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ESTUDO DAS ECTONUCLEOTIDASES EM CÉLULAS ESTRELADAS HEPÁTICAS: RELAÇÃO ENTRE A EXPRESSÃO, ATIVIDADE E SIGNIFICADO FISIOLÓGICO

CLÁUDIA M.B. ANDRADE

Orientadora: Profa. Dra. Fátima T.C.R. Guma

Co-orientadora: Profa. Dra. Ana Maria O. Battastini

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"Um quadro nunca é terminado — ele simplesmente pára em lugares interessantes"

Paul Gardner

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APRESENTAÇÃO

Esta tese encontra-se apresentada no formato de artigos científicos e organizada da seguinte forma:

PARTE I

Introdução, contendo uma revisão da literatura sobre os aspectos que fundamentaram este trabalho.

Objetivos gerais e específicos do trabalho.

PARTE II

Capítulo I, contendo um manuscrito submetido ao periódico Cell and Tissue Research.

Capítulo II, contendo um manuscrito submetido ao periódico FEBS Journal.

Capítulo III, contendo um artigo científico publicado no periódico Life Sciences.

Capítulo IV, contendo um manuscrito a ser submetido ao periódico The Journal of Biological Chemistry.

Capítulo V, contendo dados preliminares.

PARTE III

Discussão, contendo uma interpretação geral dos resultados apresentados nos diferentes capítulos.

Conclusões finais e perspectivas originadas a partir deste trabalho.

REFERÊNCIAS

Referências bibliográficas citadas nas Partes I e III.

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PARTE I

RESUMO

As células estreladas hepáticas (HSCs) são a principal fonte de componentes de matriz extracelular em doenças crônicas do fígado e, por esta razão, exercem um papel fundamental no desenvolvimento e na manutenção da fibrose hepática. Os nucleotídeos e nucleosídeos são moléculas sinalizadoras que regulam diversos processos no fígado e têm um importante papel na patogênese da fibrose hepática. As ecto-nucleosídeo trifosfato difosfoidrolases (E-NTPDases), ecto-nucleotídeo pirofosfatase fosfodiesterases (E-NPPs), ecto-5'-nucleotidase (eNT/CD73) e a fosfatase alcalina tecido inespecífica (TNALP) são enzimas localizadas na superfície celular que regulam a concentração dos nucleotídeos no meio extracelular e, desse modo, modulam os efeitos biológicos mediados por eles via ativação dos receptores P1 e P2. Assim, neste estudo nós comparamos o metabolismo extracelular de nucleotídeos e o nível transcricional das ectoenzimas em HSCs quiescentes e ativadas usando uma linhagem de células estreladas hepáticas murina GRX. Estas células expressam o fenótipo miofibroblástico em meio basal e o tratamento com retinol ou indometacina induz a mudança das células GRX para o fenótipo quiescente das HSCs. Os dois fenótipos das células GRX expressam as NTPDase3 e 5, NPP1, 2 e 3, ecto-5'-nucleotidase e TNALP. Entretanto, apenas as HSCs ativadas expressam a NTPDase6. Nas HSCs quiescentes, a hidrólise de nucleosídeos trifosfatados foi significativamente mais alta e foi correlacionada com o aumento na expressão do mRNA da *Entpd3* e *ENPP3*. Os nucleosídeos difosfatados foram hidrolisados de maneira similar pelos dois fenótipos das células GRX e esta atividade foi associada com o aumento da expressão do mRNA da *Entpd5* nas células quiescentes e com a expressão da *Entpd6* apenas nas HSCs ativadas. A atividade AMPásica foi maior nas células quiescentes do que nas células ativadas e foi relacionada com o aumento da atividade e da expressão do mRNA da ecto-5'-nucleotidase. O tratamento com retinol também envolve a ativação transcricional da TNALP. Para analisar o papel fisiológico da eNT/CD73 nas HSCs, o mRNA e a expressão desta proteína foram reduzidos nas células GRX utilizando a técnica de RNAi. O *knockdown* da eNT/CD73 aumentou a expressão do mRNA da TNALP e do colágeno I e alterou a adesão e a migração celular através de um mecanismo independente da hidrólise de nucleotídeos. Nós também avaliamos o efeito da adenosina na regulação de marcadores de ativação das HSCs. Este nucleosídeo diminuiu a proliferação, a migração, a transcrição de colágeno I e das metaloproteinases 2 e 9 e diminuiu a transcrição da eNT/CD73. As diferenças na atividade e expressão das ectonucleotidases entre os dois fenótipos das células GRX sugerem que estas enzimas modulam a concentração de nucleotídeos/nucleosídeos e geram efeitos distintos na sinalização purinérgica nos dois fenótipos e podem ser importantes alvos na regulação das funções das HSCs.

ABSTRACT

Hepatic stellate cells (HSC) are recognized as a primary cellular source of matrix components in chronic liver disease and, therefore, play a critical role in the development and maintenance of liver fibrosis. Nucleotides and nucleosides are signaling molecules that regulate a variety of activities within the liver and play a role in the pathogenesis of hepatic fibrosis. Ecto-nucleoside triphosphate diphosphohydrolases (E-NTPDases), ecto-nucleotide pyrophosphatase/phosphodiesterases (E-NPPs), ecto-5'-nucleotidase (eNT/CD73) and tissue non-specific alkaline phosphatase (TNALP) are enzymes that are located at the cell surface and regulate the concentration of extracellular nucleotides, thereby modulating their biological effects by the activation of P1 and P2 receptors. Thus, in the study we compared the extracellular metabolism of nucleotides and transcriptional levels of ectoenzymes in activated and quiescent HSC of the mouse hepatic stellate cell line GRX. This cell line expresses a myofibroblast phenotype in basal medium and both retinol and indomethacin treatment induced a phenotypic change of GRX cells to quiescent HSC. Two phenotypes of GRX cells expressed NTPDase3 and 5, NPP1, 2 and 3, ecto-5'-nucleotidase/CD73 and TNALP. However, only activated HSC expressed NTPDase6. In quiescent HSC, the hydrolysis of triphosphonucleosides was significantly higher and was related to an increase in *Entpd*3 and *ENPP3* mRNA expression. The diphosphonucleosides were hydrolyzed at a similar rate by two phenotypes of GRX cells and this hydrolysis was associated with an up-regulation of *Entpd*5 mRNA expression in quiescent-like HSC, whilst *Entpd*6 mRNA expression was observed only in activated HSC. The AMPase activity in quiescent HSC was higher than myofibroblasts and was related to an increase in ecto-5'-nucleotidase activity and its mRNA expression. The treatment with retinol also involves transcriptional activation of TNALP. To analyze the biological significance of eNT/CD73 in HSC, the expression of eNT/CD73 mRNA and protein was reduced in GRX cell line using the technique of RNAi. ENTCD73 knockdown leads to an increase in mRNA expression of TNALP and collagen I, and a clear alteration of cell adhesion and migration by a mechanism not dependent of changes in nucleotide metabolism. We also tested the effect of adenosine on the regulation of activation markers of in HSCs. This nucleotide decreased proliferation, migration, transcription of collagen I and matrix metalloproteinases 2 e 9 and increased transcription of eNT/CD73. The differential ectonucleotidases activities and expressions in two phenotypes of GRX cells suggest that these enzymes modulate the concentration of nucleotides/nucleosides and affect purinergic signaling differently in the two phenotypes and may play a role in the regulation of HSC functions.

LISTA DE ABREVIATURAS

ACR – regiões conservadas da apirase

α – SMA – alfa actina de músculo liso

bGlyc - beta-glicerofosfato

cAMP – AMP cíclico

CD39 – antígeno de ativação celular linfóide

Col Iα1- cadeia alfa 1 do colágeno tipo I

dsRNA – RNA fita dupla

eNT/CD73 – ecto-5'-nucleotidase

Ecto-ADA – ecto-adenosina deaminase

E-NTPDase - ecto-nucleosídeo trifosfato difosfoidrolase

E-NPP - ecto-nucleotídeo pirofosfatase fosfodiesterase

ERK – quinase regulada por sinal extracelular

GCALP – fosfatase alcalina das células germinativas

Gli-6-P – glicose -6-fosfato

GPI – glicosilfosfatidilinositol

GRX – linhagem murina de células estreladas hepáticas

HPLC – cromatografia líquida de alta eficiência

HSCs – células estreladas hepáticas

IALP – fosfatase alcalina intestinal

KD - *knockdown*

MAPK – proteína quinase ativada por mitógeno

MMP-2 – metaloproteinase 2

MMP-9 – metaloproteinase 9

MT1-MMP - metaloproteinase de membrana tipo I

P2X – receptor purinérgico ionotrópico

P2Y – receptor purinérgico metabotrópico

Pi – fosfato inorgânico

PPi – pirofosfato inorgânico

PKA – proteína quinase A

PLALP – fosfatase alcalina da placenta

qPCR – reação em cadeia da polimerase quantitativa

RAR – receptor para ácido retinóico

RARE - elemento de resposta ao ácido retinóico

RISC - complexo de silenciamento induzido por RNA

RNAi – RNA de interferência

RXR – receptor X de retinóides

shRNA – pequeno RNA em grampo (do inglês "short hairpin RNA")

siRNA – pequeno RNA de interferência (do inglês "small-interfering RNA")

TIMP – inibidor tecidual de metaloproteinase

TNALP – fosfatase alcalina tecido inespecífica

INTRODUÇÃO

1. Fibrose Hepática

O fígado é a maior glândula do corpo humano, localiza-se na cavidade abdominal e divide-se anatomicamente em dois lobos: direito e esquerdo, separados anteriormente pelo ligamento falciforme, inferiormente pela fissura do ligamento de teres e posteriormente pelo ligamento venoso. O lóbulo direito é ainda subdividido em lobo quadrado inferiormente e lobo caudado posteriormente. Funcionalmente o fígado é dividido em oito segmentos (I a VIII) segundo as subdivisões dos sistemas vasculares arterial e venoso, assim como sua drenagem biliar. Os segmentos I, II, III e IV correspondem ao lobo esquerdo, já os segmentos V, VI, VIII e VIII correspondem ao lobo direito (Rappaport & Wanless, 1993). Histologicamente, este órgão é composto por unidades estruturais chamadas de lóbulos hepáticos, onde os hepatócitos (principal tipo celular residente no fígado) estão separados dos sinusóides por um estreito espaço subendotelial, chamado de espaço de Disse. Os sinusóides são capilares revestidos por células endoteliais e macrófagos, que no fígado recebem o nome de células de Kupffer. O espaço de Disse é composto por uma matriz reticular constituída principalmente por colágeno tipo IV e VI que permite o fluxo de moléculas entre os sinusóides e hepatócitos e promove a integridade estrutural do parênquima hepático (Sasse, Spornitz & Maly, 1992). No espaço de Disse também se encontram as células estreladas hepáticas, produtoras de matriz extracelular e também o principal sítio de armazenamento de retinóides no organismo (Gressner & Weiskirchen, 2006).

A fibrose hepática é caracterizada como um processo de cicatrização tecidual em resposta a diversos agentes capazes de gerar um dano hepático. A reação fibrótica gera modificações na matriz reticular do espaço de Disse devido ao depósito de colágeno tipo I e III, tornando-a mais fibrilar e, assim, prejudicando o fluxo de moléculas e o funcionamento dos hepatócitos (Yang et al., 2003). O progresso da fibrose é variável e depende da causa da doença hepática, de fatores ambientais e do estado de saúde do indivíduo. A cirrose é o estágio mais avançado da fibrose, no qual a perpetuação da resposta fibrótica resulta em excessivo acúmulo de componentes da matriz extracelular, distorção do parênquima hepático e alterações no fluxo sangüíneo (Figura 1).
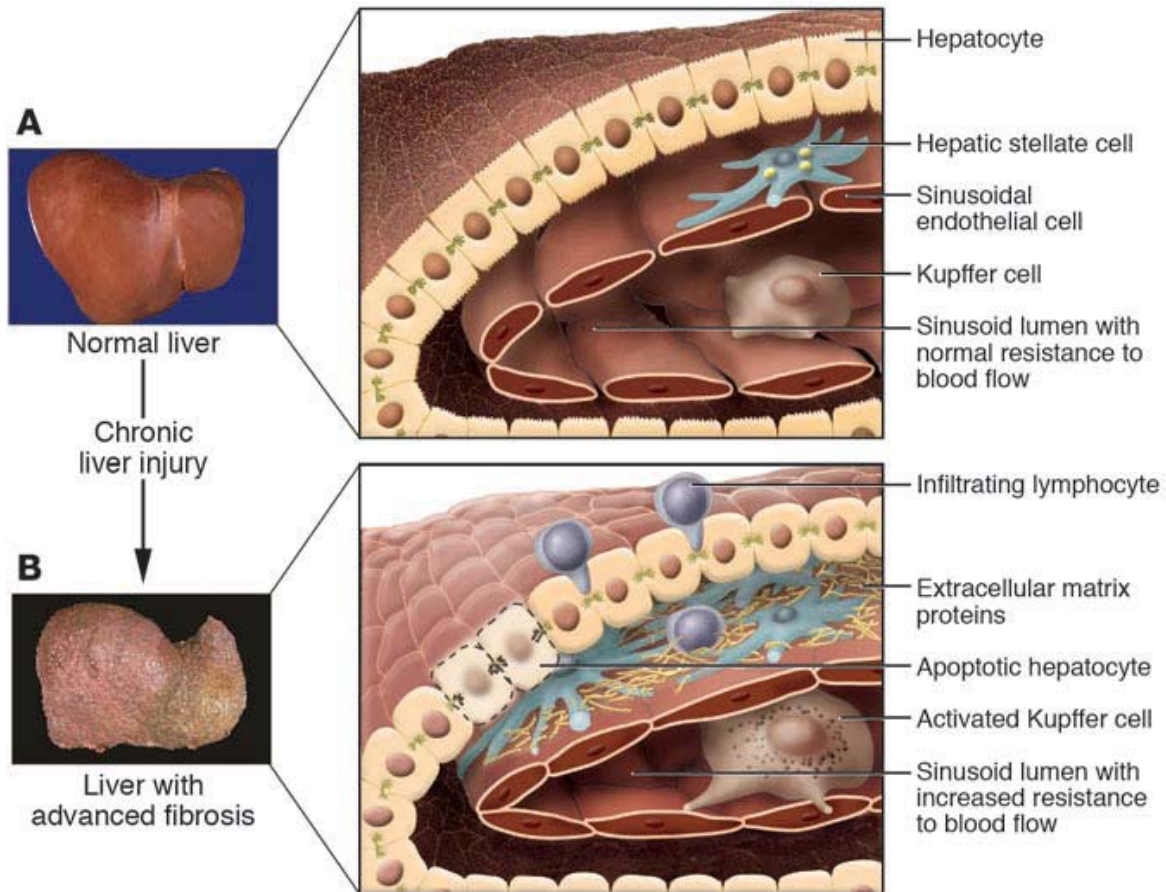
Figura 1. Mudanças na arquitetura hepática associadas com a progressão da fibrose hepática. (A) Esquema representativo do lóbulo hepático no fígado normal. (B) Após um dano crônico, ocorre infiltração de linfócitos para dentro do parênquima hepático, apoptose de alguns hepatócitos, ativação das células de Kupffer e das células estreladas hepáticas, que se tornam proliferativas e secretam grandes quantidades de proteínas de matriz extracelular. As células sinusoidais perdem suas frenestas e, juntamente com a contração das células estreladas, aumentam a resistência ao fluxo sanguíneo dentro dos sinusóides. Adaptado de Bataller & Brenner (2005).

As maiores conseqüências clínicas da cirrose são a perda de função dos hepatócitos e, conseqüente, falência do fígado, aumento da resistência intra-hepática

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ou hipertensão portal e o desenvolvimento de carcinoma hepatocelular (Schuppan & Afdhal, 2008). As principais causas desta patologia são: o consumo excessivo de álcool e drogas, o uso de certos medicamentos, doenças virais, como hepatite B e C, doenças autoimunes e desordens metabólicas, como obesidade, diabetes tipo 2 e hiperlipidemia. A exata prevalência da cirrose não é conhecida. No entanto, estima-se que mais de 10% da população mundial apresenta alguma doença hepática como hepatite crônica, esteatose alcoólica, fibrose/cirrose ou hepatocarcinoma (Luk et al., 2007).

2. Células Estreladas Hepáticas

As células estreladas hepáticas (HSCs), também conhecidas como células de Ito, células perisinusoidais, lipócitos e células acumuladoras de gordura, correspondem a 1,4% do volume total do fígado e encontram-se na proporção de uma para cada vinte hepatócitos. Elas apresentam uma forma estrelar devido aos seus proeminentes processos dendríticos citoplasmáticos e, embora represente 5 a 8% do total de células do fígado, sua distribuição e extensão espacial permitem que cubram toda a rede microcirculatória sinusoidal (Moreira, 2007). No fígado normal, as HSCs encontram-se no estado quiescente, caracterizado pela presença de gotas lipídicas no citoplasma contendo vitamina A na forma de éster de retinila. O armazenamento de vitamina A confere a estas células uma de suas principais funções fisiológicas: a manutenção da homeostasia do retinol no organismo. Outras características importantes das HSCs

quiescentes são as baixas taxas de mitose e de síntese de proteínas de matriz extracelular (Friedman, 2008a).

Na fibrogênese hepática as HSCs são as principais células responsáveis pela produção excessiva de matriz extracelular. Em resposta ao dano hepático, as HSCs sofrem um processo conhecido como ativação ou transdiferenciação e passam a expressar um fenótipo miofibroblastóide. As HSCs ativadas perdem a capacidade de armazenar retinóides, mudam a morfologia e a organização do citoesqueleto, passam a expressar alfa actina de músculo liso (α-SMA), aumentam a taxa de proliferação, migração e adesão, aumentam a contratilidade, produzem substâncias quimiotáticas capazes de recrutar células inflamatórias e outras HSCs e tornam-se fibrogênicas. O processo de ativação é classicamente dividido em duas fases (Figura 2). Na primeira, ou fase inicial, as HSCs aumentam a expressão de receptores de membrana tornando-se mais responsivas à estimulação parácrina dos hepatócitos, células de Kupffer, células endoteliais e plaquetas. O segundo estágio do processo de ativação é conhecido como perpetuação, nesta fase as HSCs ativadas aumentam a proliferação, contratilidade, quimiotaxia, expressão de componentes da matriz extracelular, de metaloproteinases (enzimas responsáveis pela degradação da matriz) e seus inibidores, resultando no remodelamento e depósito de matriz extracelular nos sítios de injúria (Friedman, 2008b).

Diversos estudos têm sido realizados nos últimos anos visando o entendimento da patofisiologia das HSCs, bem como as rotas que levam a sua ativação com o objetivo de auxiliar no desenvolvimento de estratégias antifibrigênicas e medicamentos

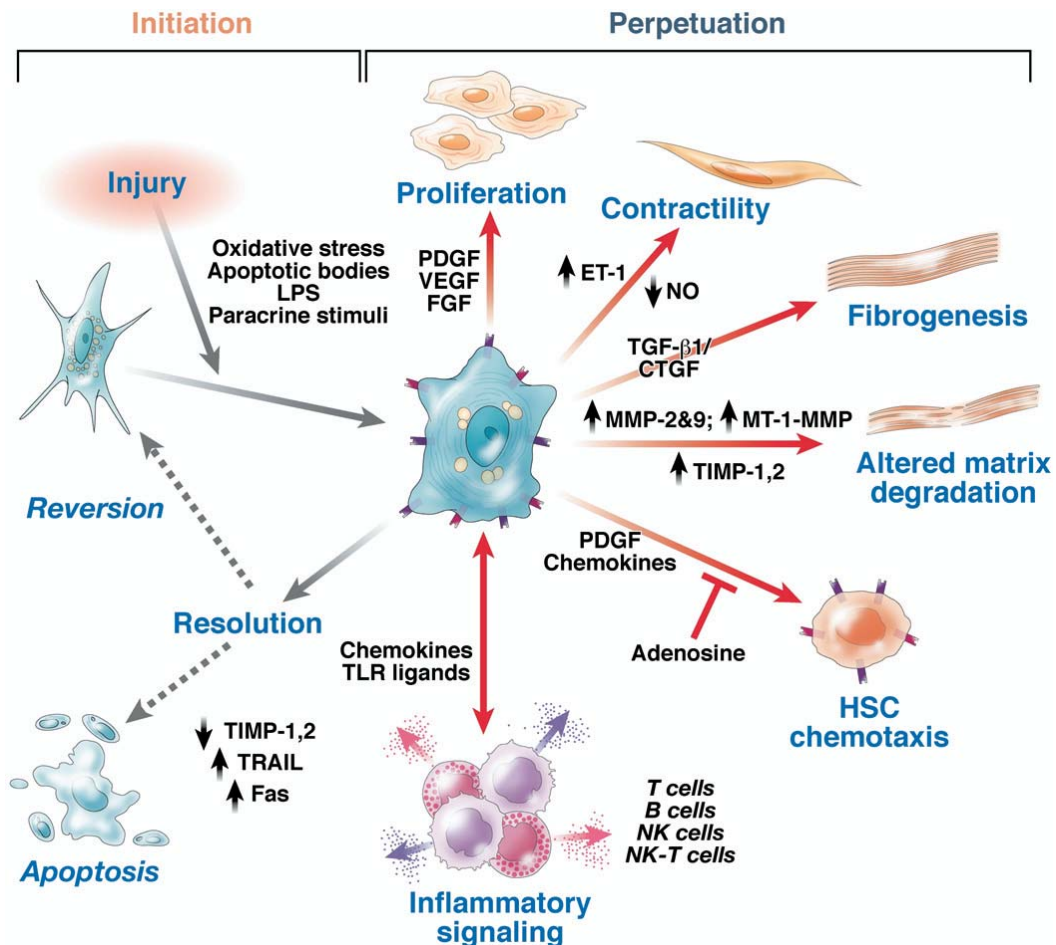para o tratamento da fibrose hepática (Bedossa & Paradis, 2003; Issa et al., 2001; Prosser, Yen & Wu, 2006).

Figura 2. Rotas de ativação das células estreladas hepáticas. O estágio inicial da ativação das HSCs é promovido por estímulos solúveis que incluem espécies reativas de oxigênio, corpos apoptóticos, lipopolissacarídeo e estímulos parácrinos das células vizinhas. A fase de perpetuação é caracterizada por mudanças fenotípicas incluindo proliferação, contração, fibrogênese, alteração na degradação da matriz extracelular, quimiotaxia e sinalização inflamatória. O destino das HSCs ativadas após a resolução do dano hepático é incerta, mas pode incluir apoptose ou a conversão ao fenótipo quiescente. Adaptado de Friedman (2008b).

3. A Linhagem Celular GRX

As culturas primárias de HSCs isoladas de modelos animais são apropriadas para o estudo do fenótipo ativado destas células, pois quando as mesmas são expostas a superfícies plásticas, recobertas ou não com filme de colágeno, rapidamente diminuem seu conteúdo lipídico e adquirem o fenótipo miofibroblastóide. Assim, a impossibilidade de manutenção do fenótipo quiescente em cultura por longo período torna as linhagens de HSCs importantes ferramentas para estudos com as HSCs quiescentes (Herrmann, Gressner & Weiskirchen, 2007).

A linhagem celular GRX foi a primeira linhagem de HSCs estabelecida (Borojevic et al., 1985). Foi isolada a partir de reações fibro-granulomatosas induzidas em fígado de camundongos através de infecção com *Schistosoma mansoni*. É representativa do tecido conjuntivo hepático, tem morfologia fibroblastóide e estrelada e é altamente proliferativa quando cultivada em vidro, plástico ou colágeno, representando assim as HSCs ativadas. Entretanto, uma importante característica das células GRX é a possibilidade de conversão fenotípica após o tratamento com retinol (Margis & Borojevic, 1989), ou indometacina (Guaragna, Trugo & Borojevic, 1991), ou β-caroteno (Martucci et al., 2004). Nestas condições, as células perdem a morfologia fibroblastóide, encurtam seus prolongamentos citoplasmáticos, progressivamente adquirem a forma poligonal, passam a acumular gotas lipídicas na região perinuclear e reduzem significativamente a taxa de proliferação, adquirindo assim caracteríticas das HSCs quiescentes. A indução do fenótipo lipocítico por retinol corresponde, *in vivo*, à hiperplasia das HSCs por hipervitaminose A, já a indução mediada por indometacina

está relacionada com a diferenciação adipogênica (Guimaraes et al., 2007; Margis & Borojevic, 1989).

Assim sendo, a linhagem celular GRX representa um modelo de estudo das HSCs por sua origem, morfologia, padrão de proliferação, capacidade de secreção de componentes de matriz e possibilidade de modulação fenotípica (Herrmann et al., 2007).

4. Nucleotídeos Extracelulares

Nucleotídeos e nucleosídeos constituem duas importantes classes de moléculas que regulam diversas funções celulares via ativação de receptores P1 e P2 expressos na superfície celular. Os receptores P1 subdividem-se em A_1, A_{2A}, A_{2B} e A_3 e são responsivos à adenosina com potencial agonista na ordem adenosina > AMP > ADP > ATP. Enquanto os receptores A_1 e A_3 são acoplados à proteína Gi e inibem a adenilato ciclase, os dois tipos de receptores A_2 são acoplados a proteína Gs e estimulam a adenilato ciclase (Fredholm et al., 2001). Os receptores P2 ligam preferencialmente nucleosídeos tri- e difosfatados, tendo uma afinidade muito baixa pelos monofosfatados. São subdivididos em dois grandes grupos, os receptores ionotrópicos P2X ($P2X_{1-7}$) e os receptores metabotrópicos P2Y ($P2Y_1$, $P2Y_2$, $P2Y_3$, $P2Y_4$, $P2Y_6$, $P2Y_{11}$, $P2Y_{12}$, $P2Y_{13}$ e $P2Y_{14}$) (Erb et al., 2006). Os efeitos promovidos pela interação dos nucleotídeos/nucleosídeos com seus receptores atingem o organismo completo e incluiem contração da musculatura lisa, neurotransmissão central e periférica, secreção

exócrina e endócrina, resposta imune, inflamação, agregação plaquetária, modulação da função cardíaca e hepática, entre outras (Novak, 2003; Robson, Sevigny & Zimmermann, 2006). A tabela 1 apresenta um resumo dos principais agonistas e antagonistas para os receptores P1 e P2, bem como a principal localização e os mecanismos de transdução de sinal envolvidos.

Desse modo, a regulação da quantidade relativa destas moléculas é fundamental para o controle da resposta mediada por estes compostos. Assim, os mecanismos que controlam a concentração dessas substâncias, como as ectonucleotidases, possuem uma posição central neste sistema.

Tabela 1. Principais agonistas e antagonistas, localização e mecanismos de transdução de sinal dos receptores purinérgicos

Receptor		Main distribution	Agonists*	Antagonists	Transduction mechanisms
P1 (adenosine)	A_1	brain, spinal cord, testis, heart, autonomic nerve terminals	CCPA, CPA, S-ENBA, CVT-510	DPCPX, N-0840, MRS1754, N-0840, WRC-0571	$G_{i/o}$ $\downarrow$cAMP
	A_{2A}	brain, heart, lungs, spleen	CGS 21680, HE-NECA, CVT-3146	KF17837, SCH58261, ZM241385, KW 6002	G_S $\uparrow$cAMP
	A_{2B}	large intestine, bladder	NECA (non-selective)	enprofylline, MRE2029-F20, MRS17541, MRS 1706	G_S $\uparrow$cAMP
	A_3	lung, liver, brain, testis, heart	IB-MECA, 2-Cl-IB-MECA, DBXRM, VT160	MRS1220, L-268605, MRS1191, MRS1523, VUF8504	$G_{i/o}$ $G_{q/11}$ $\downarrow$cAMP $\uparrow$IP$_3$
P2X	$P2X_1$	smooth muscle, platelets, cerebellum, dorsal horn spinal neurons	α,β-meATP = ATP = 2-MeSATP, L-β,γ-meATP (rapid desensitisation),	TNP-ATP, Ip$_5$I, NF023, NF449	intrinsic cation channel (Ca^{2+} and Na^+)
	$P2X_2$	smooth muscle, CNS, retina, chromaffin cells, autonomic and sensory ganglia	ATP$\geq$ATPγS$\geq$2-MeSATP$>>\alpha,\beta$-meATP (pH + zinc-sensitive)	suramin, isoPPADS, RB2, NF770, NF279	intrinsic ion channel (particularly Ca^{2+})
	$P2X_3$	sensory neurones, NTS, some sympathetic neurons	2-MeSATP$\geq$ATP$\geq\alpha,\beta$-meATP$\geq$Ap$_4$A (rapid desensitisation)	TNP-ATP, PPADS, A317491, NF110, Ip$_5$I, phenol red	intrinsic cation channel
	$P2X_4$	CNS, testis, colon	ATP$>>\alpha,\beta$-meATP, CTP, Ivermectin potentiation	TNP-ATP (weak), BBG (weak), phenolphthalein	intrinsic ion channel (especially Ca^{2+})
	$P2X_5$	proliferating cells in skin, gut, bladder, thymus, spinal cord	ATP$>>\alpha,\beta$-meATP, ATPγS	suramin, PPADS, BBG	intrinsic ion channel
	$P2X_6$	CNS, motor neurons in spinal cord	(does not function as homomultimer)	–	intrinsic ion channel
	$P2X_7$	apoptotic cells in, for example, immune cells, pancreas, skin	BzATP$>$ATP$\geq$2-MeSATP$>>\alpha,\beta$-meATP	KN62, KN04, MRS2427, O-ATP Coomassie brilliant blue G	intrinsic cation channel and a large pore with prolonged activation
P2Y	$P2Y_1$	epithelial and endothelial cells, platelets, immune cells, osteoclasts	2-MeSADP= ADPβS$>$2-MeSATP = ADP$>$ATP, MRS2365	MRS2179, MRS2500, MRS2279, PIT	G_q/G_{11}; PLC-β activation
	$P2Y_2$	immune cells, epithelial and endothelial cells, kidney tubules, osteoblasts	UTP=ATP, UTPγS, INS 37217, INS 365	suramin > RB2, AR-C126313	G_q/G_{11} and possibly G_i; PLC-β activation
	$P2Y_4$	endothelial cells	UTP$\geq$ATP, UTPγS, INS 37217	RB2 > suramin	G_q/G_{11} and possibly G_i; PLC-β activation
	$P2Y_6$	some epithelial cells, placenta, T cells, thymus	UDP$>$UTP$>>$ATP, UDPβS, IDP	MRS2578	G_q/G_{11}; PLC-β activation
	$P2Y_{11}$	spleen, intestine, granulocytes	AR-C67085MX$>$BzATP$\geq$ATPγS$>$ATP	suramin > RB2, NF157, 5'-AMPS	G_q/G_{11} and G_S; PLC-β activation
	$P2Y_{12}$	platelets, glial cells	2-MeSADP$\geq$ADP$>>$ATP	CT50547, AR-C69931MX, INS49266, AZD6140, PSB0413, ARL66096, 2-MeSAMP	$G_{i/o}$; inhibition of adenylate cyclase
	$P2Y_{13}$	spleen, brain, lymph nodes, bone marrow	ADP=2-MeSADP$>>$ATP=2-MeSATP	MRS2211, 2-MeSAMP	$G_{i/o}$
	$P2Y_{14}$	placenta, adipose tissue, stomach, intestine, discrete brain regions	UDP glucose = UDP-galactose	–	G_q/G_{11}

Adaptado de Burnstock (2007)

5. O Nucleotídeo ATP

O ATP é uma molécula pertencente à família das purinas que atua tanto como fonte energética intracelular, bem como um mensageiro extracelular. Este nucleotídeo exerce diversos efeitos fisiológicos através da interação com receptores P2X e P2Y localizados na membrana plasmática, incluindo neurotransmissão, respostas vasomotoras, agregação plaquetária, resposta imune, regulação do volume celular, proliferação e mitogênese, apoptose, transporte de água e íons, metabolismo hepático e inflamação, entre outras (Novak, 2003; Omatsu-Kanbe et al., 2006). Em HSCs, a ligação do ATP com receptores P2Y resulta no aumento da transcrição de colágeno I e contração celular (Dranoff et al., 2004; Kruglov et al., 2007).

A concentração intracelular de ATP é extremamente elevada (3 a 10 mM), já sua concentração no meio extracelular é consideravelmente mais baixa, não ultrapassando a faixa micromolar. Em situações patofisiológicas a liberação de ATP e a expressão de receptores purinérgicos pelas células são consideravelmente aumentadas (Guido et al., 2008). Como este nucleotídeo não é capaz de atravessar as membranas biológicas por difusão ou transporte ativo, o controle de sua concentração extracelular é realizado pela ação das ectonucleotidases que catalisam sua degradação à adenosina (Schetinger, et al., 2007).

6. O Nucleosídeo Adenosina

Diversos trabalhos têm mostrado o envolvimento da adenosina com a fibrose hepática sugerindo a participação deste nucleosídeo na regulação da síntese de colágeno e quimiotaxia das HSCs (Chan et al., 2006; Hashmi et al., 2007).

A adenosina pode ser formada nos espaços intracelular e extracelular. Existem duas vias principais de formação da adenosina a nível intracelular: a clivagem da S-adenosil-homocisteína pela enzima S-adenosil-homocisteína-hidrolase e a degradação de AMP a adenosina por ação de uma 5'-nucleotidase citosólica. Depois de formada, a adenosina pode passar através da membrana celular por difusão facilitada, através de transportadores de nucleosídeos. Estes transportadores são bidirecionais e equilibram as concentrações de intracelulares e extracelulares de adenosina. Além disso, seus níveis intracelulares podem ser controlados pela sua fosforilação até 5'-AMP, catalisada por uma adenosina quinase e pela desaminação até inosina, catalisada pela adenosina deaminase (Dunwiddie & Masino, 2001). Em concentrações fisiológicas (1µM), a adenosina é principalmente fosforilada pela adenosina quinase que possui o K_m de 40 nM. Já quando as concentrações de adenosina alcançam níveis mais altos, a adenosina deaminase é ativada por possuir K_m e atividade específica maior que a adenosina quinase (Borowiec et al., 2006).

A adenosina também pode ser sintetizada e degradada no espaço extracelular. O ATP liberado no meio extracelular pode originar adenosina pela ação conjunta de ectoenzimas. Após interagir com receptores específicos, a adenosina pode ser

degradada pela ecto-adenosina deaminase ou removida do espaço extracelular através de sua captação pelo sistema transportador de nucleosídeos (Spychala, 2000).

7. Ectonucleotidases

As ectonucleotidases são ectoenzimas que hidrolisam nucleosídeos trifosfatados até seus respectivos nucleosídeos. Na última década, membros de diversas famílias de ectonucleotidases têm sido descobertos, clonados e funcionalmente caracterizados (Yegutkin, 2008). A cadeia das ectonucleotidases inclui as ecto-nucleosideo trifosfato difosfoidrolase (E-NTPDases), ecto-nucleotideo pirofosfatase fosfodiesterase (E-NPPs), fosfatase alcalina e ecto-5'-nucleotidase (Figura 3).
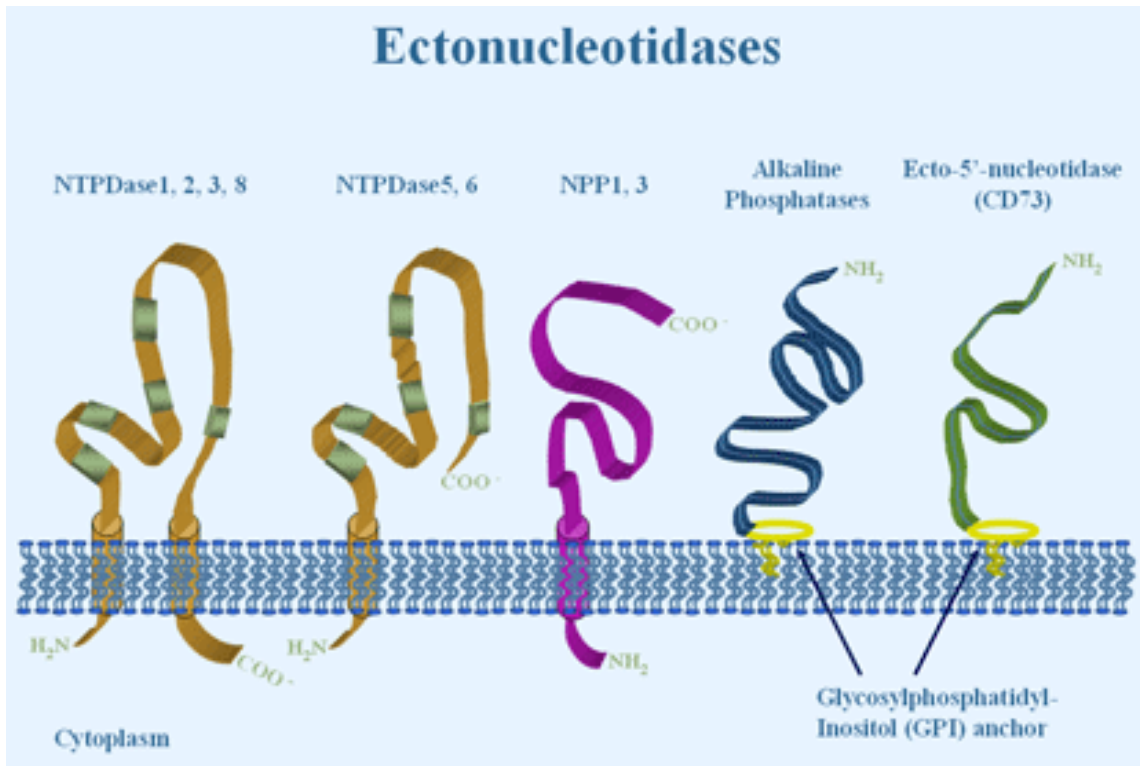
Figura 3. Topografia de membrana proposta para as ectonucleotidases. As E-NTPDases 1, 2, 3, e 8 são ligadas à membrana plasmática por dois domínios transmembrana, N e C-terminal. E-NTPDase 5 e NTPDase 6 não possuem o domínio transmembrana C-terminal e podem ser liberadas pela clivagem próximo ao domínio N-terminal, formando uma proteína solúvel. Membros da família das E-NPPs também estão ligadas à membrana pelo domínio N-terminal, podendo também ser clivadas e liberadas no meio extracelular. As quatro isoformas da fosfatase alcalina (tecido inespecífica, predominante nos rins, ossos e fígado, placental, intestinal e a das células germinativas) e a ecto-5'-nucleotidase são ancoradas à membrana por glicosilfosfatidilinositol, que pode ser clivado por fosfolipases endógenas, resultando na liberação das enzimas no meio extracelular. Todas as ectonucleotidases representadas são glicoproteínas. Fonte: http://www.crri.ca/sevigny.html (acesso em 25/06/08).

7.1. A Família E-NTPDase

Até o momento, oito membros da família das E-NTPDases foram clonados e funcionalmente caracterizados: NTPDase1 a NTPDase8 (Bigonnesse et al., 2004). A nomenclatura inicialmente proposta para estas enzimas estava relacionada com similaridade entre as seqüências de aminoácidos da apirase solúvel de batata e o antígeno de ativação celular linfóide, CD39. Em 1999, em Diepenbeek na Bélgica, durante o Segundo Workshop Internacional sobre Ecto-ATPases uma nova nomenclatura foi adotada e todos os membros da família das E-NTPDases passaram a ser referidos como NTPDases e classificados de acordo com a ordem de descoberta e caracterização. Assim, CD39 passou a chamar-se NTPDase1, CD39L1 é a NTPDase2, CD39L3 é a NTPDase3, CD39L4 é a NTPDase5 e a CD39L2 é a NTPDase6 (Zimmermann, 2001).

As NTPDases são codificadas por oito diferentes genes (*ENTPDs* em humanos e *Entpd* em camundongos) e todos os membros desta família são proteínas glicosiladas com massa molecular entre 70 e 80 kDa (Robson et al., 2006). As NTPDases 1, 2, 3, e 8 são ligadas à membrana plasmática por dois domínios transmembrana, N e C-terminal, e apresentam seu sítio catalítico voltado para o meio extracelular. As NTPDases 4 a 7 estão ancoradas à membrana de organelas intracelulares por um (NTPDase5 e 6) ou dois (NTPDase4 e 7) domínios transmembrana com seus sítios catalíticos voltados para lúmem do complexo de Golgi e retículo endoplasmático. As NTPDases 5 e 6 também estão presentes na membrana plasmática ou solúveis no meio extracelular, após clivagem próximo ao domínio N-

terminal. As NTPDases têm em comum a presença de cinco regiões conservadas, as quais são conhecidas como "regiões conservadas da apirase" (ACR1 a 5), sendo que a ACR1 e ACR4 são similares aos domínios de ligação de fosfato β e γ de proteínas como a actina, HSP70 (heat shock protein) e hexoquinase que, apesar de apresentarem funções e estruturas primárias diversas, têm em comum a habilidade de ligar e hidrolisar ATP (Kukulski et al., 2005; Yegutkin, 2008).

Todos os membros desta família hidrolisam nucleosídeos trifosfatados e difosfatados e necessitam de íons Ca^{++} ou Mg^{++} para máxima atividade. No entanto, estas enzimas diferem em suas razões de hidrólise e preferência por substrato. A NTPDase1 hidrolisa ATP e ADP igualmente, as NTPDases 3 e 8 hidrolisam mais ATP do que ADP (aproximadamente 3:1 a 2), a NTPDase2 hidrolisa preferencialmente ATP (30:1). A NTPDase4 tem baixa preferência por ATP e ADP, hidrolisando preferencialmente UDP. A NTPDase5 tem maior afinidade por nucleosídeos difosfatados do que pelos trifosfatados (UDP>GDP>CDP>ADP>ATP). A NTPDase6 também tem preferência por nucleosídeos difosfatados, sendo que a sua máxima atividade foi encontrada com GDP como substrato. A NTPDase7 tem maior afinidade por UTP, GTP e CTP (Zimmermann, 2001).

As ações biológicas das NTPDases estão relacionadas com seus efeitos sobre a sinalização mediada pelos receptores P2 e afetam diversos processos celulares, tais como proliferação, migração, adesão, diferenciação e apoptose. A identificação molecular de cada NTPDase, as análises mutacionais e a geração de anticorpos específicos têm propiciado importantes descobertas sobre a estrutura e função destas

enzimas e seu papel em diferentes tecidos, gerando muitas perspectivas para o desenvolvimento de novos tratamentos para doenças neurológicas, gastrointestinais, cardiovarculares, entre outras (Robson et al., 2006).

7.2. A Família E-NPP

A família das E-NPPs é composta por sete ectoenzimas estruturalmente relacionadas (NPP1 a NPP7) as quais são classificadas de acordo com sua ordem de descoberta. Com exceção da NPP2, todas NPPs estão ligadas à membrana por um único domínio transmembrana N-terminal e apresentam um domínio para clivagem proteolítica, sugerindo que possam ocorrer como enzimas solúveis. A NPP2 é sintetizada como pré-pró-enzima e apenas existe na forma solúvel. O peso molecular destas proteínas varia entre 110 e 125 kDa. Estas enzimas hidrolisam ligações pirofosfato e fosfodiéster em nucleotídeos e seus derivados, ácidos nucléicos, lisofosfatidilcolina e ácido lisofosfatídico (Stefan, Jansen & Bollen, 2005; 2006). No entanto, apenas os três primeiros membros desta família (NPP1, NPP2 e NPP3) são capazes de hidrolisar nucleotídeos e desempenhar relevante papel na cascata de sinalização purinérgica. A atividade catalítica *in vitro* é dependente de cátions divalentes, o pH ótimo é o alcalino e o p-nitrofenil-5'-timidina-monofosfato é usado como substrato artificial específico para as NPPs (Bollen et al., 2000).

A ampla distribuição tecidual e o envolvimento das NPPs em diversos processos fisiológicos, como mineralização óssea, câncer, proliferação e migração celular e

resistência à insulina fazem destas enzimas possíveis alvos para intervenção terapêutica (Goding, Grobben & Slegers, 2003). No entanto, muitas questões ainda precisam ser elucidadas no que se refere às suas propriedades enzimáticas, regulação e diversidade funcional.

7.3. Fosfatase Alcalina

As fosfatases alcalinas são enzimas encontradas em diversos organismos, desde bactérias até o homem. Representam uma família de ectofosfomonoesterases não específicas que atuam sobre diversos substratos fosfatados, incluindo nucleosídeos fosfatados, pirofosfato, fosfatidatos com diversas cadeias de ácido graxo, polifosfato inorgânico, glicose-6-fosfato, β-glicerofosfato e proteínas, com liberação de fosfato inorgânico (Picher et al., 2003). O pH ótimo para a atividade catalítica encontra-se na faixa alcalina, variando de 8 a 11. Quatro isoformas de fosfatase alcalina são expressas em humanos, três delas, a placental (PLALP), a intestinal (IALP) e das células germinativas (GCALP), apresentam entre 90 e 98% de homologia e são tecido-específicas. A quarta isoforma é expressa predominantemente no fígado, rins e ossos, apresenta apenas 50% de homologia com as outras isoenzimas, e é conhecida como fosfatase alcalina tecido-inespecífica (TNALP). Em camundongos as isoformas de fosfatase alcalina encontradas são a intestinal (IAP), a embrionária (EAP) e a TNALP (Millan, 2006).

A importância da TNALP no controle da mineralização óssea, através da hidrólise de PPi, um inibidor deste processo, é bem documentada (Addison et al., 2007; Hessle et al., 2002). No entanto, as funções destas enzimas em outros tecidos ainda são pouco exploradas.

7.4. Ecto-5'-nucleotidase/CD73

A ecto-5'-nucleotidase (eNT/CD73, EC 3.1.3.5) é uma proteína glicosilada ancorada à superfície externa da membrana plasmática por glicosilfosfatidilinositol e consiste de duas subunidades de 60 a 70 kDa. É expressa em diferentes tecidos, principalmente em fígado, rins, cérebro, coração e pulmão. Esta enzima hidrolisa eficientemente nucleosídeos monofosfatados, mas o AMP é seu principal substrato fisiológico, sendo que os valores de K_m estão na faixa de micromolar (Bianchi & Spychala, 2003; Strater, 2006).

A principal função fisiológica da ecto-5'-nucleotidase parece estar relacionada com sua atividade catalítica devido ao fato de ser a principal enzima responsável pela produção de adenosina no meio extracelular e, conseqüentemente, pela regulação da sinalização mediada pelos receptores P1. Isto justifica o interesse de diversos estudos sobre a modulação da expressão e atividade desta proteína em diversos tipos celulares (Babiychuk & Draeger, 2006; Hunsucker, Mitchell & Spychala, 2005; Narravula et al., 2000). Entretanto, outras funções não relacionadas com a atividade enzimática têm sido propostas, como sua atuação como molécula de adesão, mediando interação entre as células e com os componentes da matriz extracelular (Sadej et al., 2008).

8. Adenosina Deaminase

A adenosina deaminase (ADA, E.C. 3.5.4.4) é uma enzima envolvida no metabolismo das purinas por catalisar a conversão da adenosina e da deoxiadenosina a inosina e deoxiinosina, respectivamente. ADA1 e ADA2 são duas isoenzimas da adenosina deaminase. A ADA1 é amplamente expressa em diversos tecidos, o pH ótimo está entre 7 e 7,5 e o K_m para adenosina é na ordem de 50 µM. Já a ADA2 é encontrada principalmente no soro, apresenta baixa afinidade pelo substrato, o K_m para adenosina é 2 mM e o pH ótimo é 6,5. Estas características tornam esta isoenzima eficiente em sítios de inflamação, onde a concentração de substrato é alta (Gakis, 1996; Yegutkin, 2008).

A ADA é encontrada como uma enzima citosólica e também pode ser expressa na superfície celular como uma ectoenzima. A ecto-ADA é associada a uma proteína de ligação específica, identificada como uma ecto-peptidase (dipeptidil peptidase IV/CD26). A CD26 foi a primeira molécula de superfície identificada como proteína ancoradora da ecto-ADA. Posteriormente, os receptores A_1 e A_{2B} de adenosina também foram identificados como moléculas capazes de interagir com a ecto-ADA. A capacidade de interação da ecto-ADA com proteínas de superfície indica que além de atuar como ectoenzima, diminuindo os níveis extracelulares de adenosina, ela também pode estar envolvida com outros mecanismos de transdução de sinal (Hashikawa et al., 2004; Herrera et al., 2001; Pacheco et al., 2005).

9. Nucleotídeos e Ectonucleotidases no Fígado

Os nucleotídeos extracelulares estão potencialmente envolvidos com várias funções hepáticas como, por exemplo, proliferação de hepatócitos, controle da glicogenólise, regulação do fluxo sangüíneo e da secreção biliar (Dranoff et al., 2002; McGill et al., 1994; Schlenker et al., 1997). Além disto, a geração extracelular de adenosina a partir da desfosforilação do ATP pelas ectonucleotidases é essencial não só para a ativação dos receptores P1, mas também para as rotas de salvação das purinas e conseqüente restauração dos estoques de ATP dentro dos hepatócitos (Che, Gatmaitan & Arias, 1997).

Diversas ectonucleotidases são expressas pelos diferentes tipos celulares presentes no fígado em várias espécies de vertebrados. A NPP1 é expressa na membrana basolateral de hepatócitos e a NPP3 é encontrada predominantemente no canalículo biliar (Goding et al., 1998; Scott et al., 1997). A NTPDase1 é encontrada nos sinusóides hepáticos, bem como nas células de Kupffer (Sevigny et al., 2000). A NTPDase2 é expressa em fibroblastos portais e nas células endoteliais (Dranoff et al., 2002). A NTPDase8 é predominantemente encontrada no fígado, principalmente no canalículo hepático (Bigonnesse et al., 2004; Fausther et al., 2007). A ecto-5'-nucleotidase é expressa na membrana apical de hepatócitos, em HSCs e também no canalículo biliar (Schell et al., 1992; Schmid et al., 1994).

Até o presente momento, existem poucos dados a respeito da expressão da ectonucleotidases em HSCs. Já foi demonstrado que a expressão destas moléculas é modulada após a ativação das HSCs e que a sinalização mediada pelos receptores

P2Y está envolvida com a regulação da transcrição do colágeno representando, assim, um possível alvo para o tratamento da fibrose hepática (Dranoff et al., 2004).

OBJETIVOS

A ativação das células estreladas hepáticas é um processo crítico no desenvolvimento da fibrose. Alterações progressivas no metabolismo, proliferação, adesão, migração e padrão de expressão gênica das HSCs resultam no estabelecimento da cirrose e conseqüente falência do fígado. Muitas investigações têm sido feitas com o objetivo de entender as rotas de sinalização que regulam a fibrogênese e promover a prevenção e/ou tratamento destas doenças hepáticas. Neste contexto, a sinalização purinérgica tem fundamental importância na fisiologia das HSCs.

Assim sendo, o objetivo geral deste estudo foi investigar a expressão e a atividade de todas as enzimas da cadeia das ectonucleotidases nos fenótipos quiescente e ativado das HSCs e o possível efeito da adenosina sobre a modulação fenotípica destas células. O modelo experimental utilizado foi a linhagem celular GRX.

1. Objetivos Específicos

1. Estudar e comparar a hidrólise extracelular de nucleotídeos em culturas de células estreladas hepáticas.

2. Caracterizar cineticamente o metabolismo do ATP e do AMP nos dois fenótipos das HSCs.

3. Identificar as ectonucleotidases responsáveis pelo metabolismo extracelular de nucleotídeos observado nos diferentes fenótipos das HSCs.

4. Quantificar e comparar a expressão das ectonucleotidases presentes nos dois fenótipos das HSCs.

5. Silenciar a ecto-5'-nucleotidase, utilizando a técnica de RNA de interferência, para avaliar seu papel na fisiologia das HSCs.

6. Avaliar o efeito da adenosina sobre diversos parâmetros relacionados com a ativação das HSCs, como proliferação, migração, adesão e expressão do mRNA da ecto-5'-nucleotidase, colágeno tipo I e metaloproteinases.

PARTE II

CAPÍTULO 1

CHANGES IN E-NTPDase EXPRESSION PROFILE AND EXTRACELLULAR NUCLEOTIDE HYDROLYSIS DURING THE PHENOTYPE CONVERSION OF A HEPATIC STELLATE CELL LINE

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CHANGES IN E-NTPDase EXPRESSION PROFILE AND EXTRACELLULAR NUCLEOTIDE HYDROLYSIS DURING THE PHENOTYPE CONVERSION OF A HEPATIC STELLATE CELL LINE

Cláudia M. B. Andrade[1], Márcia R. Wink[2], Rogério Margis[1], Radovan Borojevic[3], Ana Maria O. Battastini[1] and Fátima C R Guma[1]

[1]Departamento de Bioquímica, Instituto de Ciências Básicas da Saúde, UFRGS. Porto Alegre, RS, Brazil

[2]Departamento de Fisiologia, Faculdade Federal de Ciências Médicas de Porto Alegre, FFFCMPA. Porto Alegre, RS, Brazil

[3]Departamento de Histologia e Embriologia, ICB, e PABCAM, Hospital Universitário Clementino Fraga Filho, UFRJ, Rio de Janeiro, RJ, Brazil

*Corresponding author: Dr. Fátima T. C. R. Guma

        Departamento de Bioquimica – ICBS – UFRGS

        Rua Ramiro Barcelos, 2600-anexo

        CEP 90035-003

        Porto Alegre, RS, BRAZIL

        Fone +55 51 3316 5546

        FAX +55 51 3316 5535

        e-mail: fatima.guma@ufrgs.br

ABSTRACT

Hepatic stellate cells (HSC) are recognized as a primary cellular source of matrix components in chronic liver disease and, therefore, play a critical role in the development and maintenance of liver fibrosis. Nucleotides and nucleosides are signaling molecules that regulate a variety of activities within the liver and play a role in the pathogenesis of hepatic fibrosis. Ecto-nucleoside triphosphate diphosphohydrolases (E-NTPDases) are ecto-enzymes that are located at the cell surface and regulate the concentration of extracellular nucleotides, thereby modulating their biological effects by the activation of P2Y and P2X receptors. We have identified and compared the expressions of E-NTPDase family members in two different phenotypes of a mouse hepatic stellate cell line (GRX). Two phenotypes of GRX cells expressed NTPDase3 and 5. However, only activated HSC expressed NTPDase6. In quiescent-like HSC, the hydrolysis of triphosphonucleosides was significantly higher and was related to an increase in *Entpd*3 mRNA expression. The diphosphonucleosides were hydrolyzed at a similar rate by two phenotypes of GRX cells and this hydrolysis was associated with an up-regulation of *Entpd*5 mRNA expression in quiescent-like HSC, whilst *Entpd*6 mRNA expression was observed only in activated HSC. The differential E-NTPDase activities and expressions in two phenotypes of GRX cells suggest that these enzymes modulate the concentration of nucleotides/nucleosides and affect P2-receptor signaling differently in the two phenotypes and may play a role in the regulation of HSC functions.

Keywords: Ecto-nucleoside triphosphate diphosphohydrolase, E-NTPDases GRX cell line, hepatic stellate cells, nucleotide metabolism

INTRODUCTION

Hepatic stellate cells (HSC) are recognized as a primary cellular source of matrix components in chronic liver disease and, therefore, play a critical role in the development and maintenance of liver fibrosis (Friedman, 2003; Moreira, 2007). In the healthy liver, HSC express a quiescent phenotype and are responsible for the storage of vitamin A located within the subendothelial space of Disse (Geerts, 2001). During fibrosis, HSC become active and transform into proliferating fibroblast-like cells that display increased proliferation and migration, enhanced matrix protein production and increased production of matrix metalloproteinases and tissue inhibitors of metalloproteinases, all of which lead to replacement by interstitial collagen or a scar matrix (Friedman, 2008; Kisseleva and Brenner, 2006). Although many studies have focused on signaling pathways that regulate fibrogenesis by HSC, there is still no effective treatment for liver fibrosis (Friedman, 2004; Gabele et al., 2003; Prosser et al., 2006).

Extracellular nucleotides and nucleosides are signaling molecules that regulate several physiological functions by interacting with specific purinoreceptors (Yegutkin, 2008). In actived HSC, nucleotides have been associated with the regulation of procollagen-1 transcription (Dranoff et al., 2004). In addition, HSC express P2Y receptors, linking extracellular ATP to inositol triphosphate-mediated cytosolic calcium signals and inducing morphological changes in cells, characteristic of cell contraction (Kruglov et al., 2007). However, adenosine inhibits the increase in HSC cytosolic calcium concentration induced by ATP and is a potent stop signal for chemotaxis, localizing HSC to the site of tissue injury and inducing HSC differentiation (Hashmi et al., 2007).

Ecto-nucleoside triphosphate diphosphohydrolase (E-NTPDases) describes a family of eight mammalian enzymes that catalyze the hydrolysis of γ and β-phosphate residues of nucleotides with different abilities (Zimmermann, 2001). They regulate the concentration of extracellular nucleotides and, thereby, modulate their biological effects by the activation of P2Y and P2X receptors (Kukulski et al., 2005).

These enzymes are tightly bound to the plasma membrane and/or to intracellular organelles by one or two transmembrane domains. NTPDases 1, 2, 3 and 8 have two plasma membrane spanning domains with an active site facing the extracellular milieu (Bigonnesse et al., 2004; Robson et al., 2006)Zimmermann, 2001). NTPDases 4-7 are anchored to the membranes of intracellular organelles by one (NTPDase5 and 6) or two (NTPDase4 and 7) transmembrane domains and their catalytic sites face the lumen of intracellular compartments, such as the Golgi apparatus and the endoplasmic reticulum. NTPDases 5 and 6 may also be found on the plasma membrane and are secreted following proteolytic cleavage (Braun et al., 2000; Hicks-Berger et al., 2000; Ivanenkov et al., 2003; Murphy-Piedmonte et al., 2005; Shi et al., 2001).

Although several studies have shown the presence of NTPDases1,2 and 8 in the liver, their expression patterns and physiological role in HSC remain unclear. Thus, in the present study, we identified and compared the expressions of E-NTPDase family members in two different phenotypes of the mouse hepatic stellate cell line, GRX, and evaluated the nucleotide hydrolysis by these cells.

MATERIALS AND METHODS

Cell cultures

GRX cells were obtained from the Rio de Janeiro Cell Bank (PABCAM, Federal University, Rio de Janeiro, Brazil). For nucleotide hydrolysis experiments, $3x10^4$ cells/well were seeded into 24-well culture plates and, for gene expression experiments, $1x10^5$ cells/well were seeded into 6-well culture plates for 7 days (Nunc, Roskilde, Denmark). Cells were maintained in Dulbecco's culture medium (DMEM) (GIBCO, Grand Island, New York, USA) with 2 g/L HEPES buffer (Sigma Chemical Company, St Louis, MO, USA), supplemented with 5% fetal bovine serum (Cultilab, Campinas, SP, Brazil), pH 7.4, in a humidified atmosphere with 5% CO_2. In the experimental series, the quiescent-like phenotype was induced by treatment for seven days with 5 μM of *all-trans*-retinol (Margis and Borojevic, 1989) dissolved in ethanol (0.1% final concentration) (both from Sigma Chemical Company, St Louis, MO, USA). The concentration of *all-trans*-retinol in the stock solution was determined by ultra-violet absorption at 325 nm, using the molar extinction coefficient (ϵ) of 52.770 $cm^{-1}M^{-1}$.

NTPDase activity assay

To determine the ATP and ADPase extecellular hydrolysis, 24 multiwell plates containing GRX cultures were incubated, as described previously (Andrade et al., 2008) in the incubation medium containing 2 mM $CaCl_2$, 120 mM NaCl, 5 mM KCl, 10 mM glucose, 20 mM Hepes, pH 7.4 and 1 mM of ATP, GTP, CTP, UTP, ADP, CDP or UDP at 37°C. The release of inorganic phosphate (Pi) was measured as previously described (Chan et al., 1986). The non-enzymatic Pi

released from nucleotide into assay medium without cells and Pi released from cells incubated without nucleotide were subtracted from the total Pi released during incubation, providing net values for the enzyme activities. All samples were run in triplicate. Specific activity is expressed as nmol Pi released/min/mg of protein.

Protein determination

Cells in the 24 multiwell plates were dried and solubilized with 100 μL of NaOH 1N and frozen overnight. An aliquot was then taken and the protein was measured by the Coomassie blue method (Bradford, 1976), using bovine serum albumin as standard.

RNA extraction, cDNA synthesis and RT-PCR analysis

RNA was isolated using the TRIzol Reagent (Invitrogen, Carlsbad, California, USA). Approximately 2 μg of total RNA were added to each cDNA synthesis reaction using the SuperScript-II RT pre-amplification system (Invitrogen). Reactions were performed at 42°C for 1 h using the primer T23V (5' TTT TTT TTT TTT TTT TTT TTT TTV). PCRs were carried out in a 20 μl final volume composed of 10 μl of each reverse transcription sample diluted 50 to 100 times, 2 μl of 10 times PCR buffer, 1.2 μl of 50 mM MgCl$_2$, 0.4 μl of 5 mM dNTPs, 0.4 μl of 10 μM primer pairs, 5.90 μl of water and 0.1 μl of Platinum Taq DNA polymerase (5 U/μl) (Invitrogen). Reaction settings were composed of an initial denaturation step of 5 min at 94°C, followed by 35 cycles of 30 s at 94°C, 30 s at 60°C, 30 s at 72°C and a final 10 min extension at 72°C. The PCR products were separated by 2.0% agarose gel electrophoresis and visualized by ethidium

bromide staining. PCR amplification was carried out using specific primer pairs for *Entpd1* (*CD39*), *Entpd2* (*CD39L1*), *Entpd3* (*CD39L3*), *Entpd5* (*CD39L4*) and *Entpd6* (*CD39L2*), described by (Vollmayer et al., 2001) and synthesized by RW-Genes (RJ, Brazil). For *Entpd*8, the sequences of the primers were 5' TAAGGGCCAGAAGGATTGTG3' and 5'CCCAGGATAGCTGACTTCCA3' and *β-actin*: 5´TATGCCAACACAGTGCTGTCTGG3´ and 5´ TACTCCTGCTTGCTGATCCACAT 3´. Negative controls were performed with water as template and positive controls were plasmids with cDNA sequences for mouse *Entpd1*, rat *Entpd2*, human *NTPDase3*, *NTPDase5* and *NTPDase6* (Wink et al., 2006) and, for *Entpd8,* cDNA from mouse liver was used.

Real-time PCR

Total RNA and cDNA were generated, as described in RT-PCR analysis. Real-time PCRs were carried out in an Applied-Biosystem 7500 real-time cycler and performed in quadruplicate. Reaction settings were constituted by an initial denaturation step of 5 min at 94°C, followed by 40 cycles of 10 s at 94°C, 15 s at 60°C, 15 s at 72°C and 35 s at 60°C for data acquisition; samples were kept for 2 min at 40°C for annealing and then heated from 55 to 99°C with a ramp of 0.1°C/sec to acquire data to produce the denaturing curve of the amplified products. Real-time PCRs were carried out in a 20 µl final volume composed of 10 µl of each reverse transcription sample diluted 50 to 100 times, 2 µl of 10 times PCR buffer, 1.2 µl of 50 mM $MgCl_2$, 0.4 µl of 5 mM dNTPs, 0.4 µl of 10 µM primer pairs, 3.95 µl of water, 2.0 µl of SYBRgreen (1:10,000 Molecular Probe), and 0.05 µl of Platinum Taq DNA polymerase (5 U/µl) (Invitrogen). The primers used for real time PCRs were the same used in RT-PCR analysis. All

results were analyzed by the 2 $^{-\Delta\Delta CT}$ method (Livak and Schmittgen, 2001) . β-actin was used as the internal control gene for all relative expression calculations (Guimaraes et al., 2006).

Immunocytochemistry

Cells were cultured until semi-confluence on glass coverslips, fixed with 4% paraformaldehyde for 30 min and then permeabilized with 0.1% Triton X-100 in PBS for 5 min. Cells were washed with PBS and incubated with 3% BSA in PBS for 1h at 37 °C to block non-specific binding sites. They were then incubated with primary anti-NTPDases polyclonal antibodies (Wink et al., 2006) (1:100) overnight at 4 °C. After washing, samples were incubated with secondary goat anti-rabbit fluorescein conjugate (Calbiochem, San Diego, California, USA) (1:1000) for 2 h at room temperature. Negative controls were performed without primary antibody. DNA was stained with 0.2 µg/ mL DAPI for 2 min. After a final washing with PBS, coverslips were mounted with FluorSave (Calbiochem). Images were captured using a Nikon Eclipse E600 microscope equipped with a digital camera Nikon DXM1200C.

Statistical Analysis

All experiments were performed in triplicate or quadruplicate. Data represent mean ± standard error of mean (SEM). Statistical analysis was performed employing Student's *t*-test, using the statistical program SPSS 15.0 for Windows. The values were considered statistically different when $P<0.05$.

RESULTS

Ectonucleotidase activity and substrate specifity

The GRX-HSC cell line, employed as an experimental model, presents a myofibroblast-like phenotype in basal medium and is known to convert to quiescent-like HSC cells when treated with retinol for 7 days. Under such conditions, neutral lipids and retinyl esters accumulate in perinuclear lipid droplets, reducing activation markers, decreasing proliferation and collagen production, and reprogramming the metabolism (Andrade et al., 2003; Guimaraes et al., 2007; Margis and Borojevic, 1989; Vicente et al., 1998; Vicente et al., 1997). During treatment with retinol, the cultures were observed daily by phase contrast microscopy to monitor morphological alterations. After 7 days, when all analyses were performed, nearly all cells exhibited the lipocyte or quiescent phenotype.

The hydrolysis of the different triphosphonucleosides (NTPs) and diphosphonucleosides (NDPs) was determined in two phenotypes of GRX cells, by measuring the amount of Pi liberated *in vitro* from the exogenous extracellular nucleotides. All conditions, such as cation dependence, substrate saturation and incubation time were previously determined in order to ensure the linearity of reaction (data not shown).

The hydrolysis of NTPs in quiescent-like HSC was significantly higher when compared with activated HSC (Fig.1). Of the NTPs, ATP was the preferred substrate. NDPs were hydrolyzed at a similar rate by the two phenotypes of GRX cells, where UDP was the preferred substrate (Fig. 1).

These results suggest either the presence of enzymes with different potentials for the hydrolysis of both NTPs and NDPs or a differential expression of the same enzymes by activated and quiescent-like HSC.

Entpd mRNA expression in GRX cells

Since tri- and diphosphonucleosides can be metabolized by different members of the E-NTPDase family (Zimmermann, 2001), we investigated the mRNA expressions of *Entpd* 1, 2, 3, 5, 6 and 8 in GRX cells. Using specific primers, oligonucleotide fragments were amplified and analyzed on agarose gels.

The two phenotypes of the GRX cells synthesized mRNA for *Entpd*3 and *Entpd*5; however, only activated HSC synthesized mRNA for *Entpd*6. The *Entpd*1, 2 and 8 expressions were undetectable in the two phenotypes of GRX cells, begin observed only in positive controls. The lengths of the oligonucleotide fragments obtained for the samples were comparable to those obtained for the positive controls and corresponded to the expected size (Fig. 2).

Quantitative real-time PCR analysis

In order to compare the level of mRNA expression of *Entpd*3 and 5 between activated and quiescent-like HSC and correlate these with the ectonucleotidase activity observed, we performed quantitative real-time PCR analysis.

The results showed that, in quiescent-like HSC, the *Entpd*3 and *Entpd*5 transcriptions were increased by 2.8 and 3.3 fold, respectively, in comparison to activated HSC (Fig. 3). These data support the hypothesis that the increase in NTPs hydrolysis observed in quiescent-like HSC may be related to an up-

regulation of the *Entpd*3 mRNA expression. On the other hand, the increase in *Entpd*5 in quiescent-like HSC, associated with the expression of *Entpd*6 mRNA only in activated HSC, may explain the similar rate of NDP hydrolysis observed in the different GRX cell phenotypes.

NTPDase protein expression in GRX cells

To analyze whether the protein level expression corresponded to the mRNA expression of E-NTPDases in GRX cells, as detected by RT-PCR, cells were fixed and analyzed by immunoflourescence.

In agreement with the RT-PCR analysis, activated HSC displayed a strong labeling by polyclonal anti-NTPDase 3, 5 and 6 antibodies, while the quiescent-like HSC displayed an immunolabelling for anti-NTPDase 3 and 5 on the surface of the cells (Fig. 4 and 5). No significant immunostaining of GRX cells was observed in control experiments, where only secondary antibody was applied. The experiments revealed an intense labeling with a punctuated and granular distribution pattern around the nucleous and a faint labeling of the cell membrane.

DISCUSSION

Hepatic stellate cells play a crucial role in the development of liver fibrosis and are important targets of liver disease therapy. However, despite great advances in the understanding of liver fibrosis, there are still gaps in the understanding of hepatic stellate cell physiology and cell biology.

The ecto-nucleoside triphosphate diphosphohydrolases are widely regarded as the major enzymes responsible for the hydrolysis of extracellular nucleotides

and, thus, as one of the control mechanisms regulating P2 receptor nucleotide signaling. As such, considering the pivotal role of HSC in the fibrotic process and the importance of purinergic signaling in this context, we identified the expressed NTPDases responsible for the nucleotide metabolism in two phenotypes of the mouse hepatic stellate cell line.

Our data demonstrate that GRX cells express members of the E-NTPDase family. We have previously shown that GRX cells express mRNA for ecto-5'-nucleotidase/CD73 and alkaline phosphatase and exhibit catalytic activity for AMP (Andrade et al., 2008). In addition, GRX cells also express members of the E-NPP family (C. M. B. Andrade, unpublished work). Taken together, these data demonstrate that these cells contain all components of the enzymatic cascade necessary for the complete metabolism of extracellular nucleotides.

Quiescent-like HSC expressed NTPDases 3 and 5 and these enzymes were able to efficiently hydrolyze all tri- and diphosphonucleosides. The hydrolysis ratio of ATP/ADP was 2.97, in accordance with the expression of NTPDase3, which catalyzes the hydrolysis of ATP and ADP, producing a ratio of 3:1. This hydrolysis differs considerably from those of NTPDase1 (approximately 1:1) and NTPDase2 (30:1) (Zimmermann, 2001) and may explain the absence of *Entpd* 1 and 2 mRNA expression in GRX cells. NTPDase5 exhibits a higher affinity for nucleoside diphosphate than for nucleoside triphosphate, with preferences for UDP, GDP and IDP (Mulero et al., 1999; Murphy-Piedmonte et al., 2005). The expression of NTPDase5 in quiescent-like HSC suggests that this enzyme could contribute to the degradation of NDPs and could be the most important enzyme responsible for the UDP hydrolysis presented by cells.

In activated HSC, the ATP/ADP rate was 1.41. However, considering the lower expression of NTPDase3 in this phenotype, it is likely that ATPase/ADPase rate is a consequence of the participation of the enzymes capable of hydrolyzing ADP. NTPDase6, like NTPDase5, prefers NDPs to NTPs as substrate (Braun et al., 2000) and, as such, these two enzymes are probably responsible for the NDP hydrolysis in activated HSC. However, since NTPDase5 demonstrates lower expression in this phenotype, it is possible that NTPDase6 is largely responsible for the NDP hydrolysis in activated HSC. In accordance with this finding, an expression of *Entpd*6 mRNA was also observed in activated rat HSC (Dranoff et al., 2004).

Thus, NTPs may be hydrolyzed mainly by NTPDase3 in GRX cells and the increase in the NTPs hydrolysis observed in quiescent-like HSC is probably related to the up-regulation of *Entpd*3 mRNA expression. Consistent with these observations, COS-7 cells transfected with plasmid encoding mouse *Entpd*3 presented similar ATP and UTP hydrolysis profiles to those observed in GRX cells (Kukulski et al., 2005). In addition, NDPs could be hydrolyzed in GRX cell line by NTPDases 3, 5 and 6; however, the similar level of NDPs hydrolysis in the two GRX cell phenotypes may be due to the presence of NTPDase5 in both phenotypes and NTPDase6 in activated HSC. Furthermore, the higher UDP hydrolysis, in relation to ADP hydrolysis, corroborates this hypothesis. Thus, the E-NTPDase expression profile reported in GRX cells is in accordance with the catalytic activity observed on the surface of cells.

The absence of *Entpd*1 and 2 mRNA expression in HSC has already been demonstrate by (Dranoff et al., 2002); however, this result contrasts to that reported in rat HSC primary cultures, where activated HSC were observed to

express NTPDase2, and both quiescent and activated cells may express NTPDase1 (Dranoff et al., 2004). Therefore, it remains unclear whether these enzymes are expressed or not in HSC primary cultures.

NTPDases 4 and 7 were not investigated in this study, since these proteins have exclusively intracellular localization and, thus, cannot be responsible for nucleotide degradation at the surface of GRX cells (Biederbick et al., 2000; Shi et al., 2001). The tissue distribution of mouse *Entpd*8 mRNA, analyzed with total RNA from various mouse tissues, showed that enzyme is predominantly expressed in liver and lower levels of expression were detected in the jejunum and kidney (Bigonnesse et al., 2004). Our results confirm the expression of *Entpd*8 mRNA in mouse total liver and demonstrate that this enzyme is not expressed in HSC.

It is known that the distribution of E-NTPDases varies with specific cell types in the liver. NTPDase1 is expressed on larger vessels, sinusoids and Kupffer cells (Sevigny et al., 2000). NTPDase2 is expressed by cells in the subendothelium of veins, adventitial cells of arteries and portal fibroblasts (Dranoff et al., 2002). NTPDase8 is present in the hepatic canalicule and low expression of *Entpd*3 mRNA has been demonstrated in the liver (Bigonnesse et al., 2004; Robson et al., 2006).

Therefore, the main finding of the present work is that the two phenotypes of HSC differentially express members of the E-NTPDase family. These data suggest that these enzymes have a different impact on the formation of adenosine in activated and quiescent-like HSC. Moreover, the modulation of the concentration of nucleotides/nucleosides in a different manner in two phenotypes may affect P2-receptor signaling distinctly and affect a number of

biological functions, such as cell proliferation, adhesion, activation and migration (Bagchi et al., 2005; Erb et al., 2006; Kaczmarek et al., 1996; Klawitter et al., 2007; Takemura et al., 1994).

In conclusion, we herein described a striking difference in the extracellular nucleotide degradation rates between quiescent-like and activated HSC and showed that this difference is related to differences in the E-NTPDase expressions presented by the two cell types. As such, our results suggest that this enzyme family may play a role in the regulation of HSC functions and may constitute possible targets for the prevention or treatment of hepatic fibrosis.

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FIGURE LEGENDS

Fig. 1 Nucleotide hydrolysis by GRX cells. Myofibroblasts (MYO) and quiescent-like HSC (ROH) were submitted to enzymatic assay, as described in Materials and Methods. Specific activity values are expressed as nmol Pi/ min/ mg protein. Data represent the means of three independent experiments performed in triplicate $\pm$ S.E.M. *significantly different from control (MYO) at $P<$ 0.05, as determined by Student's t-test.

Fig. 2 RT-PCR analysis of *Entpd* gene expressions by GRX cells. Total RNA was isolated from myofibroblasts (MYO) and quiescent-like HSC (ROH) and cDNA was analyzed, as described in Materials and methods. The length (bp) of the PCR products obtained with each pair of primers is given in the figure. (C+) positive control; (C-) negative control.

Fig. 3 Quantitative real-time PCR analysis of *Entpd* genes by GRX cells. The expressions of *Entpds* members in myofibroblast (MYO) and quiescent-like HSC (ROH) were analyzed by real-time PCR, as described in Materials and Methods. Data represent mean $\pm$ S.E.M. *significantly different from MYO at $P<$ 0.05, as determined by Student's t-test.

Fig. 4 Immunofluorescence analysis of E-NTPDase expressions in myofibroblasts. Cells were cultured on glass coverslips, fixed in paraformaldehyde and incubated with polyclonal antibodies (1:100), as described in Materials and Methods. NTPDases (green) and DAPI/DNA (blue). Magnification: 100x. Scale bar = 20 μm
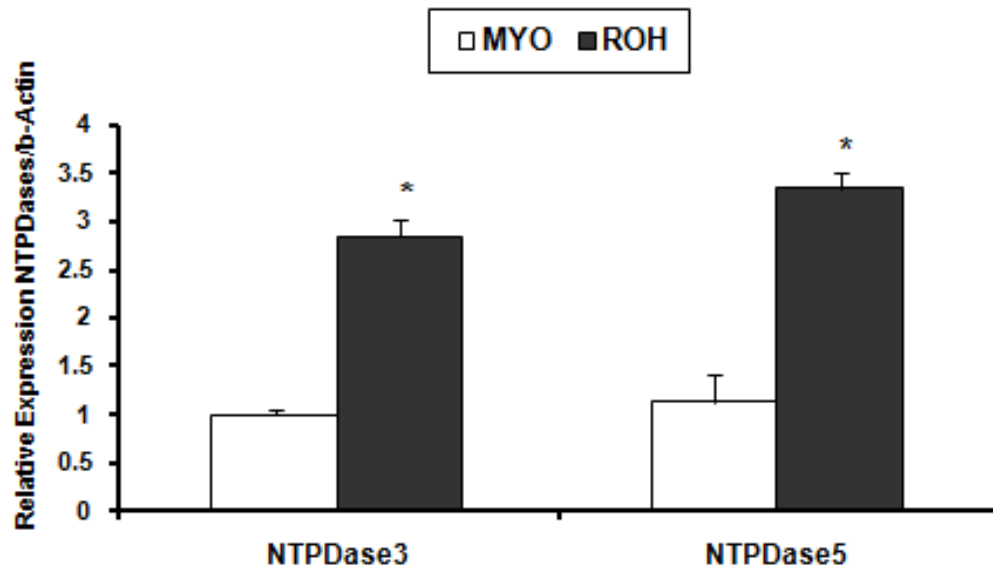
Fig. 5 Immunofluorescence analysis of E-NTPDase expressions in quiescent-like HSC. Cells were cultured on glass coverslips, fixed in paraformaldehyde and incubated with polyclonal antibodies (1:100), as described in Materials and Methods. NTPDases (green) and DAPI/DNA (blue). Magnification: 100x. Scale bar = 20 μm.

FIG. 1

FIG. 2

NTPDase1 (CD39)

NTPDase2 (CD39L1)

NTPDase3 (CD39L3)

NTPDase5 (CD39L4)

NTPDase6 (CD39L2)

NTPDase8
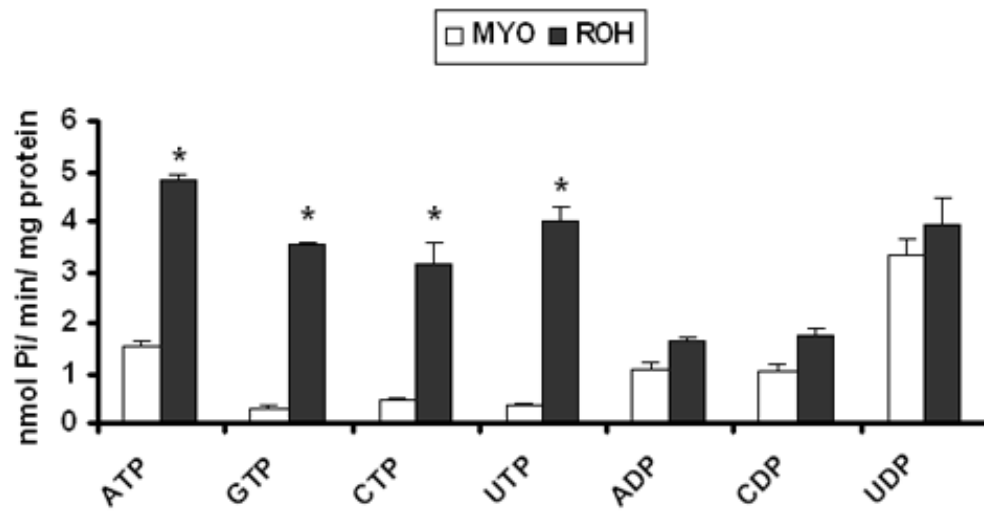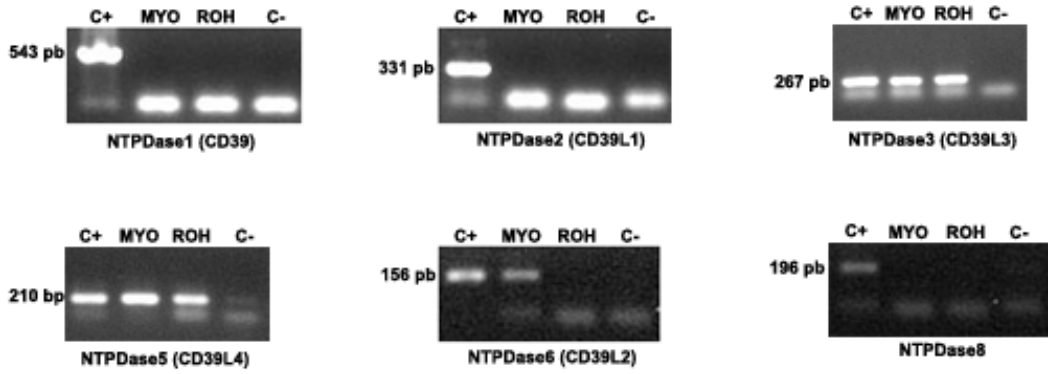
FIG. 3

FIG. 4
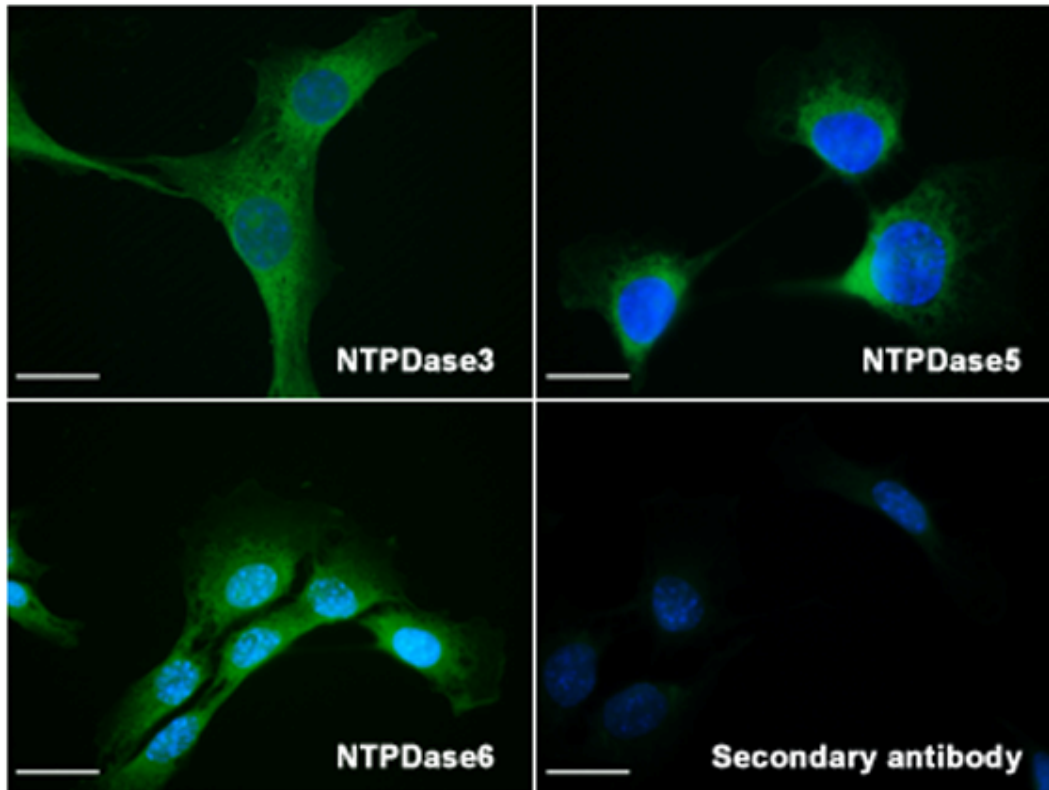
FIG. 5
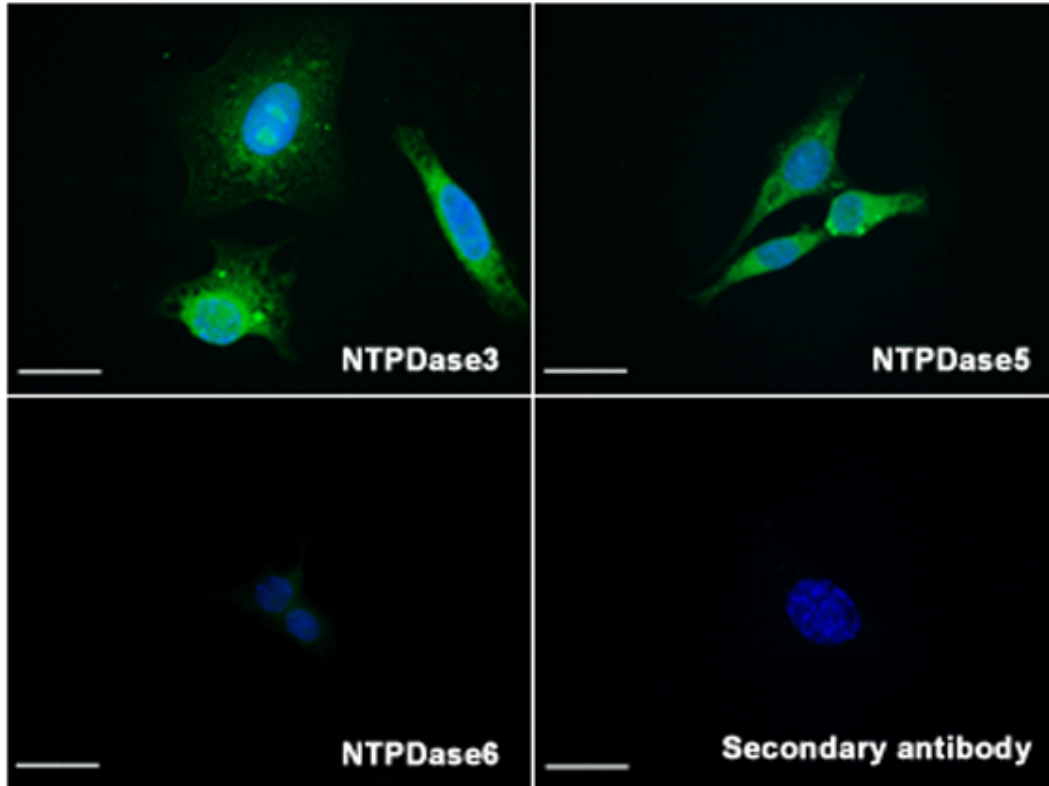
CAPÍTULO 2

ACTIVITY AND EXPRESSION OF ECTO-NUCLEOTIDE

PYROPHOSPHATE/PHOSPHODIESTERASES IN A HEPATIC STELLATE CELL

LINE

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ACTIVITY AND EXPRESSION OF ECTO-NUCLEOTIDE PYROPHOSPHATE/PHOSPHODIESTERASES IN A HEPATIC STELLATE CELL LINE

Cláudia M. B. Andrade[1], Márcia R. Wink[2], Rogério Margis[1], Radovan Borojevic[3], Ana Maria O. Battastini[1] and Fátima C R Guma[1]

[1]Departamento de Bioquímica, Instituto de Ciências Básicas da Saúde, UFRGS. Porto Alegre, RS, Brazil

[2]Departamento de Fisiologia, Faculdade Federal de Ciências Médicas de Porto Alegre, FFFCMPA. Porto Alegre, RS, Brazil

[3]Departamento de Histologia e Embriologia, ICB, e PABCAM, Hospital Universitário Clementino Fraga Filho, UFRJ, Rio de Janeiro, RJ, Brazil

Running Title: **E-NPPs IN A HEPATIC STELLATE CELL LINE**

* Corresponding author: Dr. Fátima T. C. R. Guma

                Departamento de Bioquimica – ICBS – UFRGS

                Rua Ramiro Barcelos, 2600-anexo

                CEP 90035-003

                Porto Alegre, RS, BRAZIL

                Fone +55 51 3316 5546

                FAX +55 51 3316 5535

                e-mail: fatima.guma@ufrgs.br

SUMMARY

Nucleotides and nucleosides represent an important and ubiquitous class of molecules that interact with specific receptors, regulating a variety of activities within the liver and play a role in the pathogenesis of hepatic fibrosis. Ecto-nucleotide pyrophosphatase/phosphodiesterases (E-NPPs) are ecto-enzymes that are located at the cell surface. NPP1-3 have been implicated in the hydrolysis of nucleotide and together other ectonucleotidases, control the events induced by extracellular nucleotides. We have identified and compared the expression of E-NPP family members in two different phenotypes of the mouse hepatic stellate cell line (GRX). In quiescent-like hepatic stellate cells (HSCs), the E-NPP activity was significantly higher, NPP2 mRNA expression decreased and NPP3 mRNA increased when compared with myofibroblasts. The differential NPPs activity and expression in two phenotypes of GRX cells suggest that they are involved in the regulation of extracellular nucleotide metabolism in hepatic stellate cells. However, the role of E-NPPs in the liver remains to be clarified.

INTRODUCTION

Nucleotides and nucleosides represent an important and ubiquitous class of molecules that interact with specific receptors, regulating a variety of functions in the extracellular environment [1,2]. The events induced by extracellular nucleotides are controlled by the action of ecto-nucleotidases (E-NTPDase, E-NPPs, ecto-5'-nucleotidase/CD73 and ecto-alkaline phosphatase), which hydrolyze ATP into adenosine in the extracellular space [3].

The family of ecto-nucleotide pyrophosphatase/phosphodiesterases (E-NPPs) consists of seven structurally-related ecto-enzymes that are located at the cell surface, either expressed as transmembrane proteins or as secreted enzymes [4]. They hydrolyze pyrophosphate or phosphodiester bonds in a variety of extracellular compounds including nucleotides, lysophospholipids and choline phosphate esters. Only NPP1-3 have been implicated in the hydrolysis of nucleotide. NPP2 has also intrinsic lysophospholipase D activity that results in the generation of lysophosphatidic acid (LPA) from lysophosphatidylcholine. Likewise, NPP6 and NPP7 are choline phosphate esterases. NPP4 and NPP5 have been identified as additional members of the E-NPP family, but little is known concerning their catalytic activity. [3-5].

NPP1-3 have been detected in almost all tissues, although individual isoforms are usually confined to specific substructures and/or cell types [5]. In addition, NPP members have been localized in different cellular compartments and differentially targeted the plasma membrane of polarized cells, suggesting a specific physiological function of NPPs in cells and tissues [6].

Liver fibrosis may be considered as a dynamic and integrated cellular response to chronic liver injury. The activation of hepatic stellate cells (HSCs) and the consequent deposition of large amounts of extracellular matrix play a role in the fibrogenic process. Although new anti-fibrotic therapies have been under investigation during recent years [7-9], many questions still remain unanswered, including the identification of novel mechanisms regulating matrix deposition and HSC proliferation.

Although it has been frequently reported that adenine nucleotides and nucleosides regulate a variety of activities within the liver and adenosine plays a role in the pathogenesis of hepatic fibrosis [10-14], data concerning the presence of ecto-nucleotide pyrophosphatase/phosphodiesterase enzymes in the liver are very limited. Thus, in the present study, we identified and compared the expression of E-NPP family members in two different phenotypes of the mouse hepatic stellate cell line, GRX, in order to provide new information about the regulation of nucleotide signaling pathways in liver.

RESULTS and DISCUSSION

Conversion of GRX cells to quiescent-like HSCs

The experimental model, GRX-HSC cell line, presents a myofibroblast-like phenotype in basal medium and is known to be converted to quiescent-like HSC cells when treated with retinol for 7 days. Under such conditions, neutral lipids and retinyl esters accumulate in perinuclear lipids droplets, reducing activation markers, decreasing proliferation and collagen production, and reprogramming the metabolism [15-23]. During treatment with retinol, the cultures were observed daily by phase contrast

microscopy to monitor morphological alterations. After 7 days, when all analyses were performed, nearly all cells exhibited the lipocyte or quiescent phenotype.

_p_-Nph-5'-TMP hydrolysis by E-NPPs in GRX cells

Members of E-NPP family generally have wider substrate specificity than intracellular pyrophosphatases and phosphodiesterases. First, we investigated the hydrolysis of _p_-Nph-5'-TMP in the two phenotypes of GRX cells. The p-nitrophenyl ester of TMP is used routinely for the _in vitro_ assay of E-NPP [5]. All conditions, such as cation dependence, substrate saturation and incubation time were previously determined in order to ensure the linearity of reaction (data not shown).

Table 1 shows that the maximal _p_-Nph-5'-TMP hydrolysis occurs at alkaline pH (8.9), which is in accordance with previously data for E-NPPs [5], while a low activity was observed at physiological pH (7.4), demonstrating the presence of E-NPP activity in GRX cells. Another important finding is that the _p_-Nph-5'-TMP hydrolysis in quiescent-like HSC was significantly higher when compared with myofibroblasts (Table 2). This enzyme activity, in association with the ecto-5'-nucletidase, also present in the GRX cell [24], may modulate the adenosine generation and the nucleotide/nucleoside signaling and may play a role in the regulation of quiescent HSC functions.

Analysis of extracellular ATP metabolism

The products of ATP hydrolysis from GRX cells were analyzed by HPLC (Fig.1). The extracellular ATP was efficiently metabolized by GRX cells with subsequent ADP, AMP and adenosine production. At the end of the incubation time an aliquot of the medium

was used for measurement of LDH activity, to assess cellular viability. No membrane damage occurred during assay (data not shown), indicating that the products found are not the consequence of leakage from cells.

At the end of the incubation time (60 min), the compounds detected in the incubation medium of activated HSC were ADP, AMP and adenosine represented 16%, 8% and 4%, respectively, of the ATP concentration added to the cells. In quiescent-like HSC the levels of ADP, AMP and adenosine detected were 19%, 20% and 9%, respectively, of the ATP concentration added to the cells, demonstrated that in quiescent-like HSC the rate of exogenous ATP decay was higher than in activated HSC. Moreover, the increase in AMP amount in 30 and 60 min for quiescent-like HSC may suggest the presence of E-NPP, which hydrolyzes ATP into AMP and PPi. This result is in agreement with the increase of p-Nph-5'-TMP hydrolysis observed in this phenotype.

mRNA expression and quantitative analyses of E-NPPs in GRX cells

As p-Nph-5'-TMP can be metabolized by three different members of the E-NPP family, we investigated its mRNA expression in GRX cells. The mRNAs for NPP1, NPP2 and NPP3 were detected in the two phenotypes of GRX cells. In order to compare the different levels of mRNA expression of E-NPPs in quiescent-like HSC and myofibroblasts, we performed quantitative real-time RT-PCR analysis. Fig. 2 shows that, after 7 days, in parallel with the establishment of the quiescent-like phenotype, retinol treatment induced a decrease in NPP2 mRNA and an increase in NPP3 mRNA. In contrast, no alteration in NPP1 transcription was detected.

NPP1-3 have a relatively broad tissue distribution in mammals and have been implicated in a variety of cellular processes. NPP1, previously known as PC-1, is likely to be involved in bone and matrix mineralization, soft tissue calcification and insulin resistance [25,26]. NPP2, or autotoxin, was revealed to be a key enzyme in the production of lysophosphatidic acid (LPA), a lipid mediator with multiple biological actions. In liver, LPA stimulates proliferation, contraction and migration and inhibits apoptosis in rat HSCs. Recent studies showed that plasma LPA level and serum autotoxin activity were increased in rats with various liver injuries in relation to their severity [27,28]. NPP2 was also shown to stimulate cell motility, cell growth and to augment the invasive and metastic potential of *ras*-transformed cells [29,30]. NPP3 (gp130 [RB13-6] or B10), expressed on the apical surface of hepatocytes, is proposed to function in the recycling of nucleotides from bile in rodents. NPP3 has also been suggested to be marker of adenocarcinoma derived from biliary cells [4,5].

The observed levels of NPPs transcripts (Fig. 2) in the two GRX phenotypes indicate that the increase in the NPPs activity (Table 2) during the phenotype conversion is due to the NPP3 isoform. The changes in NPP2 expression, in combination with the increase in NPP3 expression and activity during the phenotype conversion of GRX cells, is in agreement with the quiescent-like cell physiological characteristics, such as, their decreased proliferation rate and migration, reorganization of the cytoskeleton and modifications of expression of a specific set of genes. Moreover, the decreases in NPP2 mRNA may represent a decrease in LPA production in quiescent phenotype resulting in modulation of signaling pathway by which LPA acts, in particular those involving protein

kinase B and small GTPases (Ras, RhoA, Rac1 and Cdc42), affecting the survival, proliferation, contraction and migration of the cells.

PPi Hydrolysis by GRX cells

Since E-NPP hydrolyzes ATP into AMP and PPi, to examine whether GRX cells can also hydrolyze PPi, we incubated the cultures with this substrate. Fig. 3 demonstrates that GRX cells hydrolyze PPi and that the hydrolysis in quiescent-like HSC was significantly higher than in myofibroblasts. It is known that tissue-nonspecific alkaline phosphatase (TNALP) is able to hydrolyze PPi into Pi [6]. Previously, we demonstrated that the transcriptional level and the activity of this enzyme were increased after treatment of GRX cells with retinol [24]. Taken together, these data suggest that quiescent-like HSC are able to hydrolyze ATP by the action of E-NPPs, generating PPi, which may be the substrate of TNALP, more efficiently than myofibroblasts.

The differential NPPs activity and expression in two phenotypes of GRX cells may be involved in the regulation of the extracellular nucleotide metabolism, directly interfering in the termination of nucleotide signaling and promoting the salvage of nucleotides and/or the generation of new messengers such as AMP, adenosine and pyrophosphate (Fig. 4).

Although the role of E-NPPs in the liver remains to be clarified, the present study describes, for the first time, the activities of these enzymes in hepatic stellate cells. As such, our results may indicate new possibilities for research into the physiological roles of this enzyme family in liver and their possible therapeutic potential in hepatic fibrosis.

MATERIALS AND METHODS

Cell cultures

GRX cells were obtained from the Rio de Janeiro Cell Bank (PABCAM, Federal University, Rio de Janeiro, Brazil). Cells were maintained in Dulbecco's culture medium (DMEM) (GIBCO, Grand Island, New York, USA) with 2 g/L HEPES buffer (Sigma Chemical Company, St Louis, MO, USA), supplemented with 5% fetal bovine serum (Cultilab, Campinas, SP, Brazil), pH 7.4, in a humidified atmosphere with 5% CO_2. In the experimental series, the quiescent-like phenotype was induced by treatment for seven days with 5 µM of *all-trans*-retinol [16] dissolved in ethanol (0.1% final concentration) (both from Sigma Chemical Company, St Louis, MO, USA). The concentration of *all-trans*-retinol in the stock solution was determined by ultraviolet absorption at 325 nm, using the molar extinction coefficient (ϵ) of 52.770 $cm^{-1}M^{-1}$.

Assay of Ecto-Nucleotide Pyrophosphatase/ Phosphodiesterase activity

The phosphodiesterase activity was assessed using *p*-nitrophenyl thymidine 5'-monophosphate (*p*-Nph-5'-TMP), an artificial substrate marker for E-NPPs, (Sigma Chemical Company, St Louis, MO, USA), as described by Vollmayer [31] . The cells were incubated with reaction medium containing 50 mM Tris-HCl buffer, 5 mM KCl, 120 mM NaCl, 0.5 mM $CaCl_2$, 60 mM glucose, pH 7.4 and 8.9, and 0.5 mM of *p*-Nph-5'-TMP. After 40 min of incubation, the reaction was stopped with 200 µL 0.2 M NaOH. Enzyme activities were expressed as nmol *p*-nitrophenol released per minute per milligram of protein.

Analysis of extracellular ATP metabolism by HPLC

After 7 days of culture, the cellular monolayers were washed three times with incubation medium containing 2 mM $CaCl_2$, 120 mM NaCl, 5 mM KCl, 10 mM glucose, 20 mM Hepes, pH 7.4. The reaction was started by adding ATP at a final concentration of 100 μM. The final volume was 300μL and the incubation was carried out at 37°C. After different times of incubation (0, 30, and 60 min), the supernatant was taken, cooled and centrifuged at 4°C for 15 min at 16,000xg. The separation of purines was carried out from 50 μL of incubation sample, according to a previously described method [32] with modifications. Briefly, purines were determined by a reverse-phase HPLC system (Shimadzu Instrument Liquid Chromatograph, Shimadzu, Japan) equipped with a C-18 column (Supelcosil[TM] LC-18, Supelco[®] 25 cm × 4.6 mm) and UV detector. The elution program consisted of an isocratic gradient of buffer A (KH_2PO_4 60 mM, 5 mM of tetrabutylammonium phosphate, pH 6.0) and buffer B (buffer A plus methanol 30%) at a flow rate of 1.25 ml/min. The amounts of purines were measured by absorption at 254 nm and all peaks were identified by their retention time and by comparison with standards. The products of ATP hydrolysis were expressed as percentage of the ATP concentration added to the cells. All incubations were carried out in triplicate. Controls to correct the non-enzymatic hydrolysis of ATP were done by measuring the peaks presents in the same reaction medium incubated without cells. Controls for cellular purine secretion were carried out by incubating the cells without substrate in the same condition described above. Under our experimental conditions, GRX cells secreted only inosine into the extracellular medium. The levels of secreted inosine in myofibroblasts (actived HSC) and quiescent-like HSC were 1.32 ± 0.076 and 2.61 ± 0.64 nmol/mg protein, respectively. These values were discounted from incubations with ATP.

Cell viability was assessed by the measurement of lactate dehydrogenase (LDH) activity in the incubation medium after the end of the experimental procedures, according to the procedure of Whitaker [33].

Inorganic Pyrophosphate (PPi) hydrolysis

GRX cells were incubated with reaction medium containing 2 mM $MgCl_2$, 120 mM NaCl, 5 mM KCl, 10 mM glucose, 20 mM Hepes, pH 7.4 and 1 mM of PPi at 37°C. After 40 min of incubation, the reaction was stopped with ice cold TCA (5% w/v) [34]. The release of inorganic phosphate (Pi) was measured by the malachite green method [35], using KH_2PO_4 as a Pi standard. The non-enzymatic Pi released and Pi released from cells incubated without nucleotide were subtracted from the total Pi released during incubation, providing net values for enzymatic activity. All samples were run in triplicate. Specific activity is expressed as nmol Pi released/min/mg of protein.

Protein determination

Protein was measured by the Coomassie blue method [36], using bovine serum albumin as standard.

RNA extraction, cDNA synthesis and Real-time PCR

RNA was isolated using the TRIzol Reagent (Invitrogen, Carlsbad, California, USA). Approximately 2 µg of total RNA were added to each cDNA synthesis reaction using the SuperScript-II RT pre-amplification system (Invitrogen, Carlsbad, California, USA). Reactions were performed at 42°C for 1 h using the primer T23V (5' TTT TTT TTT TTT

TTT TTT TTT TTV). Real-time PCR amplification was carried out using specific primer pairs designed with Oligo Calculator version 3.02 (http://www.basic.nwu.edu/biotools/oligocalc.html) and synthesized by RW-Genes (RJ, Brazil). The sequences of the primers used are listed in Table 2. Real-time PCRs were carried out in an Applied-Biosystem 7500 real-time cycler and performed in quadruplicate. Reaction settings were composed of an initial denaturation step of 5 min at 94°C, followed by 40 cycles of 10 s at 94°C, 15 s at 60°C, 15 s at 72°C and 35 s at 60°C for data acquisition; samples were kept for 2 min at 40°C for annealing and then heated from 55 to 99°C with a ramp of 0.1°C/sec to acquire data to produce the denaturing curve of the amplified products. Real-time PCRs were carried out in a 20 µl final volume composed of 10 µl of each reverse transcription sample diluted 50 to 100 times, 2 µl of 10 times PCR buffer, 1.2 µl of 50 mM $MgCl_2$, 0.4 µl of 5 mM dNTPs, 0.4 µl of 10 µM primer pairs, 3.95 µl of water, 2.0 µl of SYBRgreen (1:10,000 Molecular Probe), and 0.05 µl of Platinum Taq DNA polymerase (5 U/µl) (Invitrogen). All results were analyzed by the $2^{-\Delta\Delta CT}$ method [37]. β-actin was used as the internal control gene for all relative expression calculations [23]. The Real-time PCR products were separated by 2.0% agarose gel electrophoresis and visualized by ethidium bromide staining.

Statistical Analysis

All experiments were performed in triplicate or quadruplicate. Data represent mean ± standard error of mean (SEM). Statistical analysis was performed by Student's *t*-test,

using the statistical program, SPSS 15.0 for Windows. The values were considered statistically different when $P<0.05$.

Acknowledgements

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Table 1. Ecto-Nucleotide Pyrophosphate Phosphodiesterase Activity. Enzyme activities were determined in GRX cultures using p-nitrophenyl thymidine 5' monophosphate (p-Nph-5'-TMP) as substrate. The results are expressed as nmol p-nitrophenol release per minute per milligram of protein.

Cell Type	pH	
	Optimal (8.9)	Physiological (7.4)
	nmol p-nitrophenol/min/mg protein	
Myo	4.7 ± 0.070	0.75 ± 0.027
ROH	6.20 ± 0.193*	1.20± 0.023*

*p-Nph-5'-TMP hydrolysis in the ROH group was significantly different from the Myo group ($P<0.05$, Student's t-test)

Table 2. Primers used for real-time polymerase chain reaction

	Forward and Reverse primers
NPP1	5' GAATTCTTGAGTGGCTACAGCTTCCTA 3'
	5'CTCTAGAAATGCTGGGTTTGGCTCCCGGCA 3'
NPP2	5' GAAAATGCCTGTCACTGCTC 3'
	5'GCTGTAATCCATAGCGGTTG 3'
NPP3	5' AGCCGCCGGTTATCTTGTTCTC 3'
	5' TGATGCCGGTGCGACTCTGGATAC 3'
β-actin	5' TATGCCAACACAGTGCTGTCTGG 3'
	5' TACTCCTGCTTGCTGATCCACAT 3'

FIGURE LEGENDS

Figure 1: Kinetics of extracellular ATP degradation in GRX cells. Activated HSC (A), quiescent-like HSC (B) were incubated with 100 μM ATP, as described in Materials and methods. The presence of ATP, ADP, AMP, and adenosine were determined after separation by HPLC. The compounds of the purine cascade were identified and measured by comparison with their respective standards. Results were expressed as percentage of the ATP concentration added to the cells. The data are mean $\pm$ S.E.M. of two different experiments performed in triplicate. In some instances, error bars are too small to be visible.

Figure 2: E-NPPs gene expression in GRX cells. Total RNA was isolated from Myofibroblast (MYO) and quiescent-like HSC (ROH) and the expression of E-NPPs was quantitatively analyzed by real-time RT-PCR, as described in Materials and Methods. Data represent mean $\pm$ S.E.M. *significantly different from control (MYO) at $P < 0.05$, as determined by Student's t-test.

Figure 3: Extracellular hydrolysis of PPi by myofibroblast (MYO) and quiescent-like HSC (ROH). GRX cells culture were incubated with reaction medium described in Materials and Methods and 1mM of PPi as substrate. Specific activity values were expressed as nmol Pi/mim/mg protein. Data represent means of three independent experiments performed in triplicate $\pm$ S.E.M. *significantly different from control (MYO) at $P < 0.05$, as determined by Student's t-test.

Figure 4: Proposed mechanism for the participation of E-NPPs in purinergic signaling in GRX cells. NPPs hydrolyze nucleoside triphosphate (NTP) into monophosphate (AMP) and pyrophosphate (PPi). PPi is hydrolyzed into Pi by tissue-nonspecific alkaline phosphatase (TNALP). AMP is hydrolyzed into adenosine (ADO) by ecto-5'-nucleotidase (5'NU). ADO activates P1 receptors or is internalized by nucleoside transporters.
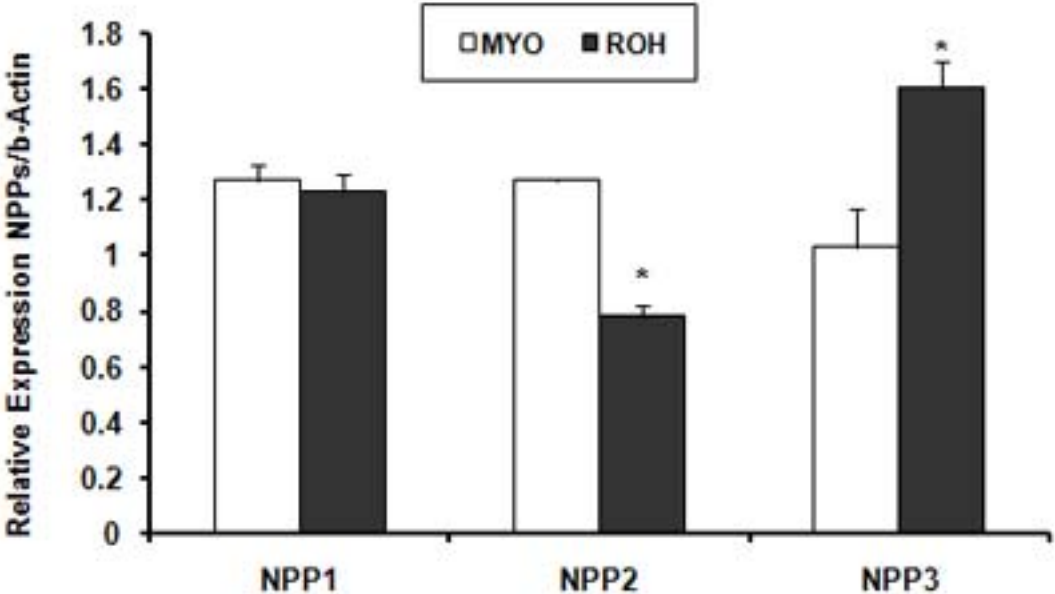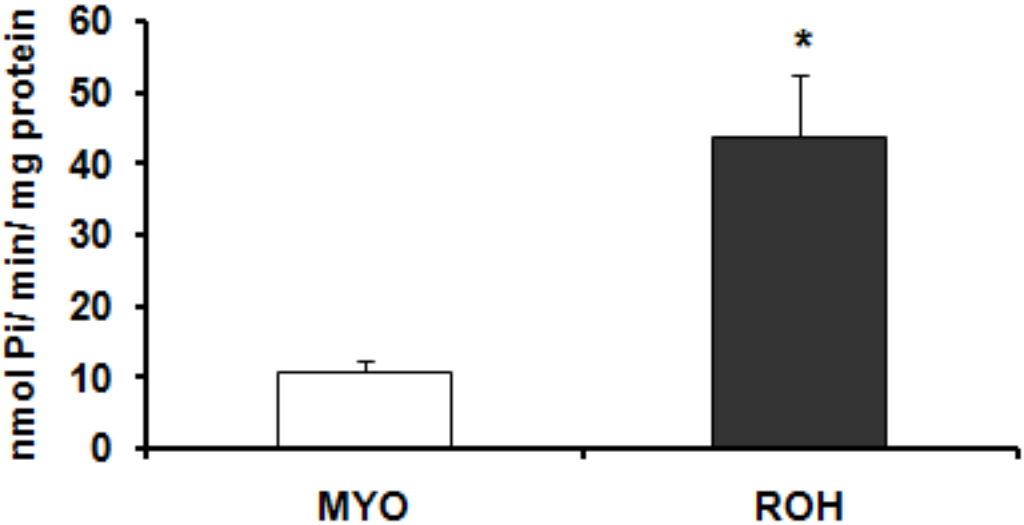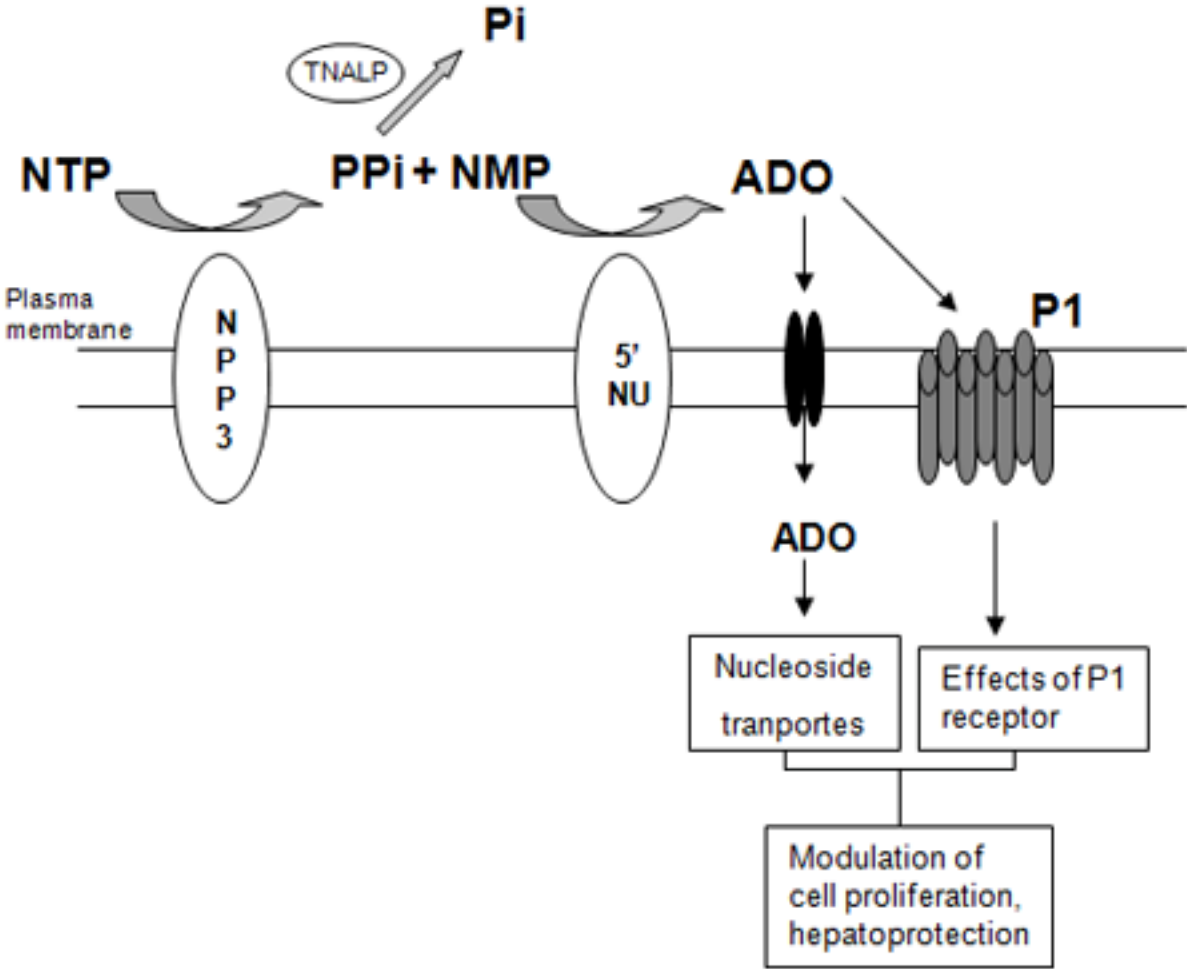
Figure 1
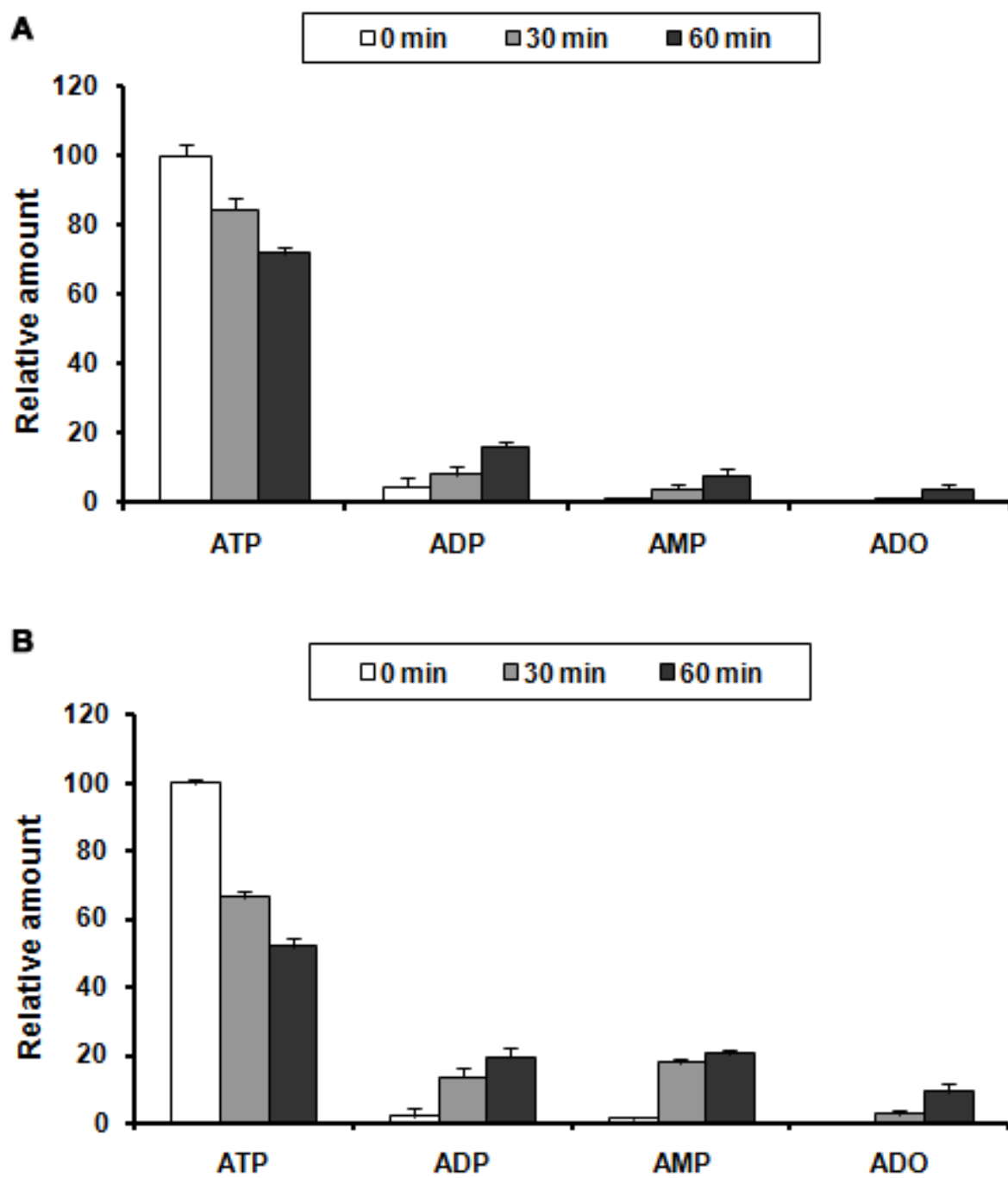
Figure 2

Figure 3

Figure 4

CAPÍTULO 3

ACTIVITY AND EXPRESSION OF ECTO-5'-NUCLEOTIDASE/CD73 ARE INCREASED DURING PHENOTYPE CONVERSION OF A HEPATIC STELLATE CELL LINE

Artigo publicado

Activity and expression of ecto-5′-nucleotidase/CD73 are increased during phenotype conversion of a hepatic stellate cell line

Cláudia M.B. Andrade [a], Gislaine C. Roesch [a], Márcia R. Wink [b], Eduardo L.M. Guimarães [a], Luiz F. Souza [a], Fernanda Rafaela Jardim [a], Regina M. Guaragna [a], Elena A. Bernard [a], Rogério Margis [a], Radovan Borojevic [c], Ana Maria O. Battastini [a], Fátima C.R. Guma [a,*]

[a] *Departamento de Bioquímica, Instituto de Ciências Básicas da Saúde, UFRGS. Porto Alegre, RS, Brazil*
[b] *Departamento de Fisiologia, Faculdade Federal de Ciências Médicas de Porto Alegre, FFFCMPA. Porto Alegre, RS, Brazil*
[c] *Departamento de Histologia e Embriologia, ICB, e PABCAM, Hospital Universitário Clementino Fraga Filho, UFRJ, Rio de Janeiro, RJ, Brazil*

Abstract

Hepatic stellate cells (HSC) play a crucial role in the development of liver fibrosis and are important targets in liver disease therapy. Adenosine acts as an extracellular signaling molecule in various tissues and in liver this nucleoside exerts protective effects. Ecto-5′-nucleotidase/CD73 is a marker for the plasma membrane and is considered to be a key enzyme in the generation of adenosine in the extracellular medium, by transforming AMP into adenosine. In addition, adenosine production from AMP is also catalyzed by alkaline phosphatase. We compared the extracellular metabolism of AMP and transcriptional levels of the ecto-5′-nucleotidase/CD73 and tissue non-specific alkaline phosphatase (TNALP) in activated and quiescent HSC of the mouse hepatic stellate cell line GRX. This cell line expresses a myofibroblast phenotype in basal medium and both retinol and indomethacin treatment induced a phenotypic change of GRX cells to quiescent HSC. Ecto-5′-nucleotidase activity and its mRNA expression were found to be higher in quiescent HSC than in activated HSC. During phenotype conversion, mediated by retinol, the AMP decay was accelerated with adenosine accumulation in extracellular medium, likely due to the decrease in adenosine deaminase activity also observed in quiescent HSC. The treatment with retinol also involves transcriptional activation of TNALP. Taken together, these data suggest that ecto-5′-nucleotidase-dependent adenosine generation may play a role in the regulation of quiescent HSC functions.
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Keywords: Ecto-5′-nucleotidase/CD73; TNALP; Adenosine; Hepatic stellate cell line

Introduction

Hepatic fibrosis is a wound-healing process after chronic liver injury, and is characterized by increased deposition and altered composition of extracellular matrix (Iredale, 2001; Yang et al., 2003). Hepatic stellate cells (HSC) are presently regarded as one of the key cell types involved in the progression of liver fibrosis. In physiological conditions, HSC synthesize low levels of matrix proteins, store retinyl esters in lipid droplets in their cytoplasm, regulate both transport and storage of retinoids, have low levels of proliferation and are named "quiescent". In pathological conditions such as in liver fibrosis, HSC lose retinoids, proliferate and transform to a myofibroblast-like phenotype, a process termed activation. Activated HSC express α-smooth muscle actin (α-SMA) and procollagen I and are known to be the major source of collagens and other matrix proteins that are deposited in fibrosis (Vogel et al., 2000; Senoo, 2004). Although new anti-fibrotic therapies have been under investigation during recent years (Issa et al., 2001; Bedossa and Paradis, 2003; Prosser et al., 2006), many questions still remain unanswered, such as the identification of novel mechanisms regulating matrix deposition and HSC proliferation.

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* Corresponding author. Departamento de Bioquimica – ICBS – UFRGS, Rua Ramiro Barcelos, 2600-anexo, CEP 90035-003, Porto Alegre, RS, Brazil. Tel.: +55 51 3308 5546; fax: +55 51 3308 5535.
E-mail address: fatima.guma@ufrgs.br (F.C.R. Guma).

In vitro models of primary HSC cultures are appropriate for the study of activation and induction of the myofibroblast phenotype, principally because the contact with the culture dish surface and the discontinuity of cell–cell contacts elicits the loss of the fat droplets, proliferation and enhanced secretion of extracellular matrix. Thus, the mechanisms that maintain the HSC in the quiescent phenotype and the formation of lipids droplets can only be studied in an established cell line. The first HSC line obtained was GRX, established from the spontaneous immortalization of HSC using limiting dilution cloning of primary cells obtained from murine liver (Borojevic et al., 1985). These cells present a myofibroblast-like phenotype in basal culture medium, which can be converted to the quiescent-like phenotype by treatment with retinoids or indomethacin, with an overall increase of lipid storage (Margis and Borojevic, 1989; Guaragna et al., 1991). The induction mechanism elicited by retinol corresponds to the hyperplasia of hepatic fat-storing cells i.e. to the relative increase of resting stellate cells observed in vivo in hypervitaminosis-A, and involves the retinoid signaling pathway via RARs (Vicente et al., 1997). The signaling mechanism involved in indomethacin treatment inducing increase in PPARγ expression and producing an increase in adipsin transcription, a typical adipogenis marker (Guimarães et al., 2007). Although both compounds mediate the accumulation of lipid droplets, this occurs by different modifications in the gene expression programme of these cells. Retinol induced lipid synthesis and storage without affecting characteristic adipocytic genes, while indomethacin treatment restores the lipocytic phenotype with increase adipsin expression.

Nucleotides and nucleosides represent an important and ubiquitous class of extracellular molecules that interact with specific receptors, regulating a variety of cellular activities in the liver (Arnaud et al., 2003, 2004; Dranoff et al., 2004). It has already been shown that adenosine can act as a hepatoprotective molecule by enhancing collagenolytic activity and stimulating hepatocyte proliferation (Hernandez-Munoz et al., 1990, 1997, 2001). Many of the effects that adenosine exerts, such as the ability to suppress proinflammatory cytokines production and release and modulate cellular proliferation, are consistent with the concept that adenosine may exert anti-fibrotic effects in the liver (Hasko and Cronstein, 2004; Borowiec et al., 2006). Recent evidence indicates that adenosine blocks cytosolic elevations in Ca^{++} concentration in HSC and is a potent stop signal for chemotaxis, identifying a novel role for adenosine in liver fibrosis by localizing HSC to the site of tissue injury and inducing HSC differentiation (Hashmi et al., 2007).

The events induced by extracellular adenine nucleotides are controlled by the action of ecto-nucleotidases (E-NTPDase, E-NPPs, ecto-5′-nucleotidase/CD73 and ecto-alkaline phosphatase), which hydrolyze ATP into adenosine in the extracellular space (Zimmermann, 2000).

Ecto-5′-nucleotidase/CD73 (ecto-5′-NT/CD73, EC 3.1.3.5) is a homodimer linked to the plasma membrane through a glycosyl-phosphatidylinositol lipid anchor, with its catalytic site exposed to the extracellular space. The enzyme hydrolyzes extracellular nucleoside monophosphates to their respective nucleosides, with AMP being considered the major physiolog-ical substrate and also the best-characterized enzymatic source of adenosine (Zimmermann, 1992).

In addition, AMP can be hydrolyzed by a family of non-specific ectophosphomonoesterases (ALP, EC 3.1.3.1), also named alkaline phosphatase (Picher et al., 2003). Human ALPs are encoded by four different gene loci and three of the four isozymes are tissue-specific, the intestinal (IALP), placental (PLALP), and germ cell (GCALP), whereas the fourth ALP is tissue non-specific (TNALP) and is expressed in relatively high amounts in bone, liver, and kidney (Martins et al., 2001). In mouse, alkaline phosphatase isoforms are intestinal (IAP), embryonic (EAP) and the tissue non-specific (TNALP) or "liver-bone-kidney type" AP (Millán, 2006).

Both ecto-5′-nucleotidase/CD73 and alkaline phosphatase are expressed in many tissues and co-localize with the detergent-resistant and glycolipid-rich membrane subdomain, called the lipid raft, which are important for controlling signal transduction and membrane trafficking (Bianchi and Spychala, 2003; Matsuoka and Ohkubo, 2004).

Thus, considering the pivotal role of HSC in the fibrotic process and the possible anti-fibrogenic effect of adenosine, the aim of this study was to compare the extracellular metabolism of AMP and transcriptional levels of the ecto-5′-nucleotidase/CD73 and the tissue non-specific alkaline phosphatase (TNALP), in the different phenotypes of the mouse hepatic stellate cell line, GRX.

Materials and methods

Cell cultures

GRX cells were obtained from the Rio de Janeiro Cell Bank (PABCAM, Federal University, Rio de Janeiro, Brazil). For nucleotide hydrolysis experiments, 3×10^4 cells/well were seeded into 24-well culture plates and for gene expression experiments, 1×10^5 cells/well were seeded into 6-well culture plates (Nunc, Roskilde, Denmark). Cells were maintained in Dulbecco's culture medium (DMEM) (GIBCO, Grand Island, New York, USA) with 2 g/L HEPES buffer (Sigma Chemical Company, St Louis, MO, USA), supplemented with 5% fetal bovine serum (Cultilab, Campinas, SP, Brazil), pH 7.4, in a humidified atmosphere with 5% CO_2. In the experimental series, the quiescent-like phenotype was induced by treatment for 7 days with 5 μM of all-*trans*-retinol (Margis and Borojevic, 1989) or 130 μM of indomethacin (Guaragna et al., 1991) both dissolved in ethanol (0.1% final concentration) (both from Sigma Chemical Company, St Louis, MO, USA). The concentration of all-*trans*-retinol in the stock solution was determined by ultra-violet absorption at 325 nm, using the molar extinction coefficient (ε) of 52,770 cm^{-1} M^{-1}. Intracellular lipid droplets were identified by standard staining with the lipotropic dye Oil-Red-O. The proliferation rate was determined by direct counting on a haemocytometer after 2, 4 and 6 days.

Ecto-5′-nucleotidase/CD73 assay

To determine AMP hydrolysis, the 24 multiwell plates, containing GRX cells cultured as indicated above, were washed

three times with phosphate free incubation medium in the absence of nucleotides. The reaction was started by the addition of 200 µL of the incubation medium containing 2 mM $MgCl_2$, 120 mM NaCl, 5 mM KCl, 10 mM glucose, 20 mM Hepes, pH 7.4 and 1 mM of AMP at 37 °C. Alternatively, AMP hydrolysis was monitored in the presence of α,β-methylene adenosine diphosphate (AOPCP) 100 µM, an inhibitor of ecto-5′-nucleotidase (Gelain et al., 2003).

After 40 min of incubation, the reaction was stopped by taking an aliquot of the incubation medium and transferring to a tube containing TCA (5% w/v) previously placed on ice (Wink et al., 2003). The release of inorganic phosphate (Pi) was measured by the malachite green method (Chan et al., 1986), using KH_2PO_4 as a Pi standard. The non-enzymatic Pi released from nucleotide into assay medium without cells and Pi released from cells incubated without nucleotide was subtracted from the total Pi released during incubation, giving net values for enzymatic activity. All samples were run in triplicate. Specific activity is expressed as nmol Pi released/min/mg of protein.

Ecto-phosphatase assay

The extracellular phosphatase activity was determined in the same medium described for AMP hydrolysis except that 1 mM of glucose-6-phosphate or β-glycerophosphate was used as substrate (Millán, 2006). Alternatively, the AMP hydrolysis was monitored in the presence of 1 mM levamisole, a specific inhibitor of tissue non-specific alkaline phosphatase (Scheibe et al., 2000).

Analysis of extracellular AMP metabolism by HPLC

After 7 days of culture, the cellular monolayers were washed three times with ecto-5′-nucleotidase incubation medium. The reaction was started by adding AMP at a final concentration of 100 µM. The final volume was 300 µL and the incubation was carried out at 37 °C. After different times of incubation (0, 10, 30, 60, 90 and 120 min), the supernatant was taken, cooled and centrifuged at 4 °C for 15 min at 16,000 ×g. The separation of purines was carried out from 50 µL of incubation sample, according to a previously described method (Gelain et al., 2003) with modifications. Briefly, purines were determined by a reverse-phase HPLC system (Shimadzu Instrument Liquid Chromatograph, Shimadzu, Japan) equipped with a C-18 column (Supelcosil™ LC-18, Supelco® 25 cm×4.6 mm) and UV detector. The elution program consisted of an isocratic gradient of buffer A (KH_2PO_4 60 mM, 5 mM of tetrabutylammonium phosphate, pH 6.0) and buffer B (buffer A plus methanol 30%) at a flow rate of 1.25 mL/min. The amounts of purines were measured by absorption at 254 nm and all peaks were identified by their retention time and by comparison with standards. The degradative products were expressed as nmol/mg of protein. All incubations were carried out in triplicate. Controls to correct the non-enzymatic hydrolysis of AMP were done by measuring the peaks presents in the same reaction medium incubated without cells. Controls for cellular purine secretion were carried out by incubating the cells without substrate in the same condition described above. Under our

experimental conditions, GRX cells secreted only inosine into the extracellular medium. The levels of secreted inosine in myofibroblasts (actived HSC) and quiescent-like HSC induced by indomethacin or retinol were 1.32 ± 0.076, 0.9 ± 0.23, 2.61 ± 0.64 nmol/mg protein, respectively. These values were discounted from incubations with AMP. Cell viability was assessed by the measurement of lactate dehydrogenase (LDH) activity in the incubation medium after the end of the experimental procedures, according to the procedure of Whitaker (1969).

Adenosine deaminase (ADA) assay

Adenosine deaminase activity was determined by HPLC, as described above for the analysis of extracellular AMP metabolism, except that 100 µM adenosine was used as substrate. All samples were run in triplicate. Specific activity is expressed as nmol inosine/min/mg of protein.

Protein determination

Cells in the 24 multiwell plates were dried and solubilized with 100 µL of NaOH 1 N and frozen overnight. An aliquot was then taken and the protein was measured by the Coomassie blue method (Bradford, 1976), using bovine serum albumin as standard.

RNA extraction, cDNA synthesis and real-time PCR

RNA was isolated from GRX cells using the TRIzol Reagent (Invitrogen). Approximately 2 µg of total RNA was added to each cDNA synthesis reaction using the SuperScript-II RT preamplification system (Invitrogen). Reactions were performed at 42 °C for 1 h using the primer T23V (5′ TTT TTT TTT TTT TTT TTT TTT TTV). Real-time PCR amplification was carried out using specific primer pairs designed with Oligo Calculator version 3.02 (http://www.basic.nwu.edu/biotools/oligocalc.html) and synthesized by RW-Genes (RJ, Brazil). The following sets of primers were used: for ecto-5′-nucleotidase/CD73: 5′ CACGGGGGGCCTCTAGCACATCA3′ and 5′GCCTGGACCACAGGCACCTG3′ (amplification product: 403 bp), tissue non-specific alkaline phosphatase (TNALP): 5′AAGGGCCAGCTACACCACAACACG3′ and 5′GCCCACGGACTTCCCAGCATC3′ (amplification product: 267 bp) and for β-actin: 5′TATGCCAACACAGTGCTGTCTGG3′ and 5′TACTCCTGCTTGCTGATCCACAT3′ (amplification product: 195 bp). Real-time PCRs were carried out in an Applied-Biosystem 7500 real-time cycler and done in quadruplicate. Reaction settings were composed of an initial denaturation step of 5 min at 94 °C, followed by 40 cycles of 10 s at 94 °C, 15 s at 60 °C, 15 s at 72 °C and 35 s at 60 °C for data acquisition; samples were kept for 2 min at 40 °C for annealing and then heated from 55 to 99 °C with a ramp of 0.1 °C/s to acquire data to produce the denaturing curve of the amplified products. Real-time PCRs were made in a 20 µL final volume composed of 10 µL of each reverse transcription sample diluted 50 to 100 times, 2 µL of 10 times PCR buffer, 1.2 µL of 50 mM $MgCl_2$,
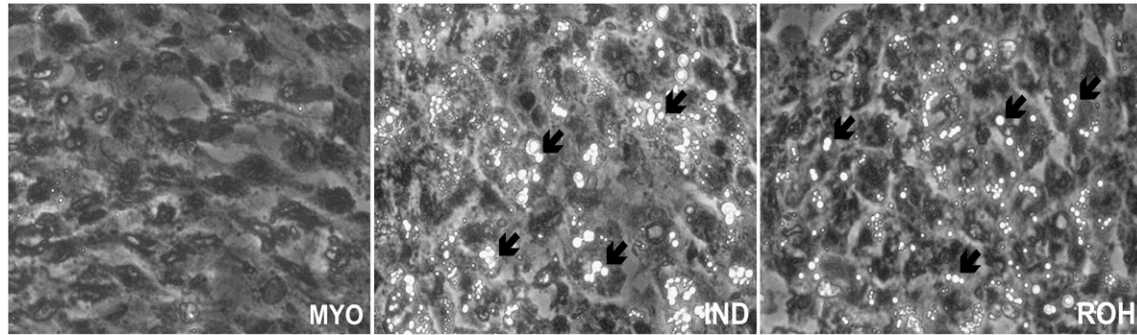
Fig. 1. Phase-contrast microscopy of GRX cells after 7 days under standard conditions ×200 (MYO), treated with 130 μM of indomethacin ×200 (IND) or treated with 5 μM of retinol ×200 (ROH). The fast droplets in GRX cells are indicated by arrows.

0.4 μL of 5 mM dNTPs, 0.4 μL of 10 μM primer pairs, 4.95 μL of water, 1.0 μL of SYBRgreen (1:10,000 Molecular Probe), and 0.05 μL of Platinum Taq DNA polymerase (5 U/μL) (Invitrogen). All results were analyzed by the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen, 2001). β-actin was used as the internal control gene for all relative expression calculations (Guimaraes et al., 2006). The real-time PCR products were separated by 2.0% agarose gel electrophoresis and visualized by ethidium bromide staining. The identity of real-time PCR products were confirmed by direct sequencing using the automatic sequencer ABI-PRISM 3100 Genetic Analyzer armed with 50 cm capillaries and POP6 polymer (Applied Biosystems). DNA templates obtained from the real-time PCR reactions were purified with a PCR clean-up system (Promega) and 30 to 45 ng were labeled with 3.2 pmol of specific forward or reverse primer and 2 μL of BigDye Terminator v3.1 Cycle Sequencing RR-100 (Applied Biosystems) in a final volume of 10 μL. Labeling reactions were performed in a GeneAmp PCR System 9700 (Applied Biosystems) thermocycler with an initial denaturing step of 96 °C for 3 min followed by 25 cycles of 96 °C for 10 s, 55 °C for 5 s and 60 °C for 4 min. Labeled samples were purified by isopropanol precipitation followed by 70% ethanol rinsing. Precipitated products were suspended in 10 μL formamide, denatured at 95 °C for 5 min, ice-cooled for 5 min and electroinjected in the automatic sequencer. Sequencing data were collected using the software Data Collection v1.0.1 (Applied Biosystems).

Statistical analysis

All experiments were performed in triplicate or quadruplicate. Data represent mean±standard error of mean (SEM). Differences were assessed by ANOVA, followed by Duncan's test, using the statistical program, SPSS 10.0 for Windows. The values obtained in the assays were considered statically different when $P < 0.05$.

Results

Conversion of activated HSC to quiescent HSC

The cultures were monitored daily by phase contrast microscopy and photographed on the seventh day (Fig. 1). Under standard culture conditions, GRX cells had a fibroblastoid morphology (Fig. 1A). When reaching confluence, they not fully contact-inhibited, and displayed the typical "hills and valleys" pattern of growth, characteristic of the smooth muscle cell lineages. When induced to convert into the fat-storing phenotype with 5 μM of retinol or 130 μM of indomethacin for 7 days, GRX cells began to lose their fibroblastoid morphology, shortened the cell extensions, and progressively acquired a polygonal shape. They accumulated refringent lipid droplets
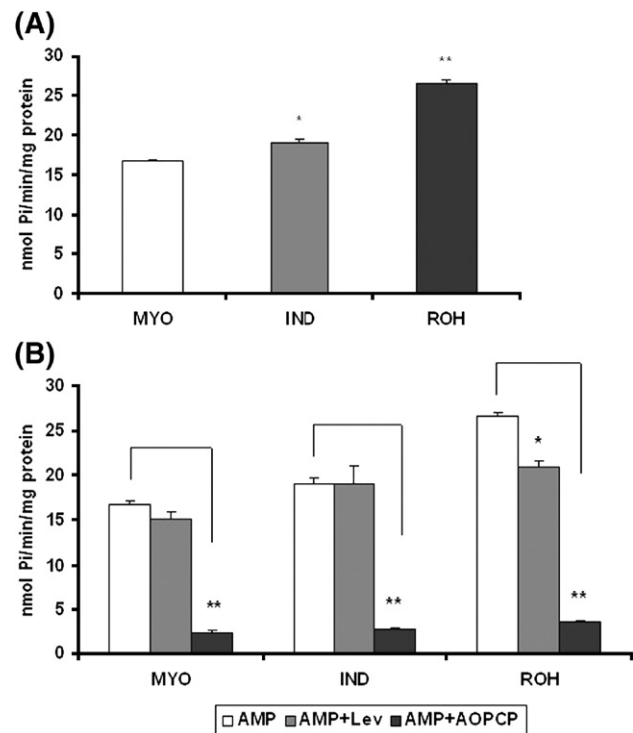

Fig. 2. AMP extracellular hydrolysis by GRX cells. Activated HSC (MYO), quiescent-like HSC induced by indomethacin (IND) and quiescent-like HSC induced by retinol (ROH) were submitted to enzymatic assay as described in Materials and methods in the absence (A) or presence of 100 μM AOPCP or 1 mM levamisole (B). Specific activity values were expressed as nmol Pi/min/mg protein. Data represent the means of three independent experiments performed in triplicate±S.E.M. In some instances, error bars are too small to be visible. Symbols denote significance $P < 0.05$ by one way ANOVA followed by Duncan's post-test. (A) *Different from control (MYO), **different from control (MYO) and IND, (B) *different from control (absences AOPCP or levamisole), **different from control and levamisole treatment.
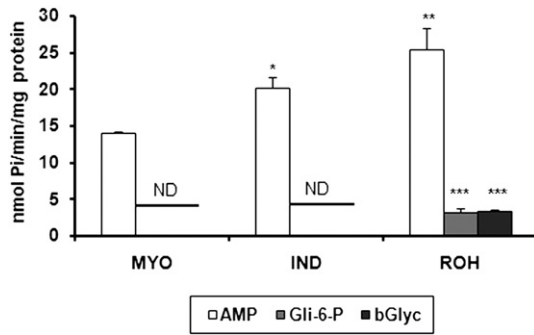
Fig. 3. Extracellular hydrolysis of AMP, glucose-6-phosphate (Gli-6-P) and β-glycerophosphate (bGlyc) by GRX cells. Specific activity values were expressed as nmol Pi/min/mg protein. Data represent means of three independent experiments performed in triplicate±S.E.M. Symbols denote significance $P<0.05$ by one way ANOVA followed by Duncan's post-test. *Different from AMP/MYO, **different from AMP/MYO and AMP/IND, ***different of AMP/MYO, AMP/IND and AMP/ROH. ND: no detected hydrolysis.

initially in the perinuclear area. The number and the size of fat droplets progressively increased until they occupied most of the cell volume (Fig. 1B and C). These droplets stained intensely with Oil-Red-O. Moreover, The proliferation rate in activated HSC (myofibroblasts) is significantly higher when compared with quiescent-like HSC. The proliferation of GRX cells in the presence of the IND or retinol attains the stationary phase after 2 days of treatment concomitantly with the beginning of fat droplets accumulation (data not shown).

AMP hydrolysis in GRX cells

In a first set of experiments, we investigated the hydrolysis of AMP in two phenotypes of GRX cells. All conditions for nucleotide hydrolysis such as cation dependence, substrate saturation and incubation time were previously determined in order to ensure the linearity of reaction (data not shown). The AMP hydrolysis in quiescent-like HSC was significantly higher when compared with activated HSC (myofibroblasts) (Fig. 2A). The specific activities for AMP hydrolysis in myofibroblasts (MYO), quiescent-like HSC induced by indomethacin (IND) and quiescent-like HSC induced by retinol (ROH) were: 16.82 ± 0.33; 19.13 ± 0.57 and 26.59 ± 0.46 nmol Pi/min/mg protein, respectively. In order to characterize the enzymatic activity responsible for AMP hydrolysis, the specific inhibitor of ecto-5′-nucleotidase (AOPCP) was tested. Fig. 2B shows that AOPCP significantly inhibited the AMPase activity in the three cell types studied. The residual AMPase activities in the MYO, IND and ROH were: 2.51 ± 0.29; 2.94 ± 0.13 and 3.75 ± 0.08 nmol Pi/min/mg protein, respectively, and represent approximately 15% of the activity in the absence of AOPCP. This inhibition supports the notion that the bulk of the determined enzyme activity was due to the action of the ecto-5′-nucleotidase.

Ecto-phosphatase assay

The extracellular alkaline phosphatase activity was also analyzed by the presence of the specific inhibitor levamisole

(Fig. 2B). No modification in the AMPase activity was observed in the presence or absence of this reagent in MYO and IND, showing that alkaline phosphatase was not active in these two cell types. On the other hand, the AMP hydrolysis decreased significantly by 20% in ROH in the presence of levamisole (20.94 ± 0.28 nmol Pi/min/mg protein) when
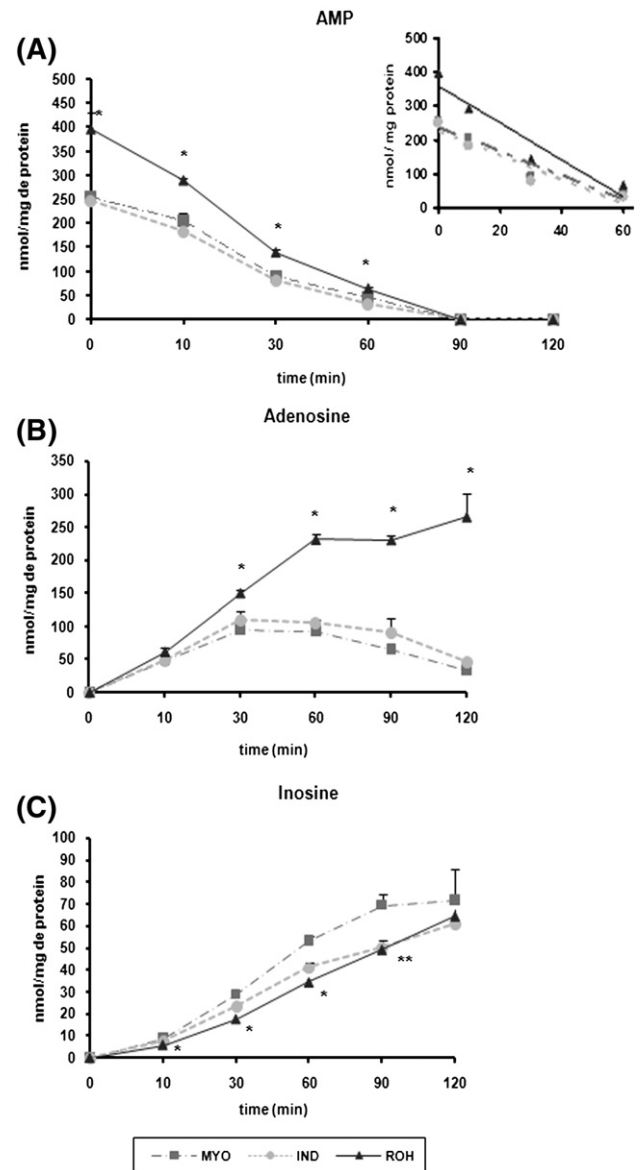

Fig. 4. Kinetics of extracellular AMP degradation in GRX cells. Activated HSC (MYO), quiescent-like HSC induced by indometacin (IND) and quiescent-like HSC induced by retinol (ROH) were incubated with 100 μM AMP, as described in Materials and methods. The presence of AMP (A), adenosine (B) and inosine (C) were determined after separation by HPLC. The compounds of the purine cascade were identified and measured by comparison with their respective standards. Results were expressed as nmol/mg protein. The data are mean±S.E.M. of two different experiments performed in triplicate. *Significantly different from MYO and IND at $P<0.05$, **significantly different from MYO at $P<0.05$ as determined by one way ANOVA followed by Duncan's test. In some instances, error bars are too small to be visible. The inset shows the tendency lines for the exogenous AMP metabolization by the different GRX phenotypes. The line equations obtained by linear regression analysis were $y=-3.54x+238.34$, $R^2=0.917$ for MYO; $y=-3.52x+224.30$, $R^2=0.913$ for IND and $y=-5.39x+357.85$, $R^2=0.914$ for ROH.
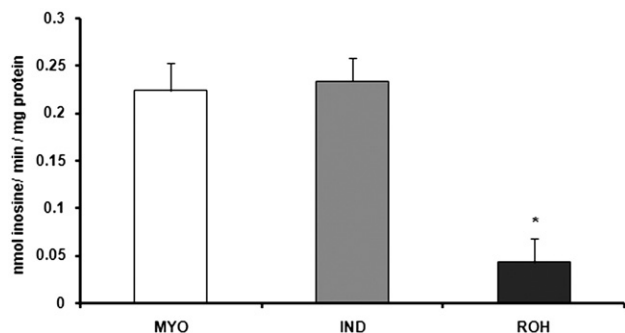
Fig. 5. Adenosine deaminase activity in GRX cells. Activated HSC (MYO), quiescent-like HSC induced by indometacin (IND) and quiescent-like HSC induced by retinol (ROH) were incubated with 100 μM adenosine and the ADA activity was determined as described in Materials and methods. Specific activity was expressed as nmol inosine/min/mg protein. The data are mean±S.E.M. of two different experiments performed in triplicate. *Significantly different from MYO and IND at $P<0.05$, as determined by one way ANOVA followed by Duncan's test.

compared with AMP hydrolysis in the absence of this inhibitor (26.59 ± 0.46 nmol Pi/min/mg protein). This result suggests that ecto-5′-nucleotidase would contribute approximately 80% of the total AMP hydrolysis in this phenotype.

Alkaline phosphatases are known to hydrolyze a variety of organic phosphates. To better investigate the presence of an ecto-alkaline phosphatase in GRX cells, the extracellular phosphatase activity was examined using glucose-6-phosphate and β-glycerophosphate as substrates. Neither MYO nor IND hydrolyzed the substrates, glucose-6-phosphate and β-glycerophosphate. However, these substrates were degraded by ROH, with specific activities that were significantly lower than AMP hydrolysis (Fig. 3). Activity values to AMP, glucose-6-phosphate and β-glycerophosphate hydrolysis in ROH were: 25.46 ± 3.0; 3.26 ± 0.55 and 3.42 ± 0.10 nmol Pi/min/mg protein, respectively. These data suggest that the absence of extracellular alkaline phosphatase activity in MYO and IND and together with the levamisole action (Fig. 2B) confirms the presence of this activity in ROH.

Analysis of extracellular AMP metabolism

The products of AMP hydrolysis from GRX cells were analyzed by HPLC (Fig. 4). In agreement with the results obtained from the AMPase activity measured by Pi released, the extracellular AMP was efficiently metabolized by GRX cells. Within 90 min, virtually all the extracellular AMP was metabolized with subsequent adenosine and inosine production. At the end of the incubation time an aliquot of the medium was used for measurement of LDH activity, to assess cellular viability. No membrane damage occurred during assay (data not shown), indicating that the products found are not the consequence of leakage from cells.

At the end of the incubation time (120 min), the compounds detected in the incubation medium of activated HSC (MYO) were adenosine (33.39 nmol/mg protein), which represented 13% of the AMP concentration added to the cells and inosine (71.87 nmol/mg protein), which represented 28% of the AMP

concentration added to the cells. In quiescent-like HSC, induced by indomethacin (IND), the levels of adenosine detected were (47.59 nmol/mg protein), which represented 19% of the AMP concentration added to the cells and inosine (61.07 nmol/mg protein), which represented 24% of the AMP concentration added to the cells. In quiescent-like HSC, induced by retinal (ROH), the compounds that were present at the end of the incubation time (120 min) were adenosine (265.25 nmol/mg protein), which represented 67% of the AMP concentration added to the cells and inosine (64.67 nmol/mg protein), which represented 16% of the AMP concentration added to the cells. The results presented in Fig. 4 also demonstrate that the rate of exogenous AMP degradation was higher in retinol-induced quiescent-like HSC. The slopes of the tendency lines (inset Fig. 4) for the exogenous AMP metabolization for the different GRX phenotypes, obtained by regression analysis, reinforce this observation.

To clarify the cause of the accumulation of adenosine in the extracellular medium of quiescent HSC, adenosine deaminase activity was studied. As shown in Fig. 5, ADA activity is significantly lower in ROH when compared with MYO and IND. These results suggest that the accumulation of adenosine in the extracellular medium of quiescent HSC, induced by retinol, is due to a decreased adenosine deaminase activity. In MYO and IND, adenosine is probably converted to inosine by
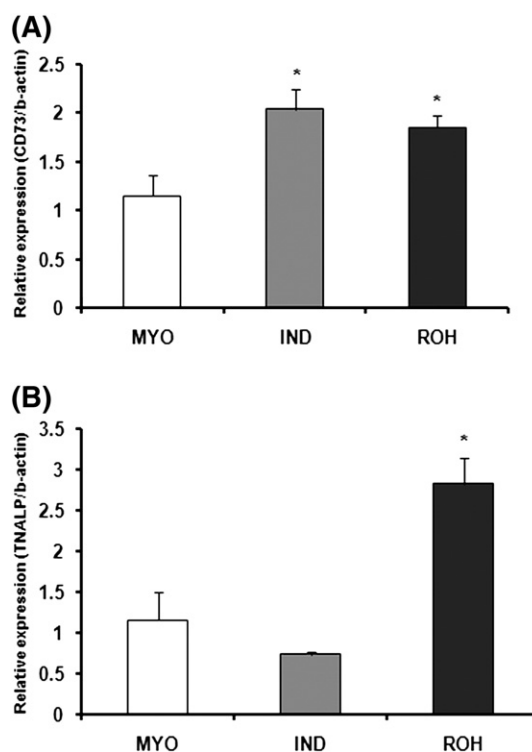

Fig. 6. Ecto-5′-nucleotidase/CD73 (A) and tissue non-specific alkaline phosphatase/TNALP (B) gene expression in GRX cells. Untreated cells, or myofibroblast phenotype (MYO) or treated for 7 days with 130 μM of indomethacin (IND) or 5 μM of retinol (ROH) were analyzed by real-time RT-PCR, as described in Materials and methods. Data represent mean±S.E.M. (A) *Significantly different from control (MYO) at $P<0.05$, (B) *significantly different from MYO and IND as determined by one way ANOVA followed by Duncan's test.

adenosine deaminase and/or by the uptake of adenosine by nucleoside transport system.

Expression of the ecto-5′-nucleotidase and tissue non-specific alkaline phosphatase (TNALP) in GRX cells

The AMP hydrolysis was higher in quiescent-like HSC than in activated HSC. Therefore, to determine if this increase in the AMP hydrolysis in quiescent-like HSC was mediated by the increase in the expression of ecto-5′-nucleotidase and/or TNALP, the mRNA levels were measured after the phenotype conversion from myofibroblasts to quiescent-like HSC with indomethacin or retinol. Quantitative analysis showed that after 7 days, in parallel with the establishment of the quiescent-like phenotype, the ecto-5′-nucleotidase transcription was increased 2 fold with indomethacin treatment and 1.7 fold with retinol in comparison to control cells (myofibroblasts) (Fig. 6A). These results support the hypothesis that the increase observed in ecto-5′-nucleotidase activity in quiescent-like HSC is related to an up-regulation of the ecto-5′-nucleotidase mRNA expression. On the other hand, real-time RT-PCR analysis revealed that only retinol treatment induces an increase in TNALP mRNA (Fig. 6B), in agreement with the increased catalytic activity presented by quiescent-like HSC, induced by retinol, and also with the blockage of AMP hydrolysis by levamisole, an uncompetitive inhibitor of TNALP (Fig. 2B).

Discussion

Hepatic stellate cells play a crucial role in the development of liver fibrosis and are important targets of liver disease therapy. The experimental model of the permanent cell line, GRX, is known to be converted to quiescent-like HSC cells when treated with indomethacin or retinol for 7 days, decreasing proliferation and extracellular matrix production, accumulating neutral lipids and retinyl esters in perinuclear lipids droplet, reducing activation markers, modifying the extracellular matrix production, reorganizing the cytoskeleton and reprogramming the metabolism (Margis and Borojevic, 1989; Margis et al., 1992; Vicente et al., 1997, 1998; Guma et al., 2001; Mermelstein et al., 2001; Andrade et al., 2003; Guimaraes et al., 2006).

In the present study, we compared the extracellular AMP degradation in activated HSC (myofibroblast-like phenotype) and in quiescent-like HSC induced by two different agents. The AMPase activity in quiescent-like HSC, independent of inductor agent, was significantly higher than in myofibroblasts and ecto-5′-nucleotidase was the main enzyme responsible for AMP hydrolysis.

In addition to ecto-5′-nucleotidase, nucleoside 5′-monophosphates can also be hydrolyzed by alkaline phosphatases. Although the maximal enzyme activity occurs at alkaline pH, a low activity was observed in quiescent-like HSC induced by retinol at physiological pH, when incubated with glucose-6-phosphate and β-glycerophosphate. This result is in agreement with the significant inhibition of AMP hydrolysis by levamisole observed in this phenotype. Taken together, these data indicate that AMP phosphohydrolase activity in quiescent-like HSC, induced

by retinol, is mainly due to ecto-5′-nucleotidase but involves the participation of the ecto-alkaline phosphatase. This may explain the increase in the AMP hydrolysis observed in these cells in comparison to quiescent-like HSC induced by indomethacin.

We also determined the expression of these enzymes on the seventh day of the GRX phenotype conversion. Indomethacin and retinol significantly increased the amount of ecto-5′-nucleotidase transcripts, suggesting that the increase in the AMPase activity is related to an up-regulation of the ecto-5′-nucleotidase mRNA expression.

The transcriptional level of alkaline phosphatase increased after treatment of GRX cells with retinol, whereas no alteration in the transcripts was detected with indomethacin. It is known that retinoic acid, a metabolic product of retinol, increases the specific activity of alkaline phosphatase in various cells during differentiation as a result of induced transcriptional activation of the alkaline phosphatase gene (San Miguel et al., 1999; Scheibe et al., 2000; Orimo and Shimada, 2005). These results reinforce the hypothesis that the increase in ecto-5′-nucleotidase expression is related to the phenotype conversion, independent of inductor agent, while the alkaline phosphatase modulation could be an effect of the treatment with retinol.

The induction of the quiescent-like phenotype in GRX cells, either by retinol or indomethacin, increases "de novo" synthesis of neutral lipids, phospholipids (Margis and Borojevic, 1989; Guaragna et al., 1991, 1992) and total ganglioside content, concomitant with a considerable change in fatty acid composition of the ceramide moiety (Andrade et al., 2003). These modifications suggest the formation of distinct membrane microdomains or rafts with specific functions on the two phenotypes of GRX cells and it could also be related to the redistribution of ecto-5′-nucleotidase and ecto-alkaline phosphatase from their intracellular pools to the plasma membrane.

The analysis of kinetics of degradation extracellular AMP demonstrated that, in quiescent-like HSC induced by retinol, the rate of exogenous AMP decay was higher and that more adenosine was accumulated in the extracellular medium than in myofibroblasts and quiescent HSC induced by indomethacin.

Adenosine generated in the extracellular space can be directed to following pathways: (i) interaction with its plasma membrane receptors (P1), eliciting several actions, such as, proliferative and cytoprotective effects; (ii) inosine formation by adenosine deaminase activity or (iii) uptake to a specialized transport system (Hasko and Cronstein, 2004).

In quiescent-like HSC induced by retinol, the accumulation of adenosine is probably the consequence of a decreased adenosine deaminase activity observed during the phenotype conversion. In MYO and IND, adenosine accumulates in extracellular medium, and a part is likely to be converted to inosine by ecto-adenosine deaminase and/or uptaken by a nucleoside transport process, which may be of further importance for salvage of extracellular nucleosides for intracellular de novo synthesis of nucleotides, such as ATP. Furthermore, the higher adenosine deaminase activity in MYO is in accordance with studies that report an increase in adenosine deaminase activity in several liver diseases, such as cirrhosis (Kobayashi et al., 1993; Le Moine et al., 1999; Fernandez et al., 2000).

Adenosine acts as an extracellular signaling molecule in various tissues through activation of specific G protein-coupled receptors. They are classified as purinergic P1-type and four subtypes have been identified: A_1, A_{2A}, A_{2B} and A_3 receptors (Fredholm et al., 2001). Studies have been shown that adenosine, through interaction with the A_{2B} receptor, was able to suppress both proinflammatory gene expression and NF-kB activation (Jijon et al., 2005). In addition, inhibited cell functions involved in fibrosis, such as deposition of extracellular matrix proteins and collagen synthesis (Dubey et al., 1998, 1999, 2001; Chen et al., 2004).

In conclusion, the results obtained here, demonstrate that quiescent-like HSC exhibit distinct AMP hydrolysis when compared with activated HSC, indicating differences in nucleotide metabolism between the two cell types. We also show that ecto-5′-nucleotidase is the main enzyme responsible for this hydrolysis in HSC, suggesting that the ecto-5′-nucleotidase-dependent adenosine generation may play a role in the regulation of quiescent HSC functions.

Acknowledgements

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CAPÍTULO 4

ECTO-5'-NUCLEOTIDASE/CD73 KNOCKDOWN INCREASES CELL ADHESION, MIGRATION AND mRNA LEVEL OF COLLAGEN I IN A HEPATIC STELLATE CELL LINE

ECTO-5'-NUCLEOTIDASE/CD73 KNOCKDOWN INCREASES CELL ADHESION, MIGRATION AND mRNA LEVEL OF COLLAGEN I IN A HEPATIC STELLATE CELL LINE

Cláudia M. B. Andrade[1], Patrícia L. C. Lopez[2], Bruno T. Noronha[1], Márcia R. Wink[3], Radovan Borojevic[4], Rogério Margis[1], Guido Lenz[2], Ana Maria O. Battastini[1] and Fátima C R Guma[1]

[1]Departamento de Bioquímica, UFRGS. Porto Alegre, RS, Brazil
[2]Departamento de Biofísica, UFRGS, Porto Alegre, RS, Brazil
[3]Departamento de Fisiologia, Universidade Federal de Ciências da Saúde de Porto Alegre, UFCSPA. Porto Alegre, RS, Brazil
[4]Departamento de Histologia e Embriologia, ICB, PABCAM, Hospital, Universitário Clementino Fraga Filho, UFRJ, Rio de Janeiro, RJ, Brazil.

Running head: Effects of eNT/CD73 knockdown in hepatic stellate cells

*Corresponding author: Dr. Fátima T. C. R. Guma. Departamento de Bioquimica/UFRGS. Rua Ramiro Barcelos, 2600-anexo. CEP 90035-003. Porto Alegre, RS, BRAZIL Fone +55 51 3316 5546. FAX +55 51 3316 5535. e-mail: fatima.guma@ufrgs.br

Ecto-5'-nucleotidase (eNT/CD73, E.C.3.1.3.5) is a glycosyl phosphatidylinositol (GPI)-linked cell surface protein with several functions, including local generation of adenosine with consequent activation of adenosine receptors and salvaging of extracellular nucleotides as well as functions apparently independent of its activity, such as mediating cell-cell adhesion. Liver fibrosis may be considered as a dynamic and integrated cellular response to chronic liver injury and the activation of hepatic stellate cells (HSCs) plays a role in the fibrogenic process. Ecto-5'-nucleotidase and adenosine were reported to play an important role in hepatic fibrosis in murine models. To analyze the biological significance of eNT/CD73 in HSC, here we show that eNTC/D73 knockdown leads to an increase in mRNA expression of tissue non-specific alkaline phosphatase (TNALP), another AMP degrading enzyme. eNT/CD73 knockdown also leads to changes in the expression of collagen I and a clear alteration of cell adhesion and migration, despite not altering total ecto-AMPase activity of the cell. We suggest that eNT/CD73 protein expression may control migration by affecting cell-substrate adhesion and collagen expression, in a mechanism not dependent of changes in nucleotide metabolism.

Ecto-5'-nucleotidase (eNT/CD73, E.C.3.1.3.5) is a glycosyl phosphatidylinositol (GPI)-linked cell surface protein expressed in different cell types and widely distributed from plants to mammals (1,2). Ecto-5'-nucleotidase catalyses the extracellular dephosphorylation of nucleosides monophosphate to their corresponding nucleosides (3). There are several proposed functions for eNT/CD73, including local generation of adenosine with consequent activation of adenosine receptors and salvaging of extracellular nucleotides as well as functions apparently independent of its activity, such as mediating cell-cell adhesion and possibly acting as a co-receptor for T-cell activation (4-7).

Liver fibrosis may be considered as a dynamic and integrated cellular response to chronic liver injury. The activation of hepatic stellate cells (HSCs) and the consequent deposition of large amounts of extracellular matrix play a role in the fibrogenic process (8). Although many studies have focused on signaling pathways that regulate fibrogenesis by HSC, there is still no effective treatment for liver fibrosis (9-11). Recently, eNT/CD73 was reported to play an important role in adenosine generation in the liver, and this nucleoside, acting at A_{2A} receptor, is a critical mediator of hepatic fibrosis in murine models (12,13). Here we show that eNT/CD73 knockdown (eNT/CD73 KD) in a HSC cell line leads to an increase in mRNA expression of tissue non-specific alkaline phosphatase (TNALP), another enzyme capable of degrading AMP. eNT/CD73 knockdown lead also to changes in the expression of collagen I and a clear alteration of cell adhesion and migration, despite not altering total ecto-AMPase activity of the cell.

EXPERIMENTAL PROCEDURES

Transduction of GRX cells with eNT/CD73 shRNA: lentiviral vectors were produced by co-transfecting shRNA-producing vectors containing sequences specific for mouse ecto-5'-nucleotidase or a non-targeting sequence (MISSION® shRNA Sigma) together with the helper vectors CMV-VSVG, RSV-REV and pMDLg/pRRE into HEK293 cells. Supernatants were used to transduce GRX cells (14). Transduced cells were selected for 10 days using 10μM puromycin. The experimental groups were named as: untreated (WT), control (non-targeting shRNA) and two ecto-5'-nucleotidase knockdowns (eNT/CD73 shRNA C1 and C2). The sequences of shRNA are listed in Table 1.

RNA extraction, cDNA synthesis and qPCR, and Ecto-5'-nucleotidase assay were carried out as described previously (15). The primers sequences are listed in Table 2.

Western blot analysis were performed as previously described (16), using a primary antibody against eNT/CD73 (ABGENT) (1:500) or β-actin (Cell Signaling) (1:1000) and horseradish peroxidase-conjugate goat anti-rabbit IGg (GE Healthcare) as secondary antibody.

Proliferation and cell adhesion assays were assessed by cell counting in a haemocytometer using trypan blue (17) and crystal violet staining (18).

Wound healing assay: cell migration was performed following standard methods (19).

Immunocytochemistry were performed as described previously (20) using rhodamine-phalloidin (Molecular Probes).

Statistical Analysis: Data represent mean ± standard error of mean (SEM). Differences were assessed by ANOVA, followed by Duncan's test. The values obtained in the assays were considered statistically different when $P<0.05$.

RESULTS AND DISCUSSION

In order to address the biological role of ecto-5'-nucleotidase in HSC, the expression of eNT/CD73 mRNA and protein were significantly reduced in a HSC cell line using RNAi (Fig. 1A and B). Early investigations have shown the association between adenosine production by eNT/CD73 and hepatic fibrosis (Peng, 2008)(21). Surprisingly, no modification in AMPase activity was observed in GRX cells silenced on eNT/CD73 (Fig. 1C). In order to characterize the enzymatic activity responsible for AMP hydrolysis, specific inhibitors of eNT/CD73 (AOPCP) and TNALP (levamisole) were tested. Fig. 1C shows that APCP significantly inhibited the AMPase activity in WT and shRNA control cells, but had no effect in eNT/CD73 knockdown (KD) cells. However, levamisole only decrease the AMP hydrolysis in eNT/CD73 KD cells. These results indicate that the main ecto-AMPase in control cells is eNT/CD73, whereas in eNT/CD73 KD cells TNALP assumes this role. Quantitative analysis of mRNA showed that TNALP transcription was increased in eNT/CD73 KD cells (Fig. 1A), confirming the hypothesis that AMP phosphohydrolase activity observed in this cells is mainly due to TNALP. In agreement, previous analysis of the substrate concentration-enzyme activity relationship revealed different kinetic properties for the two ectoenzymes. AMP concentration in the range of mM is eliminated more rapidly by TNALP than by eNT/CD73 (22), which may explain the maintenance of the activity even with lower amount of TNALP transcripts in eNTCD73 KD cells when compared to eNT/CD73 transcript in WT cells.

To further evaluate the effect of eNT/CD73 KD in liver fibrosis, we characterized the impact of eNT/CD73 KD in cell proliferation, adhesion, migration and mRNA expression of type I collagen. The ability of migration and adhesion of GRX cells was increased after KD of eNT/CD73 (Fig. 2A, B, C and D). However, no differences were observed with respect the cell proliferation (Fig.2E). Some studies suggest that eNT/CD73 is involved in adhesion and migration of different cell types due to its enzymatic activity or presence on the surface of the cell. The observations that on lymphocytes, several ecto-5'-nucleotidase/CD73 molecules together form larges clusters that facilitates the adhesion mediated by this molecule argues in favor to the second hypothesis (23). Alternatively, on lymphocytes eNT/CD73 could function as a co-regulatory molecule, leading to activation of other adhesion molecules (7). In human breast cancer cells, eNT/CD73 interacts strongly and specifically with tenascin C and this interaction influences in cell adhesion, migration and generation of local adenosine, but this study was inconclusive as to whether the effects of CD73 were due to its enzymatic activity or presence on the surface of the cell (24). Moreover, eNT/CD73 directly

interacts with laminin and fibronectin and these interactions may be the underlying mechanism of eNT/CD73-mediated change in adhesion and migration (25).

In addition, we found that the disruption of eNT/CD73 expression increased the levels of collagen I mRNA. During hepatic fibrosis, type I collagen is up-regulated and induces migration of HSC (26). The increase in collagen expression is mediated primarily by a post-transcriptional mechanism that stabilizes the mRNA and increases the half-life of collagen mRNA molecule from 1.5h in quiescent HSCs to greater 24h in activated HSCs (27). In conclusion, the knockdown of eNT/CD73 affected migration by changing cell-substrate adhesion and collagen expression, in a mechanism not dependent of changes in nucleotide metabolism.

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FOOTNOTES

The abbreviations used are: eNT/CD73, ecto-5'-nucleotidase; TNALP, tissue non-specific alkaline phosphatase; HSCs, hepatic stellate cells; KD, knockdown; AOPCP, α,β-methylene adenosine diphosphate

FIGURE LEGENDS

Fig. 1. **Analysis of eNT/CD73 and TNALP expression and activity.** (A) mRNA analysis of eNT/CD73 and TNALP by q PCR. Mouse β-actin was determined in parallel and used as internal standard. eNT/CD73 shRNA decreases eNT/CD73 mRNA expression and increases TNALP mRNA expression in GRX cells, (B) eNT/CD73 protein levels analyzed by Western blot. ENT/CD73 protein expression decreases significantly in cells transduced with eNT/CD73 shRNA, (C) eNT/CD73 and TNALP enzyme activities. Surface enzyme activities were measured by AMP hydrolysis in the presence of the eNT/CD73 inhibitor AOPCP and the TNALP inhibitor levamisole. Data represent the mean ± s.e.m. of three independent experiments. Differences were assessed by ANOVA, followed by Duncan's test and considered statistically different when $P<0.05$.

Fig. 2. **Effect of knockdown of eNT/CD73 on cell migration, adhesion and collagen I mRNA expression**. (A) Phase contrast images of wound-healing assay of WT, control shRNA and eNT/CD73 KD cells at 0 and 18h after wounding (100x), (B) Cells were stained with rhodamine-phalloidin after wounding to examine cell morphology at the wound edge. Bar, 20 μm. The images show the spreading of the cells into the cell-free space and the formation of actin-rich lamellipodia at the leading edge, (C) Quantification of cell migration using wound healing assay. The percentage of the migration was higher in eNT/CD73 KD cells, (D) Cell adhesion was measured on plastic culture plates by crystal violet stain. The adhesive potential of GRX cells was increased after knockdown of eNT/CD73, (E) Cell proliferation was assessed by counting of the cell and no differences were observed after the knockdown of eNT/CD73, (F) mRNA level of collagen I was analyzed by qPCR and β-actin was used as internal standard. Knockdown of eNT/CD73 increased mRNA expression of collagen I. Data represent the mean ± s.e.m. of three independent experiments. Means with different letters are statistically different, $P<0.05$ as determined by ANOVA, followed by Duncan's test.

**Table 1:** The sequences of short hairpin RNA targeting sequences.

Name	Sequences
eNT/CD73 shRNA C1	CCATTCATAGTCACCGCAGAT
eNT/CD73 shRNA C2	GACATTAACCAATGGAGGAT
Non-targeting	CAACAAGATGAAGAGCACCAA

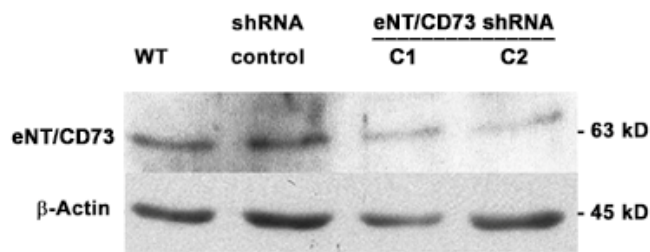**Table 2:** Primers used for qPCR (quantitative polymerase chain reaction)

	Forward primer	Reverse primer
eNT/CD73	5'CACGGGGGCCTCTAGCACATCA	5'GCCTGGACCACAGGCACCTG
TNALP	5'AAGGGCCAGCTACACCACAACACG	5'GCCCACGGACTTCCCAGCATC
Col I1a	5'TCACCTACAGCACCCTTGTG	5'GGTGGAGGGAGTTTACACGA
β-Actin	5'TATGCCAACACAGTGCTGTCTGG	5'TACTCCTGCTTGCTGATCCACAT

Figure 1
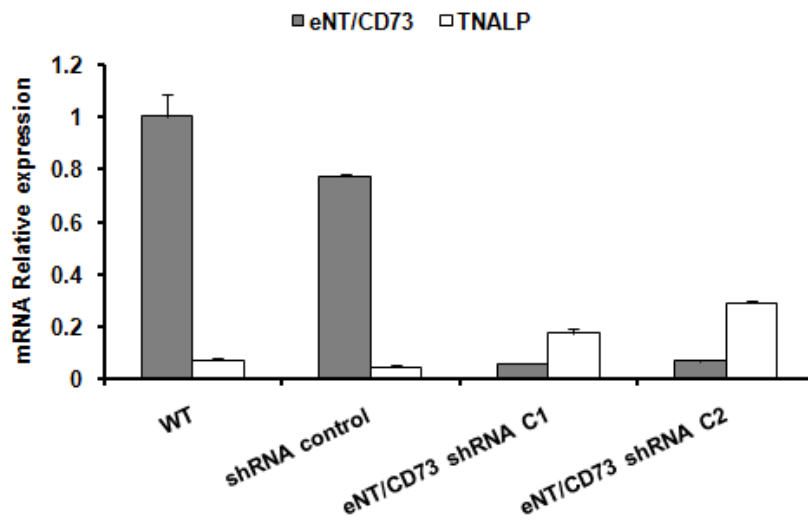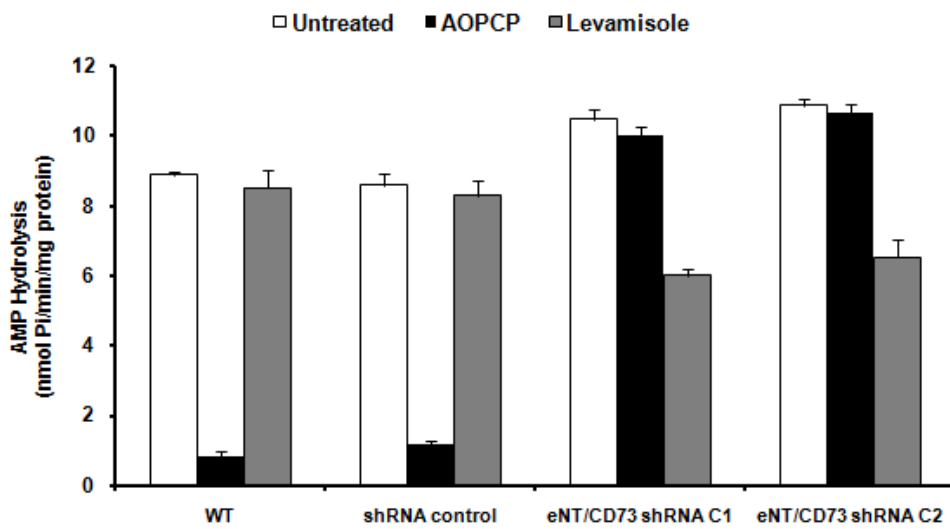
Figure 2A, 2B and 2C
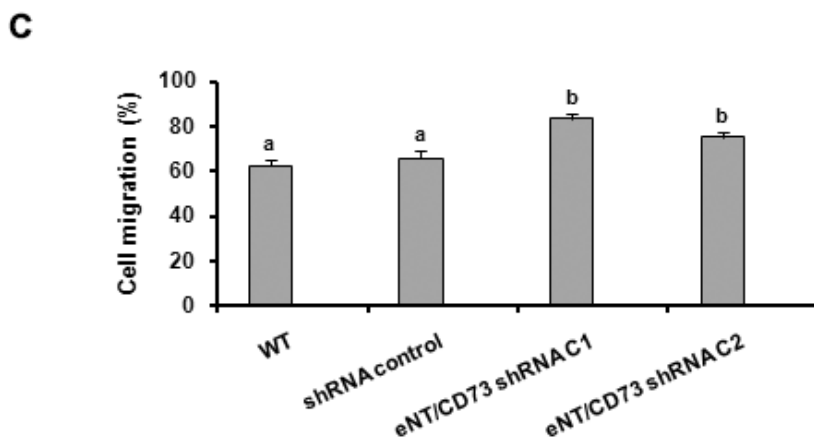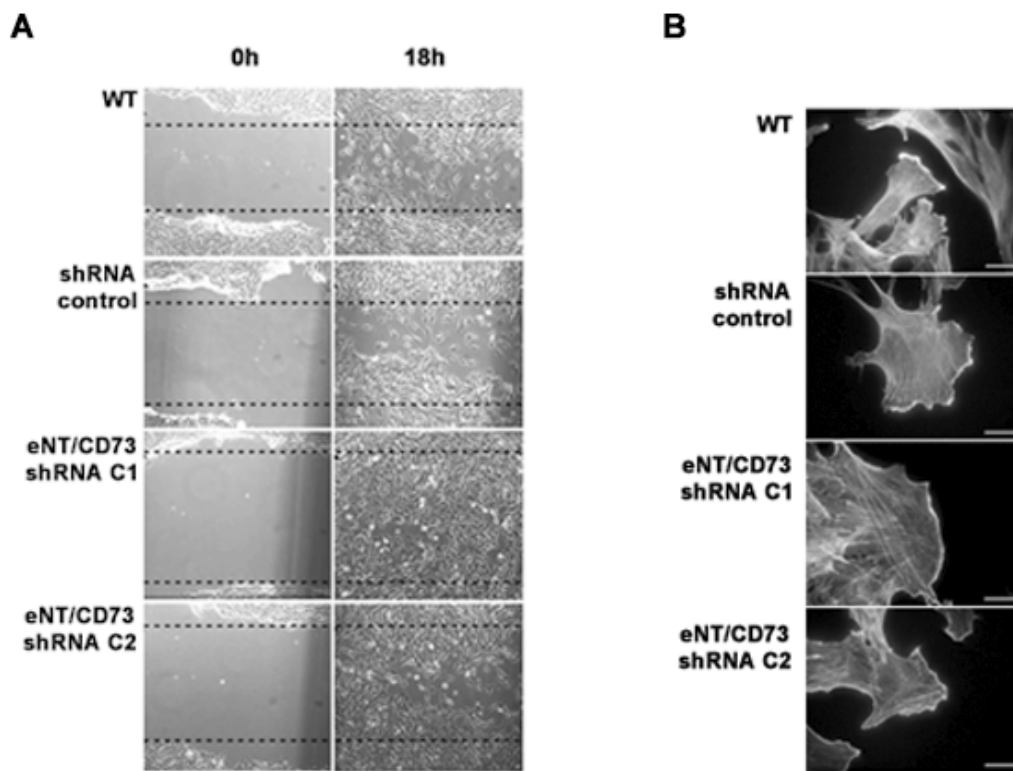
Figure 2D, 2E and 2F
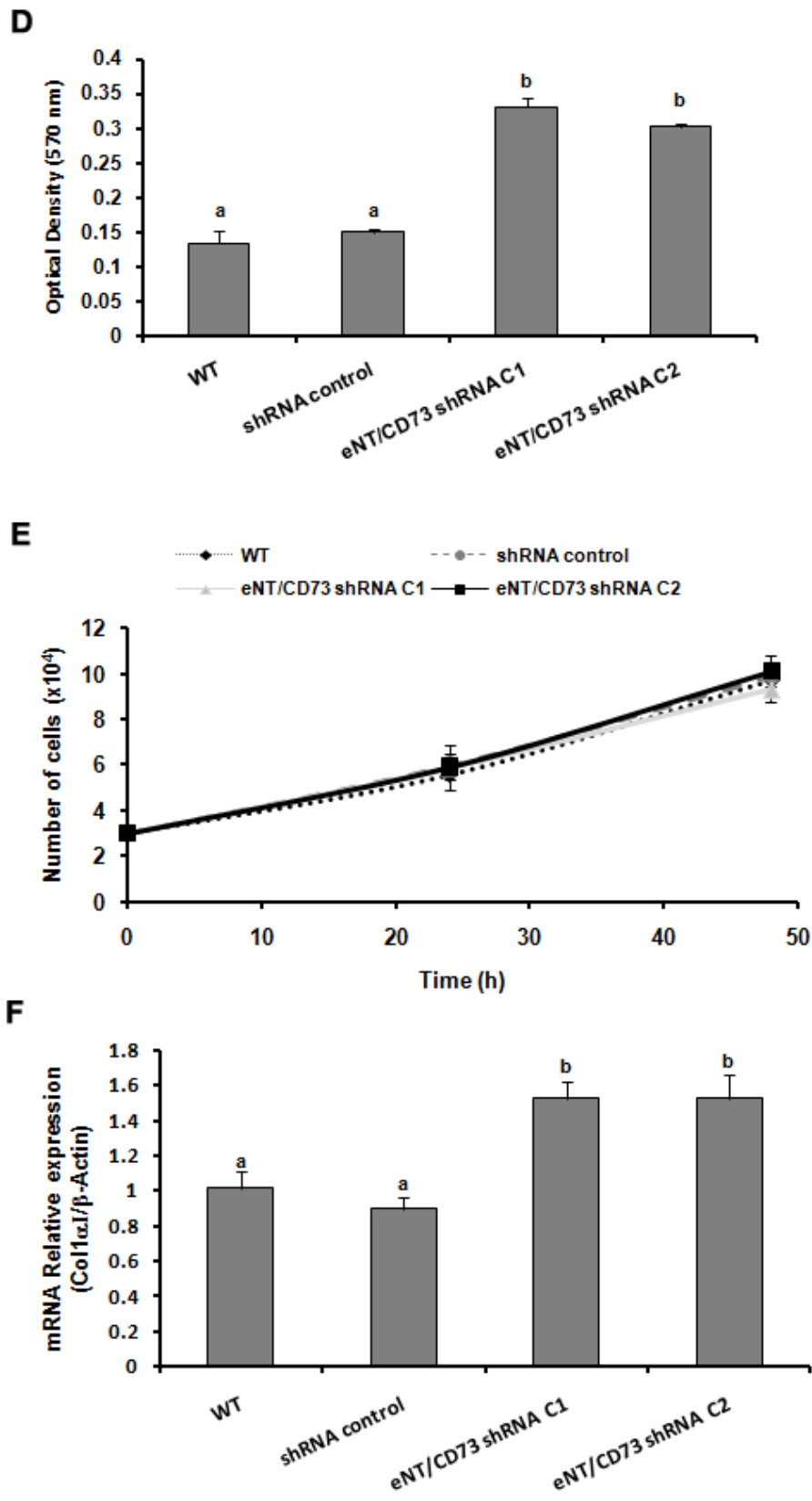
CAPÍTULO 5

Investigação sobre o efeito da adenosina sobre parâmetros de ativação das células estreladas hepáticas

(Dados preliminares)

INTRODUÇÃO

A adenosina é um nucleosídeo purínico endógeno que pode ser liberado pelas células ou formado extracelularmente através da completa desfosforilação do ATP por ação de ectoenzimas. Este nucleosídeo atua de forma autócrina e parácrina no controle de diversos processos fisiológicos, tais como: inibição da agregação plaquetária, supressão da inflamação, promoção da angiogênese, reparo tecidual e vasodilatação (Borowiec et al., 2006; Hasko & Cronstein, 2004). Os efeitos da adenosina são mediados por uma família de quatro receptores acoplados à proteína G (A_1, A_{2A}, A_{2B} e A_3), os quais apresentam diferente distribuição tecidual, perfil farmacológico e reposta após ativação (Linden, 2001).

O efeito hepatoprotetor da adenosina foi demonstrado em experimentos em que a cirrose hepática foi induzida por tetracloreto de carbono (CCl_4). A administração intraperitoneal de adenosina bloqueou a atividade fibrogênica e aumentou a degradação de colágeno, provavelmente, por diminuir os níveis dos inibidores teciduais de metaloproteinases (TIMPs). Além disto, o nucleosídeo promoveu efetiva proliferação dos hepatócitos no fígado cirrótico, acelerando a regeneração funcional e diminuindo os níveis de estresse oxidativo (Hernandez-Munoz et al., 1997; 2001). Por outro lado, alguns estudos sugerem que a adenosina, liberada endogenamente em resposta a hepatotoxinas, contribui para o desenvolvimento da fibrose hepática via ativação de receptores A_{2A} (Chan et al., 2006; Che, Chan & Cronstein, 2007).

Recentemente demonstramos que os dois fenótipos das células estreladas hepáticas apresentam diferenças no metabolismo extracelular de nucleotídeos e nossos dados sugerem o envolvimento da adenosina na modulação das funções relacionadas com a fibrogênese (Andrade et al., 2008). Assim, para melhor entender o papel da adenosina neste processo, nós avaliamos o efeito da adenosina sobre a proliferação, migração, adesão e nível de transcrição de ecto-5'-nucleotidase (CD73), colágeno tipo I e metaloproteinases 2 e 9 (MMP-2 e MMP-9) em células estreladas hepáticas ativadas.

METODOLOGIA

Cultura de células

A linhagem celular GRX (CR001) foi obtida do banco de células do Rio de Janeiro (PABCAM, Rio de Janeiro, Brasil). As células foram mantidas em meio Dulbecco's (DMEM) (GIBCO) com 2g/L de tampão HEPES (Sigma Chemical Company), suplementado com 5% soro fetal bovino (Cultilab), pH 7,4, a 37°C e atmosfera de 5% CO_2. Alternativamente, para estudar os possíveis efeitos da adenosina sobre a proliferação, adesão, migração e expressão gênica nas células GRX, as culturas foram tratadas com 100μM de adenosina (Sigma Chemical Company).

Proliferação celular

A proliferação celular foi analisada através da contagem de células utilizando azul de *trypan* para exclusão das células não viáveis. As células GRX foram semeadas em placas de 24 poços ($3x10^4$ células por poço) e cultivadas com DMEM suplementado ou não com adenosina. Após os períodos indicados, as células foram tripsinizadas, coletadas e centrifugadas. Os pellets celulares foram ressuspendidos com $100\mu L$ de PBS contendo 0,2% de azul de *trypan* e o número de células foi imediatamente determinado pela contagem em hemocitômetro. Os dados representam a média ± o erro padrão de três experimentos independentes (média± EPM).

Ensaio de adesão celular

O ensaio de adesão celular foi realizado em placas de cultura de 96 poços contendo $2x10^4$ células por poço (Wang et al., 2008b). As células foram incubadas em presença ou ausência de adenosina por 30, 60 e 120 minutos. Após, as células não aderentes foram cuidadosamente removidas através de lavagem com PBS. As células aderentes foram fixadas com paraformaldeído 4% por 10 min, lavadas com PBS e incubadas com 100 μL de cristal violeta 0,5% por 10 min. As células foram então lavadas e lisadas com 100 μL de etanol contendo 1% de ácido acético. A leitura foi realizada em espectrofotômetro (SpectraMax 190) em 570nm. Os resultados foram expressos como a média das densidades ópticas ± EPM de três experimentos independentes.

Ensaio de migração celular

O efeito da adenosina sobre a migração das células GRX foi avaliado de acordo com um método padrão, conhecido como ensaio do dano e cicatrização (*assay wound healing*) (Valster et al., 2005). Inicialmente 10^5 células foram semeadas em placas de 12 poços e cultivadas até alcançarem 90% de confluência. Após, com uma microponteira, foi criada uma ranhura (dano) no centro da monocamada celular e a migração das células (cicatrização do dano) foi analisada através de microscopia de contraste. As imagens foram capturadas imediatamente após o estabelecimento do dano a monocamada (0h), 10h e 18h após, em presença ou ausência de adenosina. A percentagem de migração foi determinada considerando-se a largura inicial da ranhura como 100% e os valores foram plotados como a percentagem de ocupação deste espaço ao longo do tempo. Os dados são representativos de dois experimentos independentes.

Extração de RNA, síntese de cDNA e PCR em tempo real

O RNA das células GRX foi isolado usando o reagente TRIzol (Invitrogen), conforme instruções do fabricante. A síntese do cDNA e as reações para quantificação dos mRNAs através de PCR em tempo real foram realizadas conforme previamente descrito por Andrade et al. (2008). As seqüências dos primers usados estão listadas na tabela 1. Todos os resultados foram analisados pelo método $2^{-\Delta\Delta CT}$ (Livak &

Schmittgen, 2001). β-actina foi usada como gene controle para todos os cálculos de expressão relativa.

Análise estatística

Todos os experimentos foram realizados em quadruplicata. Os dados representam a média ± o erro padrão da média (EPM). As diferenças foram analisadas por ANOVA, seguida pelo teste de Duncan.usando SPSS 15.0. Os valores foram considerados estatisticamente diferentes quando $P<0.05$.

RESULTADOS E DISCUSSÃO

Tendo em vista nossos resultados prévios que sugerem o envolvimento da adenosina na modulação fenotípica das células estreladas hepáticas e no intuito de obter evidências sobre a possível ação antifibrogênica deste nucleosídeo, neste estudo nós analisamos o efeito da adenosina sobre parâmetros diretamente relacionados com a ativação da HSCs e com a fibrogênese. As HSCs ativadas aumentam a taxa de proliferação, migração, síntese e depósito de proteínas de matriz extracelular, principalmente colágeno tipo I, e a expressão de moléculas de adesão, além de secretarem citocinas pró-inflamatórias e produzirem inibidores de metaloproteinases, gerando um desequilíbrio entre a síntese e a degradação dos componentes da matriz extracelular (Kisseleva & Brenner, 2006; Reif et al., 2003). O tratamento das culturas de

células GRX com adenosina reduziu a taxa de proliferação celular (Fig. 1). O potencial de adesão das células ao substrato não foi alterado (Fig. 2), no entanto, a migração celular foi reduzida após o tratamento com adenosina (Fig. 3A e 3B). As alterações observadas na mobilidade celular em presença da adenosina podem ser justificadas pela sua interação com receptores A_{2B}, único subtipo de receptor P1 expresso pelas células GRX (informação pessoal da Dra Elena Bernard). É bem conhecido que a ativação dos receptores A_{2B} aumentam a atividade da adenilato ciclase e conseqüentemente os níveis de cAMP, que através da fosforilação da proteína quinase A (PKA), desencadeia a ativação de diversos fatores de transcrição que controlam a expressão de genes relacionados com a migração celular (Howe, 2004). Além disto, cAMP também pode reduzir a migração celular por interferir na formação de lamelipódios, estruturas subcelulares importantes para a mobilidade celular (Chen, Zhang & Huang, 2008).

Considerando que durante o estabelecimento da fibrose hepática ocorrem alterações no padrão de expressão gênica das HSCs, nós avaliamos o efeito da adenosina sobre a expressão do mRNA de genes envolvidos com o remodelamento da matriz extracelular. Os resultados obtidos demonstram que a adenosina diminui a transcrição de colágeno I e das metaloproteinases 2 e 9 (Fig. 4). Resultado semelhante foi relatado em um estudo a respeito da fibrose cardíaca no qual a adenosina foi capaz de inibir a síntese de colágeno via ativação de receptores A_{2B} (Dubey, Gillespie & Jackson, 1998). Interessantemente, durante a conversão fenotípica das células GRX com retinol ocorre diminuição da transcrição de colágeno I e acúmulo de adenosina no meio extracelular   (Andrade et al., 2008). As metaloproteinases são enzimas

responsáveis pelo *turnover* de todos os componentes da matriz extracelular. O aumento na atividade e expressão destas enzimas é uma das principais causas da fibrogênese, tornando-as alvos importantes para as terapias de prevenção desta patologia (Han et al., 2004; Zhen et al., 2006). Neste contexto, a adenosina parece exercer ação benéfica, visto que reduziu os níveis de mRNA expressos pelas células GRX. No entanto, estas enzimas são secretadas como pró-enzimas inativas e sua ativação depende da expressão de inibidores de metaloproteinases (TIMPs) e da metaloproteinase de membrana tipo I (MT1-MMP) (Wang et al., 2008a). Assim sendo, a análise do efeito da adenosina sobre a expressão dos TIMPs e da MT1-MMP permitiria verificar se este nucleosídeo atua somente a nível da transcrição ou também é capaz de inibir a atividade enzimática.

Uma vez que a ecto-5'-nucleotidase é a principal enzima responsável pela geração de adenosina no meio extracelular e nossos resultados anteriores demonstram que sua atividade e expressão estão aumentadas em HSCs quiescentes (Andrade et al., 2008), resolvemos analisar a possibilidade da adenosina estar envolvida com a regulação transcricional desta enzima. Em concordância com nossos resultados prévios, o nível de transcrição da ecto-5'-nucleotidase aumentou após o tratamento com adenosina (Fig. 4). A clonagem da região promotora do gene da ecto-5'-nucleotidase humana revelou que próximo ao códon de iniciação há um elemento responsivo a cAMP (CRE) responsável pela regulação da transcrição. Deste modo, a alteração nos níveis de cAMP intracelular interfere na expressão desta proteína (Hansen et al., 1995). Narravula et al (2000) demonstraram que a adenosina via ativação de receptores A_{2B}, análogos de adenosina e outros agonistas capazes de

elevar a concentração de cAMP aumentam a transcrição da ecto-5'-nucleotidase em células endoteliais. Deste modo, é provável que o aumento do mRNA da ecto-5'-nucleotidase observado nas células GRX possa estar relacionado com a ativação dos receptores A_{2B} após o tratamento das culturas com adenosina.

Concluindo, a análise conjunta dos resultados aqui apresentados, contribui para a hipótese de que a adenosina exerça ação antifibrogênica sobre as células estreladas hepáticas, mas a comprovação deste efeito necessita estudos adicionais.

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Tabela 1. Seqüências dos primers usados para PCR em tempo real

	Forward primer	Reverse primer
eNT/CD73	5'CACGGGGGCCTCTAGCACATCA	5'GCCTGGACCACAGGCACCTG
Col 1αI	5'TCACCTACAGCACCCTTGTG	5'GGTGGAGGGAGTTTACACGA
MMP-2	5'ATGCCATCCCTGATAACCTG	5'TGTGCAGCGATGAAGATGAT
MMP-9	5'CATTCGCGTGGATAAGGAGT	5'CACTGCAGGAGGTCGTAGGT
β-Actin	5'TATGCCAACACAGTGCTGTCTGG	5'TACTCCTGCTTGCTGATCCACAT
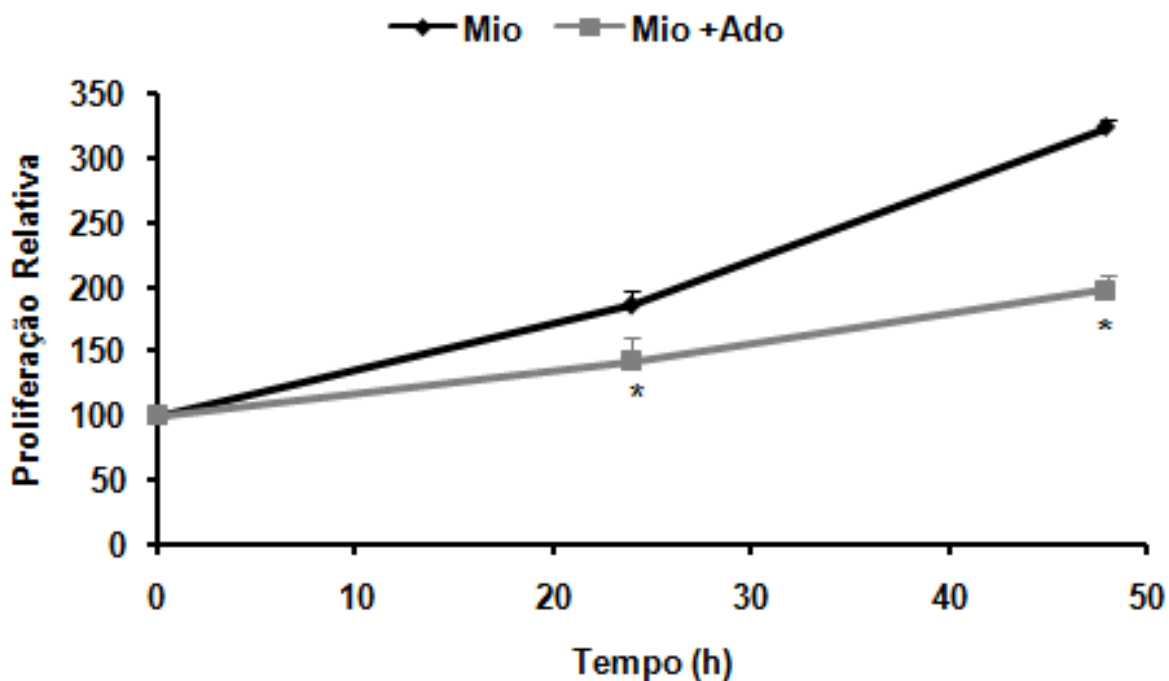
Figura 1: Efeito da adenosina sobre a proliferação das células estreladas hepáticas. Culturas semi-confluentes das células GRX foram tratadas com 100 µM de adenosina por 24h e 48h. Após estes períodos, as células foram tripsinizadas e o número de células foi determinado pela contagem em hemocitômetro. Os valores representam a média ± EPM de três experimentos independentes realizados em quadrupli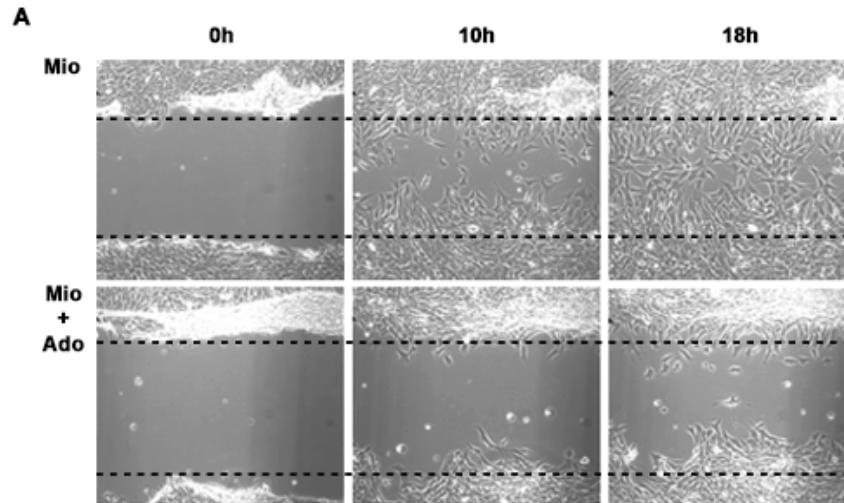cata. As diferenças foram analisadas por ANOVA, seguida pelo teste de Duncan. *Valores estatisticamente diferentes do grupo controle, *P*<0,05.
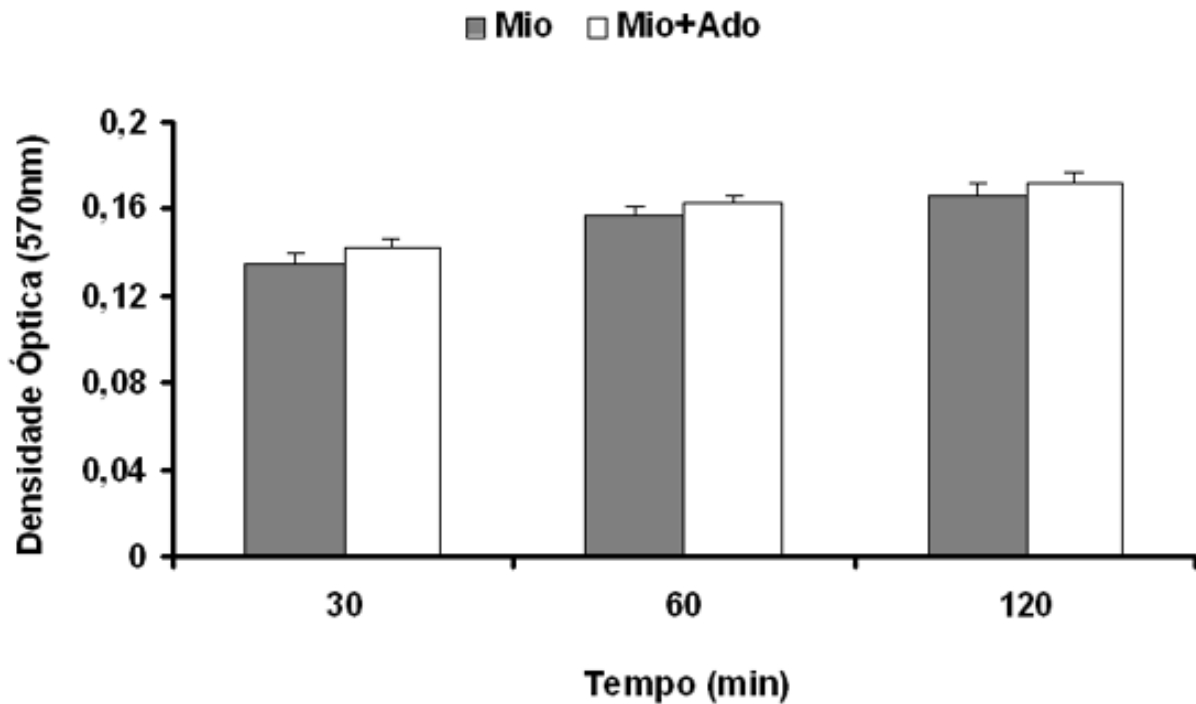
Figura 2: Análise da adesão das células estreladas hepáticas ao plástico da placa de cultura na presença de adenosina. As células GRX foram tripsinizadas e plaqueadas na presença de 100 µM de adenosina. Após 30, 60 e 120 minutos, as células não aderentes foram removidas e o potencial de adesão ao substrato foi determinado pela coloração com cristal violeta. Os valores representam a média das densidades opticas em 570nm ± EPM de três experimentos independentes realizados em quadruplicata. As diferenças foram analisadas por ANOVA, seguida pelo teste de Duncan.
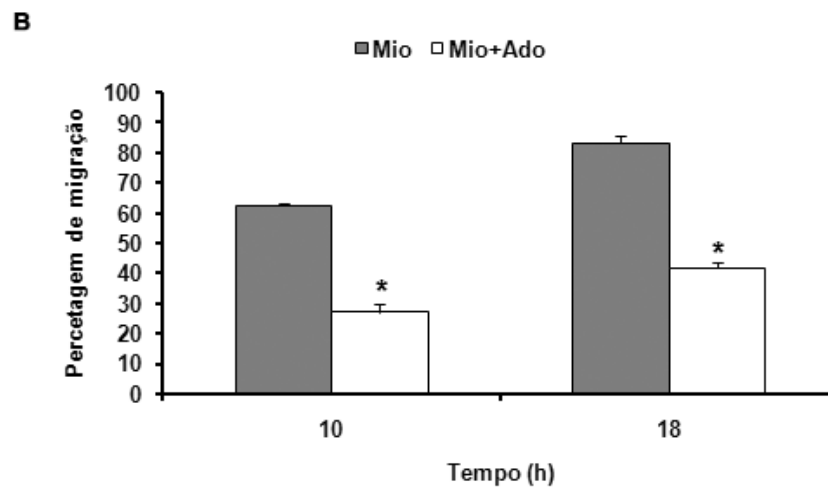
Figura 3: Efeito da adenosina na migração das células estreladas hepáticas. (A) Imagens das culturas de GRX obtidas por microscopia de contraste de fase após o dano a monocamada (0h) e 10h e 18h após, em presença ou ausência de adenosina. (B) O percentual de migração celular, após 10 e 18h, foi calculado considerando-se a largura inicial da ranhura (0h) como 100%. Os valores representam o percentual médio ± EPM de dois experimentos independentes realizados em quadruplicata. As diferenças foram analisadas por ANOVA, seguida pelo teste de Duncan. *Valores estatisticamente diferentes do grupo controle, $P<0,05$.
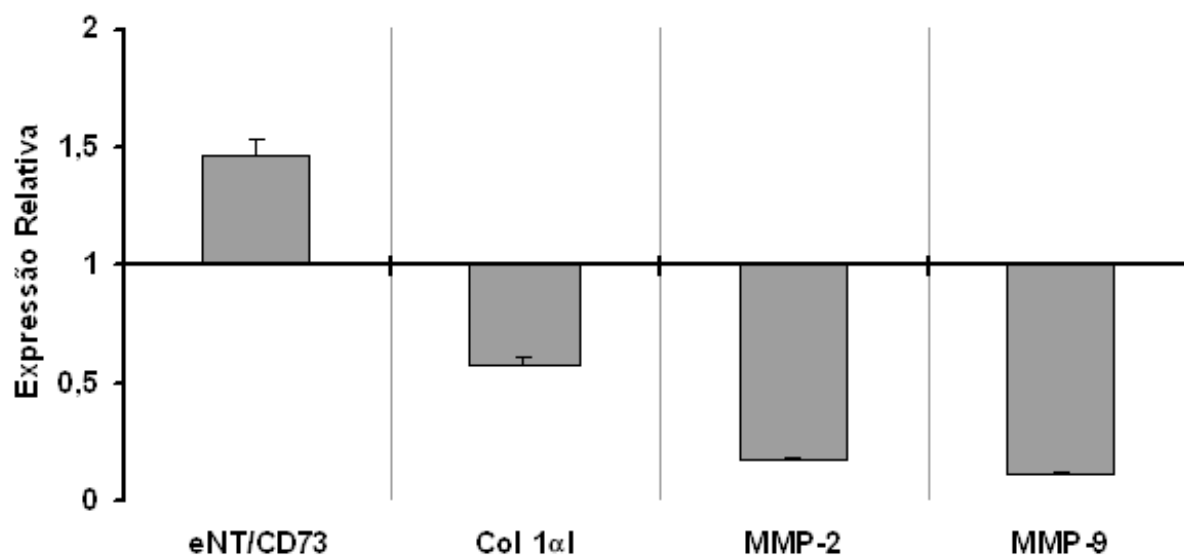
Figura 4: Análise da expressão do mRNA da ecto-5'-nucleotidase (eNT/CD73), colágeno 1 (Col 1αI) e metaloproteínases 2 e 9 (MMP-2 e MMP-9). O nível de mRNA foi determinado por PCR em tempo real e a quantificação relativa foi normalizada pela expressão de β-actina e calculada pelo método $2^{-\Delta\Delta CT}$.

PARTE III

DISCUSSÃO

As células estreladas do tecido conjuntivo hepático participam do metabolismo da vitamina A, são reguladoras do fluxo sangüíneo no sinusóide hepático, têm importância na manutenção da homeostase da matriz extracelular do lóbulo hepático bem como atuam no reparo e regeneração de danos hepáticos com o aumento na síntese de matriz extracelular. Em condições normais, estas células representam menos de 10% de todas as células do fígado, no entanto são capazes de estocar 80% da vitamina A do organismo e de mobilizar estes estoques mantendo a concentração plasmática constante (Senoo, 2004). O fato de estar em tão baixo número no fígado torna tecnicamente difícil, além de oneroso, o isolamento de quantidades suficientes de células para estudos *in vitro*. Igualmente limitante é o duplo fenótipo que as HSCs exibem. Estes dois fenótipos estão em constante equilíbrio e apresentam funções fisiológicas distintas (Friedman, 2008a). Em situações normais, no fígado saudável, há predomínio das HSCs em seu fenótipo quiescente, caracterizado pela baixa velocidade de proliferação e a presença de gotas lipídicas no citoplasma. O segundo fenótipo, com características miofibroblastóides, é produtor de componentes de matriz extracelular, mantendo a estrutura do lóbulo hepático, porém, predominante em situações patológicas. Quando as HSCs são isoladas e mantidas em cultura, há perda progressiva e rápida dos retinóides, aumento da capacidade de proliferação e expressão de marcadores de células fibroblásticas. Para transpor estas limitações, várias linhagens de HSCs têm sido estabelecidas e o número de estudos usando estes modelos tem aumentado drasticamente nos últimos anos (Herrmann et al., 2007). A

linhagem permanente GRX é um miofibroblasto capaz expressar o fenótipo lipocítico, característico das HSCs quiescentes, por longos períodos de tempo e por esta razão constitui um importante modelo para o estudo da modulação fenotípica das HSCs. Neste estudo, o fenótipo quiescente foi induzido pelo tratamento das culturas com como retinol (Margis & Borojevic, 1989) ou indometacina (Guaragna et al., 1991).

O conjunto de resultados aqui apresentado demonstra que os dois fenótipos das células estreladas hepáticas da linhagem GRX expressam todos os componentes da cascata enzimática necessários para a completa metabolização extracelular dos nucleotídeos. No entanto, células quiescentes e ativadas apresentam diferenças nas atividades dessas enzimas. As GRXs quiescentes foram mais eficientes na hidrólise de todos os nucleotídeos trifosfatados quando comparadas com as GRXs ativadas, sendo o ATP o principal substrato trifosfatado hidrolisado pelos dois tipos celulares. A avaliação da hidrólise dos nucleosídeos difosfatados mostrou que ambos os fenótipos hidrolisam igualmente estes compostos, sendo o UDP o substrato preferencial (capítulo 1). A atividade AMPásica foi consideravelmente maior do que a ATPásica e ADPásica nos dois fenótipos, entretanto as GRXs quiescentes foram mais eficazes na degradação extracelular do AMP (capítulos 1 e 3). É importante ressaltar que os resultados observados foram semelhantes tanto nas células quiescentes induzidas com retinol como com indometacina, sugerindo que estão relacionados com a conversão fenotípica e não com o agente indutor da diferenciação. A análise conjunta destes dados demonstra que nas GRXs ativadas a degradação extracelular de nucleotídeos é menor, indicando um aumento no tempo de meia vida destes compostos no meio extracelular, que pode estar relacionado com os efeitos mediados por eles em cada um

dos fenótipos. Culturas primárias de HSCs mantidas por um dia, que são representativas do fenótipo quiescente expressam os receptores $P2Y_2$ e $P2Y_4$, já as culturas de sete dias, representativas das células ativadas, expressam receptores $P2Y_6$. Os ligantes preferenciais dos receptores $P2Y_2$ e $P2Y_4$ são ATP e UTP, sendo o UDP para os $P2Y_6$ (Dranoff et al., 2004). A interação do ATP com os receptores P2 em HSCs eleva a concentração intracelular de cálcio através da ativação do receptor de IP3 tipo I, resultando no aumento da transcrição de pró-colágeno I, proliferação e contração destas células (Kruglov et al., 2007). Embora não se conheça o perfil de expressão dos receptores P2 nas células GRX, interessantemente os substratos tri- e di-fosfatados preferencialmente hidrolisados por elas foram ATP, UTP e UDP (capítulo 1), os mesmos que ativam os receptores expressos pelas células HSCs, corroborando com a hipótese de que a sinalização mediada pelo ATP e UTP é importante na regulação funcional das HSCs ativadas.

Pelo fato das ectonucleotidases serem reguladores críticos das respostas fisiológicas mediadas pelos receptores purinérgicos, a expressão diferencial das E-NTPDases (capítulo 1), E-NPPs (capítulo 2), ecto-fosfatase alcalina e ecto-5'-nucleotidase (capítulo 3) foi investigada nas GRXs quiescentes e ativadas. Os dois fenótipos expressam as NTPDases3 e 5, as NPPs 1, 2 e 3, a ecto-5'-nucleotidase e a fosfatase alcalina tecido inespecífica (TNALP). A NTPDase6 somente é expressa pelas GRXs ativadas. A expressão funcional destas enzimas está em concordância com o perfil de hidrólise de nucleotídeos acima discutido. Nas GRXs quiescentes a razão de hidrólise ATP/ADP foi 2,97, a qual é compatível com a presença da NTPDase3. A NTPDase5 hidrolisa preferencialmente nucleotídeos difosfatados, sendo o UDP seu

principal substrato e a expressão desta enzima pelas células GRXs quiescentes pode justificar a atividade UDPásica observada. Já nas células ativadas, a transcrição das NTPDases 3 e 5 é menor do que nas células quiescentes, além disto há a expressão da NTPDase6. A razão de hidrólise ATP/ADP é 1,41 e pode ser justificada pela menor expressão da NTPDase3 e conseqüentemente menor hidrólise de ATP, juntamente com a expressão das NTPDases 5 e 6, que hidrolisam os substratos difosfatados, aumentando a razão de degradação dos mesmos.

Em função do padrão de hidrólise de nucleotídeos e do perfil de expressão de ectonucleotidases observado nas células GRX, parece relevante a ausência das NTPDases 1 e 2 nestas células, uma vez que a NTPDase1 hidrolisa ATP e ADP em igual proporção e a NTPDase2 tem alta preferência por ATP. Resultados semelhantes, com relação à expressão destas duas últimas enzimas em culturas de células estreladas hepáticas, já foram demonstrados por Dranoff et al. (2002). No entanto, em 2004, utilizando um modelo de cirrose induzido por CCl_4 em ratos, foi demostrado a co-localização da NTPDase2 com alfa-actina de músculo liso, sugerindo sua expressão nas HSCs ativadas (Dranoff et al., 2004).

A expressão da NTPDase8 não foi detectada nas células GRX. Bigonesse e colaboradores (2004) mostraram que esta enzima é expressa em fígado, rins e jejuno de camundongos. Nossos resultados complementam esta informação, confirmando sua presença no fígado, mas demonstram que não é expressa pelas células estreladas hepáticas. As NTPDases 4 e 7 não foram investigadas neste trabalho por se tratarem

de enzimas intracelulares e por esta razão não participarem da degradação extracelular de nucleotídeos (Biederbick et al., 2000; Shi et al., 2001).

O estudo das E-NPPs na linhagem GRX demonstrou que ambos os fenótipos destas células expressam o mRNA das NPP1, NPP2 e NPP3 (capítulo 2). No entanto, a quantificação da expressão por PCR em tempo real mostrou que nas células quiescentes ocorre redução na transcrição da NPP2 e aumento na quantidade de transcritos da NPP3 em relação às GRXs ativadas. Já, a expressão da NPP1 não foi alterada. A análise da hidrólise do p-nitrofenil-5'-timidina-monofosfato, um substrato artificial comumente usado para investigar a atividade das E-NPPs, mostrou que tanto em pH alcalino, como no pH fisiológico a atividade é maior nas células quiescentes, o que pode estar relacionado com o aumento da transcrição da NPP3 neste fenótipo. As E-NPPs hidrolisam diversos substratos incluindo os nucleotídeos. Neste contexto, é provável que o aumento na expressão da NPP3 em células quiescentes também possa estar contribuindo com o aumento da hidrólise de nucleotídeos trifosfatados observado nestas células, embora, a NTPDase3 pareça ser a principal enzima com esta função em condições fisiológicas. O perfil de metabolização do ATP, avaliado por HPLC, reforça a hipótese da participação das NPPs na hidrólise de ATP e conseqüente formação de AMP e PPi.

A expressão da NPP2 pelas GRXs pode estar relacionada com o papel desta enzima na ativação de rotas que estimulam a proliferação e migração celular. Esta enzima foi identificada pela primeira vez no meio condicionado de células de melanoma, como uma proteína estimulante da motilidade tumoral e foi denominada de

autotaxina (Stracke et al., 1992). Os efeitos estimulatórios da NPP2 na proliferação, contração e migração celular têm sido atribuídos à sua habilidade de produzir ácido lisofosfatídico a partir de lisofosfatidilcolina. Entretanto, estes efeitos também podem ser atribuídos à sua capacidade de hidrolisar nucleotídeos (Stefan et al., 2006). Deste modo, a redução da expressão desta proteína nas GRXs quiescentes está em concordância com a menor taxa de proliferação e migração destas células quando comparadas com as GRXs ativadas.

A expressão da NPP3 na membrana apical de hepatócitos e colangiócitos está relacionada com a formação da bile e a expressão da NPP1 na membrana basolateral dos hepatócitos está envolvida com a estimulação da proliferação celular (Yano et al., 2004). Até o presente momento, os dados apresentados aqui são os primeiros acerca da expressão e atividade da E-NPPs em células estreladas hepáticas e mostram que estas enzimas fazem parte do conjunto de ectonucleotidases responsáveis pelo controle dos nucleotídeos na superfície destas células.

A atividade AMPásica foi a mais expressiva nas células GRX (capítulo 3). A rápida metabolização do AMP, observada na análise por HPLC, está em concordância com a atividade de hidrólise deste nucleotídeo determinada pela quantificação da liberação de fosfato inorgânico. A comparação da degradação do AMP entre GRXs quiescentes e ativadas mostrou que nas células quiescentes a atividade específica é maior e a metabolização do AMP é mais rápida. As principais enzimas responsáveis pela metabolização do AMP são a ecto-5'-nucleotidase e a fosfatase alcalina. No entanto, em condições fisiológicas, onde a concentração de AMP é baixa, a ecto-5'-

nucleotidase é mais eficiente na hidrólise deste nucleotídeo por apresentar baixo K_m para este substrato (Yegutkin, 2008).

O estudo da expressão da ecto-5'-nucleotidase e da fosfatase alacalina tecido inespecífica mostrou que células ativadas e quiescentes expressam mRNA para ambas as enzimas (capítulo 3). Para investigar se o aumento observado na hidrólise do AMP nas células quiescentes foi devido a maior expressão da ecto-5'-nucleotidase e/ou da TNALP, os níveis de mRNA das duas enzimas nos dois fenótipos das HSCs foram comparados. Os resultados mostraram que nas células quiescentes, induzidas por indometacina ocorre aumento do mRNA da ecto-5'-nucleotidase e nas induzidas por retinol ocorre aumento da transcrição da ecto-5'-nucleotidase e da TNALP.

A alta variabilidade no nível de expressão da ecto-5'-nucleotidase em diferentes tipos celulares e tecidos, em humanos e animais, e o número de potenciais elementos regulatórios no promotor do gene que codifica esta proteína, sugere a existência de mecanismos de controle tecido-específico e um elevado nível de complexidade na interação com ligante (Spychala, Zimmermann & Mitchell, 1999). O aumento na atividade enzimática e na transcrição da ecto-5'-nucleotidase também foi relatado em culturas de gliomas após o tratamento com 100μM de indometacina (Bernardi et al., 2007). Uma hipótese para explicar o efeito da indometacina na modulação da transcrição da ecto-5'-nucleotidase é através da ativação da proteína Sp1. Sp1 é um fator de transcrição essencial na regulação de diversos processos celulares que pode ser modulado por fatores que ativam ERK/MAPK (Lomberk & Urrutia, 2005). A presença de cinco sítios para ligação de Sp1 no gene da ecto-5'-nucleotidase, logo

após o códon de iniciação (Spychala et al., 1999), associado ao fato de que a indometacina é capaz de ativar ERK (Bernardi et al., 2006) reforçam a hipótese de que este fármaco pode ativar o promotor da ecto-5'-nucleotidase através da indução da proteína Sp1.

O all-trans ácido retinóico e seu estereoisômero 9-cis-ácido retinóico são os retinóides biologicamente mais ativos. Estão presentes em vários tecidos e em quantidades menores que as do all-trans retinol e são formados a partir da oxidação do retinol, retinaldeído ou do beta-caroteno (Ross & Ternus, 1993). O ácido retinóico regula a expressão de vários genes através da ativação de receptores específicos (RAR), os quais formam heterodímeros com os receptores RXR e este complexo liga-se às regiões promotoras dos genes alvos chamados elementos de resposta ao ácido retinóico (RARE) (Chen et al., 2004). O aumento da transcrição da ecto-5'-nucleotidase observado após a conversão fenotípica das GRXs também foi demonstrado após o tratamento de células de neuroblastoma humano com ácido retinóico (Kohring & Zimmermann, 1998), sugerindo que este composto está envolvido na regulação da transcrição da ecto-5'-nucleotidase. No entanto, o aumento no mRNA desta enzima e provavelmente da proteína, visto que há um aumento também na atividade enzimática, após a indução fenotípica das células GRX por dois agentes distintos, indometacina e retinol, sugere que a ecto-5'-nucleotidase deve ter um papel funcional na modulação fenotípica das HSCs.

É conhecido que o ácido retinóico aumenta a transcrição da TNALP em linhagens de fibroblastos, osteoblastos e teratocarcinomas e este efeito é via ativação

de RARE, presente na região promotora do gene da TNALP (Orimo & Shimada, 2005). Assim, o aumento da transcrição desta enzima observado nas células quiescentes parece ser modulado pelo ácido retinóico, mas não pela indometacina que não provocou o mesmo efeito.

Outro resultado divergente entre a indução mediada pelo retinol em relação à indometacina é o acúmulo de adenosina no meio de incubação após a hidrólise do AMP (capítulo 3). Para investigar as razões do acúmulo de adenosina nestas células, a atividade da ecto-adenosina deaminase e a liberação de nucleotídeos para o meio extracelular pelas GRXs quiescentes foram determinadas por HPLC. Os resultados obtidos mostram que nas células tratadas com retinol há diminuição da degradação de adenosina e que não há liberação de quantidades detectáveis deste nucleotídeo para o meio extracelular. Estes dados sugerem que o acúmulo de adenosina pode ser devido à diminuição da atividade da ecto-adenosina deaminase, mas não exclui a possibilidade de o tratamento com retinol estar inibindo os transportadores de nucleotídeos e conseqüentemente a captação da adenosina. No entanto, é necessário esclarecer se o controle da concentração de adenosina extracelular está relacionado com a regulação das ações da adenosina na fibrogênese ou se faz parte do mecanismo de ação do retinol durante a conversão fenotípica das células.

A ecto-5'-nucleotidase, além de ser fundamental para a geração de adenosina e conseqüente regulação da sinalização via receptores P1, também apresenta funções independentes de sua ação catalítica, atuando como molécula de adesão no contato entre células ou com proteínas de matriz extracelular (Yegutkin, 2008). Assim, com o

objetivo de avaliar o papel da ecto-5'-nucleotidase nas HSCs, o mRNA e a expressão desta proteína foram reduzidos utilizando a técnica de interferência por RNA (RNAi) (capítulo 4).

O silenciamento gênico pós-transcricional consiste na degradação citoplasmática do mRNA do gene alvo, utilizando pequenas moléculas guia de RNA, com aproximadamente 21 nucleotídeos, que apresentam homologia com a seqüência do gene alvo. Em plantas este é um mecanismo natural de defesa contra vírus degradando RNAs relacionados a tais patógenos. Em 1998, demonstrou-se que a inoculação de moléculas de RNA dupla fita em *Caenorhabditis elegans* era capaz de silenciar genes homólogos de maneira potente e específica (Fire et al., 1998). O termo interferência por RNA (RNAi) foi então criado e a metodologia adotada para reduzir a expressão de genes em diversos organismos, especialmente em mamíferos, e também em cultura de células. Desse modo, quando RNAs dupla fita (dsRNAs) são introduzidos nas células, eles são processados pela ribonuclease Dicer gerando pequenos RNAs (siRNAs), os quais se associam ao complexo de silenciamento induzido por RNA (RISC) e direcionam a destruição do mRNA complementar a uma das fitas do siRNA (Kuhn, Streif & Wurst, 2007; Mello & Conte, 2004). Com esta técnica é possível obter até 90% de redução na expressão do gene em estudo, mas os resultados variam de acordo com o tipo de células e RNA utilizado (dsRNA, RNA duplas fita ou shRNA, short hairpin RNA produzidos por plasmídios). Além disto, a supressão parcial do gene (knockdowns) apresenta vantagens em relação à supressão total (knockouts), pois siRNAs com diferentes eficiências podem ser usados para produzir animais ou linhagens celulares com diferentes níveis de expressão do mesmo gene, tornando

possível o estudo dos efeitos de pequenas alterações na expressão de um dado gene (Lenz, 2005).

A redução na expressão da ecto-5'-nucleotidase nas células GRX, surpreendentemente não alterou a atividade AMPásica, no entanto, aumentou a adesão das células ao substrato e a migração celular (capítulo 4). A manutenção da hidrólise de AMP observada nas células transduzidas provavelmente se deve ao aumento na transcrição da TNALP e às diferentes propriedades cinéticas destas duas enzimas. É importante salientar que o aumento na transcrição da TNALP não parece ser um efeito associado ao uso de RNAi, ou efeito *off-target*, porque três diferentes seqüências para o mesmo mRNA alvo foram eficazes na redução da expressão da proteína e as duas que produziram a redução mais efetiva foram usadas nos demais experimentos. Além disto, a seqüência utilizada como controle, a qual ativa RISC, mas não tem nenhum mRNA alvo, não afetou a transcrição da TNALP. No entanto, o fato da redução da expressão de ecto-5'-nucleotidase levar ao aumento da transcrição de outra proteína com funções semelhantes sugere que esta atividade enzimática é importante para estas células. Além disto, evidencia a necessidade de novos estudos para o esclarecimento do mecanismo deste processo compensatório.

A migração celular é um evento complexo e essencial para diversos fenômenos fisiológicos, incluindo cicatrização tecidual. Este processo envolve a polarização das células, formação de lamelipódios ou protusões de membrana na direção da migração, ciclos de adesão e desligamento da matriz extracelular, contração e translocação do corpo celular, gerando mudanças coordenadas no citoesqueleto de actina e a interação

dinâmica entre as células e a matriz extracelular (Chen, Zhang & Huang, 2008; Katoh, Hiramoto & Negishi, 2006). Já foi demonstrado que a ecto-5'-nucleotidase pode se ligar a diversos componentes de matriz, como laminina, fibronectina e tenacina C, sugerindo sua participação nos processos de adesão e migração celular (Sadej et al., 2008). Além disto, por ser uma proteína ancorada a GPI, a ecto-5'-nucleotidase pode ser expressa na superfície celular associada a outras moléculas de adesão e também com proteínas da família Src quinases em microdomínios de membrana, conhecidos com *rafts*. Acredita-se que esta associação de proteínas possa ativar rotas de sinalização envolvidas com a adesão celular (Airas et al., 1997; Sadej, Spychala & Skladanowski, 2006). As alterações na adesão e migração das células GRX, observadas após o *knockdown* da ecto-5'-nucleotidase não parecem estar relacionadas com a atividade catalítica desta proteína, uma vez que a hidrólise de AMP foi mantida, e corroboram com a hipótese de que a ecto-5'-nucleotidase desempenha outras funções além da geração de adenosina.

Dentro deste contexto, o efeito deste nucleosídeo sobre diversos parâmetros das células estreladas hepáticas ativadas foi avaliado (capítulo 5). O tratamento com adenosina reduziu a taxa de proliferação e migração celular, diminuiu a transcrição de colágeno I e das metaloproteínases 2 e 9 e aumentou a transcrição da ecto-5'-nucleotidase. O efeito antimitogênico da adenosina está em concordância com os efeitos observados em células de músculo liso vascular, onde a ativação de receptores A_{2B} inibiu o crescimento celular (Dubey et al., 2000). A redução da migração das HSCs em presença de adenosina pode estar relacionada com o papel deste nucleosídeo no direcionamento e concentração destas células nos sítios de injúria, onde a

concentração de adenosina é elevada (Hashmi et al., 2007). Além disto, o aumento na transcrição da ecto-5'-nucleotidase pode estar relacionado com a diminuição da migração, pois quando a expressão desta proteína foi reduzida obteve-se efeito contrário, reforçando a hipótese do envolvimento da mesma na mobilidade celular.

Diversos trabalhos têm sido realizados com o objetivo de investigar a relação da adenosina com a fibrose hepática. Chan *et al.* (2006) demonstraram que a ativação de receptores A_{2A} aumenta a expressão de colágeno em uma linhagem de células estreladas humana (LX-2), no entanto o efeito não foi observado com a ativação do receptor A_3. O mesmo grupo de pesquisadores demonstrou que o aumento na expressão de colágeno mediado pelo receptor A_{2A} é um processo complexo, que ativa múltiplas rotas de sinalização, incluindo G_s/cAMP/PKA e p38 MAPK (Che, Chan & Cronstein, 2007). Uma vez que as células GRX expressam somente o $A_{2B,}$ os resultados divergentes, aqui apresentados, com relação à transcrição de colágeno I devem estar relacionados com o subtipo de receptor de adenosina. Assim sendo, embora preliminares, estes dados sugerem que a sinalização mediada pela adenosina é complexa e os efeitos sobre as células estreladas hepáticas são dependentes do tipo de receptor ativado.

Até o momento, não existe nenhum tratamento específico para a fibrose hepática e muitas questões permanecem com respeito ao destino das HSCs ativadas após a resolução do dano hepático. Postula-se que possa ocorrer a reversão para o fenótipo quiescente e também a apoptose destas células (Friedman, 2008b). Assim sendo, os resultados apresentados nesta tese contribuem para o entendimento da

modulação fenotípica das HSCs bem como geram novos paradigmas a respeito do reparo tecidual e da fisiologia deste fascinante tipo celular.

CONCLUSÕES

1. As células GRXs quiescentes e ativadas apresentam um perfil diferenciado na hidrólise extracelular de nucleotídeos.

2. As células GRXs quiescentes degradam nucleotídeos trifosfatados e o AMP com uma atividade específica significativamente maior que as células ativadas.

3. A conversão fenotípica das células GRXs altera quantitativamente o padrão de expressão das ectonucleotidases pertencentes à família das E-NTPDases, E-NPPs, além da ecto-5'-nucleotidase e da fosfatase alcalina tecido inespecífica.

4. As diferenças no metabolismo de nucleotídeos e na transcrição das ectonucleotidases parecem estar relacionadas com a conversão fenotípica e não com o agente indutor, pois resultados semelhantes foram obtidos tanto após a indução mediada por retinol, como por indometacina.

5. A redução da expressão da ecto-5'-nucleotidase por RNAi sugere que esta proteína altera a adesão e a migração das células GRX por um mecanismo independente da atividade catalítica.

6. O efeito inibitório da adenosina sobre a fibrogênese parece estar relacionado com o estado de ativação da célula e com o tipo de receptor P1 expresso.

7. A adenosina parece desencadear respostas antifibrogênica por alterar diversos parâmetros relacionados com a ativação das HSCs.

As conclusões acima nos levaram a formular uma hipótese para o envolvimento da cadeia das ectonucleotidases na modulação fenotípica das HSCs.

MODELO PROPOSTO PARA O ENVOLVIMENTO DAS ECTONUCLEOTIDASES NA MODULÇÃO FENOTÍPICA DAS HSCs

As células GRX constituem uma importante ferramenta para o estudo de fatores que regulam a modulação fenotípica das HSCs por representar o estado de ativação intermediário destas células, o qual pode ser induzido ao fenótipo quiescente e também a um estado mais ativado através ação de diferentes agentes indutores. Propomos, então, que o controle da expressão e atividade das ectonucleotidases nas HSCs representa um dos mecanismos relacionados com sua modulação fenotípica. A diminuição na expressão destas proteínas nas células ativadas contribui para a manutenção da alta concentração de ATP nos sítios de injúria e a manutenção das células no seu estado ativado. Por outro lado, o aumento na expressão destas enzimas pode representar uma estratégia das HSCs que se encontram em um estado intermediário de ativação retornar para o fenótipo quiescente após cessar os estímulos que levam a ativação das mesmas, corroborando assim com a hipótese de que *in vivo* possa ocorrer a reversão ao estado quiescente e regeneração hepática após a cicatrização tecidual.
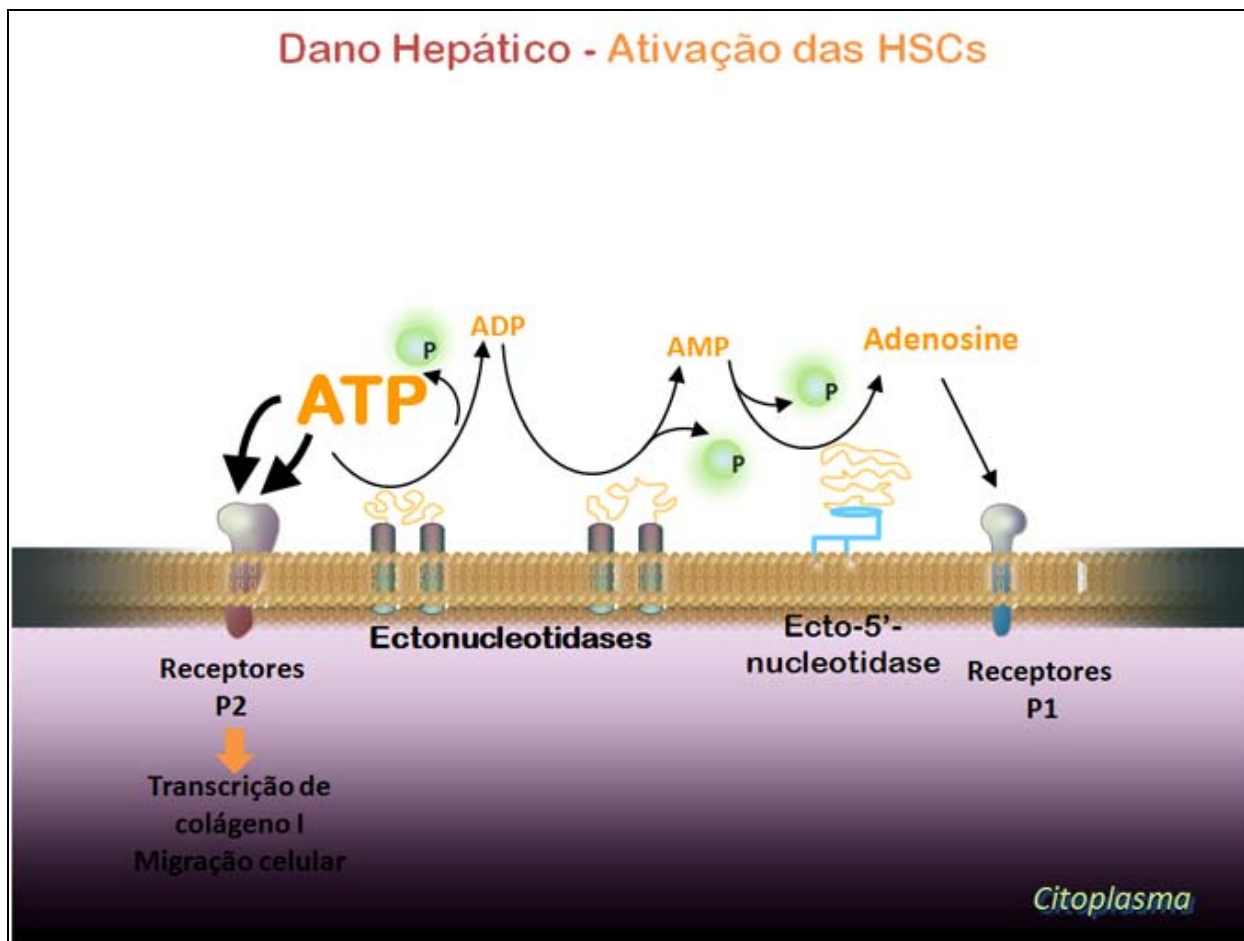
Figura 1. Modelo proposto para o envolvimento das ectonucleotidases na modulação fenotípica das HSCs durante um dano hepático. A diminuição na expressão das ectonucleotidases nas HSCs contribui para a manutenção da alta concentração de ATP nos sítios de injúria e a ativação dos receptores P2, desencadeando estímulos fibrogênicos, como o aumento da transcrição de colágeno e a migração celular, favorecendo, assim, a manutenção das células no seu estado ativado.
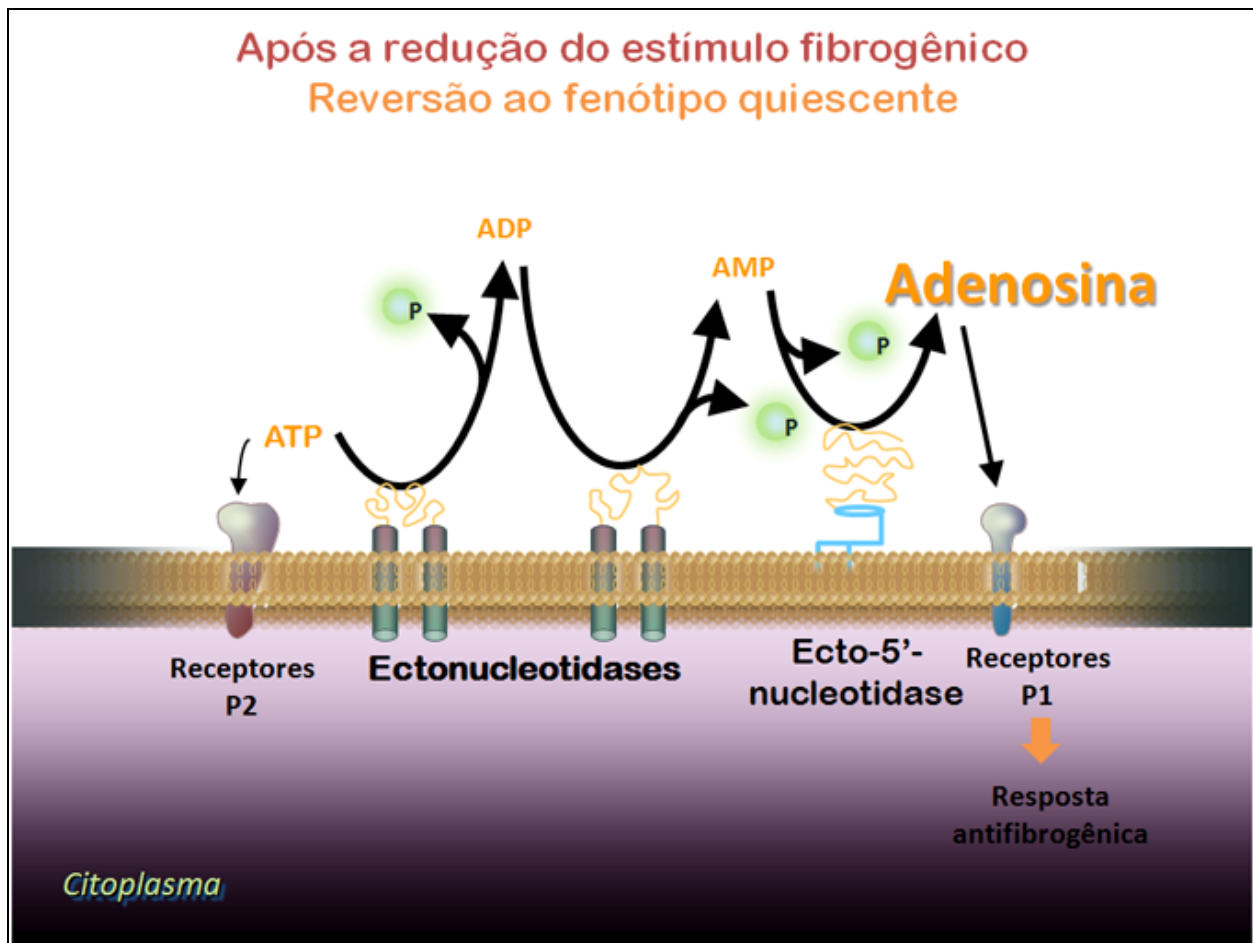
Figura 2. Modelo proposto para o envolvimento das ectonucleotidases na modulação fenotípica das HSCs após a redução do estímulo fibrogênico. O aumento na expressão das ectonucleotidases nas HSCs que se encontram em um estado intermediário de ativação aumenta a formação de adenosina e ativa sinalização via receptores P1, desencadeando respostas antifibrogênicas e favorecendo a reversão ao estado quiescente e regeneração hepática após a cicatrização tecidual.

PERSPECTIVAS

Os resultados obtidos neste estudo originaram uma proposta de participação das ectonucleotidases na modulação fenotípica das HSCs. No entanto, muitas questões ainda necessitam ser esclarecidas, as quais geram as seguintes perspectivas de trabalho:

1. Determinar quais subtipos de receptores P2 são expressos pelas células GRX.

2. Avaliar o efeito do ATP sobre parâmetros de ativação das HSCs.

3. Avaliar a hidrólise de ATP e p-nitrofenil-5'-timidina-monofosfato na presença de inibidores da fosfatase alcalina (levamisole) e de E-NTPDases (cloreto de gadolínium) para determinar possíveis associações enzimáticas na hidrólise destes dois substratos.

4. Determinar a atividade das metaloproteinases 2 e 9 nas células GRX na presença de adenosina.

5. Avaliar os efeitos de um agonista do receptor A_{2B} (BAY 60-6583) e comparar com os efeitos da adenosina.

6. Reduzir a expressão da fosfatase alcalina tecido inespecífica com siRNA e comparar com os efeitos encontrados após a redução da expressão da ecto-5'-nucleotidase.

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