1999; Wink et al., 2006) reduziu fortemente a hidrólise de ATP, no entanto, o evans blue, que tem sido demonstrado ser mais eficiente na inibição da atividade apirásica (NTPDase1), praticamente aboliu ambas atividades. Esses resultados indicam fortemente a participação de mais de um membro da família das NTPDases no processo de hidrólise dos nucleotídeos na fração estudada.

Também foram testados inibidores clássicos de fosfatase alcalina, levamisole e tetramisole, devido ao elevado pH ideal para hidrólise do AMP. Neste caso, a hidrólise do AMP não foi afetada, demonstrando a ausência desta enzima nas condições estudadas. Além disso, foi testado o molibdato de amônio, que tem sido considerado inibidor de ecto-5'-nucleotidase, para avaliarmos a presença da enzima na preparação. Este composto foi responsável por inibir em 82% a hidrólise de AMP, demonstrando que esta enzima possivelmente é a responsável por esta hidrólise na fração estudada (Capítulo 1, Tabela 2).

As propriedades cinéticas revelaram que os valores de K_M ficaram na faixa de micromolar, o usualmente encontrado para estas enzimas. Os valores de V_{max} foram 536.93 ± 35.02, 219.48 ± 36.46 and 110.53 ± 10.02 nmol de fosfato inorgânico.min^{-1}.mg de $proteína^{-1}$ para ATP, ADP e AMP, respectivamente (Capítulo 1, Figura 3).

Uma vez que ATP, ADP e AMP podem ser metabolisados por diferentes membros da família das ectonucleotidases (Zimmermann, 2001) e que os resultados das análises bioquímicas indicavam a participação de mais de uma enzima, nós investigamos a expressão de mRNA das NTPDases1, 2, 3, 5, 6 e 8 da ecto-5'-nucleotidase/CD73 em ventrículo esquerdo de rato por RT-PCR. Neste caso foi necessário fazer a análise no tecido cardíaco, em vista da ausência de material genético nas vesículas sinaptossomais. Entre os mRNAs analisados, todos os membros estudados foram identificados, exceto a NTPDase8. De acordo com a literatura, a NTPDase8 apenas parece estar envolvida em eventos relacionados ao sistema hepático, jejuno e rim (Bigonnesse et al., 2004; Fausther

et al., 2007). Os resultados encontrados demonstram que o tecido de VE cardíaco apresenta várias ectonucleotidases com potencial para hidrolisar nucleotídeos extracelulares a seus respectivos nucleosídeos. Já os níveis de intensidade de mRNA detectados para as enzimas estudas neste tecido foram diferentes. Através da técnica de PCR em tempo real, observamos que entre os genes estudados o mRNA mais expresso foi o da NTPDase2, seguida da ecto-5'-nucleotidase/CD73 e NTPDase6. A elevada expressão da NTPDase2, juntamente com o elevado valor de hidrólise de ATP, em comparação com ADP e AMP, sugere a presença desta enzima na preparação sinaptossomal. Uma vez que tem sido sugerido que o ATP possa ser liberado juntamente com a NE em terminais nervosos simpáticos, a presença desta enzima se torna extremamente relevante, principalmente como mecanismo de término para a sinalização purinérgica (Todorov et al., 1997). Além disso, foi demonstrado que o ATP é capaz de modular a liberação de NE através da ligação com receptores P2 (Bohem & Huck, 1997). Um aumento da atividade adrenérgica e liberação de NE são fatores relacionados a algumas disfunções cardíacas, incluindo arritmias e morte súbita durante isquemia cardíaca (Benedict et al., 1996). Já foi descrito que se ATP se liga aos receptores P2X em culturas de neurônios ganglionares cervicais superiores, ocorre um aumento na liberação de NE, enquanto que, quando ocorre ligação aos receptores P2Y, esta liberação é atenuada. Desta forma, as ectonucleotidases podem estar diretamente relacionadas à cardioproteção, visto que são capazes de controlar os níveis de nucleotídeos/nucleosídeos e consequentemente a modulação dos receptores purinérgicos.

Através de um estudo de imunohistoquímica em nervos cardíacos porcinos, foi sugerido que a principal enzima participante deste processo seria a NTPDase1 (Machida et al., 2005). No entanto, a presença de outros membros da família das NTPDases não foi investigada, sendo que o trabalho também não avaliou especificamente o VE. Em nosso estudo observamos uma baixa expressão para NTPDase1 em tecido de VE de coração de rato, o que nos sugere a participação de outras enzimas no processo de hidrólise dos nucleotídeos estudados. Entretanto a presença da NTPDase1 não pode ser descartada, pois mesmo não sendo prevalente no tecido total do VE, poderia ser enriquecida especificamente nos terminais sinápticos.

Sevigny e colaboradores já haviam demonstrado através da análise bioquímica de tecido cardíaco de murinos e de suínos, razões de hidrólise ATPase/ADPase de 10, sugerindo a expressão de enzimas com atividade preferencialmente ATPásica (Sevigny et al, 2002). Devido aos altos níveis de RNA mensageiro (mRNA) para NTPDase2 expressos em coração de humanos e murinos encontrados por outros grupos, esta enzima pode ser uma forte candidata a responsável pela alta atividade ATPásica neste tecido (Chadwick & Frischauf, 1997; Kegel et al., 1997; Chadwick & Frischauf, 1998). Além disso, nosso grupo já havia demonstrado uma ectoenzima que possui preferência por nucleosídeos trifosfatados em sarcolema cardíado de ratos (Menezes de Oliveira et al., 1997), o que seria mais uma evidência da possível participação da NTPDase2 na hidrólise de ATP em preparações de sinaptossoma cardíaco.

As NTPDase5 e 6 também foram identificadas no tecido do VE. Estas são enzimas que apresentam localização intracelular e podem ser secretadas depois

de expressão heteróloga, além de apresentarem mais afinidade por nucleotídeos difosfatados do que por trifosfatados. A NTPDase6, depois da NTPDase2, foi o membro das E-NTPDases mais expresso em VE. Como já foi descrito, ectonucleotidases podem ser liberadas de terminais nervosos simpáticos juntamente com ATP para terminação dos mecanismos de sinalização (Tododov et al., 1997). Assim, esta enzima poderia estar participando na hidrólise do nucleotídeo ADP, formado a partir do ATP liberado dos terminais nervosos em conjunto com a NE. Além do mais, já está bem descrito a presença desta enzima em coração, principalmente em células do músculo cardíaco e células endoteliais capilares (Hicks-Berger et al., 2000; Braun et al., 2000, Yeung et al., 2000). A NTPDase5, que também tem preferência por NDPs, foi encontrada em menor quantidade, não podendo-se também descartar sua participação na hidrólise dos nucleotídeos.

Já a NTPDase3, enzima encontrada com baixos níveis de expressão, pode agir como regulador pré-sináptico dos níveis de ATP. Quando fizemos a razão de hidrólise ATPase/ADPase, encontramos um valor de 2.9, o que poderia sugerir a participação de uma NTPDase3. Entretanto, tendo-se em vista a alta expressão de NTPDase2 e a baixa expressão de NTPDase3, este valor de razão, poderia ser uma média do resultado de uma mistura das razões de hidrólises das enzimas, já que provavelmente mais de um membro esteja participando do processo de degradação dos nucleotídeos. Como nossa análise foi feita em tecido cardíaco e não em sinaptossoma cardíaco, vale ressaltar que estamos apenas extrapolando para uma possível participação destas enzimas na fração estudada.

A segunda enzima mais expressa encontrada neste primeiro estudo foi a ecto-5'-nucleotidase. Já está bem descrito que esta enzima é expressa em diversos tecidos, dentre eles, o coração (Moriwaki et al., 1999). Juntamente com E-NTPDases e E-NPPs, ela faz parte da cascata para término da ação dos nucleotídeos na sinalização extracelular (Zimmermann, 2000). Além disso, a enzima gera Ado, molécula importante para o sistema cardiovascular, que age através da ligação com receptores P1 (Sträter, 2006), que também possuem extensa distribuição no sistema cardiovascular (Shryok & Belardinelli, 1997). A primeira sugestão de que as purinas poderiam agir como moléculas cardioprotetoras veio com a demonstração de que a Ado era capaz de mediar uma vasodilatação durante hipóxia, para aumentar o fluxo sangüíneo e assim, manter a oxigenação cardíaca (Stoner & Green, 1950; Berne, 1963). Hoje está bem descrito que a Ado possui diversos papéis na fisiologia do sistema cardiovascular, dentre os quais podemos citar a diminuição dos batimentos cardíacos, redução da contração atrial, diminuição da condução átrio-ventricular, antagonismo dos efeitos estimulatórios das catecolaminas e redução da atividade de células marca-passo (Shryok & Belardinelli, 1997). Assim a Ado tem sido considerada uma molécula cardioprotetora, já que é capaz de (1) diminuir a freqüência atrial e ventricular por depressão do estímulo do nodo sinoatrial e da condução elétrica do nodo átrio-ventricular, (2) atenuar os efeitos estimulatórios das catecolaminas e (3) inibir a liberação de NE de terminais nervosos. Estas ações podem contribuir para a redução do trabalho cardíaco durante eventos de isquemia e reperfusão, redução da captação de cálcio pelos miócitos e redução de arritmias (Shryok & Belardinelli, 1997). Uma elevada expressão desta enzima em tecido de VE parece ser lógica

em vista da importância da presença desta enzima para formação da Ado, uma molécula que participa tanto de eventos fisiológicos, quanto patológicos.

O estudo dos diferentes padrões de expressão e de atividades das ectonucleotidases é importante em condições fisiológicas, para que posteriormente possa ser comparado e compreendido em situações patológicas. Sem dúvida estas enzimas podem contribuir para a função cardiovascular normal, mas também podem estar envolvidas na sinalização purinérgica de doenças cardiovasculares.

1.1.2 Análise da presença das E-NPPs em ventrículo esquerdo de ratos adultos e medida da hidrólise do marcador artificial 5'-TMP em sinaptossoma cardíaco

Em 2001, Vassort publicou uma revisão onde ele cita a falta de estudos sobre outras enzimas, que não são ATPases, mas que sejam capazes de hidrolisar o ATP em tecido cardíaco, dentre elas as E-NPPs (Vassort, 2001). As E-NPPs influenciam muitos processos fisiológicos e alterações na sua atividade podem estar relacionadas com a fisiopatologia de várias doenças (Goding et al., 2003). Alguns trabalhos mostram a presença destas enzimas em coração, no entanto, sem a análise específica de quais são expressas no ventrículo esquerdo (Stefan et al., 1999; Bollen et al., 2000). Assim, investigamos as propriedades bioquímicas das E-NPPs em sinaptosomas de VE e seu padrão de expressão gênica, no tecido de VE (capítulo 2).

A atividade enzimática encontrada em sinaptossoma cardíaco nas condições estudadas mostrou propriedades bioquímicas já descritas para E-NPPs

em outros estudos (Kelly et al., 1975; Sakura et al., 1998; Hosoda et al., 1999; Bollen et al., 2000; Grobben et al., 2000; Vollmayer et al., 2003; Furstenau et al., 2006).

A presença de mRNA das NPP1, NPP2 e NPP3 em tecido de VE foi investigado por RT-PCR, sendo que dos três membros da família das E-NPPs analisados, somente a NPP1 não foi detectada (Capítulo 2, Figura 4A). A presença de NPP1 já foi descrita em tecido de coração total (Stefan et al., 1999; Goding et al., 2003), provavelmente sendo expressa em compartimentos específicos de acordo com suas funções fisiológicas.

A NPP2 já foi descrita em tecido cardíaco de humanos, porém, esta expressão não havia sido demonstrada apenas em ventrículo esquerdo de tecido cardíaco de ratos. No entanto, esta expressão foi menor do que a encontrada para a NPP3 (Capítulo 2, Figura 4B). De acordo com a literatura, a NPP3 é uma enzima muito expressa em tecido cardíaco (Stefan et al., 1999). No VE foi a mais expressa, sendo provavelmente a principal enzima responsável pelos resultados de hidrólise de 5'-TMP encontrados em sinaptossoma do mesmo tecido (Capitulo 2, Figura 4B).

A exata contribuição destas enzimas em sistema cardíaco ainda permanece pouco ou nada estudada. A presença destas enzimas pode fazer parte da terminação da sinalização purinérgica e/ou gerar outras moléculas mensageiras como ADP, adenosina e pirofosfato (Stefan et al., 2006). O que sabemos é que ectonucleotidases específicas são expressas pelas células para regular o metabolismo dos nucleotídeos em microambientes. Devido à importância desta regulação, esta deve ser finamente modulada. Como um dos nossos alvos de

estudo foi a teminação nervosa cardíaca, podemos fazer algumas inferências em função de estudos já realizadas em neurônios.

O ATP é estocado em vesículas nos terminais nervosos em concentrações milimolares e sua liberação ocorre a partir de estímulos nas células nervosas. A neuromodulação de terminais pré e pós-sinápticos já foi demonstrada estar relacionada com ATP e seus produtos de degradação, como a Ado, através da ativação de receptores específicos (Zimmermann, 1996; Sneedon et al., 1999; Goding et al., 2003). Como já discutido anteriormente nesta tese, desde que o ATP é co-liberado com a norepinefrina em terminais nervosos simpáticos, muito provavelmente estas enzimas estejam fazendo parte desta regulação.

Um outro ponto importante a ser observado é o porquê de estas células estarem expressando tantas enzimas com funções semelhantes, já que demonstramos que as E-NTPDases também são expressas. Isto também já foi mostrado em outros sistemas, como plaquetas e sistema nervoso central (Furstenau et al., 2006; Cognato et al., 2008) e se sabe que células e tecidos são capazes de co-expressar distitintas ectonucleotidases que possuem características semelhantes (Heine et al., 1999; Kukulski & Komozynski, 2003; Oses et al., 2004). Nos últimos anos, vários trabalhos têm sido realizados na tentativa de encontrar inibidores específicos para cada membro da família das ectonucleotidases, na tentativa de se conseguir estudar separadamente a participação de cada enzima nos mais diferentes sistemas (Escalada et al., 2004; Levesque et al., 2007; Mukonda et al., 2007). Estudos ainda estão em andamento, mas parece que apenas para a NTPDase3 existe algum avanço com a descoberta de um anticorpo monoclonal capaz de inibir 60 a 90% a atividade da enzima e de

um derivado de antraquinona, o Azul Reativo 2 (RB-2), considerado um potente inibidor de E-NTPDases, com alguma seletividade para NTPDase3 (Munkonda et al., 2009; Bagy et al., 2009). No entanto, acredita-se que estas enzimas possam fazer parte de um complexo multienzimático com a capacidade de regular finamente os níveis de nucleotídeos/nucleosídeos (Oses et al., 2004; Furstenau et al., 2006).

Assim, podemos sugerir que a atividade E-NPP encontrada possa participar em conjunto com as E-NTPDases e ecto-5'-nucleotidase, do controle da hidrólise de nucleotídeos nos terminais simpáticos cardíacos e consequentemente da liberação de NE. Em VE, a estimulação simpática pode ser responsável pelo desenvolvimento de arritmias e morte súbita em função de taquiarritmias ventriculares (Cao et al., 2000). Danos aos nervos extrínsecos, como gânglio estrelado, ou em nervos instrínsecos cardíacos a partir de infecções virais, ou a partir de doenças que causam danos cardíacos, podem produzir a cardioneuropatia (Rubart & Zipes, 2005).

Em conclusão, os dois primeiros capítulos desta tese mostram uma expressão de NTPDases, NPPs e ecto-5'-nucleotidase em tecido de VE de ratos e sugerem uma co-expressão destas enzimas em sinaptossoma cardíaco preparados a partir do tecido de VE de ratos adultos. Estes resultados podem indicar um possível papel cardioprotetor do controle da hidrólise de nucleotídeos, em termos de controle da liberação de NE, ação do ATP e produção de Ado.

1.2 Alterações no sistema purinérgico cardiovascular de ratos diabéticos por estreptozotocina

Apesar da importância que as doenças cardiovasculares relacionadas ao diabetes possuem, a natureza complexa e multifatorial das perturbações celulares e moleculares que alteram a estrutura e função cardíaca permanece incompletamente entendida (Poormina et al., 2005). Desta forma, nos capítulos 3 e 4 desta tese, objetivamos estudar os efeitos do diabetes sobre o sistema purinérgico em coração e soro de ratos diabéticos por estreptozotocina, na tentativa de correlacionar possíveis alterações encontradas nesta patologia.

1.2.1 Ectonucleotidases em sinaptossoma cardíaco de ventrículo esquerdo de ratos

Uma das complicações relacionadas ao diabetes é a neuropatia autonômica, que pode levar a disfunção cardiovascular (Giudice et al, 2002; Howarth et al., 2006; Malone et al., 2007). A redução na habilidade de regular finamente os batimentos cardíacos (HR) têm sido atribuída a alterações na função autonômica cardíaca (McLeod, 1992) e isto tem sido relacionado a um desbalanço do sistema simpático/parassimpático, com prevalência da atividade simpática (Kleiger et al., 1987; Tsuji et al., 1994; Manzella D & Paliosso G., 2005).

No terceiro capítulo desta tese, primeiramente fizemos a caracterização do modelo de diabetes induzido por estreptozotocina, medindo parâmetros já descritos como glicemia, peso, batimento cardíaco e pressão sanguínea (Capítulo 3, Tabela 1). A elevada glicemia, a perda de peso, a redução dos batimentos cardíacos e a redução da pressão sanguínea validaram o modelo de diabetes escolhido. Conjuntamente, avaliamos estes parâmetros nos animais tratados com

insulina e verificamos uma reversão destas alterações. Os mecanismos da diminuição encontrada para batimentos cardíacos e pressão sangüínea ainda permanecem desconhecidos, porém este é um modelo conhecido para estudo de problemas cardiovasculares do diabetes, dentre eles a neuropatia autonômica cardiovascular (NAC) (Schmid et al., 1999; Giudice et al., 2002). Como já descrito anteriormente, a neuropatia é uma das complicações comuns do diabetes e pode afetar ambos os sistemas somáticos e sistema nervoso autônomo. É caracterizada pela diminuição de velociade de condução, reflexos cardiovasculares anormais e disfunção vascular (Dyck et al., 1986, Johnson et al., 1986). A perda do controle de reflexos autonômicos do sistema cardiovascular pode ser devastador ao paciente, resultando em diminuição da tolerância ao exercício, hipotensão ortostática, infarto do miocárdio indolor e morte súbita. O paciente diabético com reflexos cardiovasculares anormais tem maior incidência de mortalidade do que o paciente com as funções autonômicas normais (Giudice et al., 2002).

Muitos autores tem atribuído as alterações cardíacas do diabetes a distúrbios no sistema simpático/parassimpático (Kleiger et al., 1987; Tsuji, 1994; Manzella & Paliosso, 2005). Está bem estabelecido que o ATP é capaz de agir como uma molécula sinalizadora extracelular em vários tecidos e está envolvido em uma variedade de processos regulatórios, dentre os quais podemos citar contração muscular, controle vascular, dor ou comunicação neuronal (Burnstock, 1997), além de servir como neurotrasmissor no sistema nervoso central e periférico (Bean, 1992; Burnstock, 1997). Desta forma, o intuito do terceiro trabalho foi avaliarmos a hidrólise de nucleotídeos em sinaptossoma de VE de ratos diabéticos por estreptozotocina e também tratados com insulina, na tentativa

de co-relacionar estas alterações cardíacas com possíveis alterações no sistema purinérgico. Desde que o ATP foi descrito ser co-liberado com a NE e de que também pode ser modulador da liberação desta molécula, parece claro que um desbalanço neste sistema possa estar relacionado com alterações no sistema que controla os níveis deste nucleotídeo. Já foi descrita uma possível co-liberação de ATP e ectonucleotidases em terminais nervosos (Todorov et al. 1997), o que poderia ser o modo de regulação dos níveis deste nucleotídeo e seus efeitos. Assim, realizamos a medida de hidrólise de nucleotídeos em sinaptossoma cardíaco e observamos uma significativa diminuição na hidrólise dos nucleotídeos ATP e ADP, mas não do AMP e substrato marcador 5'-TMP, em ratos com 30 dias de indução de diabetes, a qual foi revertida com o tratamento com insulina. Com isso, podemos inferir uma possível participação do sistema purinérgico nas alterações cardiovasculares encontradas no diabetes. Em 1929, Drury e Szent-György reportaram um efeito cronotrópico negativo das purinas. Estudos seguintes sugeriram que o ATP poderia ter efeitos dose-dependentes, ou seja, pequenas doses produziriam taquicardia, enquanto doses elevadas poderiam ocasionar diminuição dos batimentos cardíacos e bloqueio na condução do nodo atrioventricular (Hollander & Webb, 1957; Stoner et al., 1948). Talvez os efeitos observados de alteração nos batimentos cardíacos e na pressão sanguínea possam estar relacionados com as alterações na hidrólise destes nucleotídeos. Com certeza outros mecanismos devam estar relacionados, como mecanismos parassimpáticos e até a participação da Ado, molécula importante para o sistema cardiovascular. Apesar de não termos tido alteração na hidrólise de AMP, não sabemos como estão realmente os níveis deste nucleotídeo no teminal sináptico e

no coração como um todo. Através dos resultados de hidrólise extracelular de ATP medidos através de HPLC, podemos observar que ocorre um retardo na hidrólise de ATP e ADP, porém os níveis de Ado são semelhantes em todos os grupos (Capítulo 3, Figura 2). No entanto, Grden e colaboradores, em 2005, publicaram um trabalho mostrando a expressão alterada de receptores de Ado em coração de ratos diabéticos (Grden et al., 2005). Sabemos que os receptores de Ado são elementos chave na ação cardioprotetora da Ado e foi demonstrado uma expressão alterada destes receptores no diabetes agudo (14 dias após injeção de STZ). Além disso, o tratamento de 4 dias com insulina foi capaz de reverter as alterações encontradas (Grden et al., 2005). Em nosso trabalho não encontramos alteração na hidrólise de AMP nem na quantidade de Ado formada com a hidrólise extracelular de ATP, porém não podemos descartar a participação da alteração de expressão de receptores P1, inclusive porque também obtivemos uma reversão das alterações encontradas em nosso trabalho com o tratamento com insulina, semelhante ao que ocorreu no estudo com receptores de Ado. No entanto, desde que obtivemos uma alteração na hidrólise de ATP e ADP, além de podermos estar diante de uma alteração no sistema que controla a hidrólise destes nucleotídeos, ou seja, ectonucletidases, também podemos relacionar a participação de receptores P2 nestas alterações. Já foi descrito que além dos efeitos pós-sinápticos do ATP, este é capaz de afetar a trasmissão adrenérgica por agir em receptores purinérgicos em terminais nervosos (Burnstock, 1999). Dependendo do tipo de receptor afetado, poderá ocorrer um estímulo de liberação de NE ou uma inibição de liberação de NE (Figura 8). No entanto, os resultados que obtivemos neste trabalho são insuficientes para determinar qual o mecanismo relacionado

com a diminuição da hidrólise do ATP e ADP. Além disso, não é descrito como estão os receptores P2 nesta condição e se sua expressão também está alterada como ocorre com os receptores P1. Técnicas moleculares para investigar a expressão de ectonucleotidases e dos receptores P2 nos VE e nos terminais nervosos são necessárias para um maior e melhor entendimento da condição estudada.

Com este intuito, no capítulo anexo desta tese, mostramos os resultados ainda preliminares, das análises moleculares que estamos realizando para melhorar o entendimento das alterações encontradas na hidrólise de ATP e ADP em ventrículo esquerdo de ratos diabéticos e tratados com insulina. A análise por PCR em tempo real (Anexo I, Figura 1) mostrou que temos um perfil semelhante ao encontrado em nossos trabalhos de caracterização (Capítulos 1 e 2), ou seja, com uma expressão de mRNA mais elevada para NTPDase2 e CD73 (Capítulo 1) e NPP3 e ausência de NPP1 (Capítulo 2). Para a NTPDase6, no entanto, foram encontrados níveis mais baixos de mRNA e mais elevados para a NTPDase1, o que deve ser ainda confirmado.

Além do perfil de expressão de mRNA praticamente ter se mantido, os resultados nos mostram que existe uma tendência de diminuição de expressão para a NTPDase2 e NTPDase6 em ratos diabéticos e um retorno da expressão para o grupo tratado com insulina, o que estaria de acordo com os resultados encontrados para as hidrólises de ATP e ADP . Além disso, não observamos uma alteração na hidrólise de AMP em ratos diabéticos, o que também estaria de acordo com a não alteração de hidrólise de AMP. No entanto, parece haver uma pequena tendência de diminuição no grupo insulina, o que precisa ser também

confirmado. Para as NPPs, confirmamos a ausência de expressão da NPP1 em VE de ratos, e uma maior expressão de NPP3. No entando, parece haver uma tendência de aumento de expressão para NPP3 e NPP2 e uma redução nos ratos tratados com insulina. Com certeza estes resultados precisam ser confirmados, pois os resultados de hidrólise do marcador artificial para a atividade fosfodiesterásica, não mostraram nenhuma diferença de hidrólise entre os grupos testados. No entanto, um trabalho mostrando a caracterização enzimática e farmacológica de nucleotidases liberadas de vaso deferente durante estimulação nervosa, sugere que as E-NPPs não contribuem para a atividade solúvel ATPásica em nervos simpáticos (Myhailova-Todorova et al., 2002).

Devido a atividade enzimática ter sido avaliada em preparações de sinaptossoma cardíaco e a análise por PCR em tempo real ter sido realizada em tecido de VE, estamos apenas extrapolando uma possível relação entre expressão de mRNA e atividade enzimática. Para avaliar se a alteração no mRNA observada através do PCR de tempo real no tecido total do VE ocorria também a nível protéico nos sinaptosomas deste tecido, realizamos a técnica de Western blotting. Através dessa análise detectamos a presença da NTPDase1 e da NTPDase2 na preparação de sinaptossoma cardíaco de ratos diabéticos e tratados com insulina (Anexo I, Figura 2). É necessário lembrar da dificuldade de quem trabalha nesse campo, tendo em vista a dificuldade de obtenção de anticorpos. Existe uma reduzida opção de anticorpos disponíveis comercialmente e os que estão disponíveis muitas vezes possuem uma falta de especificidade, reconhecendo regiões conservadas, o que torna difícil a análise dos resultados.

A confirmação dos resultados apresentados é essencial para podermos sugerir quais os membros da família das ectonucleotidases poderiam estar participando da alteração das hidrólises de ATP e ADP. No entanto, serão necessários estudos adicionais para elucidarmos possíveis alterações do sistema purinérgico que poderiam estar participando das complicações cardíacas encontradas no diabetes, principalmente o estudo da participação de receptores P2 nestas condições.

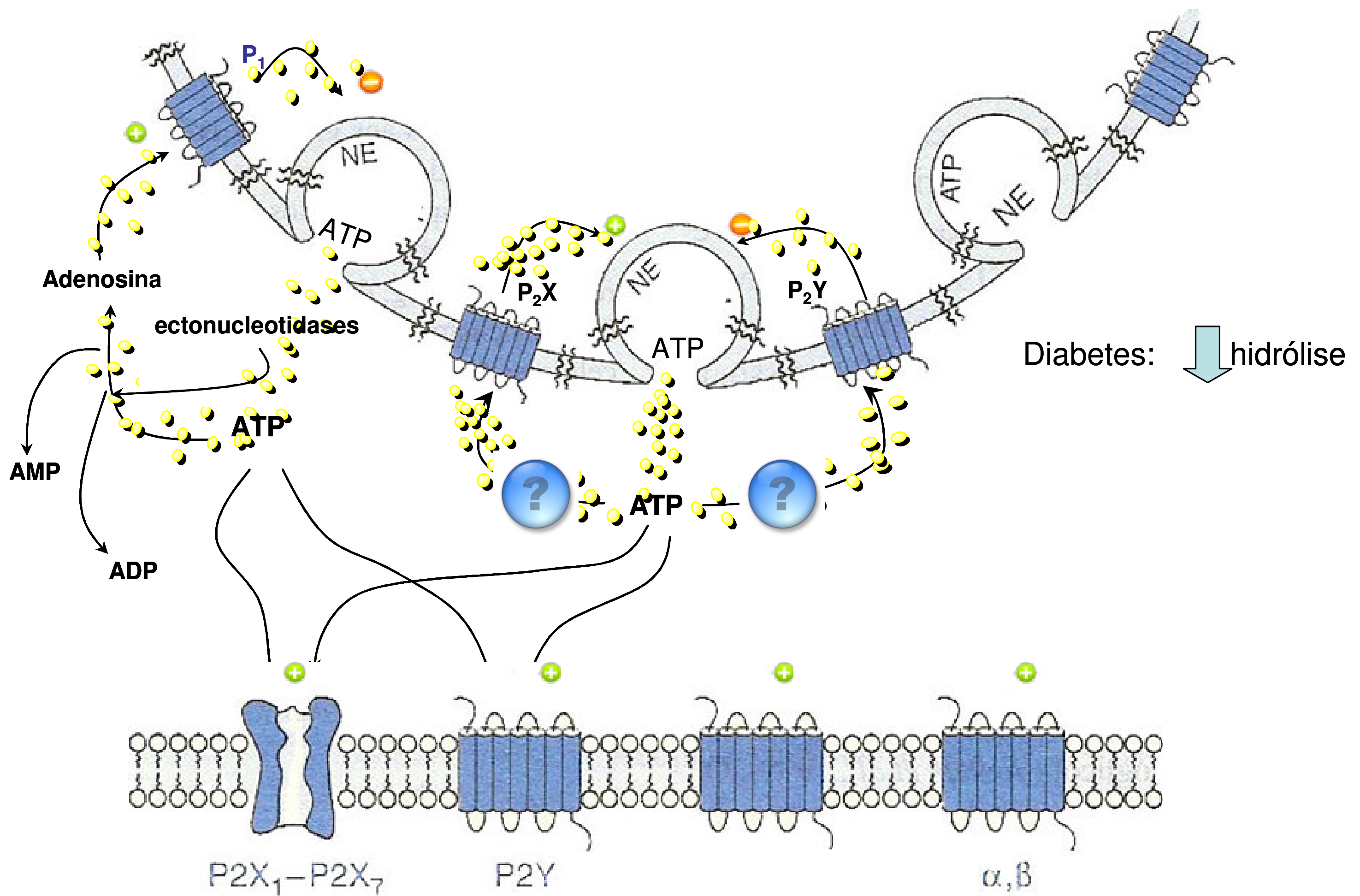


Figura 8. Modelo proposto para o envolvimento das ectonucleotidases e do ATP nas alterações cardíacas encontradas no diabetes. O ATP pode ser co-liberado com a NE e também pode ser modulador da liberação desta molécula. Um desbalanço neste sistema, como o encontrado no diabetes, pode estar relacionado com alterações no sistema que controla os níveis deste nucleotídeo, como as ectonucleotidases, mas também poderia haver a participação de receptores P2 nestas alterações. Dependendo do tipo de receptor afetado, poderia ocorrer um estímulo de liberação de NE ou uma inibição de liberação desta molécula. *Adapatado de Westfall & Westfall, 2006.*

1.2.2 Hidrólise de nucleotídeos em soro de ratos diabéticos e tratados com insulina

Além dos efeitos cardíacos relacionados ao diabetes já relatados nesta tese, é sabido que o diabetes pode promover um estado de hipercoagulabilidade, sendo capaz de acelerar e exacerbar o desenvolvimento de aterosclerose e a incidência de eventos trombóticos arteriais (Carr, 2001).

Para investigar o envolvimento das ectonucleotidases em soro sangüíneo de ratos diabéticos e tratados com insulina, medimos a hidrólise dos nucleotídeos da adenina após 30 dias de indução do diabetes e após 6 dias de tratamento com insulina. Os resultados obtidos demonstram que o diabetes é capaz de alterar o metabolismo dos nucleotídeos extracelulares em soro e que o controle glicêmico com insulina é capaz de reverter esta alteração. Os ratos diabéticos tiveram as hidrólises de ATP, ADP, AMP e 5'-TMP aumentadas significativamente (Capítulo 4, Figura 1). Além disso, através da análise por HPLC, foi possível observar uma hidrólise mais rápida de ATP nos grupos diabetes e salina, quando comparados

aos grupos citrato e insulina (Capítulo 4, Figura 2). Estes resultados podem indicar a participação das ectonucleotidases no controle dos níveis de nucleotídeos/nucleosídeos em soro de ratos diabéticos.

Nucleotídeos extracelulares podem ser liberados para o sangue por células vasculares como, por exemplo, eritrócitos, plaquetas, leucócitos e pelo endotélio (Dubyak & Elmoatassin, 1996; Atkinson et al., 2006) (Figura 9). Em sítios de inflamação, estresse vascular oxidativo ou mecânico pode ocorrer um aumento significativo dos nucleotídeos e nucleosídeos liberados de céluals locais (Atkinson et al., 2006). Já está bem estabelecido um sistema multienzimático para hidrólise dos nucleotídeos, constituído pelas e-NTPDases, E-NPPs e pela ecto-5'-nucleotidase, presentes no soro (Sakura et al., 1998; Oses et al., 2004). Estas enzimas seriam as responsáveis por regular os níveis destes nucleotídeos/nucleosídeos, tendo um papel importante para a manutenção adequada dos níveis fisiológicos circulantes destas moléculas.

Foi demonstrado que altos níveis de glicose podem causar liberação de ATP em células endoteliais e células β pancreáticas (Parodi et al., 2002; Hellman et al., 2004), e o aumento de ATP pode causar ativação de receptores P2 gerando uma sinalização para ativação das células musculares lisas vasculares (VSMC). Além disso, altos níveis de glicose induzem a liberação de nucleotídeos extracelulares, que agem nos receptores P2Y para estimular o crescimento de VSMC, via ativação de NFAT (fator nuclear de célula T ativada) (Nilsson et al., 2006). É importante lembrar que o ATP hidrolisado a ADP tem como principal efeito funcional a ativação da agregação plaquetária, que é uma parte importante

do processo hemostático (Boarder & Hourani, 1998). Portanto, o controle dos níveis extracelulares de ADP, principalmente por ectonucleotidases de membrana ou na forma solúvel, é fundamental para os processos trombóticos (Ralevick & Bunstock, 2003). Além disso, com o aumento da atividade de hidrólise do ATP até AMP, muito provavelmente estará ocorrendo uma maior formação de Ado, que é tanto um precursor, quanto um metabólito dos nucleotídeos da adenina. As fontes de Ado na circulação incluem tanto o ATP e o ADP liberados pelas plaquetas, o AMP liberado pelos neutrófilos, o ATP liberado pelas células endoteliais, a Ado que é carreada das células sanguíneas para o plasma por transportadores de nucleosídeos e a Ado do interstício que se difunde para o espaço vascular através de fendas entre as células endoteliais adjacentes na parede dos capilares (Shryok & Belardinelli, 1997). A Ado exerce múltiplos efeitos protetores em diferentes processos fisiopatológicos, tais como: proteção tecidual contra isquemia em casos de reperfusão; diminuição da pressão arterial através da vasodilatação em casos de hipertensão; proteção contra arteriosclerose por meio da vasodilatação, da proliferação celular e do aumento na expressão de fatores de crescimento endoteliais; inibição da agregação plaquetária, entre outros (Mubagwa et al., 1996; Shryok & Belardinelli, 1997; Ralevic & Burnstock, 2003).

Lunkes e colaboradores (2003) também demonstraram um aumento na atividade NTPDásica e 5'-nucleotidásica em plaquetas em paciente com diabetes tipo 2. Os autores sugerem um envolvimento de respostas fisiológicas ao diabetes, ou seja, um mecanismo compensatório para evitar possíveis danos ao sistema vascular no caso de um aumento dos níveis de nucleotídeos. O mesmo grupo demostrou um aumento da hidrólise de ATP, ADP e AMP quando altos níveis de

glicose são adicionados *in vitro* em uma preparação de plaquetas, sugerindo um efeito direto da glicose na atividade enzimática (Lunkes et al., 2008). Como obtivemos o mesmo perfil de hidrólise de nucleotídeos em soro, resolvemos testar se o mesmo poderia ocorrer em nossa fração (Figura 3). Neste caso não foi possível observarmos a mudança na hidrólise dos nucleotídeos em soro, o que pode sugerir não ser um efeito direto da glicose nas enzimas solúveis, pelo menos na condição *in vitro*.

Desde que altos níveis de glucose podem induzir uma série de modificações metabólicas que são capazes de gerar tanto complicações microvasculares, quanto danos macrovasculares (Hermans, 2007), acreditamos que os altos níveis de glicose *in vivo* podem induzir liberações locais de nucleotídeos extracelulares e isto poderia ocasionar um aumento da liberação de nucleotidases ou um aumento da atividade enzimática com a função de prevenir injúria vascular ou interferir em mecanismos hemostáticos (Figura 9) .

Assim, na tentativa de avaliarmos a interferência *in vivo* dos altos níveis de glicose na atividade de ectonucleotidases solúveis, medimos a hidrólise de ATP, ADP, AMP e 5'-TMP em ratos diabéticos 30 dias após indução do modelo e tratados mais 6 dias com insulina. Como podemos observar nas figuras 1 e 2 (Capítulo IV), o tratamento com insulina foi capaz de restaurar a atividade de ectonucleotidases. Porém resta a dúvida se este efeito é devido ao controle glicêmico ou aos efeitos da insulina. Sakowick and Pawelczyk (2002) demonstraram que a insulina pode restaurar a expressão da Ado quinase em fígado, rins e coração de ratos diabéticos por estreptozotocina e que este efeito depende da insulina e não está relacionado com o controle glicêmico. Neste

sentido, os efeitos da insulina sobre a hidrólise de nucleotídeos em soro de ratos diabéticos precisam ser investigados com mais detalhes.

Os resultados apresentados neste capítulo mostram que o sistema purinérgico e as doenças vasculares diabéticas muito provavelmente estejam interligadas. Devemos considerar que enzimas presentes no soro, nas plaquetas e na parede vascular podem trabalhar em conjunto para evitar agregação plaquetária e formação espontânea de trombos (Oses et al., 2004; Frasseto et al., 1993; Yegutkin et al., 2000). Como já foi proposto por Oses e colaboradores (2004), as enzimas do soro, como E-NTPDases e E-NPPs, podem contribuir para remover o ATP e ADP, e, juntamente com a 5'-nucleotidase, poderiam aumentar a concentração de Ado, conhecido vasodilatador. Além disso, as E-NPPs podem atuar como "cães de guarda" a fim de prevenir a subversão da célula, destruindo o DNA e o RNA liberados sob condições de injúria tecidual ou morte celular. Acreditamos que as ectonucleotidases possam participar da inativação local de elevações de nucleotídeos, podendo representar um importante sistema auxiliar de regulação dos níveis dos nucleotídeos e de seus efeitos em uma patologia que vêm aumentando sua incidência a cada ano.

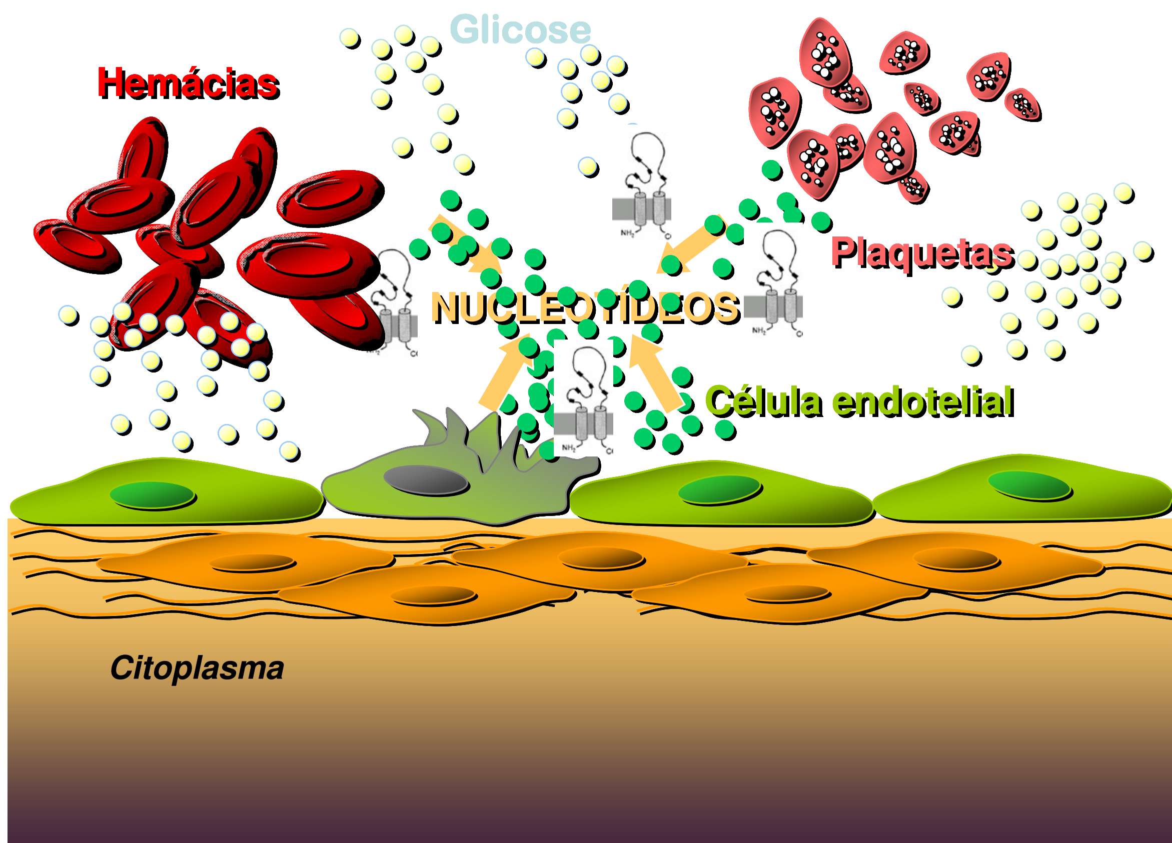


Figura 9. Modelo proposto para a participação das ectonucleotidases e os nucleotídeos da adenina nas alterações vasculares encontradas no diabetes. Nucleotídeos extracelulares podem ser liberados para o sangue por células vasculares como, por exemplo, eritrócitos, plaquetas, leucócitos e pelo endotélio. Altos níveis de glucose podem induzir, *in vivo*, liberações locais de nucleotídeos extracelulares. Este aumento de nucleotídeos pode ocasionar um aumento da liberação de nucleotidases e/ou um aumento da atividade enzimática com a função de prevenir a injúria vascular ou interferir em mecanismos hemostáticos. *Adaptado de Sevigny J., 2005 (figura não publicada).*

2. CONCLUSÕES GERAIS

De maneira geral, esta tese traz a caracterização cinética, bem como o estudo de algumas propriedades bioquímicas das ectonucleotidases em sinaptossoma cardíaco, além do estudo da expressão destas enzimas em tecido de ventrículo esquerdo de ratos adultos. Consideramos que isto possa fazer parte de um complexo sistema de hidrólise de nucleotídeos e representa uma nova abordagem de sinalização purinérgica cardíaca, podendo contribuir para o entendimento das doenças cardiovasculares, dentre elas, as que ocorrem no diabetes.

Em adição, mostramos uma alteração na hidrólise de nucleotídeos em sinaptossoma cardíaco e em soro de ratos diabéticos. A alteração encontrada em coração parece fazer parte da etiologia da doença cardiovascular diabética, no entando, a alteração encontrada em soro parece participar de um sistema de proteção a possíveis danos vasculares que o diabetes pode causar, devido à diminuição de ADP e aumento de adenosina encontrada em ratos diabéticos:

2.1 Conclusões específicas:

1) A preparação de sinaptossoma cardíaco foi capaz de hidrolisar ATP, ADP e AMP em um pH ótimo em torno de 8.0 e 9.5 e requer cátions divalentes para a sua atividade de hidrólise. A preparação necessita de inibidores de ATPases mitocondriais para excluir a atividade destas na medida de hidrólise de ATP.
2) Os mRNAs de todos os membros da família das E-NTPDases estudados (1, 2, 3, 4, 5 e 6) e da ecto-5'-nucleotidase/CD73 foram detectados, em diferentes níveis de expressão, em VE de ratos.
3) Não foi detectado mRNA para a NTPDAse8 em VE de ratos.
4) Os genes dominantes expressos foram das NTPDases 2 e 6 e ecto-5'-nucleotidase.
5) Entre os membros da família das E-NPPs, a NPP3 foi a mais expressa em VE e a NPP1 não foi detectada.
6) A preparação de sinaptossoma cardíaco hidrolisa 5'-TMP, marcador artificial para E-NPPs, em um pH ótimo em torno de 8,9 e requer cátions divalentes para a sua atividade de hidrólise. O inibidor de fosfatase alcalina, levamisole, não teve efeito na atividade de hidrólise do substrato marcador nas condições testadas, indicando uma forte participação das E-NPPs nesta atividade de hidrólise.
7) O modelo de diabetes por estreptozotocina foi confirmado pelas medidas da glicemia, peso, batimentos cardíacos e pressão sanguínea em ratos adultos. Uma injeção intraperitoneal de 65mg/kg de STZ foi capaz de aumentar a glicemia, diminuir o peso, diminuir a freqüência cardíaca e a

pressão sanguínea de ratos 30 dias após a injeção. O tratamento com insulina, durante 6 dias, foi capaz de reverter as alterações nestes parâmetros.

8) As hidrólises de ATP e ADP em sinaptossoma cardíaco foram diminuídas em ratos 30 dias diabéticos e o tratamento com insulina foi capaz de reverter esses valores no nível de controle.
9) Não houve alteração nas hidrólises de AMP e 5'-TMP, tanto em ratos diabéticos, quanto em ratos tratados com insulina na preparação de sinaptossoma cardíaco.
10) A análise por HPLC da hidrólise extracelular do ATP confirmou o retardo na hidrólise do ATP em sinaptossoma cardíaco de ratos diabéticos quando comparados ao controle citrato. O tratamento com insulina foi capaz de reverter este retardo, voltando ao padrão de hidrólise do controle citrato.
11) A análise por PCR de tempo real indica uma alteração na expressão das ectonucleotidases, principalmente da NTPDase2.
12) A análise por western blotting sugere que em sinaptossoma cardíaco possam ser expressas pelo menos as NTPDase1 e NTPDase2.
13) No soro de ratos diabéticos ocorreu um aumento na hidrólise dos nucleotídeos da adenina e também do marcador artificial 5'-TMP.
14) A análise por HPLC da hidrólise extracelular do ATP em soro confirmou o aumento na hidrólise do ATP e diminuição de ADP mais rapidamente em ratos diabéticos quando comparados ao controle citrato, além de ocorrer um acúmulo de adenosina nos ratos diabéticos. O tratamento com insulina

foi capaz de reverter as alterações, voltando aos padrões de hidrólise do controle citrato.

15) A adição *in vitro* de 20 mM de glicose no soro de ratos controle não foi capaz de alterar a hidrólise dos nucleotídeos, mostrando que este efeito talvez ocorra apenas *in vivo*, já que o tratamento com insulina foi capaz de reverter a atividade ao níveis de controle.

3. PERSPECTIVAS

1) Verificar a possível presença das demais E-NTPDases em sinaptossoma de ventrículo esquerdo de ratos pelo método de Western Blotting;
2) Verificar a presença dos receptores P2X e P2Y em sinaptossoma de ventrículo esquerdo de ratos pelo método de Western Blotting;
3) Estudar o efeito do diabetes em 15 dias (efeitos agudos) e em 60 dias (efeitos crônicos) na atividade de ectonucleotidases de soro e sinaptossoma cardíaco;
4) Verificar os efeitos agudos e crônicos do diabetes na expressão das E-NTPDases em sinaptossoma cardíaco;
5) Padronizar a preparação de sinaptossoma cardíaco para ventrículo direito e estudar atividade enzimática e possíveis diferenças na expressão das ectonucleotidases em ventrículo esquerdo e ventrículo direito;
6) Estudar o efeito do diabetes na atividade e expressão de ectonucleotidases de linfócitos de ratos adultos, em 15, 30 e 60 dias de indução de diabetes;
7) Avaliar o efeito do exercício físico nas alterações já encontradas para as ectonucleotidases em ratos diabéticos por estreptozotocina, em sinaptossoma cardíaco e soro;

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LISTA DE FIGURAS

FIGURA 1. Vias de sinalização purinérica.. 3

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