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BRUNA JUBER DE ARAÚJO

EFEITO DO ÓXIDO NÍTRICO SOBRE A ATIVIDADE E EXPRESSÃO DA NTPDase1 E DA ECTO-5'-NUCLEOTIDASE EM LINHAGENS DE CÉLULAS VASCULARES

PATOS DE MINAS – MG

FEVEREIRO DE 2019

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Dissertação de Mestrado apresentada ao Programa de Pós-graduação em Biotecnologia como requisito parcial para obtenção do título de Mestre em Biotecnologia.

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Resumo

Compreender os mecanismos que atuam na vasculatura representa um caminho promissor para o desenvolvimento e entendimento de novos estudos para as desordens vasculares. Os nucleotídeos extracelulares (ATP, ADP) e o nucleosídeo adenosina modulam o vasorelaxamento e a vasoconstrição, e estão envolvidos na produção do vasodilatador óxido nítrico (NO). Permanece por ser investigado se o NO é capaz de modular as ectonucleotidases, enzimas que hidrolisam os nucleotídeos, controlado sua disponibilidade no meio extracelular. As NTPDases 1 e 2 hidrolisam ATP e ADP; e a ecto-5'-nucleotidase metaboliza AMP até adenosina, também um vasodilatador. Para determinar o efeito do NO sobre as ectonucleotidases vasculares, células musculares lisas vasculares A7r5 e células endoteliais tEnd.1 foram tratadas com agente doador de NO (nitroprussiato de sódio (SNP)), inibidor da síntese de NO (L-NAME) e mobilizadores de NO (ATP, ADP, bradicinina (BK) e 2-Cl-adenosina). Após 24 horas, através do ensaio de MTT, notou-se uma diminuição de 35% na viabilidade das células A7r5 tratadas com 2-Cl-adenosina (10^{-4}M). Os níveis de nitrito, dosados pelo reagente de Griess, aumentaram nos dois tipos celulares em resposta ao SNP, indicando um aumento indireto dos níveis de NO. As atividades enzimáticas foram detectadas colorimetricamente pelo fosfato inorgânico liberado. As hidrólises de ATP e ADP não foram alteradas. Entretanto, a hidrólise de AMP foi significativamente aumentada pelo ATP e 2-Cl-adenosina em A7r5; e pelo ATP e ADP em tEnd.1. O aumento na atividade da ecto-5'-nucleotidase parece ser importante para aumentar os níveis de adenosina circulante, já que ela é fundamental para garantir a regulação da homeostase e a manutenção do tônus vascular. A tentativa de avaliação da expressão gênica das ectonucleotidases em resposta aos tratamentos em células A7r5 não foi bem-sucedida. Ainda, não foram encontradas alterações em nível proteico, avaliado por Western blotting. Porém, o aumento na atividade da enzima poderia ser explicado pela presença de sítios específicos altamente fosforiláveis em sua estrutura, principalmente em resíduos de serina. Embora os resultados obtidos nos levem a crer que as ectonucleotidases sejam moduladas independentemente do NO em A7r5, experimentos adicionais são necessários para confirmar a concentração de NO no meio de cultivo de tais células e os níveis de regulação sofridos pelas enzimas em questão. A compreensão de tais funções e mecanismos de ação do NO e outras moléculas vasoativas sobre as ectonucleotidases vasculares são fundamentais para a compreensão das desordens vasculares.

Abstract

Understanding the mechanisms that act in the vasculature represent a promising path for the development and understanding of future studies about vascular disorders. It is known that extracellular nucleotides (ATP, ADP) and nucleoside adenosine are able to modulate vasorelaxation and vasoconstriction and are involved in the production of the vasodilator nitric oxide (NO). It remains to be investigated whether NO is able to modulate the expression and activity of ectonucleotidases, enzymes that hydrolyze nucleotides controlling their availability in the extracellular environment. NTPDases 1 and 2 hydrolyze ATP and ADP and ecto-5'-nucleotidase metabolizes AMP to adenosine, also a vasodilator. To determine the effect of NO on vascular ectonucleotidases, vascular smooth muscle cells A7r5 and endothelial cells tEnd.1 were treated with NO donor (sodium nitroprusside (SNP)), inhibitor of NO synthesis (L-NAME) and mobilizers of NO (ATP, ADP, bradykinin (BK) and 2-Cl-adenosine). After 24 hours, a 35% decrease in cell viability of A7r5 cells was observed by MTT assay in response to 2-Cl-adenosine (10^{-4}M). Nitrite levels, measured by Griess's reagent, significantly increased in the two cell types treated with SNP, indicating an indirect increase in NO levels. The enzymatic activities were detected colorimetrically by the inorganic phosphate released. The hydrolysis of ATP and ADP was not altered. However, AMP hydrolysis was significantly increased by ATP and 2-Cl-adenosine in A7r5 cells and by ATP and ADP in tEnd.1 cells. The increase in ecto-5'-nucleotidase activity seems to be important to increase levels of circulating adenosine, since it is of great importance to ensure regulation of homeostasis and maintenance of vascular tone. The attempt to evaluate the gene expression of ectonucleotidases in response to treatments in A7r5 cells was not successful. Besides, no changes were found in protein level, as assessed by Western blotting. However, the increase in enzyme activity could be explained by the presence of specific highly phosphorylable sites, especially in serine residues, in the structure of the ecto-5'-nucleotidase. Although the results obtained lead us to believe that the ectonucleotidases are modulated independently of the NO in A7r5, additional experiments are required to confirm the concentration of NO in the culture medium of such cells and the levels of regulation undergone by the enzymes in question. The understanding of the functions and mechanisms of action of NO and other vasoactive molecules on vascular ectonucleotidases are of fundamental relevance for the understanding of vascular disorders.

Lista de ilustrações

Capítulo 1

Lista de abreviaturas

ADP: Adenosina-difosfato

AMP: Adenosina-monofosfato

AngII: Angiotensina II

ATP: Adenosina-trifosfato

BK: Bradicinina

Ca^{2+}: Cálcio

CE: Células endoteliais

cGMP: Monofosfato de guanosina cíclico, do inglês *cyclic guanosine monophosphate*

CMLV: Células musculares lisas vasculares

CMP: Citidina monofosfato

EDHF: Fator hiperpolarizante derivado do endotélio, do inglês *endothelium-derived hyperpolarizing factor*

eNOS: Óxido nítrico sintase endotelial

E-NPP: Ecto-nucleotídeo pirofosfatase/ fosfodiesterase

E-NTPDase: Ecto-nucleosídeo trifosfato difosfoidrolase

GMP: Guanosina-monofosfato

iNOS: Óxido nítrico sintase indutível

K^{+}: Potássio

L-NAME: Nω-nitro- L- arginina metil éster

mRNA: RNA mensageiro

nNOS: Óxido nítrico sintase neuronal

NO: Óxido nítrico, do inglês *Nitric oxide*

NOS: Óxido nítrico sintase, do inglês *Nitric oxide Synthase*

PGI_2: Prostaciclina

SNP: Nitroprussiato de sódio

UDP: Uridina-difosfato

UMP: Uridina-monofosfato

Sumário

1. Introdução

A sinalização purinérgica tem sido apontada como um importante regulador da fisio(pato)logia cardiovascular, uma vez que, na vasculatura, o ATP, o ADP e a adenosina podem influenciar as respostas vasomotoras, a ativação plaquetária e a função cardíaca, entre outros aspectos (BURNSTOCK, 2017a). As ectonucleotidases são enzimas capazes de hidrolisar os nucleotídeos extracelulares, inativando seus efeitos sinalizadores. Portanto, membros da família das ectonucleotidases, comumente expressas na vasculatura, tais como a NTPDase1 e 2, capazes de hidrolisar ATP e ADP; e a ecto-5'-nucleotidase, que metaboliza os monofosfonucleotídeos até adenosina, uma molécula reconhecidamente vasodilatadora, regulam a concentração de nucleosídeos e nucleotídeos na circulação (ZIMMERMANN, 1996; FUENTES & PALOMO, 2015).

É sabido que os nucleotídeos extracelulares estão implicados na ativação da enzima óxido nítrico sintase endotelial (eNOS), levando à produção de óxido nítrico (NO) e à vasodilatação (da SILVA et al., 2009). Recentemente, viu-se que a eNOS ao ser ativada por ATP extracelular leva à geração de NO também nos glóbulos vermelhos (ULKER et al., 2018). O NO participa de uma série de processos fisiológicos vasculares relacionados à regulação do tônus e vasodilatação; angiogênese; regulação da transcrição de genes; tradução de mRNA e neurotransmissão (MUTCHLER & STRAUB, 2015).

O aprofundamento dos estudos sobre o papel dos nucleotídeos em diferentes contextos da fisiopatologia cardiovascular tem particular relevância. Os componentes da sinalização purinérgica estão presentes em todos os sistemas biológicos animais, e muitos dos quais têm, inclusive, sido empregados na clínica. Como exemplo, destacam-se as tienopiridinas, ticlopidina e clopidogrel, que são largamente utilizadas como agentes anti-plaquetários, já que antagonizam de maneira irreversível os receptores de plaquetas (LORGA FILHO et al., 2013).

Em um trabalho prévio, nosso grupo demonstrou que as atividades das ectonucleotidases foram diminuídas em soro e em plaquetas de animais submetidos à condição hipertensiva pela administração prolongada de L-NAME, um bloqueador da síntese de NO. Mostramos, ainda, que os níveis de ADP, AMP e outros componentes da via de sinalização purinérgica estiveram paradoxalmente diminuídos na circulação desses animais (FURSTENAU et al., 2008). Como os dados prévios foram observados em frações

solúveis na circulação, hipotetizamos que a redução dos níveis circulantes de purinas em animais hipertensos depende de uma (des)regulação das atividades ectonucleotidases, cuja identidade permanece por ser investigada, presentes na superfície dos vasos.

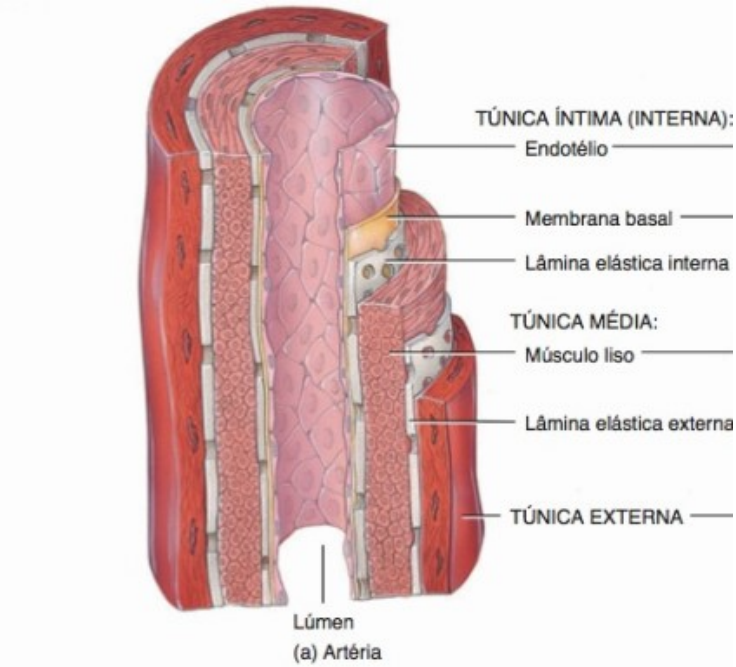
Em conjunto, essas observações denotam a existência de uma interlocução entre os sistemas purinérgico e nitrérgico, em que as purinas modulam de maneira importante a produção de NO nos vasos sanguíneos. Permanece por ser investigado se o NO é capaz de influenciar a expressão e atividade das ectonucleotidases presentes na vasculatura, regulando a taxa de remoção dos nucleotídeos circulantes e modulando o tônus vascular. Dessa maneira, o objetivo desse trabalho é determinar o efeito do NO sobre a atividade e expressão das enzimas NTPDase1 e ecto-5'-nucleotidase em linhagens de células musculares lisas vasculares (CMLV) A7r5 (ATCC® CRL-1444™) e em células endoteliais (CE) t.End1. Tal elucidação apresenta-se como um campo extremamente fértil de investigação no comportamento das desordens vasculares bem como na proposição de novas abordagens terapêuticas.

Capítulo 1

2. Referencial teórico

O sistema cardiovascular é responsável por levar nutrientes e oxigênio para todas as células do organismo, composto por sangue, coração e vasos sanguíneos (GUYTON & HALL, 2011). Os vasos sanguíneos transportam sangue do coração em direção aos tecidos do corpo e retornam o sangue dos tecidos para o coração. São, basicamente, compostos por três camadas celulares, também denominadas túnicas: 1) a camada mais interna que entra em contato com o lúmen do vaso e o fluxo sanguíneo é composta por uma monocamada de células endoteliais (CE) e é conhecida como íntima; 2) a camada média, que é responsável pelos mecanismos de contração e relaxamento, é composta por células musculares lisas vasculares (CMLV); e 3) a camada mais externa que reveste, dá suporte e inervação e é composta basicamente por tecido conjuntivo (GUYTON & HALL, 2011) (Figura 1).

Figura 1. Estrutura da parede arterial (TORTORA, 2013).



As CE são metabolicamente ativas e desempenham um papel importante em inúmeras funções fisiológicas, desde o transporte de células sanguíneas ao controle do tônus vascular, equilíbrio homeostático intravascular, permeabilidade de moléculas, imunidade adaptativa e controle da coagulação e inflamação (AIRD, 2007). Substâncias vasodilatadoras, como o óxido nítrico, e vasoconstritoras são produzidas equilibradamente para proteger a parede vascular (SNYDER & BREDT, 1992).

As CMLV, que revestem as paredes de vários órgãos e vasos, são compostas por fibras contráteis de ação involuntária. Os mecanismos de contratilidade e vasodilatação, são dependentes da ativação do cálcio (Ca^{2+}) e da atividade da fosfatase de miosina. Em resposta a estímulos específicos, elevados níveis de Ca^{2+} interagem com a calmodulina e uma cadeia leve de miosina é fosforilada via sinalização de RhoA/Rho quinase, inibindo a atividade enzimática da fosfatase de miosina, permitindo que a cadeia leve da miosina permaneça fosforilada, promovendo a contração. A remoção de Ca^{2+} do citosol estimula a fosfatase da miosina, que remove o fosfato de alta energia da cadeia leve de miosina para promover o relaxamento do músculo liso (WEBB, 2003).

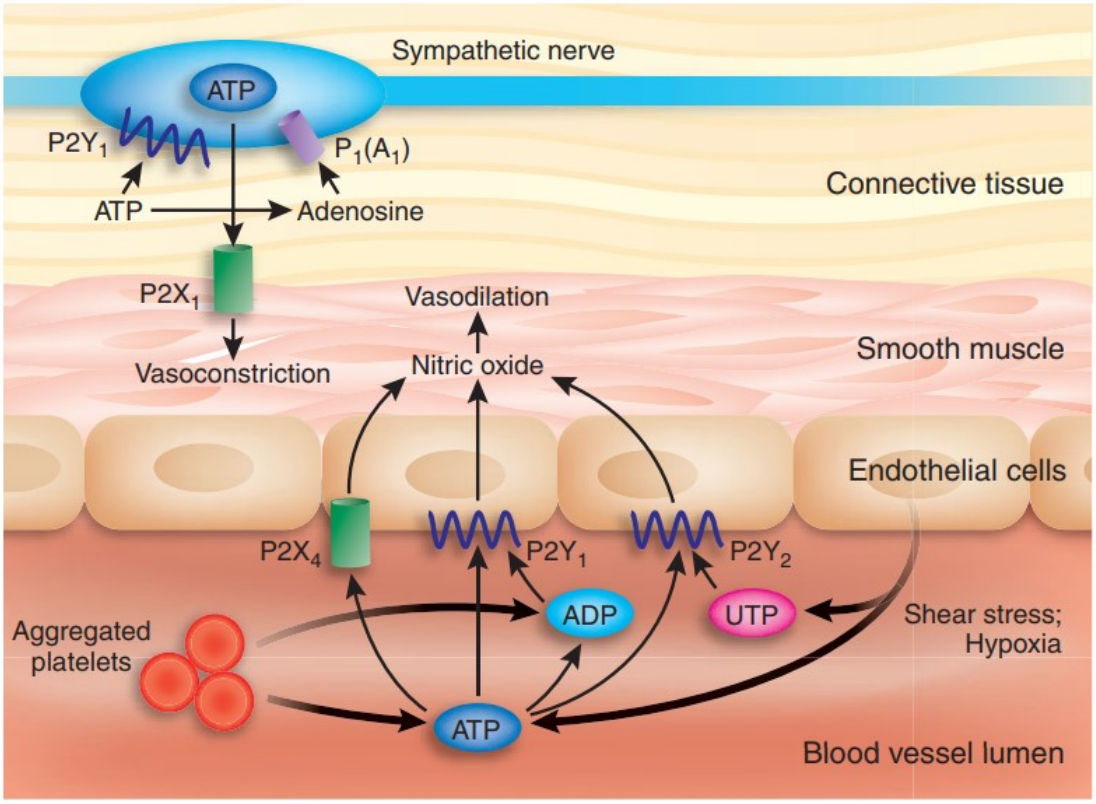
As ações do ATP e da adenosina sobre o coração e os vasos sanguíneos como agentes bradicárdicos e redutores da pressão arterial global foram descritas pela primeira vez em 1929 (DRURY e SZENT-GYÖRGYI, 1929). Atualmente, a sinalização purinérgica é amplamente aceita como um sistema primitivo e altamente conservado entre os animais, sendo atribuídos importantes papéis aos nucleotídeos e nucleosídeos como sinalizadores extracelulares em tecidos neurais e não neurais (BURNSTOCK, 2017b). Nesse sentido, no sistema vascular, a sinalização purinérgica é capaz de estimular a vasoconstrição e o vasorelaxamento, o crescimento das CMLV e a angiogênese; está envolvida no remodelamento vascular; estimula a agregação plaquetária; regula a coagulação, a inflamação e diversos aspectos da função cardíaca (BURNSTOCK e KENNEDY, 1986; OLSSON e PEARSON, 1990; RALEVIC e BURNSTOCK, 1991; ERLINGE, 1998; BURNSTOCK, 2002; DI VIRGILIO e SOLINI, 2002; GACHET, 2006; BURNSTOCK, 2017a).

Na circulação, as purinas são liberadas pelos eritrócitos, CE, plaquetas e neutrófilos ativados em reposta a diversos estímulos, tais como estresse de cisalhamento, hiperóxia, hipóxia ou estimulação por agonista (BODIN & BURNSTOCK, 2001). Essas exercem seus efeitos através de duas principais famílias de receptores P2, os quais compreendem tanto os canais iônicos ativados por ATP ($P2X_{1\text{-}7}$), quanto os receptores metabotrópicos acoplados às proteínas G ($P2Y_{1,2,4,6,11,12,13,14}$) (KING, et al., 1998; BURNSTOCK, 2017a). São conhecidos, ainda, quatro receptores purinérgicos do tipo P1 (A_1, A_{2A}, A_{2B} e A_3), os quais são responsivos à adenosina (PALMER e STILES, 1995).

A ativação de receptores P2 endoteliais induz à vasodilatação local através da produção de óxido nítrico (NO), prostaciclina (PGI_2) e fator hiperpolarizante derivado do endotélio (EDHF). Os receptores $P2Y_1$ e $P2Y_2$ são os responsáveis pelo relaxamento

induzido por ATP e UTP, respectivamente (BURNSTOCK, 2006a). Contrariamente, a ativação dos receptores P2 em CMLV promove a vasoconstrição através dos receptores $P2Y_{12}$, que ativam a agregação plaquetária por ADP ou dos receptores P2X sensíveis às pirimidinas (GITTERMAN e EVANS, 2001; WIHLBORG et al., 2004) (Figura 2).

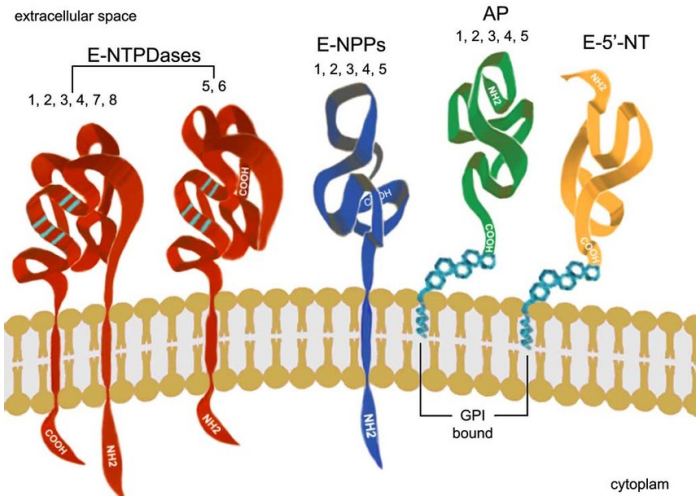
Figura 2. Mecanismo geral da sinalização purinérgica na vasculatura (BURNSTOCK, 2006b).



As vias de sinalização intracelulares, geralmente, requerem mecanismos de inativação de sinal. O grande número de receptores purinérgicos presentes em uma mesma célula ou em duas células vizinhas sugere uma fina regulação desta sinalização. Dessa forma, os nucleotídeos são hidrolisados por uma cascata enzimática extracelular, que resulta na formação do respectivo nucleosídeo e fosfato livre, orquestrando a ativação dos receptores celulares P1 e P2 via liberação controlada de nucleotídeos e nucleosídeos. As metaloectoenzimas capazes de hidrolisar os nucleotídeos extracelulares são conhecidas como ectonucleotidases e, incluem membros das famílias E-NTPDase (ecto-nucleosídeo trifosfato difosfoidrolase), E-NPP (ecto-nucleotídeo pirofosfatase/ fosfodiesterase),

fosfatases alcalinas e ecto-5'-nucleotidase, as quais apresentam uma ampla distribuição tecidual (ZIMMERMANN, 2000; AL-RASHIDA et al., 2017) (Figura 3).

Figura 3. Famílias de ectonucleotidases (COGNATO & BONAN, 2010).



Entre as ectonucleotidases conhecidas e caracterizadas, a família E-NTPDase parecer ser a mais importante. Em mamíferos, oito diferentes genes codificam membros desta família de proteínas (NTPDase1 a 8), as quais apresentam diferentes preferências por substratos e distinta distribuição tecidual (ROBSON et al., 2006). As NTPDases 1, 2, 3 e 8 são enzimas tipicamente localizadas na superfície celular com o sítio catalítico voltado para o meio extracelular. Por outro lado, as NTPDases 5 e 6 exibem uma localização intracelular e podem ser secretadas. Finalmente, as NTPDases 4 e 7 estão localizadas intracelularmente, tendo o seu sítio catalítico voltado para o lúmen das organelas citoplasmáticas (ROBSON et al., 2006). As razões de hidrólise entre os nucleosídeos tri e difosfatos varia consideravelmente entre os subtipos e, juntamente com ferramentas de biologia molecular, são utilizadas na identificação e classificação dessas enzimas: a NTPDase1 hidrolisa o ATP e o ADP igualmente bem (razão de hidrólise ATP:ADP=1:1); as NTPDases 3 e 8 revelam uma preferência pelo ATP sobre o ADP como substrato (razão de hidrólise ATP:ADP=3:1 e 2:1, respectivamente); e a NTPDase2 apresenta uma alta preferência pelos nucleosídeos trifosfatos (razão de hidrólise ATP:ADP=30:1), tendo sido previamente classificada como uma ecto-ATPase (ROBSON et al., 2006).

A NTPDase1 é a principal ectoenzima responsável pela hidrólise dos nucleotídeos na superfície dos vasos sanguíneos (KACZMAREK et al., 1996; SÉVIGNY et al., 1997), sendo dominantemente expressa em CE e em CMLV (SÉVIGNY et al., 2002). Essa enzima

tem um importante papel nas propriedades antitrombóticas das CE, uma vez que hidrolisa o ADP, uma molécula pró-agregante plaquetária que atua através dos receptores $P2X_1$, $P2Y_1$ e $P2Y_{12}$ (ROBSON et al., 2006). Ainda, foi apontada a participação da NTPDase1 na regulação local do tônus vascular pelos nucleotídeos, ajustando a disponibilidade destas moléculas na superfície das CMLV (KAUFFENSTEIN et al., 2010a); e também na modulação do relaxamento dependente dos receptores P2Y endoteliais, uma vez que controla o nível de agonistas e a reatividade dos receptores (KAUFFENSTEIN et al., 2010b).

A NTPDase2 está localizada na camada adventícia de vasos muscularizados e nos pericitos dos capilares (SÉVIGNY et al., 2002), e sua preferência em hidrolisar o ATP leva ao acúmulo de ADP, o qual é essencial para o processo de agregação plaquetária (MAFFRAND et al., 1998), importante para estancar o sangramento durante episódios de lesão vascular, por exemplo. Outra ectonucleotidase de suma importância na vasculatura é a ecto-5'-nucleotidase, que finaliza a cascata de hidrólise e atua em conjunto com as NTPDases, catalisando a conversão dos monofosfonucleotídeos (CMP, UMP, GMP e AMP) até seus respectivos nucleosídeos (ZIMMERMANN, 1996). Está predominantemente associada ao endotélio (KOSZALKA et al., 2004), e tem como principal função a produção extracelular de adenosina a partir de AMP, com a capacidade de suprimir a reação inflamatória e mediar a vasodilatação e mecanismos cardioprotetores (ZUKOWSKA et al., 2015).

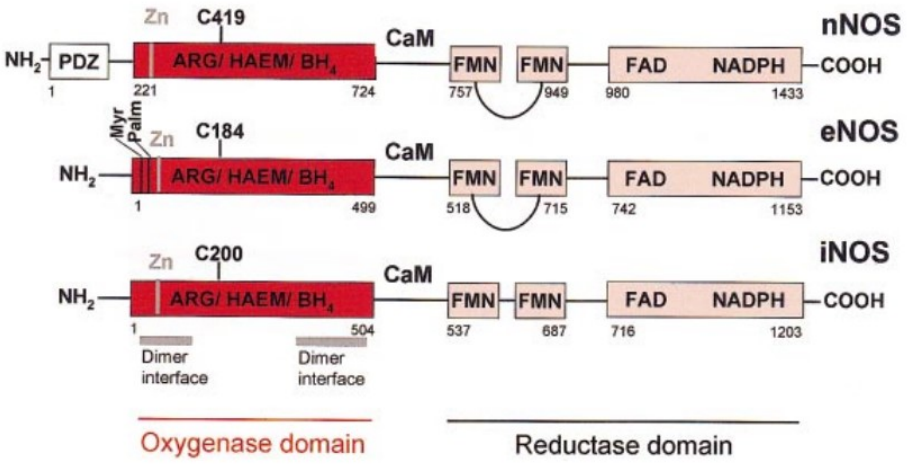
A adenosina atua por meio de uma família de receptores P1 também acoplados à proteína G (A1, A2A, A2B e A3) na superfície celular, tendo maior afinidade por A1 e A2A (SHRYOCK & BELARDINELLI, 1997). Tais receptores operam via ativação ou inibição de adenilato ciclase, e são classificados de acordo com sua afinidade pela adenosina, sendo de fundamental importância para diversos eventos fisiológicos de regulação do tônus vascular, cardioproteção, vasodilatação, regulação inflamatória e inibição da agregação plaquetária (FREDHOLM, 2007), além de estar fortemente envolvida na migração e proliferação de CMLV (YANG et al., 2015). Portanto, a ativação da ecto-5'-nucleotidase vem sendo fortemente abordada como um novo alvo estratégico para tratamentos específicos das patologias cardiovasculares, melhorando a proteção endotelial (ZUKOWSKA et al., 2015).

Os nucleotídeos no meio extracelular também estão envolvidos na ativação da enzima óxido nítrico sintase, levando à produção de óxido nítrico (NO) e consequente vasodilatação (DA SILVA et al., 2009). O NO consiste de um mediador gasoso que participa

de uma série de processos fisiológicos em todo o organismo. Na vasculatura, por exemplo, caracteriza-se por ser uma das mais importantes moléculas de sinalização, relacionado à regulação do tônus vascular e à vasodilatação; angiogênese; regulação da transcrição de genes; tradução de mRNA e neurotransmissão (MUTCHLER & STRAUB, 2015). Na circulação sanguínea, o NO está intimamente implicado em processos de coagulação e, uma vez que apresenta propriedades antitrombóticas, é capaz de inibir a adesão celular e a agregação plaquetária na parede vascular (LOSCALZO, 2001). Ainda, o NO atua na regulação da pressão sanguínea e na contratilidade do músculo cardíaco, podendo ser produzido tanto em CE quanto em CMLV (MURREL et al., 1996). Por isso, o entendimento do mecanismo da síntese de NO, pode representar um caminho promissor para o desenvolvimento e entendimento de futuras estudos sobre as desordens vasculares.

O NO endógeno é derivado de fontes enzimáticas e não enzimáticas na vasculatura. As proteínas sanguíneas por exemplo, podem preservar e liberar a bioatividade do NO. Condições de hipóxia ou baixo pH podem fazer com que os nitritos sejam reduzidos para formação de NO (CHEN, et al., 2008). A síntese deste gás por ação enzimática se dá pela ação da enzima óxido nítrico-sintase (NOS), que apresenta três isoformas: óxido nítrico-sintase neuronal (nNOS), óxido nítrico-sintase indutível (iNOS) e óxido nítrico-sintase endotelial (eNOS). Todas as isoformas se ligam à calmodulina, e utilizam L-arginina e oxigênio molecular como substratos. Em nNOS e eNOS essa ligação com a calmodulina é provocada por um aumento de Ca^{2+} intracelular, já no caso da iNOS, a mesma ligação ocorre em baixas concentrações de Ca^{2+} por ter um sítio de ligação com uma diferente estrutura de aminoácidos (CERQUEIRA e YOSHIDA, 2002) (Figura 4).

Figura 4. Estrutura dos domínios de ligação das isoformas de NOS (ALDERTON, et al., 2001).



A isoforma nNOS está relacionada com neurônios específicos do cérebro e eventos de sinalização sináptica. Quando anormal, sua sinalização pode contribuir para uma variedade de patologias neurodegenerativas, tais como esclerose múltipla, doença de Alzheimer e Parkinson (FÖRSTERMANN & SESSA, 2012). Por outro lado, a iNOS não é comumente expressa em células, mas pode ser estimulada por lipopolissacarídeos bacterianos, citocinas e outros agentes, e, devido à sua afinidade por ferro ligado a proteínas, é capaz de inibir enzimas chave que possuem o ferro em seu centro catalítico, interferindo diretamente no DNA de células-alvo causando ruptura de suas cadeias. A regulação da produção de NO por iNOS está implicada no desenvolvimento de patologias como câncer, artrites reumatoides, entre outros mecanismos de apoptose celular (LI, et al., 2014).

Já a isoforma eNOS, se caracteriza por ser a principal responsável pela maior parte do NO sintetizado na vasculatura, expressa principalmente em células endoteliais, de fundamental importância devido à sua capacidade de sinalização e interação com uma variedade de proteínas reguladoras e estruturais em vários processos fisiológicos e fisiopatológicos, de maneira tanto inibitória quanto permissiva (SU, 2014). O produto da eNOS atua em várias enzimas e proteínas-alvo, desencadeando respostas fisiológicas diversas, tal como o mecanismo de relaxamento celular, que acontece quando o NO com sua propriedade gasosa atravessa o endotélio e alcança a musculatura lisa vascular, estimulando a enzima guanilato ciclase solúvel a gerar monofosfato cíclico de guanosina (cGMP) intracelular (CONGER, 1994).

A atividade e expressão da eNOS pode ser regulada por diferentes interações. A principal delas, assim como no caso das demais isoformas, é a interação com a calmodulina induzida por Ca^{2+}, que quando ativada por altas concentrações, desbloqueia o fluxo de elétrons dependentes de NADPH da porção C- para a N-terminal e, então, à porção heme para a produção de NO. Conforme mencionado anteriormente, a atividade da eNOS é altamente dependente do nível de cálcio intracelular e de sua ligação com a calmodulina (FULTON, 2016). Os nucleotídeos ATP, ADP, UTP e UDP e os peptídeos endotelina (1 e 3), angiotensina II (angII) e bradicinina (BK) foram relatados por aumentar os níveis de cálcio intracelular (NAKAI et al., 1999) e, por isso, podem atuar como moléculas mobilizadoras da síntese de NO mediando a ligação da eNOS com a calmodulina.

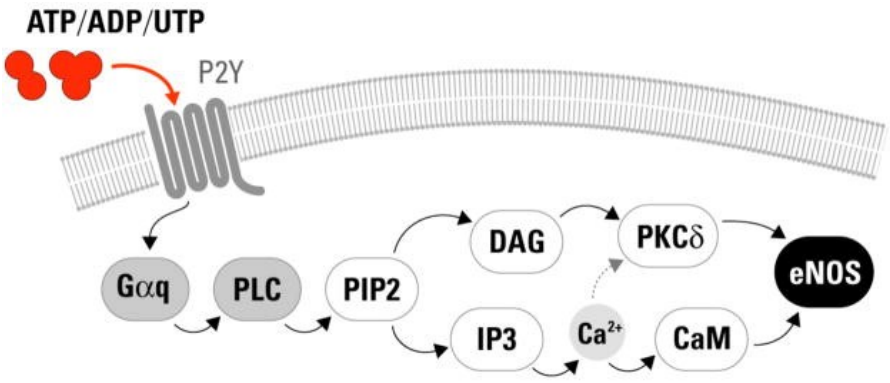
No entanto, existem ainda outros mecanismos pós traducionais que fazem com que outras proteínas também possam interagir com tal enzima, regulando sua atividade. Esse é o caso para a proteína de choque térmico 90 (Hsp90), que atua como modulador alostérico na

ativação da enzima; e também a caveolina-1, que ao interagir com eNOS, atua como regulador negativo da atividade enzimática e consequente síntese de NO (GARCIA-CADEÑA et al., 1998). O nitroprussiato de sódio (SNP) é um doador de NO que induz relaxamento vascular devido à liberação de NO nas células musculares lisas vasculares, agindo na ativação de canais de potássio (K^+) que controlam a entrada de cálcio através de canais de cálcio voltagem-dependentes e, portanto, a contratilidade através de mudanças no cálcio intracelular (BONAVENTURA et al., 2011; FUJII et al., 2016). Apesar de possuir um mecanismo de ação bem rápido, o SNP é um doador de NO amplamente utilizado em pesquisas moleculares, devido ao seu potencial efeito vasodilatador que se assemelha aos do NO endógeno derivado do endotélio (HIRAI, et al., 2012). Ainda, existem outros estímulos independentes de Ca^{2+} intracelular que podem induzir a liberação de NO, como o estresse de cisalhamento e outros mecanismos que podem desencadear a fosforilação da eNOS em resíduos de serina, treonina e tirosina (McCABE et al., 2000).

O NO endotelial, além de ser essencial para a oxigenação tecidual regulando o consumo mitocondrial de O_2 pela inibição da citocromo *c* oxidase (MONCADA & HIGGS, 2006), está fortemente envolvido com a homeostase e regulação do fluxo sanguíneo, uma vez que apresenta o poder de estimular a guanilil ciclase solúvel e aumentar o GMP cíclico nas células musculares lisas, resultando em vasodilatação (RAPOPORT, et al., 1983). Shesely e colaboradores (1996) mostraram que a deleção do gene da eNOS induz elevações dos níveis pressóricos. Além disso, o NO se caracteriza como um potente inibidor da agregação plaquetária, de fundamental importância para processos trombóticos (ALHEID, et al., 1987).

Em 2009, um estudo mostrou que o tratamento das CE com ATP, UTP e ADP levou à fosforliação da eNOS e à geração de NO pela ativação de receptores P2Y, aumentando os níveis intracelulares de cálcio e ativando a PKCδ (da SILVA et al., 2009) (Figura 5). Em um trabalho prévio, nosso grupo mostrou que as atividades ectonucleotidases foram diminuídas em soro e em plaquetas de ratos hipertensos pelo tratamento com éster metílico de Nω- nitro-L-arginina (L-NAME) (FURSTENAU et al., 2008), um inibidor crônico não seletivo da NOS capaz de produzir vasoconstrição sistêmica e hipertensão (MANEESAI et al., 2016). Mostramos ainda que os níveis séricos de ADP, AMP e hipoxantina apresentaram-se paradoxalmente diminuídos nestes animais (FURSTENAU et al., 2008).

Figura 5. Fosforilação da eNOS em células endoteliais induzida por nucleotídeos extracelulares (da SILVA et al., 2009).



Com isso, já se sabe que os nucleotídeos no ambiente extracelular são modulados pela ação das ectonucleotidases e ainda, são capazes de influenciar a produção de NO pela ligação à receptores específicos que desencadeiam a ativação de eNOS. Contudo, ainda permanece obscuro se o NO, um potente vasodilatador, pode influenciar na disponibilidade desses nucleotídeos no meio extracelular, modulando tais ectoenzimas e consequentemente regulando as respostas vasoativas de vasoconstrição e vasorelaxamento, regulando o tônus vascular e agregação plaquetária. Neste contexto, investigar a provável modulação do NO sobre a atividade e expressão da NTPDase1 e da ecto-5'-nucleotidase na superfície de células musculares lisas vasculares e células endoteliais é de grande relevância para a elucidação da contribuição da sinalização purinérgica no controle das respostas vasomotoras.

Referências

Aird, W.C. (2007). Phenotypic Heterogeneity of the Endothelium: I. Structure, Function, and Mechanisms *Circulation Research,* 100: 158–173. https://doi.org/10.1161/01.RES.0000255691.76142.4a

Alderton, W.K., Cooper, C.E., Knowles, R.G. (2001). Nitric oxide synthases: structure, function and inhibition. *Biochemistry Journal* 357 (3): 593:615. https://doi.org/10.1042/bj3570593

Alheid U, Frölich JC, Förstermann U. (1987). Endothelium-derived relaxing factor from cultured human endothelial cells inhibits aggregation of human platelets. *Thromb Res.* 47:561–571. https://doi.org/10.1016/0049-3848(87)90361-6

Al-Rashida, M., Qazi, S.u., Batool, N., Hameed, A., Iqbal, J. (2017). Ectonucleotidase inhibitors: a patent review (2011-2016), *Expert Opinion on Therapeutic Patents*, 27(12): 1291-1304. https://doi.org/10.1080/13543776.2017.1369958

Bodin, P. & Burnstock, G. (2001). Evidence that release of adenosine triphosphate from endothelial cells during increased shear stress is vesicular. *J Cardiovasc Pharmacol*, 38 (6): 900-908. https://doi.org/10.1097/00005344-200112000-00012

Bonaventura, D., de Lima, R.G., da Silva, R.S., Bendhack, L.M. (2011) NO donors-relaxation is impaired in aorta from hypertensive rats due to a reduced involvement of K(+) channels and sarcoplasmic reticulum Ca(2+)-ATPase. *Life Science*, 89 (17-18): 595-602. https://doi.org/10.1016/j.lfs.2011.07.022

Burnstock, G. (2002). Purinergic signaling and vascular cell proliferation and death. *Arteriosclerosis, Thrombosis, and Vascular Biology*, 22(3): 364-73. https://doi.org/10.1161/hq0302.105360

Burnstock, G. (2006a). Pathophysiology and Therapeutic Potential of Purinergic Signaling. *Pharmacological Reviews*, 58(1): 58-86. https://doi.org/10.1124/pr.58.1.5

Burnstock, G. (2006b). Vessel tone and remodeling. *Nat Med*, 12 (1): 16-17. https://doi.org/10.1038/nm0106-16

Burnstock, G. (2017a). Purinergic Signaling in the Cardiovascular System. *Circulation Research, 120*(1), 207-228. https://doi.org/10.1161/CIRCRESAHA.116.309726

Burnstock, G. (2017b). The therapeutic potential of purinergic signalling. *Biochemical Pharmacology*, 150 *in press*. https://doi.org/10.1016/j.bcp.2017.07.016

Burnstock, G., & Kennedy, C. (1986). A dual function for adenosine 5'-triphosphate in the regulation of vascular tone: Excitatory cotransmitter with noradrenaline from perivascular nerves and locally released inhibitory intravascular agent. *Circulation Research*, 58(3): 319-30. https://doi.org/10.1161/01.RES.58.3.319

Cerqueira, N., & Yoshida, W. (2002). Óxido nítrico: revisão. *Acta Cirurgica Brasileira, 17*(6), 417-423. https://doi.org/10.1590/S0102-86502002000600011

Chen, K., Pittman, R. N., POPEL, A.S. (2008). Nitric oxide in the vasculature: where does it come from and where does it go? A quantitative perspective. *Antioxid Redox Signal*. 10(7):1185-98. https://doi.org/10.1089/ars.2007.1959

Cognato, G. deP. & Bonan, C. D. (2010). Ectonucleotidases and Epilepsy. *The open neuroscience journal*, 4: 44-52. https://doi.org/10.2174/1874082001004010044

Conger, J. (1994). Endothelial regulation of vascular tone. Hospital practice, 15; 117-126. https://doi.org/10.1080/21548331.1994.11443095

Da Silva, C., Specht, A., Wegiel, B., Ferran, C., & Kaczmarek, E. (2009). Mechanism of purinergic activation of endothelial nitric oxide synthase in endothelial cells. *Circulation*, 119(6): 871-9. https://doi.org/10.1161/CIRCULATIONAHA.108.764571

Di Virgilio, F., & Solini, A. (2002). P2 receptors: new potential players in atherosclerosis. *British Journal of Pharmacology, 135*, 831-842. https://doi.org/10.1038/sj.bjp.0704524

Drury, A., & Szent-Györgyi, A. (1929). The physiological activity of adenine compounds with especial reference to their action upon the mammalian heart. *The Journal of Physiology*, 68(3): 213-237. https://doi.org/10.1113/jphysiol.1929.sp002608

Erlinge, D. (1998). Extracellular ATP: A Growth Factor for Vascular Smooth Muscle Cells. *General Pharmacology: The Vascular System, 31*(1), 1-8. https://doi.org/10.1016/S0306-3623(97)00420-5

Förstermann, U. & Sessa, W.C. (2012). Nitric oxide synthases: regulation and function. *European heart journal*. 33 (7): 829-837, 837a-837d. https://doi.org/10.1093/eurheartj/ehr304

Fredholm, B.B. (2007). Adenosine, an endogenous distress signal, modulates tissue damage and repair. *Cell death & Differentiation*, 14: 1315-1323. https://doi.org/10.1038/sj.cdd.4402132

Fuentes, E., & Palomo, I. (2015). Extracellular ATP metabolism on vascular endothelial cells: A pathway with pro-thrombotic and anti-thrombotic molecules. *Vascular Pharmacology, 75*, 1-6. https://doi.org/10.1016/j.vph.2015.05.002

Fujii, N., Meade, R.D., Minson, C.T., Brunt, V.E., Boulay, P., Sigal, R.J., Kenny, G.P. (2016). Cutaneous blood flow during intradermal NO administration in young and older adults: roles for calcium-activated potassium channels and cyclooxygenase? *Am J Physiol Regul Integr Comp Physiol*, 310 (11): R1081-7. https://doi.org/10.1152/ajpregu.00041.2016

Fulton, D. J. R. (2016). Transcriptional and Posttranslational Regulation of eNOS in the Endothelium. *Endothelium*, 29–64. https://doi.org/10.1016/bs.apha.2016.04.001

Fürstenau, C., Trentin, D., Gossenheimer, A., Ramos, D., Casali, E., Barreto-Chaves, M., & Sarkis, J. (2008). Ectonucleotidase activities are altered in serum and platelets of l-NAME-treated rats. *Blood Cells, Molecules, and Diseases*, 41(2): 223-9. https://doi.org/10.1016/j.bcmd.2008.04.009

Gachet, C. (2006). Regulation of platelet functions by P2 receptors. *Annual Review of Pharmacology and Toxicology, 46*, 277-300. https://doi.org/10.1146/annurev.pharmtox.46.120604.141207

Garcia-Cardena, G., Fan, R., Shah, V., Sorrentino, R., Cirino, G., Papapetropoulos, A., Sessa, W. C. (1998). Dynamic activation of endothelial nitric oxide synthase by Hsp90. *Nature*, 392(6678), 821–824. https://doi.org/10.1038/33934

Gitterman, D., & Evans, R. (2001). Nerve evoked P2X receptor contractions of rat mesenteric arteries; dependence on vessel size and lack of role of L-type calcium channels and calcium induced calcium release. *British Journal of Pharmacology*, 132(6): 1201-8. https://doi.org/10.1038/sj.bjp.0703925

Guyton & Hall, J. E. (2011). Excitação e Contração do Músculo Liso. Em: *Tratado de Fisiologia Médica* (12 ed). Elsevier, Rio de Janeiro. 95-98.

Hirai, D.M., Copp, S.W., Ferguson, S.K., Holdsworth, C.T., Musch, T.I., Poole, D.C. (2013). The NO donor sodium nitroprusside: evaluation of skeletal muscle vascular and metabolic dysfunction. *Microvascular research*, 85: 104-111. https://doi.org/10.1016/j.mvr.2012.11.006

Kaczmarek, E., Koziak, K., Sé, J., Siegel, J., Anrather, J., Beaudoin, A., Robson, S. (1996). Identification and Characterization of CD39/Vascular ATP Diphosphohydrolase. *Journal of Biological Chemistry*, 271, (51), 33116-33122. https://doi.org/10.1074/jbc.271.51.33116

Kauffenstein, G., Drouin, A., Thorin-Trescases, N., Bachelard, H., Robaye, B., D'Orléans-Juste, P., Sévigny, J. (2010a). NTPDase1 (CD39) controls nucleotide-dependent vasoconstriction in mouse. *Cardiovascular Research*, 85(1): 204-13. https://doi.org/10.1093/cvr/cvp265

Kauffenstein, G., Fürstenau, C., D'Orléans-Juste, P., & Sévigny, J. (2010b). The ecto-nucleotidase NTPDase1 differentially regulates P2Y1 and P2Y2 receptor-dependent vasorelaxation. *British Journal of Pharmacology*, 159(3): 576-85. https://doi.org/10.1111/j.1476-5381.2009.00566.x

King, B., Townsend-Nicholson, A., & Burnstock, G. (1998). Metabotropic receptors for ATP and UTP: exploring the correspondence between native and recombinant nucleotide receptors. *Trends in pharmacological sciences, 19*(12): 506-14. https://doi.org/10.1016/S0165-6147(98)01271-1

Koszalka, P., Özüyaman, B., Huo, Y., Zernecke, A., Flögel, U., Braun, N., Schrader, J. (2004). Targeted disruption of cd73/ecto-5′-nucleotidase alters thromboregulation and augments vascular inflammatory response. *Circulation Research*, 95(8): 814-21. https://doi.org/10.1161/01.RES.0000144796.82787.6f

Li, Y., Sarkar, O., Brochu, M., & Anand-Srivastava, M. (2014). Natriuretic peptide receptor-C attenuates hypertension in spontaneously hypertensive rats: role of nitroxidative stress and Gi proteins. *Hypertension, 63*(4), 846-855. https://doi.org/10.1161/HYPERTENSIONAHA.113.01772

Lorga Filho, A.M., Azmus, A.D., Soeiro, A.M., Quadros, A.S., et al. (2013) Diretrizes Brasileiras de Antiagregantes Plaquetários e Anticoagulantes em Cardiologia. *Arquivos Brasileiros de Cardiologia,* 10 1(3): 56-57. http://dx.doi.org/10.5935/abc.2013S009

Loscalzo, J. (2001). Nitric oxide insufficiency, platelet activation, and arterial thrombosis. *Circulation Research*, 88(8), 756-762. https://doi.org/10.1161/hh0801.089861

Maffrand, J., Bernat, A., Delebassée, D., Defreyn, G., Cazenave, J., & Gordon J.L. (1988). ADP plays a key role in thrombogenesis in rats. *Thrombosis Haemostasis , 59*(2), 225-230. https://doi.org/10.1055/s-0038-1642759

Maneesai, P., Prasarttong, P., Bunbupha, S., Kukongviriyapan, U., Kukongviriyapan, V., Tangsucharit, P., Pakdeechote, P. (2016). Synergistic antihypertensive effect of carthamus tinctorius L. extract and captopril in L-NAME-induced hypertensive rats via restoration of eNOS and AT1R expression. *Nutrients* 8(3): 122. https://doi.org/10.3390/nu8030122

McCabe, T. J., Fulton, D., Roman, L. J., Sessa, W. C. (2000). Enhanced electron flux and reduced calmodulin dissociation may explain "calcium-independent" eNOS activation by phosphorylation. *The Journal of Biological Chemistry*, 275(9), 6123–6128. https://doi.org/10.1074/jbc.275.9.6123

Moncada, S. & Higgs, E.A. (2006). Nitric oxide and the vascular endothelium. *Handb Exp Pharmacol.* 176: 213-254. https://doi.org/10.1007/3-540-32967-6

Murrel, G.A.C.; Dolan, M.M.; Jang, D.; Szabo, C.; Warren, R.F.; Hannafin, J. (1996). Nitric oxide: an important articular free radical. *The Journal of Bone and Joint Surgery. American, 78*(A), 265-74. https://doi.org/10.2106/00004623-199602000-00014

Mutchler, S., & Straub, A. (2015). Compartmentalized nitric oxide signaling in the resistance vasculature. *Nitric Oxide*, 15(49), 8–15. https://doi.org/10.1016/j.niox.2015.05.003

Nakai, S., Furuya, K., Miyata, S, Kiyohara, T. (1999). Intracellular Ca2+ responses to nucleotides, peptides, amines, amino acids and prostaglandins in cultured pituicytes from adult rat neurohypophysis. *Neuroscience Letters*, 266 (3): 185-8. https://doi.org/10.1016/S0304-3940(99)00286-4

Olsson, R., & Pearson, J. (1990). Cardiovascular purinoceptors. *Physiological Reviews , 70*(3), 761-845. https://doi.org/10.1152/physrev.1990.70.3.761

Palmer, T., & Stiles, G. (1995). Adenosine receptors. *Neuropharmacology, 34*(7), 683-694. https://doi.org/10.1016/0028-3908(95)00044-7

Ralevic, V., & Burnstock, G. (1991). EResearch Advances Series Roles of P2-Purinoceptors in the Cardiovascular System. *Circulation*, 84(1): 1-14. https://doi.org/10.1161/01.CIR.84.1.1

Rapoport RM, Draznin MB, Murad F. (1983). Endothelium-dependent relaxation in rat aorta may be mediated through cyclic GMP-dependent protein phosphorylation. *Nature*. 306:174–176. https://doi.org/10.1038/306174a0

Robson, S., Sévigny, J., & Zimmermann, H. (2006). The E-NTPDase family of ectonucleotidases: Structure function relationships and pathophysiological significance. *Purinergic Signalling*, 2(2): 409-430. https://doi.org/10.1007/s11302-006-9003-5

Sévigny, J., Levesque, F., Grondin, G., & Beaudoin, A. (1997). Purification of the blood vessel ATP diphosphohydrolase, identification and localisation by immunological techniques. *Biochimica et Biophysica Acta (BBA) - General Subjects, 1334*(1), 73-88. https://doi.org/10.1016/S0304-4165(96)00079-7

Sévigny, J., Sundberg, C., Braun, N., Guckelberger, O., Csizmadia, E., Qawi, I., Robson, S. (2002). Differential catalytic properties and vascular topography of murine nucleoside triphosphate diphosphohydrolase 1 (NTPDase1) and NTPDase2 have implications for thromboregulation. *Blood*, 99(8): 2801-9. https://doi.org/10.1182/blood.V99.8.2801

Shesely EG, Maeda N, Kim HS, Desai KM, Krege JH, Laubach VE, Sherman PA, Sessa WC, Smithies O. (1996). Elevated blood pressures in mice lacking endothelial nitric oxide synthase. *Proc Natl Acad Sci USA*. 93:13176–13181. https://doi.org/10.1073/pnas.93.23.13176

Shryock, J.C., Belardinelli, L. (1997) Adenosine and adenosine receptors in the cardiovascular system: biochemistry, physiology, and pharmacology. *Am J Cardiol*, 79: 2–10. https://doi.org/10.1016/S0002-9149(97)00256-7

Snyder, S.H. & Bredt, D.S. (1992). Biological role of nitric oxide. *Scientific American*, 266: 68-77. https://doi.org/10.1038/scientificamerican0592-68

Su, Y. (2014). Regulation of Endothelial Nitric Oxide Synthase Activity by Protein-Protein Interaction. *Current Pharmaceutical Design, 20*(22), 3514-3520. https://doi.org/10.2174/13816128113196660752

Tortora, Gerard J. (2013). Princípios de anatomia humana. Tradução de: Principles of human anatomy, Rio de Janeiro: *Guanabara Koogan*. 12, 523.

Ulker, P., Özen, N., Abdullayeva, G., Köksoy, S., Yaraş, N., & Basrali, F. (2018). Extracellular ATP activates eNOS and increases intracellular NO generation in Red Blood Cells. *Clinical Hemorheology and Microcirculation, 68*(1), 89-101. https://doi.org/10.3233/CH-170326

Webb, R. C. (2003). Smooth muscle contraction and relaxation. *Advances in physiology education*, 27(4), 201–206. https://doi.org/10.1152/advan.00025.2003

Wihlborg, A., Wang, L., Braun, O., Eyjolfsson, A., Gustafsson, R., Gudbjartsson, T., & Erlinge, D. (2004). ADP receptor P2Y12 is expressed in vascular smooth muscle cells and stimulates contraction in human blood vessels. *Arteriosclerosis, Thrombosis, and Vascular Biology* 24, 1810-1815. https://doi.org/10.1161/01.ATV.0000142376.30582.ed

Yang, Y., Yu, T., Yi, Lian, Ma, R., Yang, S., Cho, J,Y. (2015) Nitric oxide synthase inhibitors: a review of patents from 2011 to the present. *Expert Opinion on Therapeutic Patents*, 25(1): 49-68. https://doi.org/10.1517/13543776.2014.979154

Zimmermann, H. (1996). Extracellular purine metabolism. *Drug Development Research, 39*, 337-352. https://doi.org/10.1002/(SICI)1098-2299(199611/12)39:3/4<337::AID-DDR15>3.0.CO;2-Z

Zimmermann, H. (2000). Extracellular metabolism of ATP and other nucleotides. *Naunyn Schmiedebergs Arch Pharmacol, 362*(4-5), 299-309. https://doi.org/10.1007/s002100000309

Zukowska, P., Kutryb-Zajac, B., Toczek, M., Smolenski, R.T., Slominska, E.M. (2015). The role of ecto-5'-nucleotidase in endothelial dysfunction and vascular pathologies. *Pharmacological reports, 67 (4), 675-681.* https://doi.org/10.1016/j.pharep.2015.05.002

Capítulo 2

Does nitric oxide modulate ectonucleotidases in A7r5 vascular smooth muscle cells?

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Does nitric oxide modulate ectonucleotidases in A7r5 vascular smooth muscle cells?

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Abstract

ATP, ADP and adenosine modulate vasoconstriction and vasorelaxation. They activate nitric oxide synthase (NOS) in the vasculature, culminating in the production of nitric oxide (NO) and vasodilation. However, little is known about the modulation of NO on ectonucleotidases, enzymes that metabolize extracellular nucleotides. In the vasculature, NTPDases1 and 2, hydrolyze ATP and ADP; and ecto-5'-nucleotidase metabolizes AMP to adenosine, a well-recognized vasodilator. To determine the effect of NO on vascular ectonucleotidases, A7r5 cells were challenged with NO donor (sodium nitroprusside, SNP), NO blocker (L-NAME) and NO mobilizing agents (ATP, ADP, bradykinin (BK) and 2-Cl-adenosine). After 24 hours of treatment, MTT assay detected that 2-Cl-adenosine diminished A7r5 viability by 35%. Nitrite levels determined by Griess reaction were increased in response to SNP, indirectly indicating increased levels of NO in this group. Enzymatic activities were detected colorimetrically by the inorganic phosphate released. ATP and ADP hydrolysis were not altered while hydrolysis of AMP was significantly increased by ATP and 2-Cl-adenosine. No changes were observed at the protein level. However, regulation at the post-translational level by phosphorylation is a possibility to explain the increase in the activity of the enzyme, since it has distinct highly phosphorylable sites in its structure, especially in serine residues. Ecto-5'-nucleotidase is crucial for the generation of adenosine and the understanding of molecular mechanisms by which NO and other molecules interfere in the activity and expression of this enzyme is fundamental for the modulation of vascular tone and the regulation of blood pressure, enabling new therapeutic approaches in cardiovascular complications.

Keywords: Purinergic signaling; Ecto-5'-nucleotidase; CD73; Nitric Oxide; Adenosine; Vascular smooth muscle cells.

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1. Introduction

The actions of adenosine triphosphate (ATP) and adenosine on the heart and blood vessels as bradycardic and blood pressure reducing agents were first described in 1929 [1]. Currently, purinergic signaling is widely accepted as a primitive and highly conserved system among animals, with important roles attributed to nucleotides and nucleosides as extracellular communicators in neural and non-neural tissues [2]. In this sense, purinergic signaling has been identified as an important regulator of cardiovascular physiology, since, in the vasculature, ATP, adenosine diphosphate (ADP) and adenosine may influence vasomotor responses, platelet activation, cardiac function, and other aspects [3].

Ectonucleotidases are enzymes capable of hydrolyzing extracellular nucleotides, inactivating their signaling effects. Therefore, members of the ectonucleotidases family, commonly expressed in vasculature, NTPDase1 and 2, capable of hydrolyzing ATP and ADP; and ecto-5'-nucleotidase, which metabolizes AMP to adenosine, regulate the availability of nucleoside and nucleotides in the circulation [4,5].

Nucleotides exert their effects through two main families of P2 receptors, which comprise both the ion channels activated by ATP (P2X1-7) and metabotropic receptors coupled to G protein (P2Y1,2,4,6,11,12,13,14) [2,6]. Activation of endothelial P2 receptors induces local vasodilation through the production of nitric oxide (NO), prostacyclin (PGI2) and endothelium-derived hyperpolarizing factor (EDHF) [7]. P2Y1 e P2Y2 are responsible for relaxation induced by ATP and UTP, respectively [8]. In contrast, the activation of P2 receptors in vascular smooth muscle cells (VSMC) promotes vasoconstriction through P2Y12 receptors or P2X receptors sensitive to pyrimidines [9,10].

Particularly, ecto-5'-nucleotidase is predominantly associated with the endothelium [11], anchored in a glycosylphosphatidylinositol (GPI) residue. The main function of ecto-5'-nucleotidase is to catalyze the conversion of monophosphonucleotides to their respective nucleosides (AMP → adenosine, for example) [4]. Adenosine acts through a family of receptors also coupled to G protein (A1, A2A, A2B e A3) on the cell surface, being of fundamental importance for several physiological events of regulation of vascular tonus, cardioprotection, vasodilatation, inflammatory regulation and inhibition of platelet aggregation [12], besides being strongly involved in the migration and proliferation of VSMC [13].

The mechanism by which adenosine exerts the vasodilator effect on VSMC, is still widely discussed. Uichi Ikeda [14], relates this effect to the stimulation of NO synthesis. NO consists of a gaseous mediator that also participates in a number of physiological processes in the body. In the vasculature, it is characterized by being one of the most important signaling molecules, related to the regulation of vascular tone and vasodilation; regulation of gene transcription; translation of mRNA and neurotransmission [15]. The mechanism of NO cell relaxation occurs when the gas crosses the endothelium and reaches vascular smooth muscle, stimulating the soluble guanylate cyclase enzyme to generate cyclic guanosine monophosphate (cGMP) intracellular [16].

It is known that extracellular nucleotides are involved in the activation of the enzyme endothelial nitric oxide synthase (eNOS), leading to the production of NO and vasodilation [17]. It has recently been found that extracellular ATP activates eNOS leading to NO generation also in red blood cells [18]. Besides, in a previous work, we demonstrated that the activities of ectonucleotidases were decreased in serum and platelets of animals submitted to the hypertensive condition by prolonged administration of L-NAME, a blocker of NO synthesis. We also showed that the levels of ADP, AMP and other components of the purinergic signaling pathway were paradoxically decreased in the circulation of these animals [19]. The above-mentioned results were obtained in soluble fractions of the circulation. We thus hypothesize that the reduction of circulating levels of purines observed in hypertensive animals depends on a modulation of ectonucleotidase activities present on the vessel surface.

Taken together, these observations denote the existence of an interlocution between the purinergic and nitrergic systems, in which purines significantly modulate the production of NO in the blood vessels. It remains to be investigated whether the opposite pathway also occurs, that is, if NO is also capable of influencing the expression and activity of ectonucleotidases present in the vasculature, which can significantly impact the control of the purinergic system on vascular tone and blood pressure. Therefore, in the present study we aimed to determine the effect of NO on the activity and expression of ectonucleotidases, with emphasis on ecto-5'-nucleotidase in A7r5 vascular smooth muscle cells.

2. Material and methods

2.1 Chemicals

A7r5 (ATCC® CRL1444™) vascular smooth muscle cells were obtained from Americam Type Culture Collection (ATCC) and kindly donated by Dr. Jamil Assreuy from Universidade Federal de Santa Catarina, Brazil. Dulbecco's modified Eagle medium (DMEM) was obtained from LGC Biotecnologia (São Paulo, Brazil). Fetal bovine serum (FBS) was from Cripion (São Paulo, Brazil) while penicillin-streptomycin was from GIBCO (Grand Island, New York, USA). Sodium nitroprusside (SNP), *Nω*-Nitro-L-arginine methyl ester hydrocloride (L-NAME), Adenosine 5′-triphosphate sodium salt (ATP), Adenosine 5′-diphosphate sodium salt (ADP), Adenosine 5′-monophosphate sodium salt (AMP), 2-Chloroadenosine, Bradykinin acetate salt (BK) and 3-(4,5-dimethyltiazol-2-yl)2,5-diphenyltetrazolium bromide) (MTT) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Griess reagent kit was obtained from Invitrogen™ (Carlsbad, CA, USA). Protease Inhibitor Cocktail was purchased from Cell Signaling Technology, Inc. (Massachusetts, US). Nitrocellulose membranes (Amersham Protran Premium) were purchased from GE Healthcare, Life Science, USA. ECL detection system (WESTAR SUN) was obtained from Cyanagen (Bologna, Italy). Primary antibodies against proteins of interest CD39 and CD73 were obtain from Santa Cruz Biotechnology Inc. (Heidelberg, Germany) and primary antibody against the control gene actin was obtain from Millipore Merck (Darmstadt, Germany). Peroxidase-conjugated IgG secondary goat anti-Rabbit was purchased from KPL (Gaithersburg, USA) and goat anti-Mouse was purchased from Thermo Scientific (Rockford, USA).

2.2 Cell culture and treatments

A7r5 cells were seeded in 75 cm^2 culture flasks and cultured in Dulbecco's modified Eagle medium (DMEM), supplemented with 10% fetal bovine serum and 100 U/ mL penicillin-streptomycin. Cells were kept in a humidified incubator (95% O_2, 5% CO_2) at 37 °C until reaching 80 to 90% confluence. Thus, approximately 1 x 10^5 cells/ mL were plated into 6, 12, 24 or 96-well plates, according to the protocol performed. Cells (used from 4^{th} to 7^{th} passage) were allowed to adhere to culture plates for 24 hours. A7r5 cells were then deprived of serum using DMEM 0,5% FBS for 3 hours and thereafter subjected to the following independent treatments: SNP 10^{-5} M (NO donor agent), L-NAME 10^{-4} M (NO synthesis inhibitor), ATP, ADP, BK 10^{-5} M (mobilizing NO synthesis agents) and 2-Cl-

adenosine 10^{-4} M (a stable analogue of adenosine, a recognized vasodilator molecule), for 24 hours. For MTT assays, treatments were also performed at 48 and 72 hours. Two to four wells were used as replicates for each treatment.

2.3 3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay

Cell viability was evaluated by MTT assay, according to Mosmann [20]. Briefly, after receiving the appropriate treatments, including positive control (untreated cells) and negative control (wells with medium only) for 24, 48 or 72 hours, the culture medium was discarded and replaced by a solution of 5% MTT in DMEM 10% FBS for 4 hours. After this time, supernatant containing excess of MTT was aspirated and 200 µL dimethylsulfoxide (DMSO) was added to each well to dissolve the formazan crystals. The optical reading was performed on an ELISA plate reader (Robonik, Readwe ll PLATE) at 590 nm [20]. Mean optical density (OD) of test wells was compared to the mean OD of control wells.

2.4 Nitrite quantification

Nitrite quantification was conducted as an indirect measurement of nitric oxide (NO) levels in A7r5 cells. The treatments were performed as before, however using DMEM without phenol red (GIBCO, Grand Island, New York, USA) to not influence the readings. Nitrite content was determined using a Griess reagent kit, according to the manufacturer's instructions. Thus, the culture medium of each well was collected, centrifuged at 16.000 rpm, 4 ºC (Hermle Labor Technik, Z 36 HK), for 10 minutes and supernatant was kept for subsequent analysis. In a 96-well microplate, 20 µL of Griess's reagent, 150 µL of the nitrite-containing sample and 130 µL of deionized water were mixed. After 30 minutes incubation in the dark at room temperature, the plate was read on an ELISA plate reader (Robonik, Readwell PLATE) at 560 nm. Nitrite concentration in the samples were calculated on basis of a standard curve of different concentrations (1, 5, 10, 30, 50 and 100 µM) of sodium nitrite.

2.5 Enzyme assays

After 24 hours of treatment with NO donor, NO inhibitor or NO mobilizing agents, A7r5 cells adhered to 24-well plates were three times washed with a reaction mix containing [final

concentration, in mM]: $CaCl_2$ 2.0, NaCl 120.0, KCl 5.0, Glucose 10.0 and Hepes 20.0, pH 7.4, at 37° C. Enzyme reaction was started with the addition of 2.0 mM substrates (ATP or ADP for NTPDase1; or AMP for ecto-5'-nucleotidase) in the reaction mix. For the hydrolysis of AMP, 2.0 mM $CaCl_2$ was replaced by 2.0 mM $MgCl_2$ [21]. After 20 minutes, the reaction was stopped by transferring an aliquot of 0.2 mL of the reaction mixture into a tube containing 0.2 mL of 10% trichloroacetic acid (TCA) previously held on ice. Then, 1.0 mL of the malachite green reagent was added to each tube. The inorganic phosphate (Pi) released was measured at a spectrophotometer (Gehaka, UV-340 G) at 630 nm [22]. Enzymatic activity was expressed in nmol of released Pi/ min/ milligram protein.

2.5.1 Protein determination

At the end of the enzyme activity assay, the reaction medium was removed and 100 µL 1.0 M NaOH was added to each well. The culture plate was then kept at -18 °C for 24 hours. Subsequently, cells were solubilized with the aid of a cell scrapper and an aliquot of 50 µL of protein suspension was collected to determine total protein content in each well. Total protein was colorimetrically determined at 595 nm by the method of Bradford [23], using bovine serum albumin as standard.

2.6 Protein expression analysis

After treatment of A7r5 cells as described above, total proteins from cells were obtained using an extraction buffer (1.0% NP-40, 135 mM NaCl, 20 mM Tris, pH 8.0 and 10% glycerol) containing protease inhibitors cocktail (1x) composed of a mix of AEBSF (Aprotinin, Bestatin, E64, Leupeptin, and Pepstatin A) to promote broad spectrum protection against endogenous proteases. The crude lysate was centrifuged (14.000 x g, 10 minutes, 4 °C) (Hermle Labor Technik, Z 36 HK). The supernatant was collected and 10 µg of total protein of each group of treatment were resolved by electrophoresis on 5% stacking gels and 10% polyacrylamide-SDS gels. After separation, proteins were transferred to nitrocellulose membranes. The membranes were blocked with 3-5% milk in TBST buffer (20 mM Tris-HCl, 120 mM NaCl, and 0.1% (v/v) Tween 20) for 1 hour at room temperature and the primary antibodies to the proteins of interest NTDPDase1 (CD39) and ecto-5'-nucleotidase (CD73) were incubated overnight at 4 °C at a dilution of 1:500 in TBST. The internal control actin was used at a dilution of 1:3000 in TBST. Blots were washed in TBST and incubated

with appropriate peroxidase-conjugated IgG secondary antibodies 1:10000 for 1 hour at room temperature. Bands were detected by chemiluminescence on X-ray film (T-MAT G/ RA Film, KODAK, São José dos Campos, São Paulo, Brazil) using an ECL detection system. Quantification of the intensity of specific bands was performed using ImageJ software.

2.7 Search for potential sites of phosphorylation

Phosphorylation is an important post-translational event of enzyme regulation. Using FASTA sequence of ecto-5'-nucleotidase from *Rattus norvegicus* (GenBank: AAH81806.2), the computational tool NetPhos 3.1, available online by the Technical University of Denmark (http://www.cbs.dtu.dk/) was used to predict potential phosphorylation sites of the enzyme ecto-5'-nucleotidase.

2.8 Data analysis

GraphPad Prism 5.03 for Windows (GraphPad Software, San Diego, CA) was used to analyze data. Results are presented as mean ± standard deviation of each of the measures performed in each of the groups studied. Sample number (n) represents the number of experiments performed with different A7r5 cultures. ANOVA was used to compare differences in mean values of all groups, followed by Dunnet's *post hoc* test and differences were considered significant at $P \leq 0,05$.

3. Results

3.1 Cell viability

MTT was used to investigate A7r5 cells viability in response to different treatments. After 24 hours, A7r5 cells remained viable in response to the treatment with different molecules when compared to non-treated cells (control), except for the treatment with 2-Cl-adenosine, which decreased cell viability by 35% (Fig1a). After 48 hours of treatment, 2-Cl-adenosine decreased cell viability by 32% while BK increased cell proliferation by 20% when compared to non-treated cells (Fig1b). In cells submitted to 72 hours of treatment, 2-

Cl-adenosine hugely decreased cell viability by 62% while BK induced A7r5 proliferation similarly to 48 hours treatment (Fig1c)

Taking together all results from MTT assay, 24 hours was chosen as the best time for cells treatment since the viability of A7r5 cells remained around 100% with most of the molecules used. Subsequent analyses were then performed with cells treated for 24 hours.

3.2 Nitrite quantification

As mentioned before, Griess reagent kit was used to indirectly determine NO levels in the medium of A7r5 cells submitted to distinct treatments. Treatment with NO donor (SNP) significantly increased nitrite levels in A7r5 cells, indirectly indicating increased levels of NO and corroborating with the expected biological activity for this molecule (Fig2). Other treatments did not change significantly NO levels. However, as stated, Griess reaction only indirectly infers NO levels by measuring nitrite content.

3.3 NTPDase1 and ecto-5′-nucleotidase activities

NTPDase1 activity in A7r5 cells was not altered by any of treatments performed, since the hydrolysis of ATP (Fig3a) and ADP (Fig3b) were not modified in comparison to control group. On the other hand, ecto-5'-nucleotidase activity was modified by NO mobilizing molecules on the surface of the blood vessels (Fig3c). ATP and 2-Cl-adenosine (ADO) increased ecto-5’-nucleotidase activity, leading to a potential increase of circulating adenosine, a vasodilator molecule.

3.4 Levels of protein expression

To test if vascular ectonucleotidases could be modified at the translational level, we investigated protein expression in A7r5 cells treated with different molecules by Western blotting. No significant modulation of both enzymes was observed by the molecules indicated at the translational level (Fig4 and Fig5).

3.5 Potential phosphorylation sites

According to Fig6, it is observed that ecto-5'-nucleotidase has many phosphorylation sites above the threshold (pink line), being 22 at serine (red peaks), 15 at threonine (green peaks) and 1 at tyrosine (blue peak) residues, which may lead to the modulation of its conformation and thus the increase in its enzymatic activity.

4. Discussion

It is known that ATP is capable of inducing the synthesis of nitric oxide (NO) synthesis in various organs and tissues, including the cardiovascular system [24]. However, the inverse pathway of this modulation, investigating the effect of NO on ectonucleotidase activities and on the availability of the purinergic agonists has not yet been elucidated. Thus, in this study, we investigated the modulation of vascular ectonucleotidases by NO donor, inhibitor and mobilizing molecules.

NO is a potent vasodilator and its mechanism of cellular relaxation of vascular smooth muscle is mediated by the action of the soluble guanylate cyclase signal transduction enzyme, generating intracellular cyclic guanosine monophosphate (cGMP) [16]. Like NO, adenosine is a well-recognized vasodilator molecule and increases in its circulating concentration may therefore be beneficial for patients with hypertension, heart failure or stroke, for example [25].

A7r5 vascular smooth muscle cells were treated with the NO donor SNP, the nitric oxide synthase (NOS) inhibitor L-NAME and with NO mobilizing agents ATP, ADP, BK and 2-Cl-adenosine. From MTT assay, we observed that A7r5 cells remained viable after 24 hours treatment with all molecules, except for 2-Cl-adenosine (Fig1a). 2-Chloro-adenosine is a stable analogue of adenosine known to inhibit the growth of smooth muscle cells via A2B receptors and may prevent vascular remodeling associated with hypertension, atherosclerosis, and restenosis following balloon angioplasty [26].

As mentioned before, to study NO effect on ectonucleotidase activities, A7r5 were challenged with distinct molecules expect to direct (SNP) or indirectly (ATP, ADP, BK and 2-Cl-adenosine) increase NO availability, as well as to diminish it (L-NAME). From Griess reaction, it was possible to observe that only SNP was able to significantly increase nitrite levels, an indirect indicative of increased levels of NO (Fig2). This activity is expected for

SNP since it is a vasodilator that decomposes into NO [27]. It would also be expected that L-NAME would decrease NO levels, since it is an inhibitor of endothelial NOS. One could conclude that L-NAME did not decrease nitrite levels because the cell in question is of the smooth muscle type and not endothelial. However, different studies have shown that vascular smooth muscle cells have the necessary machinery for NO production, which would be important for local control of vascular function [28,29]. As with L-NAME, no difference in nitrite levels was observed for the NO mobilizing molecules in relation to the control group. Again, it is necessary to consider that nitrite levels may not reflect the exact levels of NO.

Regarding the measurements of ectonucleotidase activities in response to different treatments, no significant changes were found on ATP and ADP hydrolyses compared to untreated A7r5 cells (Fig3a and Fig3b). AMP hydrolysis, however, was significantly increased in response to ATP and 2-Cl-adenosine (ADO) treatments in A7r5 cells (Fig 3c) compared to the control group. A similar behavior was observed with respect to endothelial cells (tEnd.1), where the hydrolysis of ATP and ADP remained unchanged against the different treatments (Online resource, Fig7a and Fig7b), while hydrolysis of AMP was significantly increased by treatments with ATP and ADP (Online resource, Fig 7c).

By analyzing the hydrolysis results together, it can be seen that ecto-5'-nucleotidase, which is responsible for the production of adenosine, appears to be more responsive than the NTPDases in the vasculature under the conditions tested. Tamajusuku and colleagues [21] showed that AMP hydrolysis was increased in vascular smooth muscle cells in response to treatment with thyroid hormone (T3), which was accompanied by an increase in ecto-5'-nucleotidase mRNA levels, while ATP and ADP hydrolyses were not modified. They concluded that overexpression of ecto-5’-nucleotidase could result in higher levels of adenosine, an important vasodilator.

In our study, we demonstrated that, despite considerably decreasing cell growth, 2-Cl-adenosine (ADO) significantly increased the AMP hydrolysis in A7r5 cells (Fig3c). Increases in ecto-5'-nucleotidase activity leads to a potential increase and availability of adenosine, since it does the hydrolysis of AMP [30]. Stefanovic and colleagues [31] reported that adenosine and its analogues, including 2-Cl-adenosine (ADO), stimulate 5’-nucleotidase activity in rat mesangial cells via A2 receptors which implies a positive feedback loop. They suggested that this event could be an adaptation of the enzyme to the amount of extracellular adenine nucleotides available. Thus, the activation of A2 receptors

in A7r5 cells by 2-Cl-adenosine could either explain the decrease observed in cell growth by MTT assay and the increase in ecto-5'-nucleotidase activity.

ATP treatment also enhanced the hydrolysis of AMP by the ecto-5'-nucleotidase in A7r5 cells (Fig3c). Substrates of NTPDases, ATP and ADP, are classically considered to be natural inhibitors of ecto-5'-nucleotidase activity [32]. However, as previously reported by Stefanovic [31] and discussed before, stimulation of ecto-5'-nucleotidase by extracellular ATP could function as a positive feedback loop to produce more adenosine. It is also worth highlighting the effects of ATP on the vasculature. It is well established that this molecule acts directly on the P2X receptors of vascular smooth muscle cells, resulting in vasoconstriction [33]. Then, stimulation of ecto-5'-nucleotidase induced by ATP in A7r5 may result in potential increased levels of circulating adenosine, as a compensatory mechanism to favor vasodilation. Adenosine is of great importance to ensure the regulation of homeostasis and maintenance of vascular tonus [34]. Higher concentrations of adenosine may also be considered as a stressful condition and the signal causes adenosine phosphorylation to AMP by the low K_M of adenosine kinase, consequently increasing the activity of ecto-5 '-nucleotidase [35], reinforcing the feedback regulation.

After observing the modulation of ecto-5'-nucleotidase in A7r5 cells submitted to the 2-Cl-adenosine and ATP treatments, we sought to investigate the molecular mechanisms involved in such activation. Protein expression analyzes by Western blotting showed that neither the NTPDase (Fig4) nor the ecto-5'-nucleotidase (Fig5) were modified at the translational level by the treatments performed.

Another possibility for regulation of ecto-5'-nucleotidase occurs at the post-translational level by covalent modification by phosphorylation. The use of the NetPhos 3.1 tool evidenced the presence of distinct sites with a strong possibility of phosphorylation in the structure of the enzyme, especially in serine residues (Fig6). Such modification, which could be induced by ATP and 2-Cl-adenosine, may alter the conformation of the enzyme, making it more active and virtually leading to the production of more adenosine. In a previous work, we have shown an up-regulation of p-PKC in kidney membranes of hypertensive rats in comparison to normotensive animals [36], indicating that such enzyme was active and could phosphorylate ecto-5'-nucleotidase increasing its activity. It may also be considered that ATP and ADP, used in the treatments of the cells in this study, are substrates potentially used by kinases responsible for the phosphorylation of cellular molecules.

It is known that extracellular nucleotides have been implicated in the activation of the enzyme endothelial nitric oxide synthase (eNOS), leading to the production of nitric oxide (NO) and vasodilation [17]. Recently, eNOS has been shown to be activated by extracellular ATP and leads to the generation of NO also in red blood cells [18]. In a previous work, we demonstrated that the activities of ectonucleotidases were decreased in serum and platelets of animals submitted to the hypertensive condition by prolonged administration of L-NAME, a blocker of NO synthesis [19]. However, the impact of NO on vasculature ectonucleotidases remains to be investigated.

Some studies have shown that L-NAME, by decreasing intracellular NO levels, has consequently increased levels of Giα proteins and blood pressure in rats [37]. In contrast, when intracellular NO levels were increased by inducers (SNPs, SNAPs) in VSMC of spontaneously hypertensive rats, there was attenuation of Giα protein overexpression and increased cell proliferation by the inhibition of signaling pathways mediated by reactive oxygen species (ROS) [38, 39]. However, our results showed that only the NO mobilizing agents modified the AMP hydrolysis by the enzyme ecto-5'-nucleotidase. The NO donor agent, SNP, and the inhibitor molecule, L-NAME, had no effect on the enzymatic activities evaluated after 24 hours treatment. Therefore, because NO is rapidly diffused and converted to nitrites, we also investigate whether the molecules inducing and inhibiting its synthesis would affect ecto-5'-nucleotidase activity in short times, 5, 30 and 60 minutes, of treatment (Online resource, Table 1). No significant changes were observed in ecto-5'-nucleotidase activity.

Finally, considering the results obtained in the present study, the question remains: does nitric oxide modulate the ectonucleotidases of the vasculature? Indirect measurement of NO did not indicate increased levels of such molecule in response to 2-Cl-adenosine and ATP. Furthermore, the molecules that act directly on the synthesis and inhibition of NO, SNP and L-NAME, had no effect on any of the parameters tested. Taken together, the data lead us to believe that ecto-5'-nucleotidase activity is modulated by 2-Cl-adenosine and ATP independently of nitric oxide under the conditions tested. However, further experiments are required to confirm NO levels in the culture medium of the A7r5 cells treated with the distinct molecules, as well as confirmation of increased levels of adenosine in response to 2-Cl-adenosine and ATP. The ecto-5'-nucleotidase is of fundamental importance for the generation of the vasodilator adenosine. In this sense, the understanding of molecular mechanisms by which nitric oxide and other molecules interfere in the activity and

expression of this enzyme in the vasculature is extremely relevant for the modulation of vascular tone and the regulation of blood pressure levels, enabling new therapeutic approaches in cardiovascular complications.

References

[1] Drury, A., & Szent-Györgyi, A. (1929). The physiological activity of adenine compounds with especial reference to their action upon the mammalian heart. *The Journal of Physiology*, 68(3): 213-237. https://doi.org/10.1113/jphysiol.1929.sp002608

[2] Burnstock, G. (2017b). The therapeutic potential of purinergic signalling. *Biochemical Pharmacology*, 150 *in press*. https://doi.org/10.1016/j.bcp.2017.07.016

[3] Burnstock, G. (2017a). Purinergic Signaling in the Cardiovascular System. *Circulation Research, 120*(1), 207-228. https://doi.org/10.1161/CIRCRESAHA.116.309726

[4] Zimmermann, H. (1996). Extracellular purine metabolism. *Drug Development Research, 39*, 337-352. https://doi.org/10.1002/(SICI)1098-2299(199611/12)39:3/4<337::AID-DDR15>3.0.CO;2-Z

[5] Fuentes, E., & Palomo, I. (2015). Extracellular ATP metabolism on vascular endothelial cells: A pathway with pro-thrombotic and anti-thrombotic molecules. *Vascular Pharmacology, 75*, 1-6. https://doi.org/10.1016/j.vph.2015.05.002

[6] King, B., Townsend-Nicholson, A., & Burnstock, G. (1998). Metabotropic receptors for ATP and UTP: exploring the correspondence between native and recombinant nucleotide receptors. *Trends in pharmacological sciences, 19*(12): 506-14. https://doi.org/10.1016/S0165-6147(98)01271-1

[7] Malmsjö, M., Erlinge, D., Högestätt, E.D., Zygmunt, P.M. (1999). Endothelial P2Y receptors induce hyperpolarisation of vascular smooth muscle by release of endothelium-derived hyperpolarising factor. *European journal of pharmacology,* 364 (2-3): 169-173. https://doi.org/10.1016/S0014-2999(98)00848-6

[8] Burnstock, G. (2006). Pathophysiology and Therapeutic Potential of Purinergic Signaling. *Pharmacological Reviews*, 58(1): 58-86. https://doi.org/10.1124/pr.58.1.5

[9] Gitterman, D., & Evans, R. (2001). Nerve evoked P2X receptor contractions of rat mesenteric arteries; dependence on vessel size and lack of role of L-type calcium channels and calcium induced calcium release. *British Journal of Pharmacology*, 132(6): 1201-8. https://doi.org/10.1038/sj.bjp.0703925

[10] Wihlborg, A., Wang, L., Braun, O., Eyjolfsson, A., Gustafsson, R., Gudbjartsson, T., & Erlinge, D. (2004). ADP receptor P2Y12 is expressed in vascular smooth muscle cells and stimulates contraction in human blood vessels. *Arteriosclerosis, Thrombosis, and Vascular Biology* 24, 1810-1815. https://doi.org/10.1161/01.ATV.0000142376.30582.ed

[11] Koszalka, P., Özüyaman, B., Huo, Y., Zernecke, A., Flögel, U., Braun, N., Schrader, J. (2004). Targeted disruption of cd73/ecto-5′-nucleotidase alters thromboregulation and augments vascular inflammatory response. *Circulation Research*, 95(8): 814-21. https://doi.org/10.1161/01.RES.0000144796.82787.6f

[12] Fredholm, B.B. (2007). Adenosine, an endogenous distress signal, modulates tissue damage and repair. *Cell death & Differentiation*, 14: 1315-1323. https://doi.org/10.1038/sj.cdd.4402132

[13] Yang, J., Jian, R., Yu, J., Zhi, X., Liao, X., Yu,J., Zhou, P. (2015) CD73 regulates vascular smooth muscle cell functions and facilitates atherosclerotic plaque formation. *IUBMB Life*, 67 (11): 853-860. https://doi.org/10.1002/iub.1448

[14] Uichi Ikeda, Kenji Kurosaki, Ken-ichi Ohya, Kazuyuki Shimada; (1997). Adenosine stimulates nitric oxide synthesis in vascular smooth muscle cells. *Cardiovascular Research*, 35 (1): 168–174. https://doi.org/10.1016/S0008-6363(97)00068-0

[15] Mutchler, S., & Straub, A. (2015). Compartmentalized nitric oxide signaling in the resistance vasculature. *Nitric Oxide*, 15(49), 8–15. https://doi.org/10.1016/j.niox.2015.05.003

[16] Conger, J. (1994). Endothelial regulation of vascular tone. *Hospital practice*, 15; 117-126. https://doi.org/10.1080/21548331.1994.11443095

[17] Da Silva, C., Specht, A., Wegiel, B., Ferran, C., & Kaczmarek, E. (2009). Mechanism of purinergic activation of endothelial nitric oxide synthase in endothelial cells. *Circulation*, 119(6): 871-9. https://doi.org/10.1161/CIRCULATIONAHA.108.764571

[18] Ulker, P., Özen, N., Abdullayeva, G., Köksoy, S., Yaraş, N., & Basrali, F. (2018). Extracellular ATP activates eNOS and increases intracellular NO generation in Red Blood Cells. *Clinical Hemorheology and Microcirculation, 68*(1), 89-101. https://doi.org/10.3233/CH-170326

[19] Fürstenau, C., Trentin, D., Gossenheimer, A., Ramos, D., Casali, E., Barreto-Chaves, M., & Sarkis, J. (2008). Ectonucleotidase activities are altered in serum and platelets of l-NAME-treated rats. *Blood Cells, Molecules, and Diseases*, 41(2): 223-9. https://doi.org/10.1016/j.bcmd.2008.04.009

[20] Mosmann, T. (1983). Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. *Journal of Immunological Methods*, 65 (1-2): 55-63. https://doi.org/10.1016/0022-1759(83)90303-4

[21] Tamajusuku, A., Carrillo-Sepúlveda, M., Braganhol, E., Wink, M., Sarkis, J., Barreto-Chaves, M., & Battastini, A. (2006). Activity and expression of ecto-5′-nucleotidase/CD73 are increased by thyroid hormones in vascular smooth muscle cells. *Molecular and Cellular Biochemistry*, 289 (1-2): 65-72. https://doi.org/10.1007/s11010-006-9148-0

[22] Chan, K.-M., Delfert, D., & Junger, K. (1986). A direct colorimetric assay for Ca2+-stimulated ATPase activity. *Analytical Biochemistry, 157*(2), 375-380. https://doi.org/10.1016/0003-2697(86)90640-8

[23] Bradford, M. (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Analytical biochemistry,* 72, 248-254. https://doi.org/10.1016/0003-2697(76)90527-3

[24] Zimmermann, H. (2016). Extracellular ATP and other nucleotides—ubiquitous triggers of intercellular messenger release. *Purinergic Signalling* 12, 25-57. https://doi.org/10.1007/s11302-015-9483-2

[25] Sousa, J.B & Diniz, C., (2017) The adenosinergic system as a therapeutic target in the vasculature: New ligands and challenges. *Molecules*, 6; 22(5). https://doi.org/10.3390/molecules22050752

[26] Dubey, R.K., Gillespie, D.G., Shue, H., Jackson, E.K. (2000). A(2B) receptors mediate antimitogenesis in vascular smooth muscle cells. *Hypertension,* 35 (1 Pt 2):267-72. https://doi.org/10.1161/01.HYP.35.1.267

[27] Blatter, L.A., Wier, W.G. (1994). Nitric oxide decreases [Ca2+]i in vascular smooth muscle by inhibition of the calcium current. *Cell calcium,* 15(2):122-3. https://doi.org/10.1016/0143-4160(94)90051-5

[28] Lee, J.H., Xia, S., Ragolia, L. (2008). Upregulation of AT2 receptor and iNOS impairs angiotensin II-induced contraction without endothelium influence in young normotensive diabetic rats. *Am J Physiol*, 295:R144–R154. https://doi.org/10.1152/ajpregu.00191.2008

[29] Carrillo-Sepúlveda, M.A., Ceravolo, G.S., Fortes, Z.B., Carvalho, M.H., Tostes, R.C., Laurindo, F.R., Webb, R.C., Barreto-Chaves, M.L. (2010). Thyroid hormone stimulates NO production via activation of the PI3K/Akt pathway in vascular myocytes. *Cardiovasc Res.* 85(3):560-70. https://doi.org/10.1093/cvr/cvp304

[30] Oyarzún, C., Salinas, C., Gómez, D., Jaramillo, K., Pérez, G., Alarcón, S., Podestá, L., Flores, C., Quezada, C., San Martín, R. (2015). Increased levels of adenosine and ecto 5′-nucleotidase (CD73) activity precede renal alterations in experimental diabetic rats. *Biochemical and Biophysical Research Communications*, 468 (1–2): 354-359. https://doi.org/10.1016/j.bbrc.2015.10.095

[31] Stefanovic, V., Vlahovic, P., Savic, V., Ardaillou, N., Ardaillou, R. (1993). Adenosine stimulates 5'-nucleotidase activity in rat mesangial cells via A2 receptors. *FEBS Letters*, 331 (1-2): 96-100. https://doi.org/10.1016/0014-5793(93)80304-D

[32] Naito, Y., Lowenstein, J.M. (1985). 5′-Nucleotidase from rat heart membranes. Inhibition by adenine nucleotides and related compounds. *The Biochemical Journal*, 226 (3): 645–651. https://doi.org/10.1042/bj2260645

[33] Ballard, H.J. (2014). ATP and adenosine in the regulation of skeletal muscle blood flow during exercise. *Acta Physiologica Sinica*, 66(1): 67-78.

[34] Costa, F., & Biaggioni, I. (1998). Role of nitric oxide in adenosine-induced vasodilation in humans. *Hypertension*, 31(5): 1061-4. https://doi.org/10.1161/01.HYP.31.5.1061

[35] Camici, M., Garcia-Gil, M., & Tozzi, M. G. (2018). The Inside Story of Adenosine. *International journal of molecular sciences*, *19*(3), 784. https://doi.org/10.3390/ijms19030784

[36] Fürstenau, C.R., Ramos, D.B., Vuaden, F.C., Casali, E.A., Monteiro, Pde. S., Trentin. Dda. S., Gossenheimer, A.N., Bogo, M.R., Bonan, C.D., Barreto-Chaves, M.L., Sarkis, J.J., Wofchuk, S.T. (2010). L-NAME-treatment alters ectonucleotidase activities in kidney membranes of rats. *Life science, 28*; 87(9-10):325-32. https://doi.org/10.1016/j.lfs.2010.07.008

[37] Di Fusco, F., & Anand-Srivastava, M. (2000). Enhanced expression of Gi proteins in non-hypertrophic hearts from rats with hypertension-induced by L-NAME treatment. *Journal of Hypertension, 18*(8), 1081-1090. https://doi.org/10.1097/00004872-200018080-00013

[38] Li, Y., Sarkar, O., Brochu, M., & Anand-Srivastava, M. (2014). Natriuretic peptide receptor-C attenuates hypertension in spontaneously hypertensive rats: role of nitroxidative stress and Gi proteins. *Hypertension, 63*(4), 846-855. https://doi.org/10.1161/HYPERTENSIONAHA.113.01772

[39] Sarkar, O., Li, Y., & Anand-Srivastava, M. (2017). Nitric oxide attenuates overexpression of Giα proteins in vascular smooth muscle cells from SHR: Role of ROS and ROS-mediated signaling. *PLoS ONE* 12(7): e0179301. https://doi.org/10.1371/journal.pone.0179301

Legends to figures

Fig1 A7r5 cells viability (%). Cells were submitted to distinct treatments: SNP 10^{-5} M, L-NAME 10^{-4} M, ATP, ADP, BK 10^{-5} M and 2-Cl-adenosine 10^{-4} M (ADO) for 24 (a), 48 (b) and 72 (c) hours. At 24 hours of treatment, 2-Cl-adenosine significantly decreased cell viability by 35%. MTT assay was followed as described in Materials and methods. Results are presented as mean ± S.D. of 3 independent measurements of each treatment performed. *refers to statistically significant difference compared to the control group for $P \leq 0,05$ and ***refers to statistically significant difference compared to control group for $P \leq 0,001$.

Fig2 Levels of nitrite (μM) in the culture media of A7r5 cells submitted to different treatments: SNP 10^{-5} M, L-NAME 10^{-4} M, ATP, ADP, BK 10^{-5} M and 2-Cl-adenosine 10^{-4} M (ADO). Nitrite levels were significantly increased by SNP, a nitric oxide donor. Data represent mean ± S.D. of 4 independent measurements for each group. *** represents a statistically significant difference in relation to the control group for $P \leq 0.001$.

Fig3 Hydrolysis of ATP (a), ADP (b) and AMP (c) in A7r5 cells submitted to different treatments: SNP 10^{-5} M, L-NAME 10^{-4} M, ATP, ADP, BK 10^{-5} M and 2-Cl-adenosine 10^{-4} M (ADO). Enzymatic assays were followed as described in Materials and methods. Results are presented as mean ± S.D. of 6 independent experiments performed for each group. No significant differences were observed for ATP (a) and ADP (b) hydrolysis in relation to control group. However, ATP and 2-Cl-adenosine (ADO) significantly increased the hydrolysis of AMP in A7r5 cells compared to the respective control group (c). *represents differences at $P \leq 0,05$ and **represents differences at $P \leq 0,01$.

Fig4 Ratio between CD39 and actin expression levels in A7r5 cells submitted to SNP 10^{-5} M, L-NAME 10^{-4} M, ATP, ADP and BK 10^{-5} M treatments for 24 hours. Graph represents mean ± S.D. of at least 3 independent experiments performed for each group. Bands are representative of the results obtained in Western blotting assays. No significant results were found at CD39 (NTPDase1) protein levels in response to any of the treatments performed under the conditions tested. A.U. = arbitrary units.

Fig5 Ratio between CD73 and actin expression levels in A7r5 cells submitted to SNP 10^{-5} M, L-NAME 10^{-4} M, ATP, ADP and 2Cl-adenosine (ADO)10^{-4} M treatments for 24 hours. Graph represents mean ± S.D. of at least 3 independent experiments performed for each. Bands are representative of the results obtained in Western blotting assays. No significant

results were found at CD73 (ecto-5’-nucleotidase) protein levels in response to any of the treatments performed under the conditions tested. A.U. = arbitrary units.

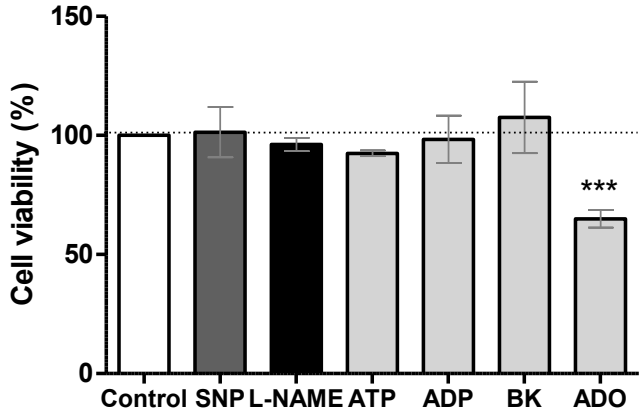
Fig6 Graph showing the potential phosphorylation sites of the ecto-5'-nucleotidase enzyme of rats, obtained by the NetPhos 3.1 Server tool. A large number of phosphorylation sites above the threshold (pink line) are observed, being 22 at serine (green peaks), 15 at threonine (red peaks) and 1 at tyrosine residues (blue peak).

Figures

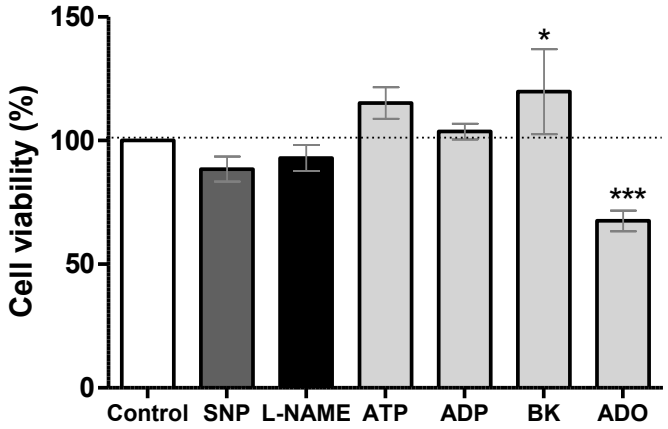
Graphic program: GraphPad Prism Version 5.03 for Windows

Fig1

a)



b)



c)

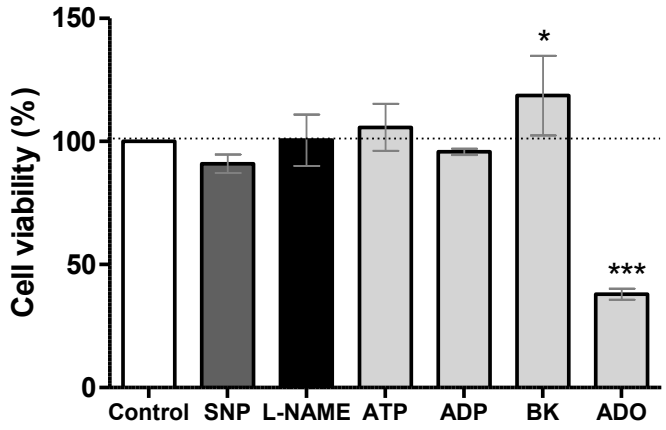


Fig2

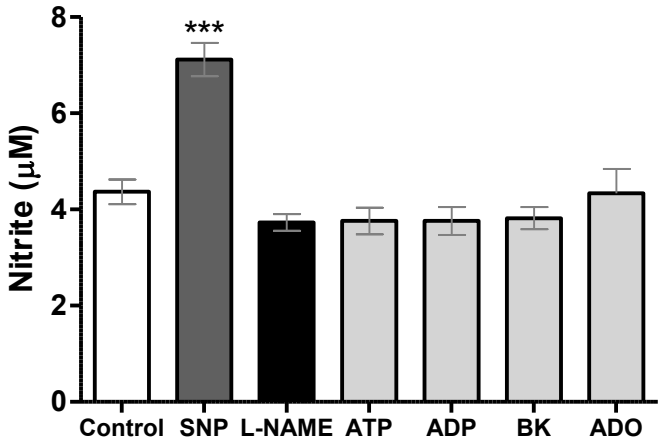
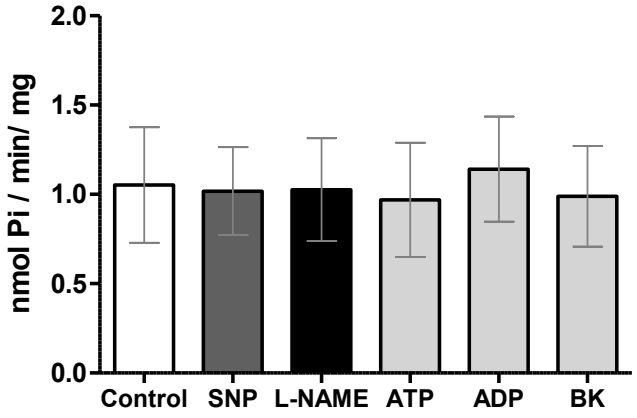
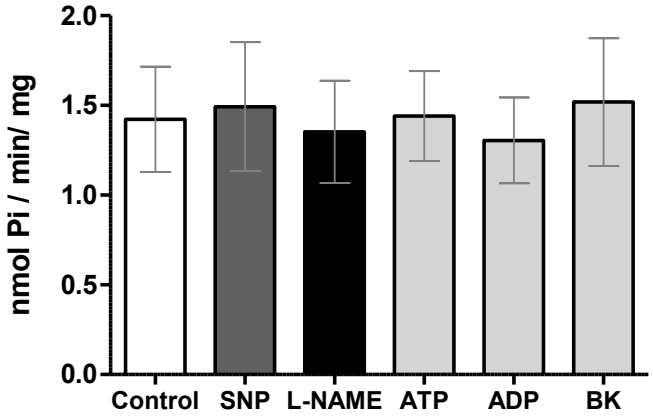


Fig3

a)


nmol Pi / min/ mg
2.0
1.5
1.0
0.5
0.0
Control
SNP
L-NAME
ATP
ADP
BK

b)


nmol Pi / min/ mg
2.0
1.5
1.0
0.5
0.0
Control
SNP
L-NAME
ATP
ADP
BK

c)

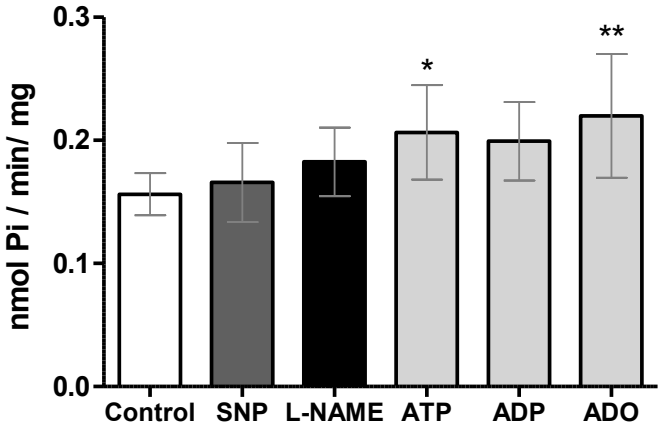

nmol Pi / min/ mg
0.3
0.2
0.1
0.0
*
**
Control
SNP
L-NAME
ATP
ADP
ADO

Fig4

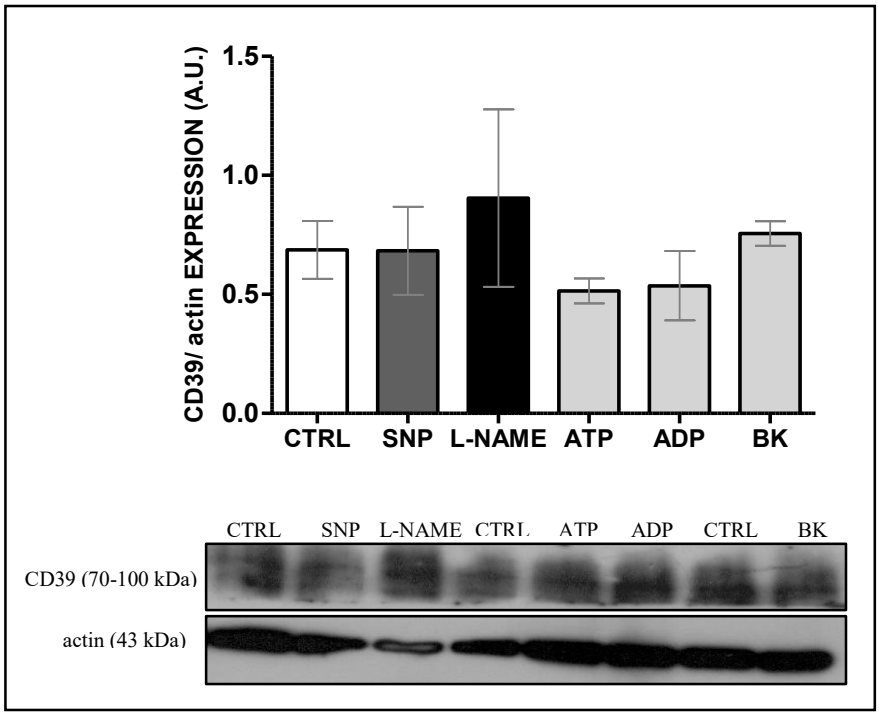

CD39/ actin EXPRESSION (A.U.)
1.5
1.0
0.5
0.0
CTRL
SNP
L-NAME
ATP
ADP
BK
CTRL SNP L-NAME CTRL ATP ADP CTRL BK
CD39 (70-100 kDa)
actin (43 kDa)

Fig5

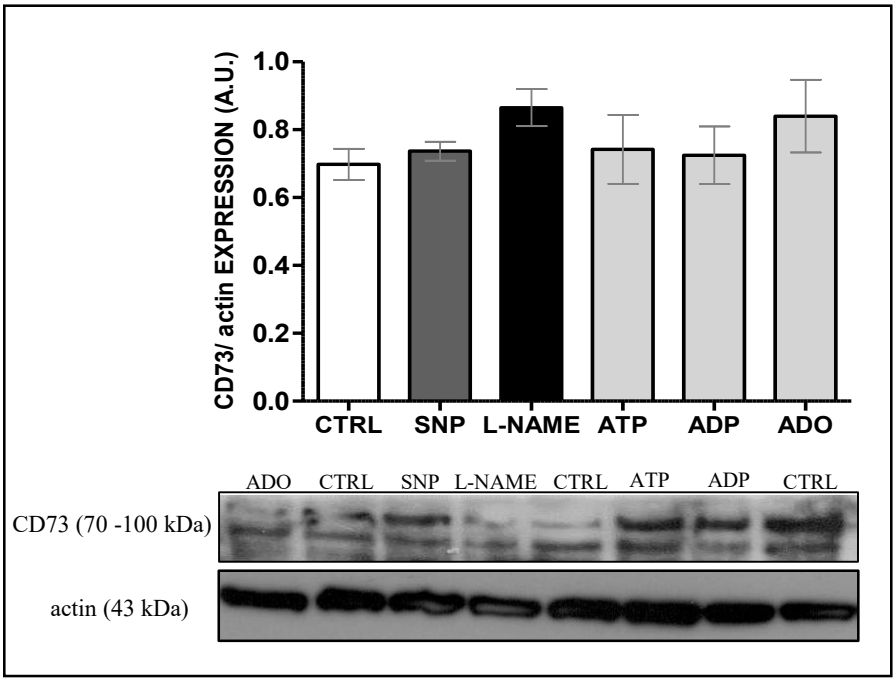
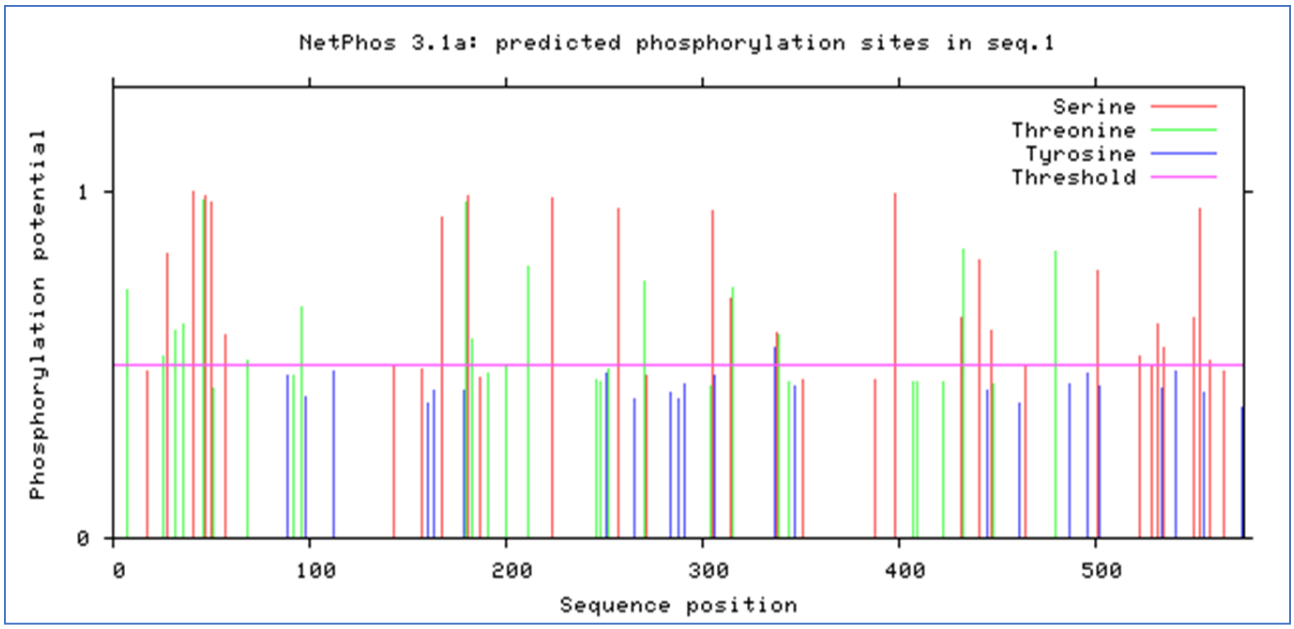

CD73/ actin EXPRESSION (A.U.)
1.0
0.8
0.6
0.4
0.2
0.0
CTRL
SNP
L-NAME
ATP
ADP
ADO
ADO
CTRL
SNP
L-NAME
CTRL
ATP
ADP
CTRL
CD73 (70 -100 kDa)
actin (43 kDa)

Fig6



	№ of potential residues of phosphorylation
Serine	22
Threonine	15
Tyrosine	1

Online Resource 1

Does nitric oxide modulate ectonucleotidases in A7r5 vascular smooth muscle cells?

Molecular and Cellular Biochemistry

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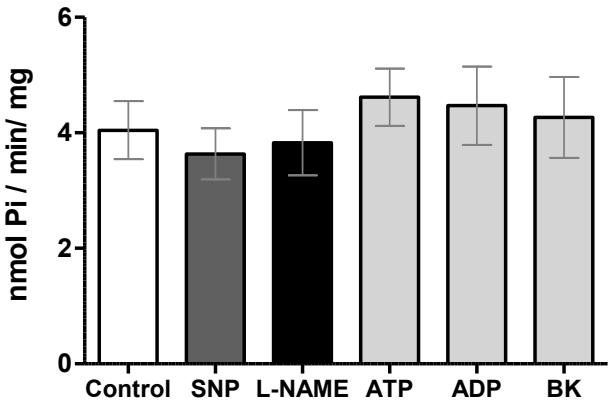
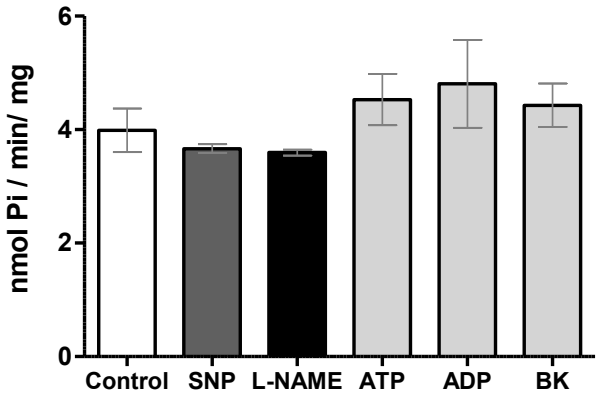
Fig7 Nucleotide hydrolysis in endothelial cells (tEnd.1)

a)



b)



c)

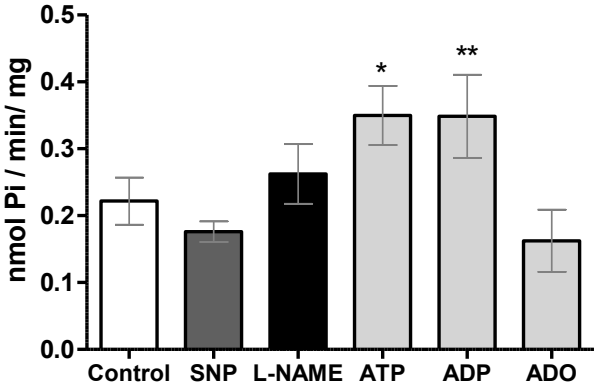


Fig7 Hydrolysis of ATP (a), ADP (b) and AMP (c) in endothelial cells (tEnd.1) submitted to different treatments: SNP 10^{-5} M, L-NAME 10^{-4} M, ATP, ADP, BK 10^{-5} M and 2-Cl-adenosine 10^{-4} M (ADO) for 24 hours. tEnd.1 cells were cultured the same way as described for A7r5. Enzymatic assays also followed the same manner as described in Materials and methods (item 2.5) for A7r5. Results are presented as means ± S.D. of 4 independent experiments performed for each group. ANOVA was used to compare differences in mean values of all groups, followed by Dunnet's *post hoc* test. No significant differences were observed for ATP (a) and ADP (b) hydrolysis in relation to the control group. However, ATP and ADP treatments significantly increased the hydrolysis of AMP in tEnd.1 cells compared to the respective control group (c). *represents differences at $P \leq 0,05$ and **represents differences at $P \leq 0,01$.

Online Resource 2

Does nitric oxide modulate ectonucleotidases in A7r5 vascular smooth muscle cells?

Molecular and Cellular Biochemistry

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Short time nucleotide hydrolysis in A7r5 cells

Table 1. AMP hydrolysis in A7R5 cells submitted to the treatment with NO donor agent (SNP 10^{-5} M) and NO synthesis inhibitor (L-NAME 10^{-4} M) for 5, 30 and 60 minutes.

5 minutes		**30 minutes**		**60 minutes**	
Control	0.08276 ± 0.0122	**Control**	0.1178 ± 0.0061	**Control**	0.1476 ± 0.0004
SNP 10^{-5} M	0.0754 ± 0.02704	**SNP 10^{-5} M**	0.1187 ± 0.0130	**SNP 10^{-5} M**	0.1459 ± 0.0229
L-NAME 10^{-4} M	0.0861 ± 0.0462	**L-NAME 10^{-4} M**	0.1070 ± 0.0194	**L-NAME 10^{-4} M**	0.1361 ± 0.0274

Results are presented as mean ± S.D. of at least 3independent experiments for each time and treatment tested**.** ANOVA was used to analyze differences in the mean values of all groups, followed by Dunnet's *post hoc* test. There were no significant differences between treatments and control group in any times tested.

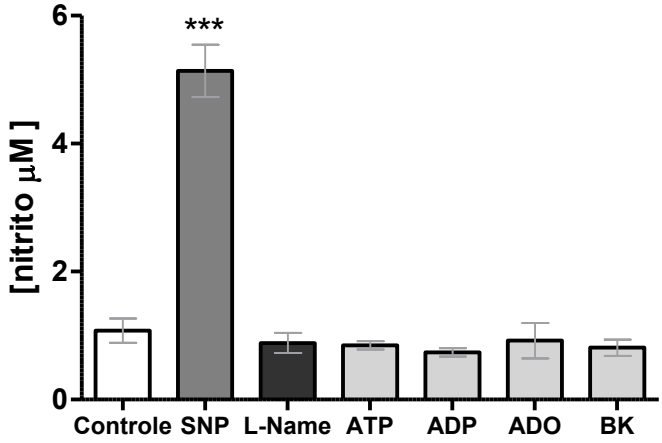
3. Conclusão

No presente estudo, investigou-se os possíveis efeitos do NO sobre a atividade e expressão das ectonucleotidases da vasculatura, quais sejam a NTPDase1 e a ecto-5'-nucleotidase. Verificou-se que, apesar dos nucleotídeos interferirem na via do NO, a atividade e expressão proteica da NTPDase1 não foi influenciada por moléculas doadora, inibidora ou mobilizadoras desse gás em A7r5. Já a ecto-5'-nucleotidase, responsável pela produção de adenosina, uma importante molécula vasodilatadora, aumentou sua atividade em resposta a tratamentos com moléculas mobilizadoras de NO - ATP e 2-Cl-adenosina em células A7r5 e ATP e ADP em células tEnd.1). Entretanto, a medida indireta dos níveis de NO, a partir da dosagem de nitritos, não foi alterada em resposta às moléculas mobilizadoras mencionadas anteriormente. Ainda, o doador direto (SNP) e o inibidor da síntese (L-NAME) de NO, testados em curtos tempos de tratamento, também não apresentou efeito sobre atividade da ecto-5'-nucleotidase. Dessa forma, é possível que a modulação observada ocorra de maneira independente da síntese de NO. No entanto, mais experimentos são necessários para confirmar os níveis de NO, bem como confirmação de níveis aumentados de adenosina em resposta ao ATP, ADP e 2-Cl-adenosina nas culturas celulares. Como abordado previamente, o aumento na atividade da ecto-5'-nucleotidase, refletido em aumentados níveis de adenosina circulante, é de grande importância para garantir a regulação da homeostase e a manutenção do tônus vascular. Os mecanismos moleculares envolvidos na ativação da ecto-5'-nucleotidase da vasculatura, como a modulação da expressão gênica ou alterações em nível pós-traducional, também precisam ser verificados e confirmados. Finalmente, compreender a interlocução entre os sistemas nitrérgico e purinérgico, e como ocorre a regulação de ambas as vias, é extremamente relevante para a modulação do tônus vascular e a regulação dos níveis pressóricos, possibilitando novas abordagens terapêuticas em complicações cardiovasculares.

Apêndice 1

Avaliação dos níveis de nitrito em células tEnd.1

Níveis de nitrito (μM) no meio de cultura de células tEnd.1 submetidas a distintos tratamentos: nitroprussiato de sódio (SNP 10^{-5} M), L-NAME 10^{-4} M, ATP, ADP, 2-Cl-adenosina 10^{-4} M (ADO) e BK 10^{-5} M. A quantificação de nitritos foi conduzida como uma medida indireta dos níveis de óxido nítrico (NO) em células tEnd.1 Os tratamentos foram realizados por 24 horas, utilizando DMEM sem vermelho de fenol (GIBCO, Grand Island, New York, USA) para não influenciar nas leituras. O conteúdo de nitritos foi determinado utilizando o kit de reagente de Griess, de acordo com as instruções do fabricante. Brevemente, o meio de cultura de cada poço foi coletado, centrifugado a 16.000 rpm, 4 ºC (Hermle Labor Technik, Z 36 HK) por 10 minutos e o sobrenadante foi recolhido. Em uma placa de 96 poços, 20 μL do reagente de Griess, 150 μL da amostra contendo nitrito e 130 μL de água deionizada foram misturados. Após 30 minutos de incubação ao abrigo da luz e à temperatura ambiente, a placa foi lida em um leitor de placas de ELISA (Robonik, Readwell PLATE) a 560 nm. A concentração de nitritos nas amostras foi calculada com base em uma curva padrão de nitritos realizada em diferentes concentrações (1, 5, 10, 30, 50 e 100 μM). Apesar de apresentar um nível geral de nitritos inferior aos resultados encontrados nas CMLV, os níveis de nitrito foram significativamente aumentados pelo tratamento com SNP, um doador de NO em uma mesma proporção. Os resultados representam média ± D.P. de 4 medidas distintas por grupo. ***representa diferença estatisticamente significativa em relação ao controle para P<0.0001.

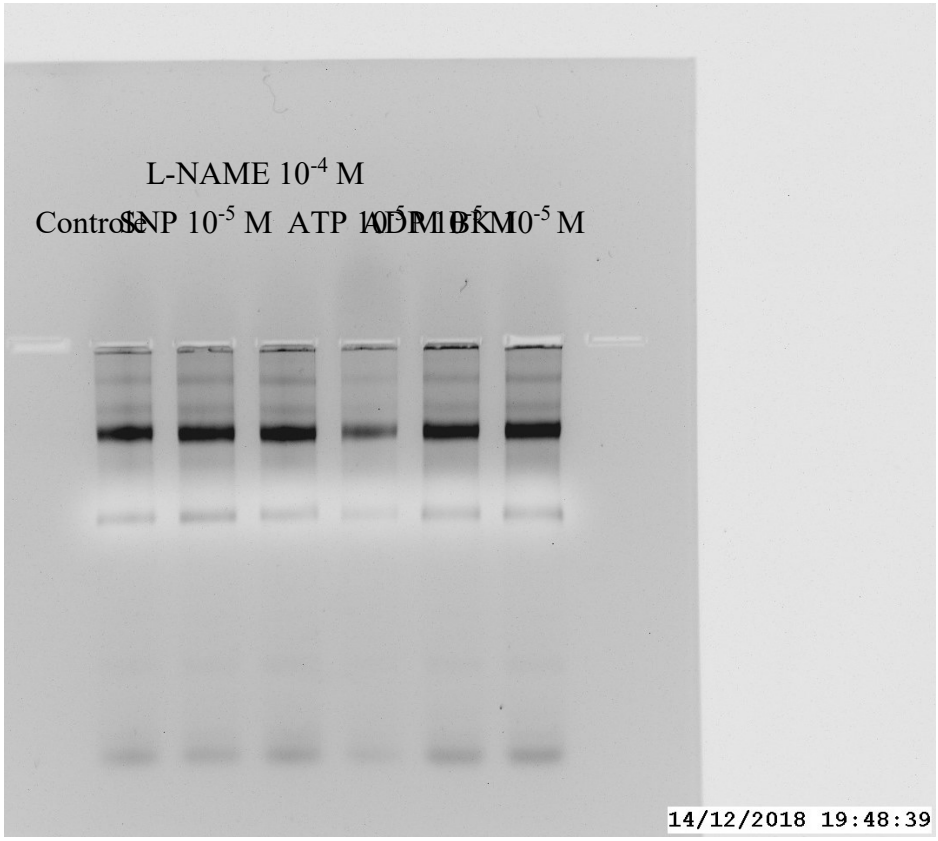


Apêndice 2

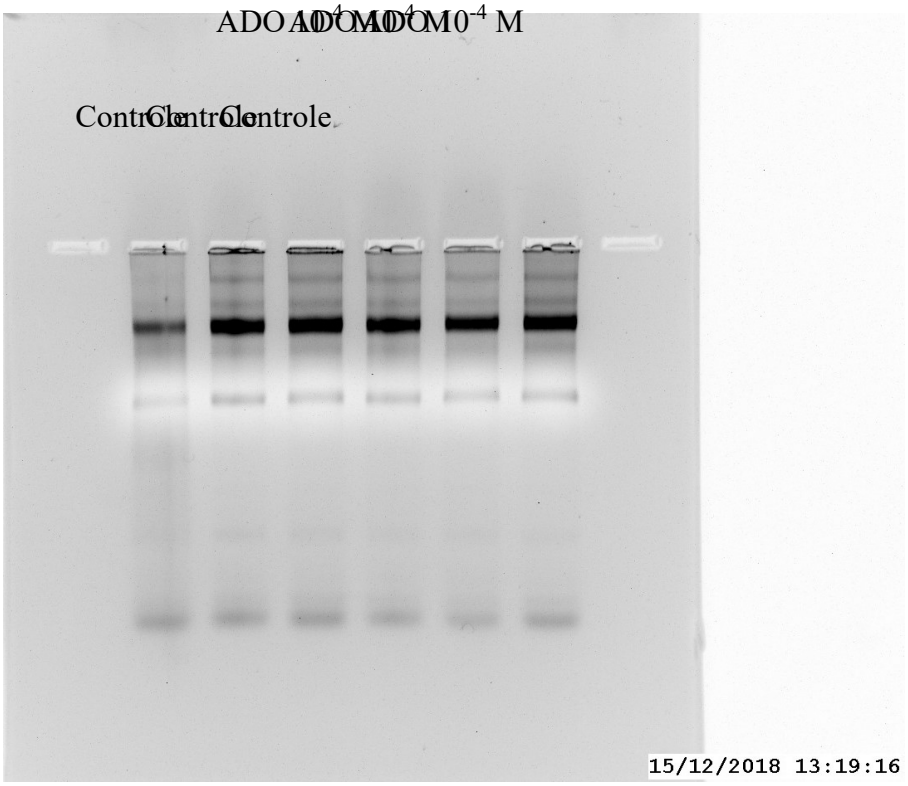
Avaliação dos genes de interesse

Células A7r5 foram tratadas com distintas moléculas: nitroprussiato de sódio (SNP 10^{-5} M), L-NAME 10^{-4} M, ATP, ADP e BK 10^{-5} M e 2-Cl-adenosina 10-4 M (ADO). Após 24 horas, o RNA total das células foi extraído utilizando-se o reagente de Trizol® (Invitrogen™, Carlsbad, CA, EUA), conforme instruções do fabricante. A qualidade e a quantidade do RNA foram acessadas em eletroforese com gel de agarose e pela razão A260/ A280 em espectrofotômetro.

Fotografia representativa de um gel de agarose para determinação da integridade do RNA em células A7r5 em reposta aos distintos tratamentos.

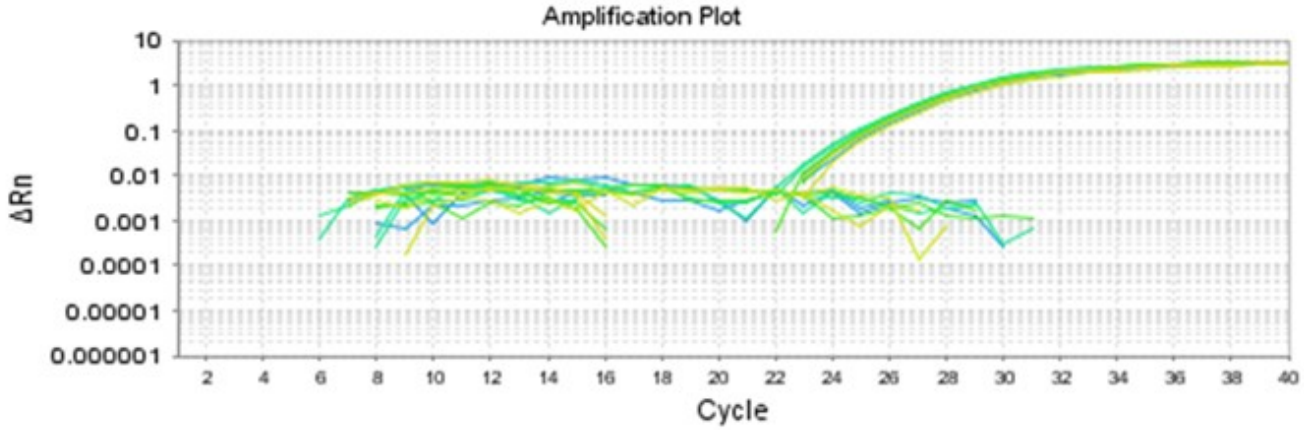


Fotografia representativa de um gel de agarose para determinação da integridade do RNA em células A7r5 em reposta à 2-Cl-adenosina (ADO).



A expressão do mRNA foi determinada utilizando-se 1 μg de RNA, o qual foi transcrito reversamente a cDNA com o kit MMLV Reverse Transcriptase (Invitrogen™, Carlsbad, CA, EUA). A qPCR foi realizado usando SYBR Green PCR blend (Thermo Scientific, Rockford, EUA) em um termociclador StepOnePlus™ Real-Time PCR System (Thermo Fisher Scientific, Rockford, EUA). Para a avaliação dos genes de interesse foram desenhados dois conjuntos de *primers*, 1: ENTPD1 (5’ TGAAAATGTCCACTGAGCGGA 3’ e 5’ CGATTGCTTGCTTTCCATTCTG 3’), NT5E (5’ GATCCGCAAGGAAGAACCC 3’ e 5’ CATGATTTCCCAGTGCCATG 3’), 2: ENTPD1 (5’ CTGGGCAGATTCACTCAGGAAC 3’ e 5’ GATCTGTGTAGAAGCTCCGCC 3’), NT5E (5’ CCTTCCTCTCAAATCCAGGGA 3’ e 5’ CCAGCAGGCACTTCTTTGG 3’) e β-actina (5' TATGCCAACACAGTGCTGTCTGG 3' e 5’ TACTCCTGCTTCCTGATCCACAT 3’). Entretanto, não foi possível detectar os níveis de RNA para as ectonucleotidases nessas células.

Amplificação para o gene da β-actina, o qual será utilizado como gene normalizador. Sua amplificação foi alcançada com êxito nas células A7r5, como pode ser observado nas curvas amplificados mais à direita do gráfico, a partir do ciclo 21.



Anexo 1

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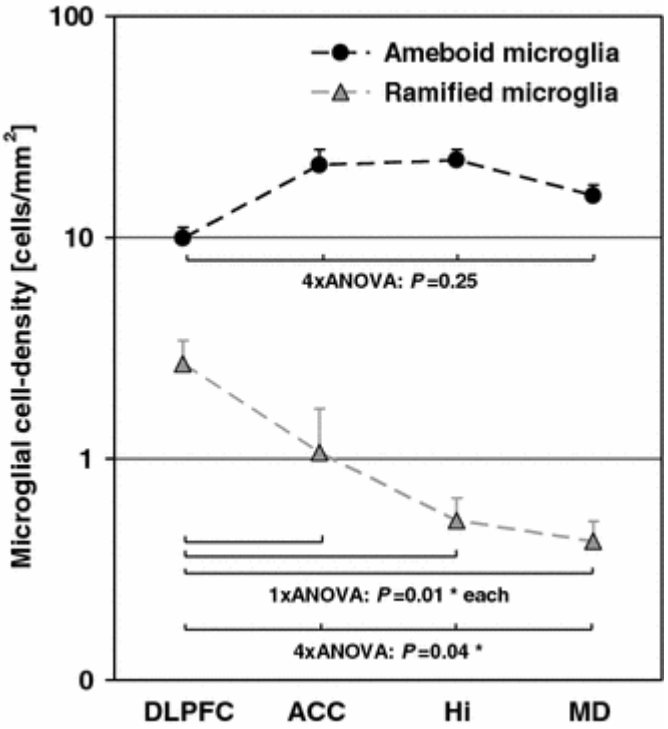

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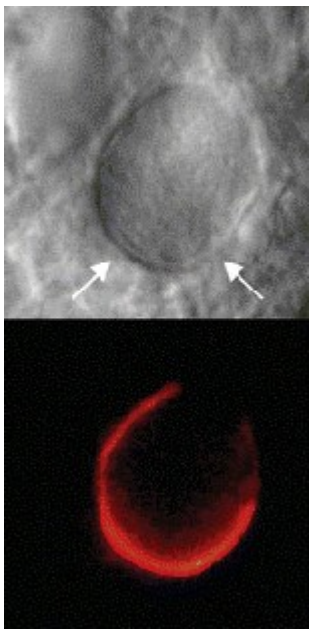

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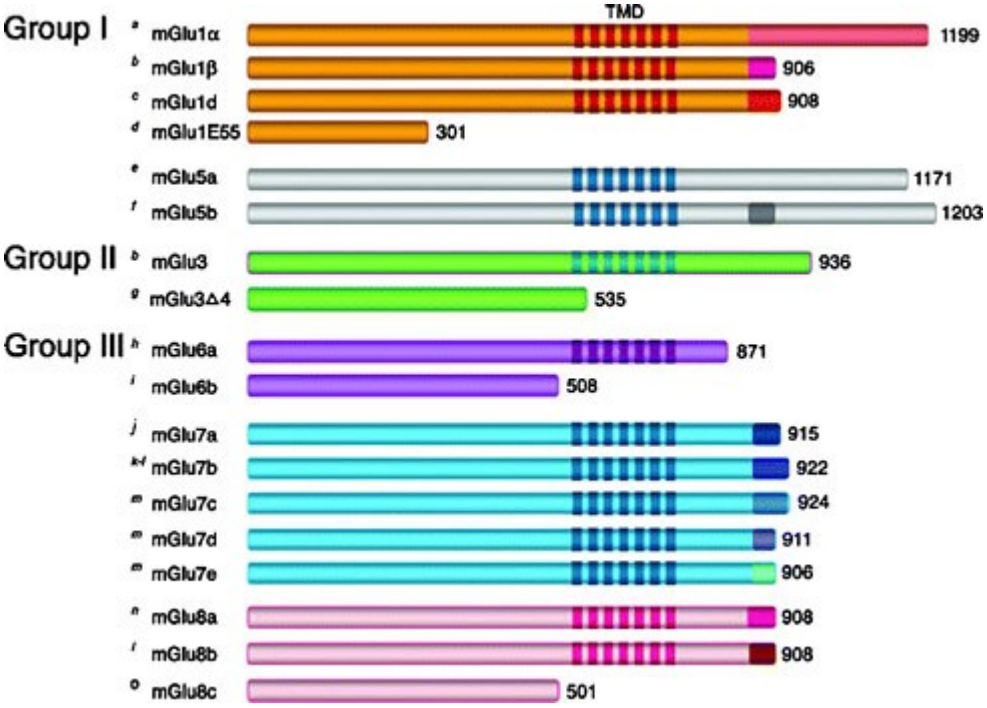

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