Pontifícia Universidade Católica do Rio Grande do Sul
Faculdade de Biociências
Programa de Pós-Graduação em Biologia Celular e Molecular

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O papel do sistema purinérgico e da via de sinalização TOR em crises convulsivas e estresse oxidativo

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

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RESUMO

A epilepsia, caracterizada pela ocorrência de crises convulsivas espontâneas e recorrentes, é umas das principais doenças neurológicas crônicas, afetando em torno de 1% da população mundial. A adenosina é um modulador endógeno da excitabilidade neuronal e apresenta propriedades anticonvulsivantes. Sendo assim, a modulação da via de sinalização adenosinérgica pode apresentar um efeito importante na epilepsia. Neste estudo, nós caracterizamos diferentes aspectos da sinalização adenosinérgica em modelo de crise convulsiva induzida por pentilenotetrazol (PTZ) em peixe-zebra. Nossos resultados demonstram um aumento nas atividades da adenosina desaminase (ADA), responsável pela desaminação de adenosina em inosina, logo após uma crise convulsiva. Além disso, foi observado que os fármacos antiepilépticos gabapentina, fenitoína e ácido valpróico preveniram o efeito estimulatório promovido pelo PTZ sobre as atividades da adenosina desaminase. Neste estudo, também analisamos o efeito de diferentes moduladores da sinalização adenosinérgica no controle do desenvolvimento de convulsões induzidas por PTZ. Nossos resultados demonstraram que a ativação de receptores de adenosina do tipo A_1 tem importante participação no controle de crises convulsivas em peixe-zebra. Além disso, observamos que as enzimas ecto-5´-nucleotidase e ADA, além dos transportadores de nucleosídeos estão diretamente envolvidos no controle dos níveis extracelulares de adenosina e, consequentemente, no controle do desenvolvimento de crises convulsivas neste teleósteo. Além disso, esclarecemos a ocorrência de dados controversos relacionados à via de sinalização mTOR em estresse oxidativo. Estudos sugeriram a ativação desta via em estresse oxidativo baseados na interpretação equivocada da fosforilação das proteínas RSK e MSK pelo anticorpo anti-fosfo-Thr389-S6K, além da fosforilação da proteína S6, regulada neste caso pela via de sinalização MAPK. Este estudo pode contribuir para um maior entendimento das vias de sinalização envolvidas nos mecanismos de controle de crises convulsivas e representar uma alternativa para o desenvolvimento de fármacos antiepilépticos, aumentando as opções terapêuticas em epilepsia. Nossos resultados também podem contribuir para futuros estudos referentes à caracterização e modulação da via de sinalização TOR em peixe-zebra.

Palavras-chave: adenosina, crise convulsiva, estresse oxidativo, peixe-zebra,TOR.

ABSTRACT

Epilepsy, characterized by the occurrence of spontaneous and recurrent seizures, is one of the main chronic neurological diseases, affecting around 1% of the world's population. Adenosine is an endogenous modulator of neuronal excitability and has anticonvulsant properties. Thus, the modulation of adenosinergic signalling pathway may presents important effects on epilepsy. In this study we characterize different aspects of the adenosinergic signaling in a model of seizures induced by pentylenetetrazole (PTZ) in zebrafish. In this study we also analyzed the effect of different modulators of adenosinergic signaling on controlling the development of seizures. Our results showed that the activation of type A_1 adenosine receptors has an important role in controlling seizures in zebrafish. Furthermore, we observed that ecto-5´-nucleotidase and ADA enzymes, in addition to nucleoside transporters, are directly involved in controlling extracellular adenosine levels and, consequently, in controlling the development of seizures in this teleost. In addition, we clarified the occurrence of controversial data related to the mTOR signaling pathway in oxidative stress. Previous studies have suggested the activation of this pathway in oxidative stress based on the misinterpretation of the phosphorylation of RSK and MSK proteins through the antibody anti-phospho-Thr389-S6K, in addition to protein S6 phosphorylation, regulated by the MAPK signaling pathway in this case. Therefore, these findings might contribute for a better understanding about the signaling pathways involved in the mechanisms of seizure control and represents na alternative for the development of antiepileptic drugs, increasing the therapeutic options in epilepsia. Our results may also contribute to future studies on the characterization and modulation of TOR signaling pathway in zebrafish.

Keywords: adenosine, seizures, oxidative stress, zebrafish, TOR.

LISTA DE ABREVIATURAS

4E-BPI - proteína de ligação do fator de iniciação eucariótico 4E 1

ADA - adenosina desaminase

ADAL - adenosina desaminase “like”

ADP - adenosina 5´- difosfato

AGC - família de proteínas quinase A,G e C

AK - adenosina quinase

AMP - adenosina 5´- monofosfato

AMPc - adenosina 5´- monofosfato cíclico

AMPCP - α,β- metileno ADP

ATP - adenosina 5´- trifosfato

Ca^{+2} - cálcio

Cl^{-} - cloreto

CPA - N^6-ciclopentil-adenosina

Deptor - proteína de interação que contém o domínio mTOR

DPCPX- [propil- 3h]8-ciclopentil-1,3-dipropilxantina

EHNA - eritro-9-[2-hidroxil-3-nonil] adenina

eIF- 4E - fator de iniciação eucariótico 4E

E-NPP - ectonucleotídeo pirofosfatase/fosfodiesterase

ERK- proteína quinase regulada por sinais extracelulares

GABA - ácido gama-aminobutírico

GPI – glicosilfosfatidilinositol

ILAE – Liga internacional contra a epilepsia

K^{+} - potássio

MAPK – proteína quinase ativada por mitógenos

Mg^{+2} - magnésio

mSin1 - proteína de interação da quinase de mamíferos ativada por estresse 1

mTOR - alvo da rapamicina em mamíferos

mTORC1/mTORC2 - complexo mTOR 1/2

MSK - quinase regulada por mitógenos e estresse

Na^{+} - sódio

NMDA - N-metil-D-aspartato

NTPDase - nucleosídeo trifosfato difosfoidrolase

PI3K – quinase fosfatidilinositol-3

PRAS40 - substrato de AKT rico em prolina de 40 KDa

Protor 1/2 - proteína observada com rictor 1/2

PTZ – pentilenotetrazol

Raptor - proteína associada reguladora de mTOR

Rictor - proteína associada insensível à rapamicina

RSK - quinase ribossomal S6 90 KDa

S6K1 – quinase ribossomal S6 1

S6 - proteína ribossômica S6

SNC - sistema nervoso central

TOR - alvo da rapamicina

TSC1 - complexo de esclerose tuberosa 1

TSC2 - complexo de esclerose tuberosa 2

Tti1/Tel2 - proteína 1 que interage com Tel2/proteína reguladora de telômero 2

LISTA DE FIGURAS

SUMÁRIO

1. INTRODUÇÃO

1.1 Epilepsia

A epilepsia é caracterizada pela ocorrência de crises convulsivas espontâneas e recorrentes, sendo uma das principais doenças neurológicas crônicas, afetando em torno de 50 milhões de pessoas no mundo (Pitkanen, 2010). As crises convulsivas ocorrem a partir de descargas neuronais excessivas e sincrônicas e são manifestadas por alguns sintomas transitórios, como perda de consciência e alterações motoras, sensitivas e sensoriais (Fisher et al., 2005). A ocorrência de crises convulsivas recorrentes é característica da epilepsia, porém crises convulsivas únicas podem ocorrer em indivíduos não epilépticos (Goldberg & Coulter, 2013).

De acordo com a *International League Against Epilepsy* (ILAE), a epilepsia pode ser de origem genética, estrutural ou metabólica, ou ainda não ter causa definida (Berg et al., 2010). Na epilepsia de origem genética, as convulsões resultam de alterações genéticas que levam principalmente às disfunções em canais iônicos dependentes de voltagem (Na^+, K^+, Ca^{2+}, Cl^-) ou ativados por ligante (receptores nicotínicos e $GABA_A$) (Deng et al., 2013). A epilepsia do tipo estrutural ou metabólica é caracterizada pelo surgimento de crises convulsivas a partir de um insulto prévio ou patologia do sistema nervoso central (SNC), como, por exemplo, trauma, infecção e tumores (Berg et al., 2010). A partir destes insultos, ocorre um evento conhecido como epileptogênese, que se trata de um processo longo, contínuo e progressivo, que pode levar meses a anos, culminando com o quadro de hiperexcitabilidade neuronal e a manifestação de convulsões (Goldberg & Coulter, 2013). A epileptogênese envolve perda neuronal, plasticidade axonal e dendrítica e reorganização das membranas celulares e da matriz extracelular, podendo ainda ocorrer formação de novos circuitos neuronais excitatórios e a perda seletiva de neurônios gabaérgicos (Jutila et al., 2002; Pitkanen, 2002; Dudek & Sutula, 2007; Dudek & Staley, 2007). As alterações genéticas, estruturais ou metabólicas são as principais causas da epilepsia, mas apesar dos amplos recursos de neurodiagnóstico atualmente disponíveis, muitos pacientes com

epilepsia não possuem nenhuma alteração neurológica claramente identificada, não sendo possível determinar a causa da patologia (Bialer & White, 2010).

As crises convulsivas que caracterizam a epilepsia podem ser classificadas como generalizadas ou focais, de acordo com a abrangência da descarga neuronal (Berg et al., 2010). As crises generalizadas acometem a área cerebral como um todo, e podem ser do tipo, clônica, tônica, tônico-clônica, mioclônica, atônica e de ausência, conforme as manifestações motoras evidenciadas. As convulsões classificadas como focais apresentam descarga neuronal restrita a um hemisfério cerebral e o indivíduo pode ter ou não alterações motoras e de consciência (Berg et al., 2010).

A epilepsia é tratada principalmente através do uso de fármacos, com o objetivo de inibir a ocorrência de crises convulsivas. Estes fármacos provocam a diminuição dos disparos neuronais repetitivos, através da modulação de canais iônicos dependentes de voltagem, potencialização da neurotransmissão inibitória gabaérgica e diminuição da neurotransmissão excitatória glutamatérgica (Bialer & White, 2010). Entre os principais fármacos antiepilépticos disponíveis atualmente, encontram-se a gabapentina, a fenitoína e o ácido valpróico.

A gabapentina atua aumentando a sinalização gabaérgica e modulando canais de Ca^{+2} (Parker et al., 2004; Rogawski & Bazil, 2008). Estudos mostram que a gabapentina liga-se a receptores $GABA_B$ e controla a síntese, liberação, degradação e recaptação do neurotransmissor GABA (Petroff et al., 2000; Bertrand et al., 2001; Errante et al., 2002; Parker et al., 2004). Sabe-se também que a gabapentina liga-se a subunidade α2δ de canais de Ca^{+2} pré-sinápticos, inibindo o influxo de Ca^{+2} e restaurando a liberação de neurotransmissores excitatórios a níveis fisiológicos, através da diminuição de sua liberação excessiva, a qual é característica em crises convulsivas (Bayer et al., 2004; Dooley et al., 2007; Rogawski & Bazil, 2008). O principal mecanismo de ação da fenitoína é a modulação dos canais de Na^{+} dependentes de voltagem (Rogawski & Loscher, 2004a,b). A fenitoína reconhece canais de Na^{+} abertos e os bloqueia, impedindo a propagação de descargas neuronais excessivas (Rogawski & Loscher 2004a,b). Este fármaco também pode agir através da ativação da liberação de GABA e diminuição da liberação de glutamato

(Cunningham et al., 2000; Yang et al., 2007). O ácido valpróico atua na modulação de canais iônicos e inibição da sinalização glutamatérgica (Owens & Nemeroff, 2003; Gobbi & Janiri, 2006). Além disso, o ácido valpróico aumenta a sinalização gabaérgica, através de diversos mecanismos, como aumento da síntese e diminuição da degradação de GABA (Johannessen, 2000; Chateauvieux et al., 2010).

Apesar da grande variedade de fármacos antiepilépticos disponíveis atualmente, somente cerca de 60-70% dos pacientes com epilepsia têm total supressão das crises convulsivas (Stefan & Hopfengartner, 2009). No caso de pacientes que não respondem a medicamentos, buscam-se alternativas não farmacológicas, como cirurgias para ressecção do foco epileptogênico e dieta cetogênica (Elger & Schimidt, 2008). Estudos têm buscado novas alternativas terapêuticas com o uso de fármacos que apresentam efeito anticonvulsivante, por meio de mecanismos independentes das vias de ação dos fármacos antiepilépticos clássicos (Wong, 2010). Entre as vias de sinalização atualmente vistas como potenciais alvos terapêuticos em epilepsia está a sinalização purinérgica, principalmente através da modulação dos níveis de adenosina (Boison, 2012).

1.2 Sistema purinérgico

A sinalização mediada por purinas constitui uma rota comum de comunicação celular, envolvida em muitas condições fisiológicas e patológicas. A adenosina e o ATP são os principais agonistas endógenos do sistema purinérgico e atuam através da ativação de receptores P1 e P2, respectivamente (Abbracchio et al., 2009). O ATP, classicamente conhecido por seu papel central no metabolismo energético, atua como um neurotransmissor excitatório de resposta rápida e apresenta efeitos tróficos importantes na proliferação, diferenciação e morte celular durante o desenvolvimento e a regeneração, assim como em condições patológicas (Burnstock, 1972; Burnstock, 2012). A adenosina atua como um neuromodulador endógeno da atividade neuronal, e está envolvida em diversos processos no SNC, como inflamação e modulação da atividade neuronal (Boison, 2007; Boison, 2010).

O ATP é sintetizado e armazenado em terminais pré-sinápticos e co-liberado na fenda sináptica por meio de estímulos nervosos (Burnstock, 2004; North & Verkhratsky, 2006). Este nucleotídeo atua como neurotransmissor de resposta rápida através dos receptores P2X, enquanto seus efeitos tróficos (proliferação, diferenciação e migração celular) são exercidos através dos receptores P2Y (Burnstock & Kennedy, 1985; Fields & Burnstock, 2006; Burnstock & Verkhratsky, 2010). O grupo de receptores P2X é composto por sete ($P2X_{1-7}$) subtipos de receptores ionotrópicos com permeabilidade rápida e seletiva para cátions (Na^+, K^+ e Ca^{2+}). Estes receptores encontram-se amplamente distribuídos em neurônios e células gliais e são ativados principalmente por ATP (Burnstock, 2012). O grupo P2Y consiste em oito subtipos de receptores metabotrópicos ($P2Y_1$, $P2Y_2$, $P2Y_4$, $P2Y_6$, $P2Y_{11}$, $P2Y_{12}$, $P2Y_{13}$ e $P2Y_{14}$) que estão presentes em vários tecidos e sistemas, como epitelial, muscular, cardíaco, nervoso e imunológico (Abbracchio et al., 2006). No sistema nervoso central e periférico, os receptores P2Y controlam canais de Ca^{2+} e K^+ e a liberação de outros neurotransmissores, como glutamato, serotonina, dopamina e noradrenalina (Hussl & Boehm, 2006). Os receptores P2Y podem ser ativados por diversos nucleotídeos, tais como ATP, ADP, UTP

e UDP (Abbracchio et al., 2006). Além de atuar em receptores do tipo P2, o ATP liberado no espaço extracelular pode ser convertido em adenosina, através de sua hidrólise por enzimas ectonucleotidases (Zimmermann, 2001; Zimmermann et al., 2012) (Figura 1).

A hidrólise dos nucleotídeos extracelulares ocorre por uma cascata de reações que resulta na formação do respectivo nucleosídeo e fosfato livre. As enzimas que catalisam essa reação são as ectonucleotidases, dentre as quais se destacam as nucleosídeo trifosfato difosfoidrolases (NTPDases) e a ecto-5'-nucleotidase (Yegutkin, 2008; Zimmermann et al., 2012). Esse grupo de enzimas inclui também a família das E-NPPs (ectonucleotídeo pirofosfatase/fosfodiesterases) e as fosfatases alcalinas (Zimmermann et al., 2012). A família das NTPDases realiza a hidrólise de nucleotídeos trifosfatados e difosfatados, produzindo o respectivo nucleotídeo monofosfatado e fosfato inorgânico e é composta por oito membros (NTPDases 1-8) (Knowles, 2011). Quatro membros das NTPDases (1, 2, 3, 8) estão localizados na superfície das células, com um sítio catalítico extracelular (Bigonesse et al., 2004; Marcus et al., 2005; Shukla et al., 2005; Belcher et al., 2006). As NTPDases 5 e 6 apresentam localização intracelular e as NTPDases 4 e 7 estão localizadas no meio intracelular com seus sítios ativos direcionados para o lúmen das organelas citoplasmáticas (Wang & Guidotti,1998; Trombetta & Helenius, 1999; Braun et al., 2000; Shi et al., 2001). As NTPDases compartilham 5 domínios altamente conservados, denominados regiões conservadas de apirase (Zimmermann, 2001; Robson et al., 2006).

A hidrólise extracelular de nucleotídeos a seus respectivos nucleosídeos é feita pela ecto-5´-nucleotidase, que hidrolisa ribo e desoxirribonucleotídeos 5´monofosfatados, incluindo AMP,CMP,UMP, IMP e GMP (Zimmermann, 1992). Esta enzima é uma proteína homodimérica ancorada à membrana plasmática por um glicosilfosfatidilinositol (GPI) e tem seu sítio catalítico voltado para o meio extracelular (Bianchi & Spychala, 2003). A ecto-5´-nucleotidase também pode ser encontrada na forma solúvel (Vogel et al., 1992; Zimmermann et al., 2012). O nucleotídeo AMP é o que tem a hidrólise mais eficiente e a produção de adenosina a partir de AMP extracelular é a principal função da enzima ecto-5´-nucleotidase. A hidrólise de AMP pela ecto-5´-

nucleotidase representa um mecanismo extremamente importante no controle dos níveis extracelulares de adenosina (Knapp et al., 2012). Além de ser produzida a partir da hidrólise do ATP, a adenosina pode ser diretamente liberada no meio extracelular através de transportadores de nucleosídeos (King et al., 2006; Bonan, 2012). Em peixe-zebra, diversos estudos identificaram e caracterizaram a atividade e expressão gênica das enzimas NTPDase e 5´-nucleotidase (Rico et al., 2003; Senger et al., 2004). Rosemberg et al. (2010) verificaram a presença de diferentes membros da família das NTPDases e demonstraram a expressão destas enzimas (NTPDases 1, 2, 3, 4, 5, 6, 8) em amostras de encéfalo, coração e fígado. As NTPDase1 e NTPDase2 também foram encontradas em fotorreceptores, células horizontais e células ganglionares em retina de peixe-zebra (Ricatti et al., 2009).

A adenosina atua como um modulador da atividade neuronal, através da ativação de receptores metabotrópicos P1, subdivididos em A_1, A_{2A}, (de maior distribuição e afinidade pela adenosina) A_{2B} e A_3 (de menor distribuição e afinidade pela adenosina) (Fredholm et al., 2005). A ativação de receptores A_1 pré-sinápticos diminui o funcionamento das sinapses excitatórias através da inibição da liberação de glutamato (Burnstock et al., 2011). A ativação pós-sináptica de receptores A_1 leva a estabilização do potencial de membrana através do controle do fluxo de Ca^{+2} e K^+ (Boison, 2005; Fredholm et al., 2005). A ativação de receptores A_{2A} leva a ativação da adenilato ciclase, levando ao aumento nos níveis intracelulares de AMPc (Popoli & Pepponi, 2012). Os receptores A_{2A} participam também do controle da liberação e recaptação de neurotransmissores, como glutamato (Lopes et al., 2002) e GABA (Cristóvão-Ferreira et al., 2009). Os receptores A_{2A} estão envolvidos em processos de neurodegeneração e imunossupressão, entre outros (Fredholm, 2010). A ativação de receptores A_{2A} pode levar a efeitos citotóxicos, devido ao aumento na sinalização glutamatérgica. Porém, a ativação destes receptores também está envolvida no aumento dos níveis de fatores neurotróficos (Sebastião & Ribeiro, 2009). A ativação de receptores A_{2B} tem efeito anti-inflamatório, através do estímulo da produção de interleucina-10 (Koscsó et al., 2013). A deficiência genética de receptores A_{2B} leva ao aumento da inflamação e diminuição da sobrevivência em modelos de sepse em roedores (Csóka et al.,

2010). Os receptores do tipo A_3 estão claramente envolvidos em processos de dano cerebral, como hipóxia e inflamação (Chen et al., 2006; Lee et al., 2006; Pugliese et al., 2007). Estudos demonstram que receptores A_3 também estão envolvidos nos mecanismos de crescimento celular e apoptose e têm sua expressão aumentada em células tumorais (Gessi et al., 2011; Di Virgilio, 2012). A ativação do receptor A_3 durante severa isquemia retarda a recuperação da transmissão sináptica, um processo que está relacionado com a internalização de receptores AMPA, o que poderia ter potencial neuroprotetor (Dennis et al., 2011). Estudos mostraram a expressão gênica de receptores de adenosina em peixe-zebra, no qual foram identificados dois genes codificantes para o receptor A_{2A} e um gene codificante para o receptor A_{2B} (Boehmler et al., 2009). Os receptores A_1, $A_{2A.1}$, $A_{2A.2}$ e A_{2B} são expressos desde 24 horas após a fertilização em peixe-zebra (Capiotti et al., 2011).

A concentração de adenosina pode ser controlada a partir de sua hidrólise pela adenosina desaminase (ADA), fosforilação a AMP pela adenosina quinase (AK) ou através de sua liberação e recaptação celular através de transportadores de nucleosídeos (King et al., 2006; Boison, 2012). A ADA é encontrada tanto no meio citosólico quanto na membrana celular e catalisa a desaminação hidrolítica da adenosina e também da 2´-deoxiadenosina em inosina e 2´-deoxinosina, respectivamente (Boison, 2012). A ADA apresenta diferentes subtipos e está presente em diferentes tecidos, principalmente no sistema gastrointestinal, encéfalo e timo (Maier et al., 2005; Sauer et al., 2012). A ADA1 e uma enzima monomérica encontrada em forma solúvel ou associada a uma proteína (CD26). O complexo ADA1/CD26 constitui uma ecto-ADA, que se encontra ancorada a membrana celular e é responsável pela hidrólise da adenosina extracelular. Alem de sua função enzimática, a ADA1 pode interagir com receptores de adenosina do tipo A_1, aumentando sua afinidade de ligação à adenosina e facilitando assim a transdução de sinal através desses receptores (Ciruela et al., 1996). A ADA2 é encontrada principalmente no plasma sanguíneo. Outro tipo de ADA, a ADAL (adenosina desaminase “like”) também participa da desaminação da adenosina, porém não está devidamente caracterizada (Maier et al., 2005). A deficiência de ADA no organismo leva ao aumento nos níveis de adenosina e está associada ao

aumento da apoptose e bloqueio da diferenciação de timócitos, causando linfopenia e anormalidades neurológicas (Nofech-Mozes et al., 2007;Gaspar et al.,2009; Poliani et al., 2009).

Estudos já identificaram a presença de atividade de desaminação de adenosina em fração citosólica e de membrana em encéfalo de peixe-zebra (Rosemberg et al., 2008). A expressão de distintos genes relacionados à ADA (ADA1, ADAL e dois ortólogos da ADA2) foi verificada em diferentes tecidos (encéfalo, brânquias, coração, fígado, esqueleto, músculo e rim) de peixe-zebra (Rosemberg et al., 2007).

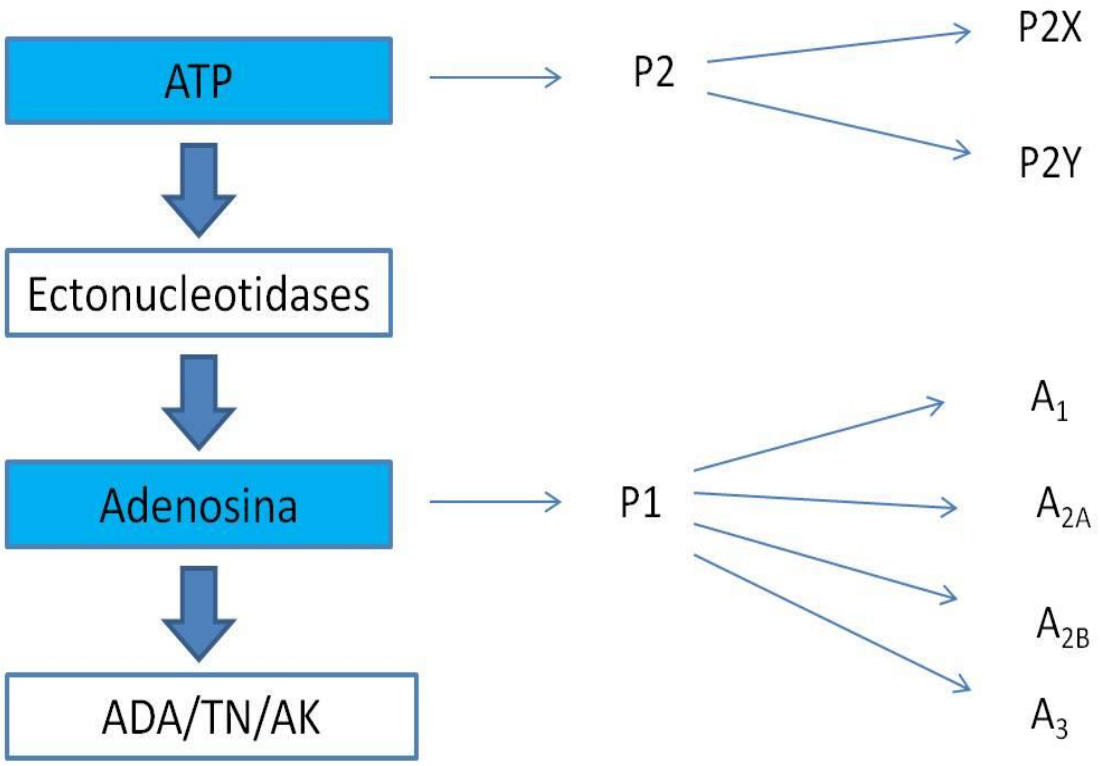

Figura 1: Representação esquemática da sinalização purinérgica. O ATP liberado liga-se a receptores P2, subdivididos em P2X e P2Y. A degradação do ATP pelas ectonucleotidases origina a adenosina, que atua em receptores P1, subdivididos em A_1, A_{2A}, A_{2B} e A_3 e tem seus níveis controlados pela adenosina desaminase (ADA), adenosina quinase (AK) e transportadores de nucleosídeos.

1.2.1 Sistema purinérgico e epilepsia

Diversos estudos têm mostrado a ação neuroprotetora da adenosina, claramente identificada como um neuromodulador endógeno da excitabilidade neuronal (Dunwiddie, 1980; Fredholm & Hedqvist, 1980; Boison, 2010). O tratamento com o análogo de adenosina N^6-(3-methoxyl-4-hydroxybenzyl) (B2) promoveu efeitos neuroprotetores em roedores submetidos a crises convulsivas induzidas por 4-aminopiridina, PTZ, picrotoxina, ácido caínico e estricnina (Li et al., 2013). Roedores pré-tratados com B2 tiveram maior latência para o início das crises convulsivas, menor ocorrência de convulsões, menor duração destas convulsões e menor mortalidade quando comparados a roedores que não receberam pré-tratamento (Li et al., 2013). O implante de células tronco modificadas para maior liberação de adenosina em hipocampo de roedores antes da indução de *kindling* induziu a uma diminuição da epileptogênese (Li et al., 2007). Além disso, o implante destas células após *status epilepticus* induzido por ácido caínico em roedores preveniu o surgimento de crises convulsivas recorrentes (Li et al., 2009).

O importante efeito anticonvulsivante da adenosina ocorre através de mecanismos independentes das vias de ação de fármacos antiepilépticos clássicos (Masino & Geiger, 2008). O efeito anticonvulsivante da adenosina ocorre principalmente através da ativação de receptores A_1, que induz a inibição pré-sináptica, através da redução do influxo de Ca^{2+}, e reduz a excitabilidade da membrana pós-sináptica, aumentando a liberação de K^+ (Fredholm et al., 2005). Foram testados os efeitos dos antagonistas A_1 (DPCPX) e A_{2A} (SCH58261) no potencial anticonvulsivante de B2 na crise convulsiva induzida por PTZ. A administração de DPCPX impediu a ação anticonvulsivante do análogo de adenosina, enquanto a aplicação de SCH58261 não mostrou influencia, evidenciando o papel dos receptores A_1 como anticonvulsivantes (Li et al., 2013). Diferentemente das características bem conhecidas dos receptores A_1, estudos já atribuíram diferentes propriedades anticonvulsivantes e pró-convulsivantes aos receptores A_{2A} (Boison, 2007). Os receptores A_{2B} e A_3, de menor afinidade e abundância, não

têm sido considerados potenciais alvos terapêuticos em epilepsia (Boison, 2005, 2007).

A ativação de receptores A_1 pela adenosina confere neuroproteção, interrompendo ou diminuindo a duração de crises convulsivas e aumentando a sobrevivência neuronal (Gouder et al., 2004; Boison, 2006). Entretanto, o ATP, através da ativação de receptores P2X7, apresenta efeitos pró- convulsivantes (Klaft et al., 2012). Em crises convulsivas, aumentam os níveis extracelulares de ATP, que é rapidamente degradado em adenosina, contribuindo significativamente para a atividade inibitória produzida pela ativação de receptores A_1 (Pascual et al., 2005). Enquanto o ATP apresenta ação convulsivante, sua subsequente degradação em adenosina pode inibir a generalização do foco epiléptico e levar à diminuição da duração da crise convulsiva (Klaft et al., 2012). Consequentemente, a modulação dos níveis de ATP e adenosina através das ectonucleotidases e transportadores bidirecionais de nucleotídeos e nucleosídeos formam um importante mecanismo no controle da epilepsia e representam alvos para terapias farmacológicas (Bonan, 2012; Cognato & Bonan, 2010).

Roedores com epilepsia de lobo temporal induzida por pilocarpina e ácido caínico apresentaram maior atividade das ectonucleotidases na fase crônica da doença (Bonan et al., 2000b; Vianna et al., 2005). Fármacos antiepiléticos, como carbamazepina, ácido valprócio e fenitoína, foram capazes de prevenir as alterações nas atividades ectonucleotidásicas induzidas pelo modelo da pilocarpina (Cognato et al., 2007). Estudos têm demonstrado um prejuízo de memória em ratos adultos previamente submetidos a uma crise no período neonatal com ácido caínico, o que foi acompanhado por um aumento na hidrólise de ATP em sinaptossomas hipocampais (Cognato et al., 2011). O aumento na hidrólise de ATP, ADP e AMP induzido pelo modelo da pilocarpina em ratos adultos não foi observado em animais jovens (de Paula Cognato et al., 2005). Em modelo de *kindling* induzido por PTZ, os ratos mais resistentes às crises convulsivas apresentavam maior hidrólise de ATP (Bonan et al., 2000a). Bruno e colaboradores (2002) observaram um aumento significativo na hidrólise de ATP, ADP e AMP em soro de ratos 24 horas após uma única injeção de PTZ. Resultados similares foram observados quando os animais

foram submetidos ao *kindling* com PTZ (Bruno et al., 2003). Oses e colaboradores (2007) sugeriram que as atividades das nucleotidases solúveis de líquido cérebro-espinhal podem ser utilizadas como marcadores bioquímicos da lesão neuronal causada por crises epilépticas agudas, uma vez que essas enzimas tiveram suas atividades aumentadas 10 minutos após crise convulsiva induzida por PTZ. Recentemente, foi demonstrado que a dieta cetogênica não preveniu as alterações da via das ectonucleotidases após *status epilepticus* induzido pelo modelo de lítio-pilocarpina (Da Silveira et al., 2013).

A concentração de adenosina pode ser controlada também através de sua fosforilação à AMP pela AK ou desaminação a inosina pela ADA (Boison, 2012) A AK é a principal enzima relacionada à eliminação de adenosina em condições fisiológicas, já a ADA contribui para a eliminação de adenosina em condições de estresse, quando os níveis de adenosina estão elevados (Latini & Pedata, 2001; Boison, 2006). A indução de um único episódio de crise convulsiva elevou a atividade da ADA em peixe-zebra. O pré-tratamento dos animais com fármacos antiepilépticos impediu esta ativação (Siebel et al., 2013, Capítulo I). Além disso, após sucessivos episódios, a atividade da ADA encontrou-se diminuída (Siebel et al., 2011). Portanto, a administração de moduladores da ADA também tem se mostrado uma estratégia de ação eficaz em diversos modelos de epilepsia (Dupere et al., 1999; Southam et al., 2002).

1.3 mTOR

A via de sinalização mTOR é modulada por diversos sinais e funciona como um mecanismo regulador central do crescimento, metabolismo e sobrevivência celular, controlando a transcrição e tradução de proteínas importantes para estes processos celulares (Wong, 2010). Hormônios, como a insulina, e nutrientes, como aminoácidos, medeiam seus efeitos celulares através desta via (Laplante & Sabatini, 2012). A mTOR é uma serina-treonina quinase atípica que pertence à família PI3K e interage com diversas proteínas, formando dois complexos distintos, mTORC1 e mTORC2 (Laplante & Sabatini, 2012). Estes dois complexos compartilham a subunidade catalítica mTOR, mLST8, DEPTOR e o complexo Tti1/Tel2 (Kim et al., 2003; Peterson et al.,

2009; Kaizuka et al., 2010). Em contraste, raptor e PRAS40 são específicos de mTORC1 (Hara et al., 2002; Wang et al., 2007), enquanto rictor, mSin1 e protor1/2 são específicos de mTORC2 (Sarbassov et al., 2004; Frias et al., 2006; Pearce et al., 2011). O mTORC1, sensível à rapamicina, é o complexo melhor caracterizado e tem como característica marcante a sensibilidade a um grande número e diversidade de sinais (Laplante & Sabatini, 2012). O mTORC1 integra as principais vias de sinalizadores intracelulares e extracelulares como fatores de crescimento, estresse, status energético e aminoácidos, no controle de processos celulares essenciais, como síntese lipídica e de proteínas e autofagia (Laplante & Sabatini, 2012).

A proteína S6K1 (quinase ribossomal S6 1) é um substrato do complexo mTORC1 (Figura 2). Este complexo regula a ativação de S6K1 através de sua fosforilação no resíduo T389 (Thr389). A ativação da S6K1 regula a fosforilação de outros substratos, como a proteína ribossomal S6 (S6), para promover a síntese protéica e o crescimento e proliferação celular (Dann et al., 2007; Magnuson et al., 2012). A proteína S6K1 é um membro da família de serina-treonina quinases conhecidas como AGC. Esta família inclui as MAPKAPKs (proteínas quinase ativadas por MAPKs), a MSK (quinase ativada por mitógeno e estresse) e a RSK (quinase ribossomal S6), que têm alta homologia com S6K1 e que também regulam a ativação da proteína S6 (Casas-Terradellas et al., 2008). Em muitos estudos da via de sinalização mTOR, a ativação de S6K1 é medida através de sua fosforilação em T389 e pelo aumento na fosforilação de seu substrato S6 (Casas-Terradellas et al., 2008). Em modelos de estresse oxidativo, alguns estudos mostram a ativação, enquanto outros mostram a inibição da via da mTOR através da análise das proteínas S6K1 e S6 (Chen et al., 2010; Gutierrez-Uzquiza et al., 2012). Estes resultados controversos podem ter ocorrido devido à fosforilação das proteínas RSK e MSK pelo anticorpo anti-fosfo-Thr389-S6K, além da fosforilação da proteína S6, que poderia estar sendo regulada pela via de sinalização MAPK, e não pela via da mTOR.

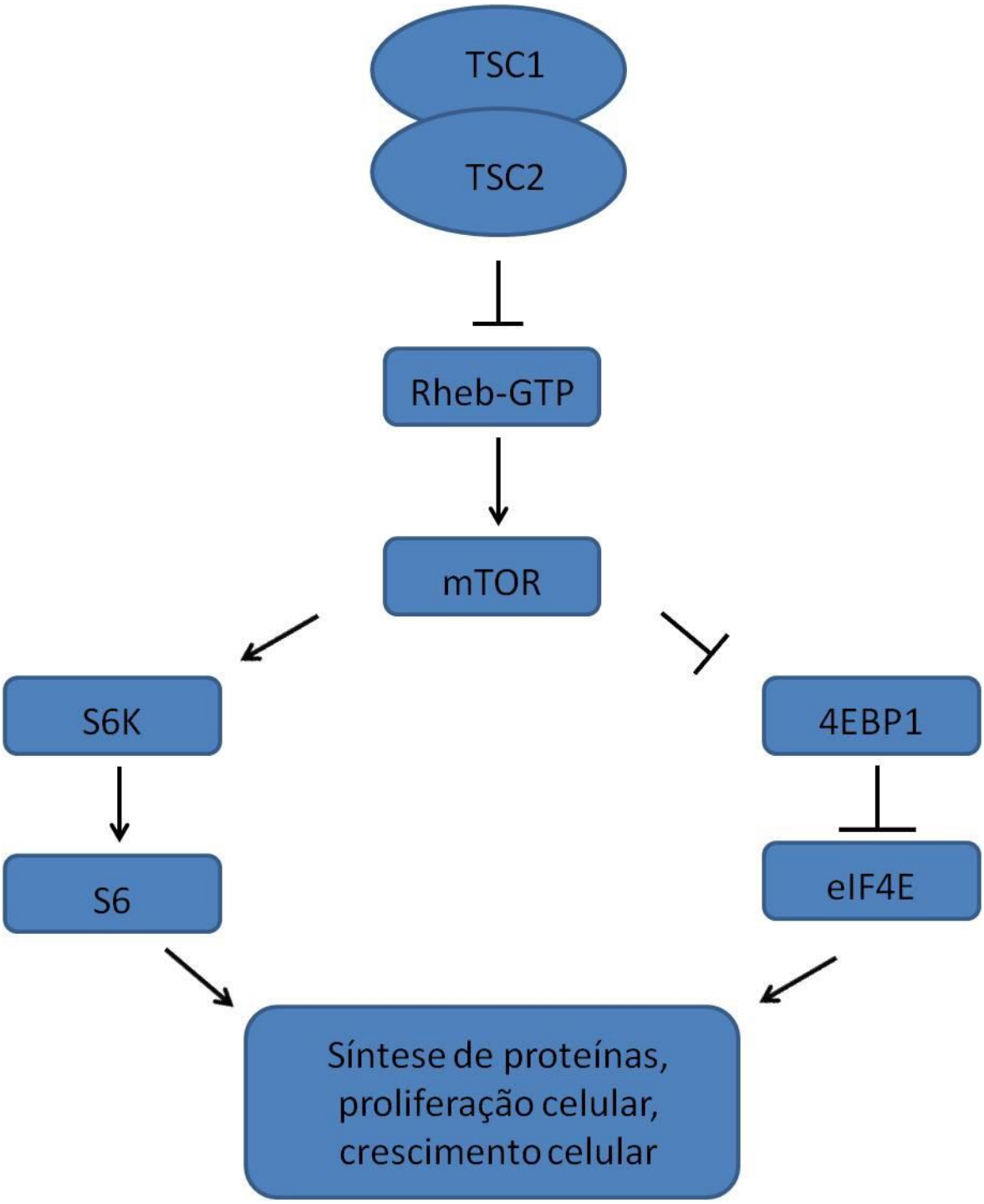

Figura 2: Via de sinalização mTOR. A proteína mTOR forma dois complexos: mTORC1 e mTORC2 (não representado). A mTOR é regulada por vários estímulos fisiológicos e patológicos através de vias de sinalização *upstream* e proteínas intermediárias (TSC1, TSC2 e Rheb). A mTOR ativa várias proteínas envolvidas na regulação da síntese protéica, crescimento e proliferação celular, plasticidade sináptica e expressão de canais iônicos.

1.4 Peixe-zebra

O peixe-zebra ou *zebrafish* (*Danio rerio*) é conhecido principalmente por seu rápido desenvolvimento e fácil manipulação genética e farmacológica, além de vantagens em manutenção em relação a outros animais modelo (Dahm & Geisler, 2006). O peixe-zebra foi inicialmente empregado no estudo da biologia do desenvolvimento e genética, além de testes farmacológicos e toxicológicos em larga escala (Goldsmith, 2004; Feitsma & Cuppen, 2008). Recentemente, seu genoma foi completamente sequenciado e comparado ao de humanos. A comparação direta entre genes codificadores de proteínas em humanos e peixe-zebra revela que aproximadamente 82% dos genes relacionados com doenças humanas tem ao menos um ortólogo em peixe-zebra (Howe et al., 2013). Além disso, modelos de diversas doenças humanas como, por exemplo, doenças neurológicas, cardíacas, e câncer, já foram desenvolvidos em peixe-zebra (Shepard et al., 2005; Lieschke & Currie, 2007; Dahme et al., 2009; Hortopan et al., 2010).

Recentemente, o peixe-zebra vem emergindo rapidamente como um importante modelo para estudos neurológicos e comportamentais. O SNC do peixe-zebra apresenta menor complexidade, porém organização geral e circuitos neuronais semelhantes aos observados em mamíferos (Sager et al., 2010). A presença dos principais neurotransmissores encontrados em mamíferos, incluindo aminoácidos (glutamato, GABA, glicina), monoaminas (histamina, dopamina, norepinefrina, epinefrina, serotonina, melatonina) e acetilcolina, entre outros, bem como seus mecanismos de ação já foram descritos (Rinkwitz et al., 2010). Esse teleósteo apresenta os sistemas motor, sensitivo e endócrino bem desenvolvidos, alta sensibilidade a alterações ambientais e manipulações farmacológicas e um amplo espectro de fenótipos comportamentais conhecidos (Egan et al., 2009; Cachat et al., 2010; Sager et al., 2010; Burne et al., 2011).

O peixe-zebra também tem se mostrado um eficiente modelo experimental para o estudo da epilepsia. As crises convulsivas induzidas pela exposição a agentes convulsivantes apresentam aspectos característicos de convulsões que ocorrem em humanos: descargas neuronais excessivas e

alterações comportamentais progressivas (Baraban et al., 2005). Larvas de peixe-zebra e também animais adultos desenvolveram padrões eletroencefalográficos correspondentes às fases ictal e inter-ictal da crise convulsiva quando expostos ao agente convulsivante PTZ (Baraban et al., 2005; Pineda et al., 2011; Afrikanova et al., 2013). Animais adultos tratados com ácido caínico também apresentaram crises convulsivas evidentes, que foram inibidas pelo pré-tratamento com antagonistas glutamatérgicos (Alfaro et al., 2011). A resposta a tratamentos farmacológicos também é evidente no peixe-zebra. Larvas que foram previamente tratadas com fármacos antiepilépticos tiveram crises convulsivas mais amenas quando expostas ao agente convulsivante (Baraban et al., 2005; Berghmans et al., 2007). Em animais adultos, o pré-tratamento com ácido valpróico teve efeito anticonvulsivante e preveniu déficits cognitivos causados pela exposição ao PTZ (Lee et al., 2010). No modelo de indução de crise convulsiva através da absorção de PTZ adicionado à água, os níveis de PTZ no encéfalo do peixe-zebra dependem da concentração e tempo de exposição à droga, perfil similar ao verificado em modelos de injeção de PTZ em roedores (Mussulini et al., 2013).

O peixe-zebra também tem sido utilizado na caracterização de novos alvos farmacológicos. A sinalização adenosinérgica em peixe-zebra também vem sendo estudada. Um estudo recente mostrou que, assim como em roedores, o bloqueio de receptores A_1 induz ansiedade enquanto o bloqueio de receptores A_{2A} provocou hiperlocomoção (Maximino et al., 2011). Em modelo de estresse agudo, ocorreu o aumento na expressão dos receptores A_1, A_{2A} e A_{2B}, sugerindo o aumento da sinalização adenosinérgica como um possível mecanismo para o restabelecimento da homeostase após episódio de estresse (Piato et al., 2011). Além disso, já foi verificada a diminuição da hidrólise de adenosina após sucessivos episódios de crises convulsivas em peixe - zebra. No entanto, após um único episódio de crise convulsiva, houve aumento na atividade da ADA, efeito que foi impedido pelo pré-tratamento com fármacos antiepilépticos. Estes resultados mostram que a ADA é diferentemente modulada em crises convulsivas em peixe-zebra, apresentando diferentes

respostas conforme o modelo experimental (Siebel et al., 2011; 2013, Capítulo I).

2. OBJETIVOS

2.1 Objetivo Geral

Considerando que a adenosina tem um importante efeito neuromodulador e que sua modulação tem se mostrado uma alternativa potencial para o tratamento em epilepsia, o objetivo geral deste estudo é verificar a participação da sinalização adenosinérgica em crises convulsivas induzidas por PTZ em peixes-zebra.

2.2 Objetivos Específicos

- Verificar o efeito de fármacos antiepilépticos sobre as alterações promovidas por crise convulsiva induzida por PTZ na atividade e expressão das ectonucleotidases e ADA em peixes-zebra adultos.
- Avaliar os efeitos de agonistas e antagonistas seletivos ou não-seletivos dos receptores de adenosina em parâmetros comportamentais de crises convulsivas induzidas por PTZ em peixes-zebra adultos.
- Avaliar os efeitos de inibidores de transportadores de nucleosídeos e das enzimas ecto-5'-nucleotidase e ADA em parâmetros comportamentais de crises convulsivas induzidas por PTZ em peixes-zebra adultos.
- Avaliar, em cultura celular, as vias de sinalização mTORC1 e MAPK em resposta ao estresse oxidativo.

3. RESULTADOS

CAPÍTULO I

ARTIGO CIENTÍFICO

Antiepileptic drugs prevent changes in adenosine deamination during acute seizure episodes in adult zebrafish

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Antiepileptic drugs prevent changes in adenosine deamination during acute seizure episodes in adult zebrafish

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ABSTRACT

Adenosine is an endogenous modulator of brain functions, which presents anticonvulsant properties. In addition, its levels can be increased during neural injury. The modulation of extracellular adenosine levels by ectonucleotidase and adenosine deaminase (ADA) activities may represent a key mechanism in the control of epileptogenesis. In the present study, we investigated the effects of acute seizure episodes and antiepileptic drug (AED) treatments on ectonucleotidases and ADA activities in adult zebrafish brain. Our data have demonstrated that pentylenetetrazole (PTZ)-induced seizures did not alter ATP, ADP, and AMP hydrolysis in brain membrane fractions. However, there was a significant increase on ecto-ADA and soluble ADA activities in PTZ-treated animals immediately after a clonus-like convulsion and loss of posture, which are typical behavioral changes observed in Stage 3. Furthermore, our results have demonstrated that AED pretreatments prevented the stimulatory effect promoted by PTZ exposure on ADA activities. The PTZ and AED treatments did not promote alterations on ADA gene expression. Interestingly, when exposed to PTZ, animals pretreated with AEDs showed longer latency to reach the clonus-like seizure status, which is an effect that matches the suppression of the increase of ADA activity promoted by the AEDs. These data suggest that the adenosine deamination could be involved in the control of seizure development in zebrafish and may be modulated by AED treatments.

1. Introduction

Epilepsy is one of the most common neurological diseases characterized by recurrent and unpredictable seizures and affects approximately 50 million people worldwide. Despite its severity and common occurrence, the cellular and molecular basis of epilepsy is still largely unknown (Banerjee et al., 2009; Elger and Schmidt, 2008). Moreover, even with the diversity of antiepileptic drugs (AEDs) currently available, approximately 30% of patients with epilepsy still suffer from seizures and the drugs that are offered nowadays are not completely without side effects. Nevertheless, ongoing studies with animal models have improved our understanding of the pathological basis of epilepsy, providing considerable knowledge into the pharmacology of the drugs employed to refrain seizures (Bialer and White, 2010). Zebrafish is a small freshwater teleost increasingly used as a model organism to understand the epilepsy mechanism. Several studies reported that PTZ-induced seizures in zebrafish larvae (Baraban et al., 2005) and adults (Pineda et al., 2011; Wong et al., 2010) caused the behavioral and electrographic alterations that would be expected from a seizure episode. Zebrafish also show AEDs response, since chemically-induced seizure-related behavioral alterations in larvae were suppressed by AED pretreatments (Baraban et al., 2005; Berghmans et al., 2007). Moreover, PTZ-induced seizures caused impairment in the passive avoidance response by adult zebrafish, which was suppressed by the treatment with AED (Lee et al., 2010).

Adenosine is a purine nucleoside that can be released during neuronal injury and has anticonvulsant properties, mediated mainly by activation of adenosine A_1 receptors (Boison, 2005). The antiepileptic role of adenosine is based on the fact that adenosine A_1 receptors are

Abbreviations: ADA, adenosine deaminase; GBP, gabapentin; AED, antiepileptic drug; NTPDase, nucleoside triphosphate diphosphohydrolase; PTZ, pentylenetetrazole; PHT, phenytoin; VPA, valproate.

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enriched in excitatory synapses, and their activation reduces glutamate release, decreases glutamatergic responsiveness and hyperpolarizes neurons (for review, see Gomes et al., 2011). It has been shown that brain extracellular adenosine levels increase during epileptic seizures in animal models (Berman et al., 2000) and also in patients with epilepsy (During and Spencer, 1992). Furthermore, rats with chronic temporal lobe epilepsy have shown upregulation of the adenosine A_1 receptors (Ohta et al., 2010). A previous study observed a decrease in adenosine deamination after 20 min of successive PTZ-induced seizures in zebrafish, suggesting a modulation of extracellular adenosine levels in the occurrence of repetitive seizures (Siebel et al., 2011).

Besides the direct cellular adenosine release, ATP hydrolysis promoted by a complex network of cell surface-located enzymes named ectonucleotidases is another important source of extracellular adenosine. ATP acts as an excitatory extracellular signal at the P2 receptor subtypes, and then it is converted to the neuromodulator adenosine through the action of ectonucleotidases. The ectonucleotidase pathway modulates ATP and adenosine availability for activation of P2 and P1 receptor subtypes, respectively, which may represent an important mechanism for epilepsy control (for review see Cognato and Bonan, 2010). Ectonucleotidases constitute an enzyme cascade system that catalyzes the successive hydrolysis of purine and pyrimidine nucleoside tri-, di-, and monophosphates to their respective nucleosides (Yegutkin, 2008). Tri- and diphosphonucleosides may be hydrolyzed by ectonucleoside triphosphate diphosphohydrolase (E-NTPDase) family members, whereas ecto-5′-nucleotidase hydrolyzes nucleoside monophosphates producing adenosine. Adenosine is metabolized by two possible pathways: deamination to inosine via adenosine deaminase (ADA), and phosphorylation to AMP via adenosine kinase (Boison et al., 2010). ADA is widely distributed among tissues and body fluids and catalyzes the hydrolytic deamination of adenosine to inosine, both in the cytosol and in the cell membrane (Franco et al., 1997; Maier et al., 2005). Ecto- and cytosolic-ADA activities and different ADA-related gene expressions were already reported in zebrafish (Rosemberg et al., 2007, 2008). Moreover, biochemical and molecular studies have also characterized NTPDase and ecto-5′-nucleotidase in zebrafish brain (Rico et al., 2003; Senger et al., 2004).

The involvement of ectonucleotidases and ADA pathway in epilepsy and acute seizures has been reported in previous studies with rodents. Changes in ectonucleotidase activities were not seen after a single convulsant PTZ injection in rats. However, rats that were more resistant to seizure development presented increased ATP hydrolysis in synaptosomes after PTZ-kindling (Bonan et al., 2000a, 2000b). Furthermore, adult rats submitted to kainate-induced seizure in the neonatal period showed increased ATP hydrolysis in hippocampal synaptosomes (Cognato et al., 2011). Concerning the ADA activity, its inhibition effectively reduced seizures in rodents (Dupere et al., 1999; Southam et al., 2002). Furthermore, PTZ-kindled mice have shown increased adenosine deamination in their brain tissue (Ilhan et al., 2005, 2006). Considering that zebrafish have been reported as an effective model for the study of epilepsy and that adenosine has evident anticonvulsant effects, the investigation of the effects of acute seizures episodes and antiepileptic drugs on ectonucleotidases and ADA in zebrafish may improve our knowledge on the role of adenosine in epilepsy. Therefore, the aim of this study was to verify whether the antiepileptic drugs phenytoin (PHT), gabapentin (GBP) and valproate (VPA) influence the alterations promoted by PTZ-induced seizures on ectonucleotidase and ADA pathways in zebrafish brain.

2. Material and methods

2.1. Animals

Adult male and female wild type zebrafish (*Danio rerio*) were obtained from a local commercial supplier (Red Fish, RS, Brazil) and acclimated for 2 weeks before the experiments. The animals were housed in a 50 L thermostated aquarium filled with unchlorinated water constantly aerated at a targeted temperature of 26 ± 2 °C. Fish were kept under a 14–10 h light/dark cycle photoperiod and fed twice a day with commercial flake fish food supplemented with live brine shrimp. The use and maintenance of zebrafish were done in accordance with the "Guide for the Care and Use of Laboratory Animals" published by the US National Institutes of Health and the experiments were designed to minimize discomfort or suffering to the animals, as well the number used. The protocol was approved by the Ethics Committee of Pontifical Catholic University of Rio Grande do Sul (PUCRS) under the number 11/00255-CEUA.

2.2. Materials

Trizma base, ammonium molybdate, polyvinyl alcohol, malachite green, nucleotides, adenosine, EDTA, EGTA, sodium citrate, Coomassie blue G, bovine serum albumin, calcium chloride, PHT, GBP, VPA and PTZ were purchased from Sigma (St. Louis, MO, USA). Magnesium chloride, phenol, and sodium nitroprusside were purchased from Merck (Darmstadt, Germany). TRIzol®, SuperScript™ III First-Strand Synthesis SuperMix, Taq Platinum, GelRed and Low DNA Mass Ladder were purchased from Invitrogen (Carlsbad, CA, USA). All other reagents used were from analytical grade.

2.3. PTZ model

To induce seizures, zebrafish were individually exposed to 7.5 mM PTZ in a 250 mL beaker. All PTZ treatments were videotaped and evaluated later by trained observers. The seizure-like behavior was classified according to each stage: stage I — dramatically increased swimming activity, stage II — whirlpool swimming behavior, and stage III — clonus-like seizures followed by loss of posture, when the animal falls to one side and remains immobile for 1–3 s, as previously reported for zebrafish larvae (Baraban et al., 2005; Berghmans et al., 2007) and adults (Wong et al., 2010). The animals were submitted to the PTZ treatment until they reached stage III. Early after reaching the stage III, each animal was gently captured from the treatment beaker and used to perform biochemical and molecular analyses. Control group animals were maintained in a 250 mL beaker with tank water for the same period and conditions as the PTZ-treated groups. Before the PTZ exposure, the animals remained exposed to AED treatments for 1 h, enough time for all drugs to achieve seizure suppressor effect. PHT (450 μM), GBP (50 mM) and VPA (3 mM) concentrations were chosen based on previous studies (Baraban et al., 2005; Berghmans et al., 2007).

2.4. Preparation of soluble and membrane fractions

The animals were cryoanesthetized, euthanized by decapitation, and brains were dissected (Wilson et al., 2009). Brain samples were prepared as previously described and each independent experiment was performed using biological preparations consisting of a "pool" of five brains (Rico et al., 2003; Rosemberg et al., 2008; Senger et al., 2004). Following the dissection, the whole zebrafish brains were homogenized in a glass-Teflon homogenizer according to the protocol for each enzyme assay. For NTPDase and ecto-5′-nucleotidase assays, zebrafish brains were homogenized in 60 vol. (v/w) of chilled Tris–citrate buffer (50 mM Tris–citrate, 2 mM EDTA, 2 mM EGTA, pH 7.4). For ADA experiments, brains were homogenized in 20 vol. (v/w) of chilled phosphate buffered saline (PBS), with 2 mM EDTA, 2 mM EGTA, pH 7.4. The brain membranes were obtained as previously described (Barnes et al., 1993). The homogenates were centrifuged at 800×*g* for 10 min and the supernatant fraction was subsequently centrifuged for 25 min at 40,000×*g*. The resultant supernatant and the pellet obtained corresponded to the soluble and membrane fractions, respectively. For soluble ADA activity experiments, the supernatant

was collected and kept on ice for enzyme assays. The pellets of membrane preparations were frozen in liquid nitrogen, thawed, resuspended in the respective buffers (to ensure the lysis of the brain vesicle membranes) and centrifuged for 20 min at 40,000×g. The final pellets were resuspended and used for enzyme assays. All samples were maintained at 2–4 °C throughout preparations.

2.5. Ectonucleotidase assays

NTPDase and 5′-nucleotidase assays were performed as previously described methods (Rico et al., 2003; Senger et al., 2004). Zebrafish brain membranes (3–5 μg protein) were added to the reaction medium containing 50 mM Tris–HCl (pH 8.0) and 5 mM $CaCl_2$ (for the NTPDase activity) or 50 mM Tris–HCl (pH 7.2) and 5 mM $MgCl_2$ (for the 5′-nucleotidase activity) at a total volume of 200 μL. The samples were preincubated for 10 min at 37 °C and the reaction was started by the addition of the substrate (ATP, ADP or AMP) to a final concentration of 1 mM. The reaction was terminated after 30 min by the addition of trichloroacetic acid at a final concentration of 5%, and then the samples were chilled on ice for 10 min. The colorimetric reagent composed by 2.3% polyvinyl alcohol, 5.7% ammonium molybdate, and 0.08% malachite green was added (1 mL) in order to determine the inorganic phosphate released (Pi) (Chan et al., 1986). The quantification of Pi released was determined spectrophotometrically at 630 nm and the specific activity was expressed as nmol of $Pi \cdot min^{-1}$ mg^{-1} of protein. Controls with the addition of the enzyme preparation after the addition of trichloroacetic acid were used to correct non-enzymatic hydrolysis of the substrates. All enzyme reactions were performed in triplicate.

2.6. Adenosine deaminase assays

Ecto- and cytosolic-ADA activities were determined as previously described (Rosemberg et al., 2008). The brain fractions (5–10 μg protein) were added to the reaction mixture containing 50 mM sodium phosphate buffer (pH 7.0) and 50 mM sodium acetate buffer (pH 5.0) for soluble and membrane fractions, respectively, in a final volume of 200 μL. The samples were preincubated for 10 min at 37 °C and the reaction was started by the addition of substrate (adenosine) to a final concentration of 1.5 mM. The reaction was stopped by the addition of 500 μL phenol-nitroprusside reagent (50.4 mg of phenol and 0.4 mg of sodium nitroprusside/ml) after 75 min (soluble fraction) and 120 min (membrane fraction). ADA activity was determined spectrophotometrically by measuring the ammonia produced over a fixed time interval using a Berthelot reaction according to Weisman et al. (1988). The reaction mixtures were mixed to 500 μL of alkaline-hypochlorite reagent (sodium hypochlorite to 0.125% available chlorine, in 0.6 M NaOH) and vortexed. Samples were incubated at 37 °C for 15 min and the colorimetric assay was carried out at 635 nm. The ADA activity was expressed as nmol of $NH_3 \cdot min^{-1}$ mg^{-1} of protein. Controls with the addition of the enzyme preparation after mixing with phenol-nitroprusside reagent were used to correct the substrates' non-enzymatic hydrolysis. All enzyme reactions were performed in triplicate.

2.7. Protein determination

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

2.8. Gene expression analysis

The expression of ADA-related genes *ada1*, *ada2.1*, and *ada2.2* was analyzed by quantitative real time RT-PCR (RT-qPCR). Total zebrafish brain RNA was isolated using TRIzol® reagent (Invitrogen, USA) according to the manufacturer's instructions. The RNA purity was quantified spectrophotometrically calculating the ratio between absorbance values at 260 and 280 nm. Afterwards, cDNA was synthesized with ImProm-II™ Reverse Transcription System (Promega) from 1 μg of total RNA, according to the supplier's instructions.

Quantitative PCR was performed using SYBR® Green I (Invitrogen) to detect double-strand cDNA synthesis. Reactions were done in a volume of 25 μL using 12.5 μL of diluted cDNA (1:50), containing a final concentration of 0.2× SYBR® Green I (Invitrogen), 100 μM dNTP, 1× PCR Buffer, 3 mM $MgCl_2$, 0.25 U Platinum® Taq DNA Polymerase (Invitrogen) and 200 nM of each reverse and forward primers (Table 1). The PCR cycling conditions were: an initial polymerase activation step for 5 min at 95 °C, 40 cycles of 15 s at 95 °C for denaturation, 35 s at 60 °C for annealing and 15 s at 72 °C for elongation. At the end of cycling protocol, a melting-curve analysis was included and fluorescence measured from 60 to 99 °C. Relative expression levels were determined with 7500 Real-Time System Sequence Detection Software v.2.0.5 (Applied Biosystems). The efficiency per sample was calculated using LinRegPCR 11.0 Software (http://LinRegPCR.nl) and the stability of the references genes, *EF1α*, and *β-actin* (M-value) and the optimal number of reference genes according to the pair wise variation (V) were analyzed by GeNorm 3.5 Software (http://medgen.ugent.be/genorm/). Relative RNA expression levels were determined using the $2^{-\Delta\Delta CT}$ method.

2.9. Statistical analysis

The results are expressed as mean ± S.D. The behavioral data were analyzed by one-way ANOVA followed by Duncan post-hoc test. The enzymatic and molecular data were analyzed by two-way ANOVA followed by Duncan post-hoc test. $P<0.05$ was considered as significant. All data were evaluated with SPSS 18.0 for Windows.

3. Results

3.1. Behavioral seizure parameters in adult zebrafish

Behavioral seizure parameters were evaluated in adult zebrafish exposed to PTZ, which have shown a sequence of progressive behavioral changes classified in stage I, II, and III. The latencies to the first episodes of the seizure activity in stages I, II, and III were analyzed for each animal during PTZ exposure. The seizure-related behavioral alterations observed in animals treated with PTZ were suppressed by their pretreatment with the antiepileptic drugs PHT, GBP, and VPA. The animals exposed to PTZ without AED pretreatments have shown the first features of stage I at 50 ± 6.6 s, whereas the animals pretreated with PHT, GBP and VPA have shown the first episode of stage I at 87.3 ± 64.72, 80.5 ± 35.65 and 91.8 ± 24.2 s, respectively. Stage II was observed at 125.8 ± 30.95, 157.6 ± 67.1, 154.1 ± 73.99 and 155.1 ± 38.88 s in PTZ, PHT, GBP and VPA groups, respectively.

Table 1
PCR primers design.

Enzymes	Primer sequences (5′–3′)	GenBank accession number (mRNA)
*β-Actin**	F-CGAGCTGTCTTCCCATCCA R-TCACCAACGTAGCTGTCTTTCTG	ENSDART00000055194
*EF1α**	F-CTGGAGGCCAGCTCAAACAT R-ATCAAGAAGAGTAGTACCGCTAGCATTAC	NSDART00000023156
*ADA1***	F-GCACAGTGAATGAGCCGGCCAC R-AATGAGGACTGTATCTGGCTTCAACG	BC076532.1
*ADA2.1***	F-TTCAACACCACACGTATCGGGCAC R-ATCAGCACTGCAGCCGGATGATC	AF384217.1
*ADA2.2***	F-TTGCAATTGTTCATCATCCCGTAGC R-TCCCGAATAAACTGGGATCATCG	XM_682627.1

* According to Tang et al. (2007).
** Designed by authors.

The animals have shown the correspondent signs of stage III at 137.5 ± 13.92 (without pretreatment), 268.2 ± 98.6 (pretreated with PHT), 253.7 ± 90.49 (pretreated with GBP) and 240 ± 38.63 s (pretreated with VPA) ($F(3,23) = 4.31$; $P < 0.05$). Our results have demonstrated that commonly used antiepileptic drugs significantly increased the latency to clonus-like convulsions in adult zebrafish (Fig. 1).

3.2. Nucleotide and nucleoside hydrolysis in zebrafish brain during PTZ-induced acute seizure

The effect of PTZ-induced acute seizure was tested on NTPDase, ecto-5′-nucleotidase, and ADA activities in adult zebrafish brain. The animals remained exposed to PTZ treatment until they reached the stage III, when the animal falls to one side and remains immobile for 1–3 s. Our results have demonstrated that PTZ-induced seizures did not alter ATP, ADP, and AMP hydrolysis in zebrafish brain membranes (Table 2). Nevertheless, PTZ treatment significantly increased ecto-ADA and soluble-ADA activities (Fig. 2). Our results have demonstrated that PHT (membrane: $F(3,31) = 3.45$, $P < 0.05$; soluble: $F(3,15) = 0.44$, $P < 0.0001$), GBP (membrane: $F(3,15) = 3.87$, $P < 0.05$; soluble: $F(3,15) = 5.98$, $P < 0.05$), and VPA (membrane: $F(3,23) = 3.18$, $P < 0.05$; soluble: $F(3,15) = 5.01$, $P < 0.05$) pretreatments prevented the stimulatory effect promoted by acute PTZ-induced seizures on ADA activity. The AED treatments themselves did not alter the ecto-ADA and soluble ADA activity (Fig. 2). Quantitative real time RT-PCR experiments were performed to verify whether the PTZ and AED treatments have altered the expression of ADA-related genes. There were no changes on *ada1*, *ada2.1*, and *ada2.2* gene expression after all treatments tested in zebrafish brain (Fig. 3).

4. Discussion

In the present study, we evaluated the effects of acute seizures episodes and AED treatments on ectonucleotidases and ADA activities in zebrafish. Our findings have demonstrated that soluble and ecto-ADA activities were increased in zebrafish brain after an acute seizure episode, an effect that was suppressed by the antiepileptic drug pretreatment. NTPDase and 5′-nucleotidase activities have not shown significant alterations after seizure occurrence.

Adenosine is an endogenous modulator of brain functions with antiepileptic actions, mainly due to the enhanced presence of adenosine A_1 receptors in excitatory synapses, where they inhibit the release of glutamate, decrease glutamatergic responsiveness and hyperpolarize neurons (for review see Gomes et al., 2011). Besides the cell adenosine release, ATP release and its degradation into adenosine via ectonucleotidases are a significant source of extracellular adenosine. Under physiological conditions, astrocytic release of ATP

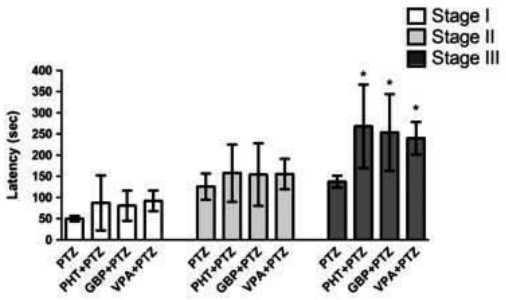

Fig. 1. Latency to the first episode of the three different seizure behavioral stages in zebrafish. Bars represent the mean ± S.D. from 6 animals for each group. The symbol (*) represents a significant difference from stage III PTZ group (one-way ANOVA, followed by Duncan test as post hoc, $P \leq 0.05$).

Table 2
Effect of PTZ treatment on ectonucleotidase activities in adult zebrafish brain membranes.

Group	n	ATP hydrolysis	ADP hydrolysis	AMP hydrolysis
Control	5	328.3 ± 33.5	122.2 ± 48.5	11.1 ± 2.5
PTZ 7.5 mM	5	313.2 ± 47.3	133.5 ± 40.6	9.6 ± 1.7

The nucleotide hydrolysis was expressed as nmol $Pi \cdot min^{-1}$ mg^{-1} protein.
Data are expressed as mean ± S.E.M.

followed by its degradation to adenosine via ectonucleotidases is a major source of adenosine (Pascual et al., 2005). In epileptic patients, a significant increase of soluble nucleotidase activity in blood serum was observed following a seizure episode (Grosso et al., 2009). Moreover, serum ATP, ADP, and AMP hydrolysis rates were increased in rats after a single PTZ-induced seizure episode (Bruno et al., 2002). It is possible to hypothesize that the higher serum nucleotidase activity and the possible increase in adenosine levels could represent an important mechanism in the modulation of epileptic events (Bruno et al., 2002; Grosso et al., 2009). Our present study shows that ectonucleotidase activities in zebrafish brain membranes were not altered upon a single acute seizure induced by PTZ. Similar results were observed after successive seizure episodes in zebrafish, with no differences in ATP, ADP, and AMP hydrolysis in brain membranes (Siebel et al., 2011). Adult rats submitted to kainic acid-induced

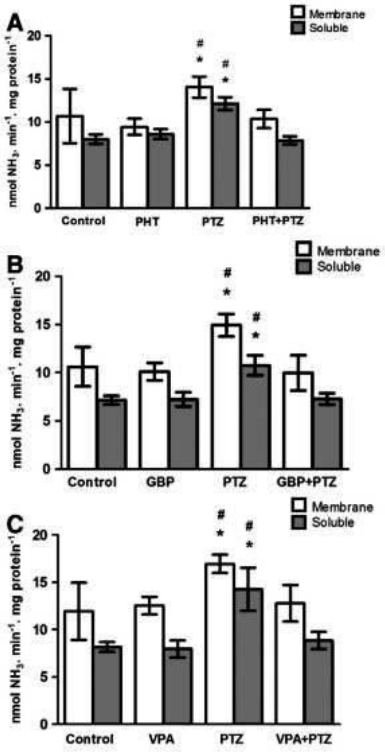

Fig. 2. Effect of the antiepileptic drugs PHT (A), GBP (B) and VPA (C) and PTZ treatments on membrane-bound and soluble ADA activity from zebrafish brain. Bars represent the mean ± S.D. from 6 different experiments. The symbols (*, #) represent a significant difference from control and AED + PTZ group, respectively (two-way ANOVA, followed by Duncan test as post hoc, $P \leq 0.05$). The specific enzyme activity is expressed as nmol of $NH_3 \cdot min^{-1}$ mg^{-1} of protein.

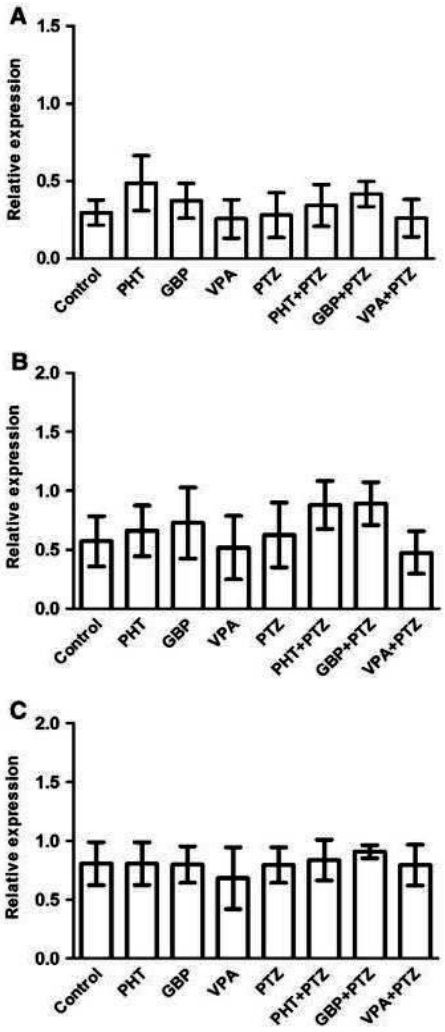

Fig. 3. Effect of PHT, GBP, VPA and PTZ treatments on ADA gene expression pattern. The figures show the expression patterns of *ada1* (A), *ada2.1* (B) and *ada2.2* (C), in adult zebrafish brain. The results were expressed as mean ± S.D. of four independent experiments performed in quadruplicate.

seizure at 7 days of age showed increased hippocampal ATP hydrolysis when compared to control animals. However, 12 h after the seizure, ATP hydrolysis was not significantly changed (Cognato et al., 2011). Furthermore, there were no differences on ectonucleotidase activities after a single PTZ exposure in rats. However, rats that were more resistant to seizures showed increased ATP hydrolysis after PTZ-kindling treatment (Bonan et al., 2000b). These findings suggest that acute seizure episodes are not able to alter ectonucleotidase activities, which are subject to late and prolonged alterations after recurrent seizures (Bonan et al., 2000a), as a consequence of an adaptive plasticity.

During seizures, brain extracellular concentrations of adenosine increase to levels that suppress epileptic activity in animal models and also in patients with epilepsy (Dunwiddie and Masino, 2001). Hippocampal adenosine levels were increased in rats submitted to seizures induced by bicuculline, kainate, and PTZ (Berman et al., 2000). During and after single spontaneous seizure episodes, humans with intractable complex partial epilepsy showed increased hippocampal extracellular adenosine range. In each patient, adenosine levels increased during the spontaneous seizure and remained elevated above basal values after the occurrence (During and Spencer, 1992). As previously reported, besides the cell uptake through bi-directional nucleoside transporters and phosphorylation to AMP by adenosine kinase, extracellular adenosine concentrations can be regulated through its deamination by ADA (Fredholm et al., 2005). ADA has an important role in the regulation of the extracellular adenosine concentration. Studies have reported that the inhibition of adenosine deamination effectively reduced seizures in diverse animal epilepsy models (Dupere et al., 1999; Southam et al., 2002).

In mice, seizures induced by a single or repeated PTZ doses cause rapid and significant increase in the density of ADA in several brain areas. The results have shown that after a single generalized convulsive seizure, ADA levels were higher than in the kindled animals, which presented several seizures (Pence et al., 2009). A previous study with zebrafish reported a decrease in ecto-ADA activity after 20 min of successive PTZ-induced seizure episodes (Siebel et al., 2011). In the present study, we showed that the soluble and ecto-ADA activities were increased at the first clonic-seizure episode induced by PTZ exposure. Our results demonstrate, for the first time, that ADA activity is early increased after a single seizure occurrence in zebrafish, suggesting a decreased extracellular adenosine levels during this period.

In this study, we have tested classical AEDs, such as PHT, GBP and VPA, which act through a variety of mechanisms, often suppressing ion channels, promoting gabaergic neurotransmission and/or decreasing glutamatergic neurotransmission. These drugs have been used for the treatment of epilepsy and partial and generalized tonic–clonic seizures (Brodie, 2010). Previous studies have also demonstrated that these drugs can interfere in the purinergic system (Borowicz et al., 1997, 2002; Cognato et al., 2007; Siebel et al., 2011). Our results have shown that antiepileptic drug treatments suppress the seizure-induced increase in ADA activity. This suppression of the increased ADA activity coincides with the longer latency to reach the stage III of seizure status showed by animals pretreated with antiepileptic drugs. The animals pretreated with PHT, GBP and VPA spent more time to reach the clonic-like seizure stage when compared with animals without AED pretreatments. In mice, the PTZ-induced increase in ADA activity was suppressed by glutathione treatment (Pence et al., 2009). Studies have shown that glutathione has anticonvulsant effect, and these results suggest that a possible ADA modulation is involved in the anticonvulsant activity of glutathione (Pence et al., 2009). In addition, the VPA treatment suppressed the PTZ-kindling-induced increase in ADA activity in mice brain tissue (Ilhan et al., 2005, 2006).

In zebrafish brain, ADA is located both in the cytosol and in the cell membrane and the regulation of brain adenosine levels might be promoted by distinct ADA members. A previous study characterized these ADA members, which have shown diverse gene expression patterns and activity properties (Rosemberg et al., 2007, 2008). The ADA1 member is a typical cytosolic enzyme that also acts as an ecto-ADA, cleaving extracellular adenosine. The ADA2 enzyme seems to act specifically in the extracellular fraction (Zavialov and Engström, 2005; Zavialov et al., 2010). Previous studies suggest that ecto-ADA has extra-enzymatic and co-stimulatory functional roles. There is evidence that ecto-ADA is bound with adenosine receptors, modulating their affinity (Ciruela et al., 1996; Herrera et al., 2001; Saura et al., 1998). In this study, we showed that one single seizure episode significantly increased cytosolic and ecto-ADA activity in zebrafish brain,

effect that was suppressed by antiepileptic drug pretreatments. However, successive convulsive episodes decreased the ecto-ADA activity, whereas they did not change adenosine deamination in the soluble fraction (Siebel et al., 2011). These results have shown that ADA activities are differently modulated early after a single seizure or successive seizure episodes. The expression profile of ADA related genes (*ada1*, *ada2.1* and *ada2.2*) in zebrafish brain was previously reported (Rosemberg et al., 2007). Our present RT-PCR results showed that single seizure episodes did not alter the ADA-related genes expression, similarly to our previous study, which showed no alteration after successive seizure episodes (Siebel et al., 2011). In view of the fact that the observed alterations in ADA activity were not caused by modifications in the transcriptional pattern of ADA family enzymes, we suggest that these changes could be attributed to seizure effects on the post-translational modulation of these enzymes. According to a previous study, ADA-related enzymes present putative regulatory sites for posttranslational mechanisms, such as phosphoylation/desphosphorylation (Rosemberg et al., 2007). Therefore, further studies are necessary to characterize the post-translational mechanisms involved in the ADA activity modulation process in zebrafish.

Nowadays, zebrafish are being increasingly used in epileptic seizure research. When exposed to convulsant agents, zebrafish show behavioral and electrographic alterations characteristic of seizure episodes (Baraban et al., 2005; Pineda et al., 2011; Wong et al., 2010) and AED response (Baraban et al., 2005; Berghmans et al., 2007). Furthermore, since caffeine, a nonselective adenosine antagonist, induced seizure-like behavior episodes in zebrafish, it is possible to suggest that adenosine signaling is associated to epilepsy in zebrafish (Wong et al., 2010). Our findings showed that antiepileptic drug pretreatments suppress the increase in adenosine deamination, which coincides with a longer period to reach the clonic-seizure status. Our results suggest that the adenosine deamination system is involved in the control of seizure occurrences in zebrafish. Furthermore, our study contributes to elucidating the mechanisms underlying the modulator effects in adenosine signaling during the occurrence of seizures in this specie.

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CAPÍTULO II

ARTIGO CIENTÍFICO

Role of adenosine signaling on PTZ-induced seizures in zebrafish

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Artigo submetido ao periódico Epilepsia

Role of adenosine signaling on PTZ-induced seizures in zebrafish

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SUMMARY

Objectives: Adenosine is a well known endogenous modulator of neuronal excitability with anticonvulsant properties. Thus, the modulation exerted by adenosine might be an effective tool to control seizures. The aim of this study was to verify the effects of drugs able to modulate adenosinergic signaling on seizure episodes induced by PTZ in zebrafish.

Methods: Zebrafish were pretreated with adenosinergic drugs through intraperitoneal injection (i.p) 30 minutes before the PTZ exposure. To induce seizures, zebrafish were individually exposed to 7.5 mM PTZ. The seizure-like behavior was classified in: stage I - dramatically increased swimming activity; stage II - whirlpool swimming behavior, and stage III- clonus-like seizures followed by loss of posture. The locomotor activity and the latency (s) to reach the seizure stages were evaluated.

Results: Caffeine, a non-selective adenosine A_1 and A_{2A} receptors antagonist, and 8-cyclopentyl- 1,3-dipropylxanthine (DPCPX), a selective A_1 receptor antagonist, decreased the latency to tonic-clonic seizure stage onset. Nevertheless, the adenosine A_1 receptor agonist cyclopentyladenosine (CPA) increased the latency to reach the tonic-clonic seizure stage. Both adenosine A_{2A} receptor agonist and antagonist, CGS 21680 and ZM 241385, respectively, did not promote changes in seizure parameters. The modulation of extracellular adenosine levels by ecto-5'-nucleotidase, adenosine deaminase (ADA) and nucleoside transporters (NTs) activities were also investigated. Pretreatment with the ecto-5´nucleotidase inhibitor Adenosine 5′-(α,β-methylene)diphosphate (AMPCP) decreased the latency to tonic-clonic seizure stage onset. However, when pretreated with the ADA inhibitor erythro-9-(2-hydroxy-3-nonyl-

adenine (EHNA) or with the NT inhibitor dipyridamole, animals showed longer latency to reach the tonic-clonic seizure status.

Significance: Our findings indicate that adenosine A_1 receptors activation is an important mechanism to control the development of seizures in zebrafish. Furthermore, the action of ecto-5´-nucleotidase, ADA, and NTs are directly involved with the control of extracellular adenosine levels and has important role on the development of seizure episodes in zebrafish.

KEY WORDS: adenosine receptors, ecto-5'-nucleotidase, adenosine deaminase, nucleoside transporters

Introduction

Epilepsy is one of the most common neurological disorders affecting approximately 1% of the population worldwide. Epilepsy is characterized by the occurrence of recurrent and unpredictable seizures[1]. In many cases, antiepileptic drugs (AEDs) can provide satisfying control of the seizures. Nevertheless, despite considerable progress in the AEDs treatments, approximately 30% of all epilepsy patients still suffer from seizures[2].

Adenosine is a well known endogenous modulator of neuronal excitability and provides anticonvulsant effects[3]. Therefore, adenosine modulation might be an effective tool to control epileptic seizures in patients resistant to conventional AEDs[4].

Adenosine is a purine nucleoside that can be produced by extracellular nucleotide hydrolysis or cell released through nucleoside transporters (NTs)[3]. Once released in the extracellular space, ATP is rapidly dephosphorylated into adenosine by ectonucleotidases, a complex network of cell surface-located enzymes[5]. Ectonucleotidases form an enzyme cascade system that catalyzes the successive hydrolysis of purine and pyrimidine nucleoside tri-, di-, and monophosphates to their respective nucleosides. Nucleoside triphosphates and diphosphates may be hydrolyzed by ectonucleoside triphosphate diphosphohydrolase (E-NTPDase) family members whereas nucleoside monophosphates are hydrolyzed by ecto-5′-nucleotidase, generating adenosine[5]. Adenosine is metabolized by two possible pathways: deamination to inosine via adenosine deaminase (ADA), and phosphorylation to AMP via adenosine kinase (AK)[6]. While AK is a key enzyme for the metabolic adenosine clearance under physiological conditions, ADA contributes to adenosine clearance under stressful situations[7]. Besides the production of adenosine through nucleotide hydrolysis, this neuromodulator can be released as adenosine *per se* by nucleoside transporters (NTs).

This transport is mediated by bidirectional equilibrative processes driven by chemical gradients and unidirectional concentrative processes driven by sodium electrochemical gradients[8,9].

Adenosine exerts its effects through activation of specific G-protein coupled receptors: A_1, A_{2A}, A_{2B}, and A_3 receptors. The A_1 and A_{2A} receptors are the most sensitive to adenosine and the most abundant in the CNS[10]. During a seizure, extracellular adenosine levels rapidly rise and are believed to play an important role in the arrest and termination of the seizure via binding to the inhibitory adenosine A_1 receptor[11,4]. The adenosine A_1 receptor activation induces presynaptic inhibition through blockade of calcium channels and postsynaptic hyperpolarization through activation of potassium channels[12]. Nevertheless, adenosine levels rise significantly during epileptic seizures that could activate all adenosine receptors, including the A_{2A} receptors, which facilitate the neuronal transmission[13,14]. In contrast to well known role of A_1 receptors, there are controversial results about the exact role of A_{2A} receptors on seizures control[15,16].

Zebrafish is a small freshwater teleost which has been used as a model organism to study the epilepsy mechanisms. Pentylenetetrazole (PTZ)-induced seizures in zebrafish larvae[17] and adults[18] caused the behavioral and electrographic alterations that would be expected from a seizure episode. In addition, zebrafish showed response to classical AEDs for seizure control[17,19]. Previous studies demonstrated adenosine receptor subtypes A_1, $A_{2A.1}$, $A_{2A.2}$ and A_{2B} in zebrafish[20,21]. The adenosine receptors are expressed since 24 h post-fertilization and caffeine exposure is able to affect the expression of A_1 and $A_{2A.1}$ receptors[21]. In addition, the blockade of A_1, but not A_2 receptors increases anxiety and arousal in zebrafish[22].

Concerning that adenosine is able to control neuronal excitability, and its signaling is regulated by nucleotide and nucleoside-metabolizing enzymes and adenosine transporters, the modulation of these mechanisms may represent an important target in epilepsy therapies. Considering that zebrafish is an effective model widely used in epilepsy studies, the investigation of the adenosine signaling in zebrafish may improve our knowledge on the role of adenosine in epilepsy. Therefore, the aim of this study was to verify the role of adenosine on seizure episodes induced by PTZ in zebrafish.

Methods

Animals

Adult male and female wild type zebrafish (*Danio rerio*) were obtained from a local commercial supplier (Red Fish, RS, Brazil) and acclimated for 2 weeks before the experiments. The animals were housed in a 50 L thermostated aquarium filled with unchlorinated water constantly aerated at a targeted temperature of 26±2 °C. Fish were kept under a 14–10 h light/dark cycle photoperiod and fed twice a day with commercial flake fish food supplemented with live brine shrimp. The use and maintenance of zebrafish were done in accordance with the "Guide for the Care and Use of Laboratory Animals" published by the US National Institutes of Health and the experiments were designed to minimize discomfort or suffering to the animals, as well as the number of animals used in the experiments. The protocol was approved by the Ethics Committee of Pontifical Catholic University of Rio Grande do Sul (PUCRS) under the number 11/00255-CEUA.

Materials

The drugs erythro-9-(2-hydroxy-3-nonyl-adenine (EHNA), 8-cyclopentyl-1,3-dipropylxanthine (DPCPX), cyclopentyladenosine (CPA), dipyridamole, caffeine, CGS 21680 hydrochloride hydrate, α, β-methylene adenosine 5'-diphosphate (AMPCP), sodium valproate (VPA), pentylenetetrazole (PTZ) and tricaine were purchased from Sigma (St. Louis, MO, USA). ZM 241385 was purchased from Tocris Bioscience (Ellisville, MO, USA).

Drug pretreatments

This study investigated the effects of different drugs able to modulate adenosine signaling on PTZ-induced seizures in zebrafish. All drugs were injected intraperitoneally (i.p.) 30 minutes before the PTZ exposure. Each drug was tested in three different doses. The doses for each drug tested are indicated in Table 1 and were chosen based on previous studies[23]. Intraperitoneal injections were conducted using a 3/10-ml U-100 BD Ultra-Fine™ Short Insulin Syringe 8mm (5/16 inch)×31G Short Needle (Becton Dickinson and Company, New Jersey, USA) as previously described[24]. Anesthesia of the animals prior to the injection was obtained by its immersion in a tricaine solution until the animal shows lack of motor coordination and reduced respiration rate. After the injection, the animals were placed in a separate tank with highly aerated unchlorinated tap water (25±2 ◦C) to facilitate the recovery of the animals from the anesthesia. After recovery from anesthesia, the animals were individually placed in glass tanks (12 x 8 x 13.5 cm, length x width x height). The locomotor activity was registered by a video camera for 30 min and further analyzed using the ANY-Maze recording software (Stoelting Co., Wood Dale, IL, USA).

PTZ- induced seizures

To induce seizures, zebrafish were individually exposed to 7.5 mM PTZ in a 250 mL beaker. All PTZ treatments were videotaped and evaluated later by trained observers. The seizure-like behavior was classified according to each stage: stage I — dramatically increased swimming activity, stage II — whirlpool swimming behavior, and stage III — clonus-like seizures followed by loss of posture, when the animal falls to one side and remains immobile for 1–3 s, as previously reported for zebrafish[17]. The animals were submitted to the PTZ treatment until they reached stage III. The latencies to the first episode of the seizure activity in stages I, II, and III were analyzed for each animal during PTZ exposure.

Statistical analysis

The results are expressed as mean ± S.D. The locomotor activity and seizure latency data were analyzed by one-way ANOVA followed by Dunnett´s post-hoc test. $P<0.05$ was considered as significant difference.

Results

Locomotor activity

The results have shown that the highest dose of EHNA tested (150 mg/kg) significantly reduced the distance traveled by animals during the 30 min drug pretreatment (Fig 5D; $F(4,27)=13.99$). Only CPA was able to reduce locomotor activity of zebrafish in all tested doses (1,2,4 mg/kg) (Fig 3D; $F(3,47)=8.021$). All other tested drugs did not alter the locomotor activity in zebrafish.

Behavioral seizure parameters

Behavioral seizure parameters were evaluated in zebrafish exposed to PTZ. In order to investigate the seizure development, we analyzed the latencies to the first behavior signal characterizing each seizure stage (I, II, and III). All animals have shown progressive behavioral alterations until reach the most severe seizure stage, the stage III, which corresponds to tonic-clonic seizure in zebrafish[17]. The classical AED VPA provided significant protection against PTZ-induced seizures, confirming the AEDs response in zebrafish (Fig. 1A). The latency to reach stage III was prolonged in animals treated with the higher VPA doses, 200 mg/kg and 500 mg/kg, when compared with the respective controls ($F(3,32)=8.33$, $P<0.05$, Fig. 1A). The animals have shown the correspondent signs of stage III at 274.5 ± 73 (saline), 349.8 ± 59.48 (100mg/kg), 401.3 ± 111.1(200 mg/kg) and 478.8 ± 97.04 s (500 mg/kg). The animals treated with VPA 500 mg/kg also showed increased latency to stage II ($F(3,16)=7.50$, $P<0.05$; Fig. 1A). Stage II was observed at 259.3 ± 68.49, 327.0 ± 34.66, 373.8 ± 65.96 and 476.0 ± 73.43 s in saline, 100 mg/kg, 200 mg/kg and 500 mg/kg VPA groups, respectively.

Effects of adenosine A_1 and A_{2A} agonist and antagonist receptors on PTZ-induced seizures

The caffeine pretreatment has shown proconvulsant effect in zebrafish, since all caffeine doses reduced the latency to stage III onset (Fig 2A, $F(3,25)=4.37$, $P<0.05$). Stage III was observed at 260.0 ± 34.51, 143.2 ± 36.19, 153.3 ± 31.88 and 164.8 ± 62.09 s in saline, 49 mg/kg, 69 mg/kg and 92 mg/kg groups, respectively (Fig. 2A).

The selective adenosine A_1 receptor antagonist DPCPX also aggravated the behavioral response to seizure. The highest DPCPX pretreatment dose (15 mg/kg) induced faster stage III onset (Fig 3A, $F(3,31)=4.13$, $P<0.05$). The animals exposed to

PTZ without DPCPX pretreatment reached the stage III at 191.7 ± 50.26 s, whereas animals pretreated with 3 mg/kg, 10 mg/kg, and 15 mg/kg DPCPX reached the stage III at 201.8 ± 72.86, 180.0 ± 44.09 and 122.2 ± 33.53 s, respectively.

The selective adenosine A_1 receptor agonist CPA provided significant protection against PTZ-induced seizures. The latency to tonic-clonic seizure stage was prolonged in animals pretreated with CPA (Fig 3C, $F(3,48)=4.37$, $P<0.05$). The animals exposed to PTZ without CPA pretreatment reached the stage III at 172.1 ± 34.86 s, whereas animals pretreated with 1 mg/kg, 2 mg/kg and 4 mg/kg CPA reached the stage III at 216.9 ± 45.56, 243.3 ± 62.66 and 231.1 ± 45.78 s, respectively.

Both the adenosine A_{2A} receptor antagonist (ZM 241385) and agonist (CGS21680) were not able to change the behavioral seizure responses (Fig. 4). Stage III was observed at 149.8 ± 28.78, 175.7 ± 19.01, 182.3 ± 36.68 and 172.6 ± 55.43 s in saline, 1 mg/kg, 2 mg/kg and 5 mg/kg ZM 241385, respectively (Fig. 4A). Similarly, the A_{2A} selective agonist CGS21680 was not able to change the behavioral seizure response (Fig 4C). Stage III was observed at 167.3 ±46.28 (saline), 152.0 ± 28.0 (1 mg/kg CGS 21680), 156.9 ± 49.7 (2 mg/kg CGS 21680) and 186.7 ± 58.64 s (5 mg/kg CGS 21680) (Fig 4C).

Effect of modulation of extracellular adenosine levels on PTZ-induced seizures

The inhibition of nucleotide hydrolysis by the ecto-5′-nucleotidase inhibitor AMPCP exacerbated the PTZ-induced seizure response in zebrafish. The latency to tonic-clonic seizure stage was faster in animals pretreated with all doses of AMPCP (Fig 5A, $F(3,46)=12.26$, $p<0.05$). The animals pretreated with AMPCP reached the stage III at 174.3 ± 36.99 (100 mg/kg), 195.5 ± 27.01 (150 mg/kg) and 126.8 ± 22.56 s (200 mg/kg) whereas saline group reached the stage III at 242.8 ± 53.25 s. The highest

AMPCP dose (200 mg/kg) also reduced the latency to stage II (Fig 5A, $F(3,28)=7.25$, $P<0.05$). Stage II was observed at 153.6 ± 11.93 (saline), 156.0 ± 33.87 (100 mg/kg), 170.4 ± 41.71 (150 mg/kg), 92.5 ± 25.88 (200 mg/kg).

The inhibition of adenosine deamination by the ADA inhibitor EHNA provided significant increased tonic-clonic seizure onset (Fig 5C, $F(3,42)=6.48$, $P<0.05$). The animals pretreated with EHNA reached the stage III at 164.9 ± 30.81 (75 mg/kg), 263.0 ± 33.94 (100 mg/kg) and 260.5 ± 91.20 s (150 mg/kg), whereas the animals without pretreatment reached the stage III at 164.9 ± 30.81 s.

Finally, the adenosine reuptake inhibition by dipyridamole provided significant protection against PTZ-induced seizures, since animals pretreated with the higher dipyridamole doses (10 mg/kg and 20 mg/kg) spent longer time to reach the tonic-clonic seizure stage (Fig 5E, $F(3,32)=10.03$, $P<0.05$). The animals pretreated with dipyridamole reached the stage III at 178.8 ± 30.8 (5 mg/kg), 209.2 ± 36.72 (10 mg/kg) and 231.3 ± 15.74 s (20 mg/kg), whereas the saline group reached the stage III at 160.9 ± 25.81 s. Animals pretreated with 20 mg/kg dipyridamole also spent longer time to reached the stage II (Fig 5E, $F(3,27)=5.20$, $P<0.05$). Stage II was observed at 148.0 ± 30.05, 164.3 ± 29.52, 196.1 ± 35.33 and 222.0 ± 40.44 s saline, 5 mg/kg, 10 mg/kg and 20 mg/kg groups, respectively.

Discussion

Adenosine signaling is an endogenous system involved in seizures control[3]. Adenosine has been described as able to suppress the seizure spread and to induce the termination of ongoing seizures[3]. Studies have demonstrated that caffeine, a non-selective antagonist of A_1 and A_{2A} receptors, is able to either lower the convulsive threshold in experimental models of epilepsy or induce seizure activity in rodents. Also,

caffeine decreases the protective effects of conventional AEDs against electroconvulsions in mice[25]. Our results indicate that caffeine decreased the latency to seizures in all doses tested, without promote effects on locomotor activity. This convulsant effect promoted by caffeine is likely related to the antagonist effect on A_1 receptors. Previous studies indicate that the proconvulsant effect of caffeine is based on antagonism of the adenosine receptors[26,27]. Acute inhibition of A_1 receptors by methylxanthines can directly contribute to ictogenesis and seizure spread[27]. The caffeine action mechanisms include the inhibition of cyclic nucleotide phosphodiesterases and inhibition of $GABA_A$ receptors[28]. However, these effects are observed only after extreme caffeine intake. Therefore, the mechanism that is considerably affected by the regular doses of caffeine intake is adenosine receptors antagonism[27,28].

Our results reinforce the role of adenosine receptors on the development and control of seizures. It is well known the anticonvulsant effect induced by activation of adenosine A_1 receptors in rodents[23]. Adenosine augmentation therapies suppress and prevent seizures[4]. Studies have shown adenosine A_1 agonist promoted increased latency of seizure onset, decreased seizure occurrence, shorter seizure duration, and reduced mortality rate in chemical-induced seizure models[29]. In our study, the A_1 receptor agonist CPA increased the latency to reach the tonic-clonic seizure stage, showing anti-convulsant properties in zebrafish. On the other hand, the A_1 antagonist DPCPX pretreatment induced a lower latency to reach tonic-clonic seizure stage, suggesting proconvulsant effects in zebrafish. These findings confirm the anticonvulsant effect induced by activation of adenosine A_1 receptors, since CPA increased the latency to seizures in all doses tested. We have also observed that CPA promoted a decrease on locomotor activity in zebrafish. Previous study support that adenosine receptor agonists,

including the selective A_1 receptor agonist CPA, produced dose-dependent suppression of spontaneous locomotor activity[30].

The extracellular adenosine levels increase during seizures and it has been proposed as a mechanism for seizure control[3,11]. Nevertheless, during a seizure, extracellular adenosine levels rise upon that could activate all types of adenosine receptors, including A_{2A}, which facilitates neuronal transmission[14]. In contrast to the well known A_1 receptor inhibitory effects, there are controversial results about the role of A_{2A} receptors in seizures. Previous study showed that the pretreatment with the selective A_{2A} antagonist ZM 241385 had no effect on seizure parameters[16]. However, the pretreatment with the selective A_{2A} receptor agonist CGS increased the after discharge duration[16]. These results are in contrast with previous report, which considers an anticonvulsant effect for selective A_{2A} receptor agonist[15]. Interestingly, in our study both A_{2A} receptor agonist and antagonist, CGS 21680 and ZM 241385, respectively, did not promote changes in seizure parameters, confirming that anticonvulsant effects of adenosine are promoted mainly through adenosine A_1 receptor activation.

Adenosine can be released from the cytoplasm through NTs or be formed by the extracellular breakdown of ATP by ectonucleotidases[3]. Cellular release of adenosine is a physiological consequence of seizures and induces seizure arrest and termination[11]. Besides cellular adenosine release, ATP release and its degradation into adenosine via ectonucleotidases represent an important source of extracellular adenosine[9]. Despite its importance, the role of adenosine production and degradation for the control of seizure episodes still remains unclear. Previously study have show that neither genetic deletion nor pharmacological inhibition of ecto-5'-nucleotidase play a role in A_1 receptor activation, suggesting that adenosine is not generated from ATP and is released directly by neurons[31]. In active spiking neurons, A_1 receptors were activated in a NTs dependent

manner[32]. However, another investigation has show a key role of ATP release from astrocytes on regulating adenosine signaling. By releasing ATP, which accumulates as adenosine, astrocytes tonically suppress synaptic transmission[33]. Acute PTZ-induced seizures did not alter ectonucleotidases activities in zebrafish brain membranes[19,34]. Furthermore, there were no differences on ectonucleotidase activities after a single PTZ exposure in rats. However, increased ATP hydrolysis was evident in rats that were more resistant to seizures after PTZ-kindling treatment[35]. Changes on ectonucleotidases activities are late and prolonged after recurrent seizures episodes[36], suggesting these effects are due to an adaptive plasticity. Our results have demonstrated that the pretreatment with ecto-5´nucleotidase inhibitor AMPCP reached tonic-clonic seizure status faster than animals without this pretreatment. These results suggest a decrease in adenosine levels by inhibition of ecto-5´-nucleotidase after PTZ-induced seizures in zebrafish. These findings reinforces the idea that extracellular AMP hydrolysis by ecto-5'-nucleotidase is an important mechanism to control adenosine levels and, consequently the development of seizures.

During stressful conditions, when the adenosine levels are exacerbated, ADA activity is important in adenosine clearance. Siebel et al. (2011) have shown that successive convulsive episodes decreased the ecto-ADA activity in zebrafish brain membranes. However, one single seizure episode significantly increased ADA activity in zebrafish brain, effect that was suppressed by AEDs pretreatments[19]. AEDs pretreatments suppressed the increase in adenosine deamination, which coincides with a longer period to reach the tonic-clonic seizure status[19]. Therefore, ADA activity is differently modulated early after a single seizure or successive seizure episodes. Evidence sustaining the approach of combating seizure by inhibiting ADA is limited. There is evidence that seizure protection conferred by the EHNA in genetically seizure-

prone epilepsy-like (EL) mice and PTZ infusion confirms that inhibition of adenosine metabolism by deamination is an effective antiseizure strategy[37]. Here we have show that inhibition of ADA activity modulates acute seizures in zebrafish. When pretreated with the ADA inhibitor EHNA, animals showed longer latency to reach the tonic-clonic seizure status. These results suggest that the control of adenosine levels by ADA activity has an important role in seizure control in zebrafish.

Another important mechanism to control extracellular adenosine levels involves the action of NTs. Our results have also demonstrated that NT inhibition by dipyridamole has protective effects during seizures in zebrafish. Animals pretreated with the NT inhibitor dipyridamole showed longer latency to reach the tonic-clonic seizure status. This NT inhibitor retards the adenosine disappearance from extracellular cleft by blocking adenosine uptake into cells, conferring protective effects until seizures[38]. Therefore, the nucleoside transport is also a mechanism able to control adenosine levels and, consequently, the development of seizures in zebrafish.

In summary, our findings indicate that adenosine A_1 receptors activation is an important mechanism to control the development of seizures in zebrafish. In addition, the action of ecto-5´-nucleotidase, ADA, and NTs are directly involved with the control of extracellular adenosine levels, which also has relevant contribution on the development of seizure episodes in zebrafish.

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Disclosure

None of the authors has any conflict of interest to disclose. We confirm that we have read the Journal's position on issues involved in ethical publication and affirm that this report is consistent with those guidelines.

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Table 1: Pretreatments with adenosinergic drugs on PTZ-induced seizures in zebrafish

Drug	Doses	Vehicle	Reference
Valproate	100,200,500 mg/kg	Saline	Cloix et al., 2009
Caffeine	46,69,92 mg/kg	Saline	Luszczk et al., 2006
DPCPX	3,10,15 mg/kg	DMSO 3%	Southam et al., 2002
CPA	1,2,4 mg/kg	Saline	Mares, 2010
ZM 241385	1,2,5 mg/kg	Saline	Mares, 2010
CGS 21680	1,2,5 mg/kg	DMSO 3%	Mares, 2010
AMPCP	100,150,200 mg/kg	Saline	Southam et al., 2002
EHNA	75,100,150 mg/kg	Saline	Southam et al., 2002
Dipyridamole	5, 10, 20 mg/kg	Saline	Akula et al., 2008

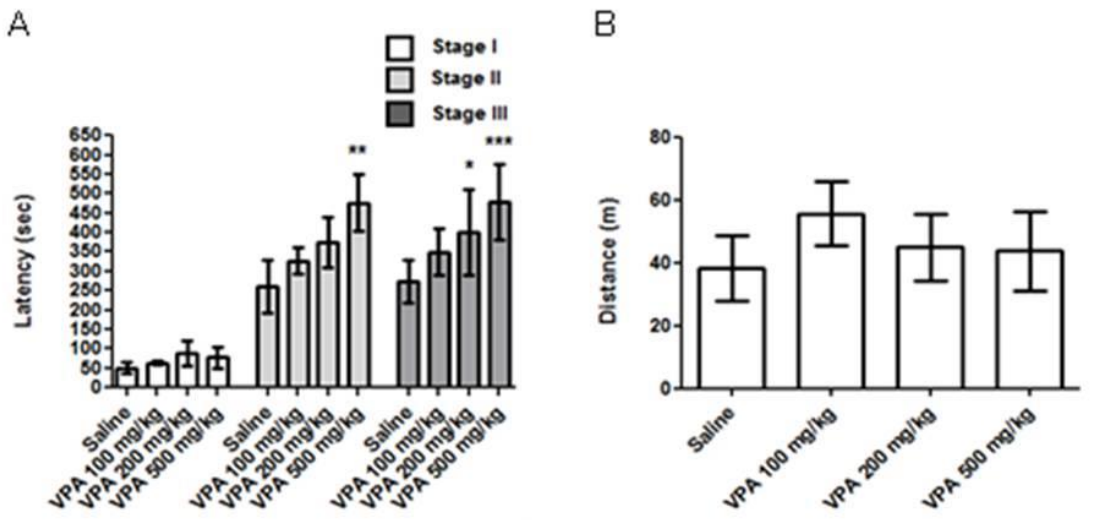

Figure 1: Effect of VPA (100,200,500 mg/kg) on PTZ-induced seizures in zebrafish. (A) Latency to the first episode of the three different seizure behavioral stages in zebrafish. (B) Locomotor activity measured as distance travelled during PTZ exposure. The data are expressed as the mean ± S.D. from 8 animals for each group and were analyzed by one-way ANOVA followed by Dunnett´s post-hoc test. * represents statistical difference ($P < 0.05$) when compared to the respective control.

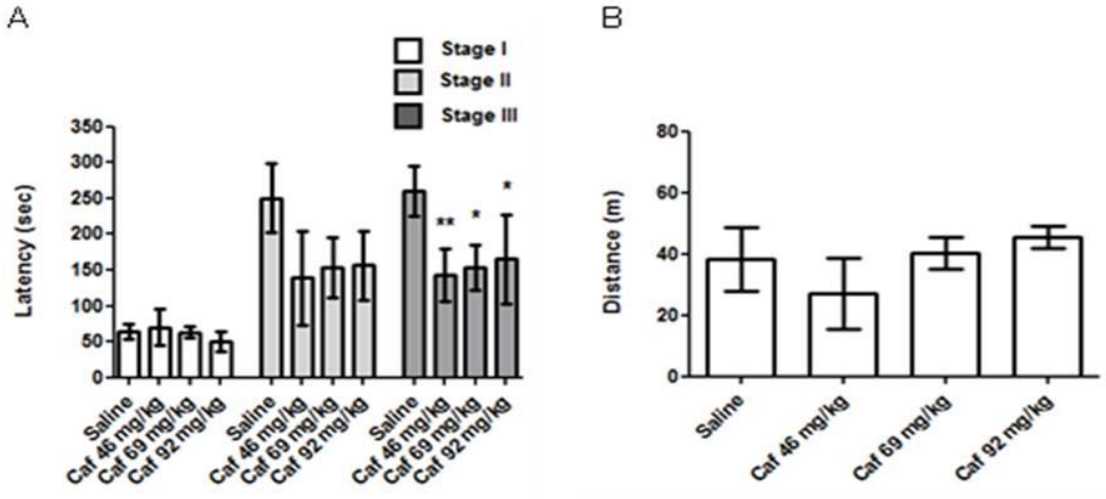

Figure 2: Effect of caffeine (46, 69, and 92 mg/kg) on PTZ-induced seizures in zebrafish. (A) Latency to the first episode of the three different seizure behavioral stages in zebrafish. (B) Locomotor activity measured as distance travelled during PTZ exposure. The data are expressed as the mean ± S.D. from 8 animals for each group and were analyzed by one-way ANOVA followed by Dunnett´s post-hoc test. * represents statistical difference ($P < 0.05$) when compared to the respective control.

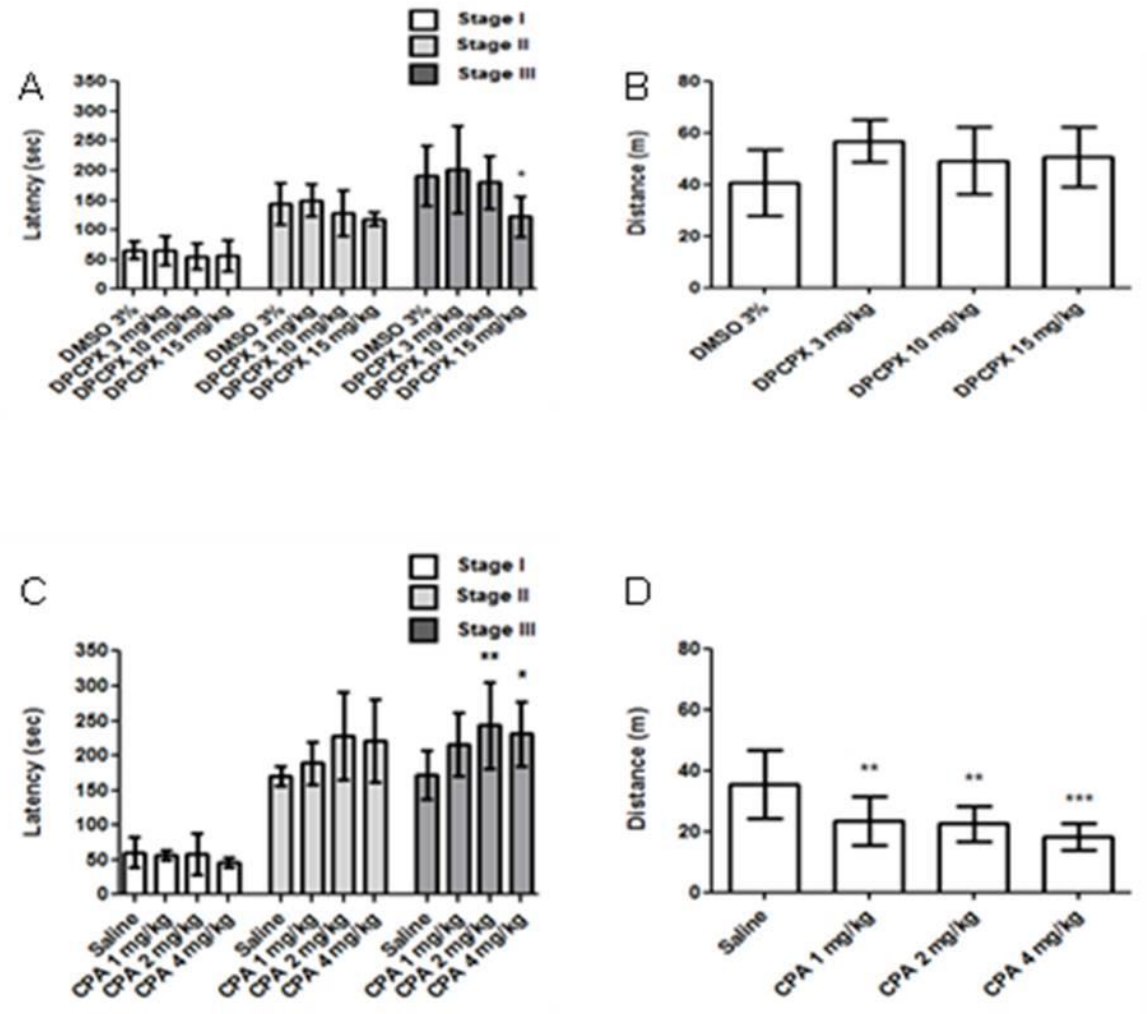

Figure 3: Effect of DPCPX (3,10,15 mg/kg) and CPA (1,2,4 mg/kg) on PTZ-induced seizures in zebrafish. (A, C) Latency to the first episode of the three different seizure behavioral stages in zebrafish. (B, D) Locomotor activity measured as distance travelled during PTZ exposure. The data are expressed as the mean ± S.D. from 8 and 12 animals for DPCPX and CPA groups, respectively, and were analyzed by one-way ANOVA followed by Dunnett's post-hoc test. * represents statistical difference ($P < 0.05$) when compared to the respective control.

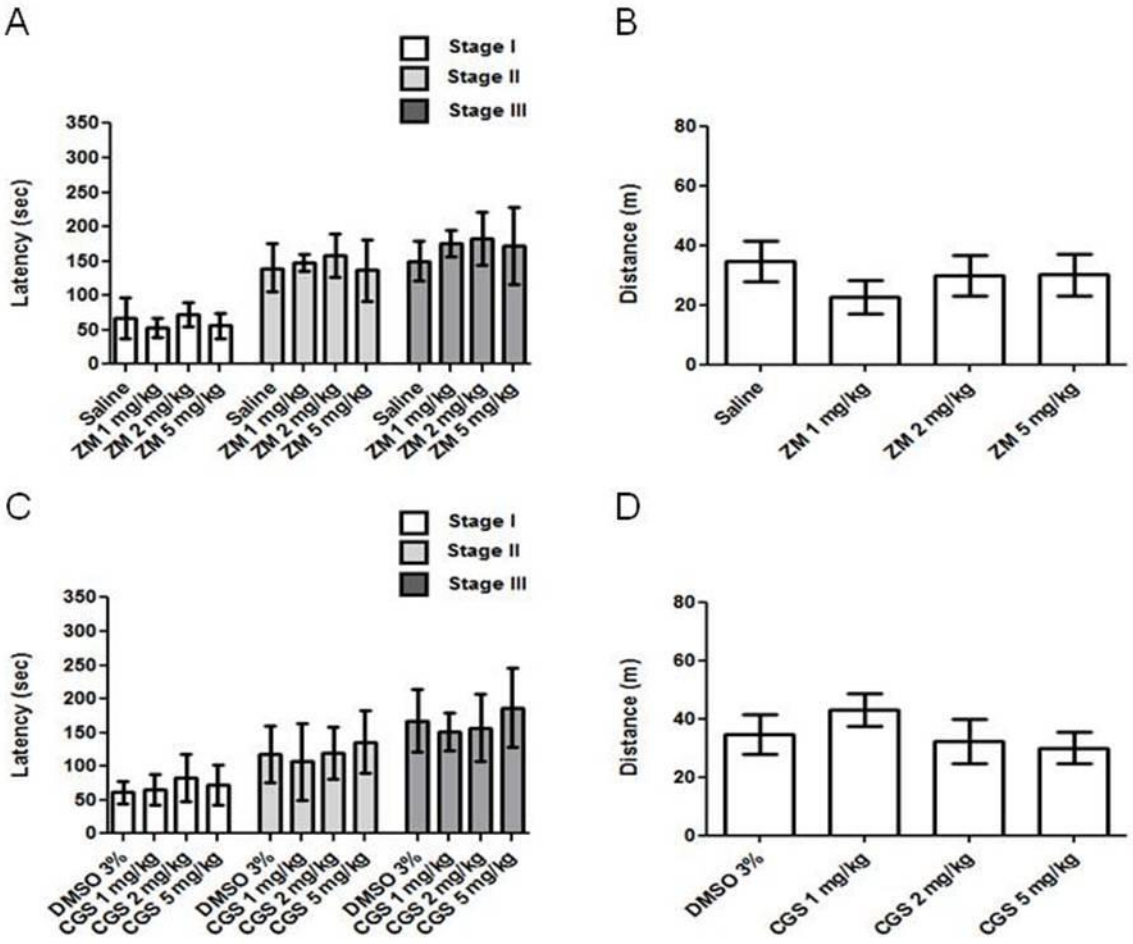

Figure 4: Effect of ZM 241385 and CGS 21680 (1,2,5 mg/kg) on PTZ-induced seizures in zebrafish. (A, C) Latency to the first episode of the three different seizure behavioral stages in zebrafish. (B, D) Locomotor activity measured as distance travelled during PTZ exposure. The data are expressed as the mean ± S.D. from 8 and 12 animals for ZM 241385 and CGS 21680 groups, respectively, and were analyzed by one-way ANOVA followed by Dunnett's post-hoc test. represents statistical difference ($P < 0.05$) when compared to the respective control.

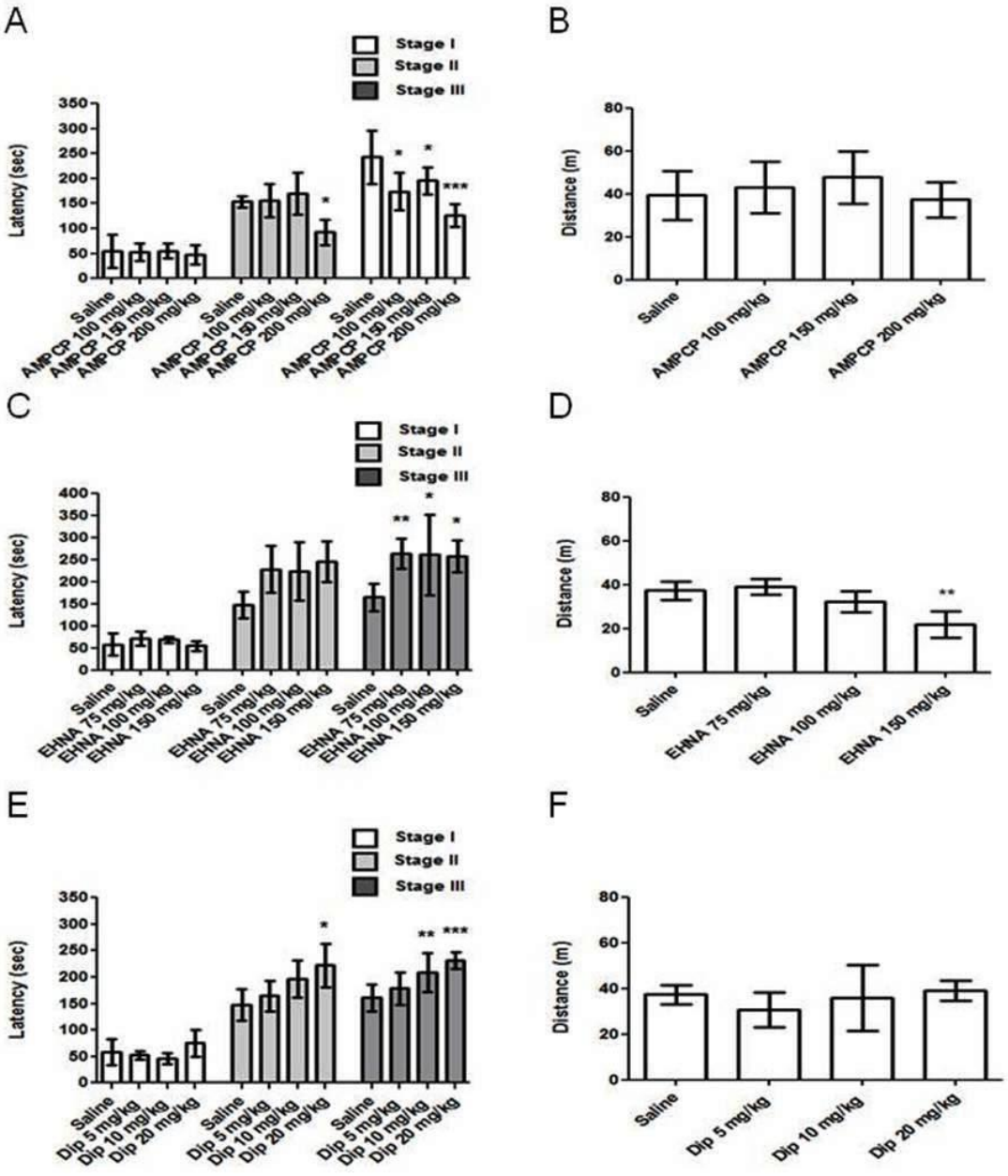

Figure 5: Effect of AMPCP (100,150,200 mg/kg), EHNA (75,100,150 mg/kg), and dipyridamole (5, 10, 20 mg/kg) on PTZ-induced seizures in zebrafish. (A, C, E) Latency to the first episode of the three different seizure behavioral stages in zebrafish. (B, D, F) Locomotor activity measured as distance travelled during PTZ exposure. The data are expressed as the mean ± S.D. from 12, 8, and 8 animals for AMPCP, EHNA, and dipyridamole and were analyzed by one-way ANOVA followed by Dunnett's post-hoc test. * represents statistical difference ($P < 0.05$) when compared to the respective control.

CAPÍTULO III

ARTIGO CIENTÍFICO

Contribution of S6K1/MAPK signaling pathways in the response to oxidative stress: Activation of RSK and MSK by hydrogen peroxide

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Contribution of S6K1/MAPK signaling pathways in the response to oxidative stress: Activation of RSK and MSK by hydrogen peroxide

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ABSTRACT

Cells respond to different kind of stress through the coordinated activation of signaling pathways such as MAPK or p53. To find which molecular mechanisms are involved, we need to understand their cell adaptation. The ribosomal protein, S6 kinase 1 (S6K1), is a common downstream target of signaling by hormonal or nutritional stress. Here, we investigated the initial contribution of S6K1/MAPK signaling pathways in the cell response to oxidative stress produced by hydrogen peroxide (H_2O_2). To analyze S6K1 activation, we used the commercial anti-phospho-Thr389-S6K1 antibody most frequently mentioned in the bibliography. We found that this antibody detected an 80-90 kDa protein that was rapidly phosphorylated in response to H_2O_2 in several human cells. Unexpectedly, this phosphorylation was insensitive to both mTOR and PI3K inhibitors, and knock-down experiments showed that this protein was not S6K1. RSK and MSK proteins were candidate targets of this phosphorylation. We demonstrated that H_2O_2 stimulated phosphorylation of RSK and MSK kinases at residues that are homologous to Thr389 in S6K1. This phosphorylation required the activity of either p38 or ERK MAP kinases. Kinase assays showed activation of RSK and MSK by H_2O_2. Experiments with mouse embryonic fibroblasts from p38 animals' knock-out confirmed these observations. Altogether, these findings show that the S6K1 signaling pathway is not activated under these conditions, clarify previous observations probably misinterpreted by non-specific detection of proteins RSK and MSK by the anti-phospho-Thr389-S6K1 antibody, and demonstrate the specific activation of MAPK signaling pathways through ERK/p38/RSK/MSK by H_2O_2.

Keywords: Hydrogen peroxide, ERK, MSK, p38, RSK, S6K1

Abbreviations used: mTOR, mammalian target of rapamycin; S6K1, ribosomal protein S6 kinase 1; RSK, 90 kDa ribosomal S6 kinase; MSK, mitogen- and stress-activated protein kinase; ERK, extracellular signal-regulated kinase; MAP, mitogen-activated protein; MAPK, mitogen-activated protein kinase; PI3K, Phosphatidylinositol 3-kinase; siRNAs, small interfering RNAs; H_2O_2, hydrogen peroxide; MEF, mouse embryonic fibroblast.

Introduction

Reactive oxygen species (ROS) function as important physiological regulators of intracellular signaling pathways [1]. High ROS levels are associated with diseases such as neurodegeneration, atherosclerosis, chronic inflammation, diabetes or cancer [1-4]. An increase in ROS is also observed with age, probably caused by the accumulation over time of free radicals from aerobic metabolism and linked to a decreased antioxidant capacity and/or mitochondrial dysfunction [1,5]. The emerging role of ROS in physiological and pathophysiological processes demonstrates the importance of understanding the cell signaling pathways involved in redox signaling [1,3,6].

The mitogen-activated protein kinase (MAPK) signaling pathways allow cells to interpret a wide range of external signals and respond by generating a plethora of different biological effects. Members of the MAPK family, including extracellular signal-regulated kinases (ERK), c-Jun N-terminal kinases (JNK) and p38, are activated by ROS. The activation of these kinases usually regulates the expression of a variety of genes involved in survival, proliferation or cell death, depending on the stimulus and the cell-type studied [1,3,7].

The ribosomal protein S6 kinase 1 (S6K1) is a common downstream target of signaling by hormones and nutrients. S6K1 is a substrate of the mammalian target of rapamycin (mTOR) complex 1 (mTORC1). This complex is a Ser/Thr kinase that regulates S6K1 activation through its phosphorylation at Thr389 (T389). Activated S6K1 regulates the phosphorylation of other substrates such as the ribosomal protein S6 to promote protein synthesis, cell growth and cell proliferation [8-10]. In recent years, several studies have also involved S6K1 in the response to oxidative stress. Thus, whereas some authors propose that mTOR inhibition is required for H_2O_2-induced cell death [11], others demonstrate that the mTOR/S6K1 pathway is not responsible for this effect [12]. In

some cases, S6K1 phosphorylation was observed [12,13], whereas in others a decrease in this phosphorylation was reported [11,14,15]. These apparently controversial findings have been justified by the complexity of the pathways involved and by the function of these pathways possibly depending on the cell type, H_2O_2 dose and duration of the stress signal [12].

S6K1 activation is measured by the increase of its phosphorylation at T389 and/or by the phosphorylation increase of its substrate, the ribosomal protein S6, at S235/S236. Thus, antibodies against these phosphorylated residues are a valuable tool for analyzing S6K1 activation. The specificity of these antibodies is crucial to interpretation of the data. S6K1 is member of a family of serine /threonine kinases named AGC. Other members of this family, such as the mitogen- and stress-activated kinases (MSK) and the p90 ribosomal S6 kinases (RSK), show a high degree of homology, in particular a serine residue within the hydrophobic motif of the RSK and MSK proteins [16]. Previous studies have shown the cross-reaction of anti-phosphorylated-T389 (P-T389)-S6K1 antibody with phosphorylated RSK and MSK proteins and that activation of these kinases also regulate the phosphorylation of the ribosomal protein S6 at S235/S236 [17].

In response to oxidative stress, MAPK signaling pathways are activated; contradictory data have been reported for the S6K1 signaling pathway. We asked whether under these conditions the anti-P-T389-S6K1 antibody detected RSK and MSK proteins and could be a motif to misinterpret these findings. In the present study, we showed that S6K1 is not involved in the fast response to incubation with H_2O_2 and that the anti-P-T389-S6K1 antibody detected the phosphorylation of RSK and MSK proteins by H_2O_2 in a p38- and ERK-dependent manner.

Materials and methods

Reagents- Insulin, wortmannin, rapamycin and anti-P-ERK1/2 antibody (Sigma-Aldrich); hydrogen peroxide solution (H_2O_2) (Panreac); U0126 and SB 203580 (Calbiochem); anti-mTOR, anti-P-T389-S6K1 (1A5), anti-P-S380-RSK, anti-P-S376-MSK, anti-P-S235/236-S6, anti-S6 (54D2) and anti-PT180/Y182-p38 antibodies (Cell Signaling Technology); anti-MSK, anti-S6K1 (C-18) and anti-RSK1 (C-21) antibodies (Santa Cruz Biotechnology, Inc.); Alexa Fluor 488, Alexa Fluor 546, TO-PRO3 (Molecular Probes); anti-P- H2AX antibody, Immobilon-P PVDF transfer membrane (Millipore Corporation); siRNA used: mTOR (CCCUGCCUUUGUCAUGCCUdTdT), S6K1 (GGGGGCUAUGGAAAGGUUUdTdT), RSK1 (GCUAUACCGUCGUGA-GAUCdTdT), RSK2 (GGAGGAGAUUAACCCACAAdTdT), MSK1 (GGAACUGG-AGCUUAUGGAAdTdT), MSK2 (UUGCACAUGAUCUCGGCCGdTdT) and non-targeting control (UAGCGACUAAACACAUCAAdTdT).

Cell Culture and transfections- WT and p38α-deficient MEFs were a gift from Dr. A. Nebreda (Institute for Research in Biomedicine, Barcelona, Spain)[18]. All cell lines were cultured at 37°C in Dulbecco's Modified Eagle medium (DMEM) (Gibco), containing 10% fetal bovine serum. siRNA transfections were carried out in MCF7 cells with the calcium phosphate transfection system. For experiments with insulin, cells were deprived of serum overnight and then incubated with 200 nM insulin for 30 min. For experiments with H_2O_2, cells were treated with 0.4 mM H_2O_2 for 30 min, without overnight serum deprivation. The specific inhibitors were added 60 min before the treatment with H_2O_2 or insulin at a final concentration of 20 nM rapamycin, 100 nM wortmannin, 5 μM U0126 and 5 μM SB 203580.

Cell lysate and immunoblotting- Previously treated cells were lysed in CHAPS lysis buffer (10 mM Tris–HCl, pH 7.5, 100 mM NaCl, 0.3% CHAPS, 50 mM NaF, 1 mM sodium vanadate, 1 mM phenylmethylsulfonyl fluoride, 5 μg/ml leupeptin, 5 μg/ml aprotinin, 1 μg/ml pepstatin A, 50 mM β-glycerophosphate, 100 μg/ml benzamidine) for 1 h at 4°C and equal amounts of proteins were separated by electrophoresis. To analyze simultaneously large and small proteins in the same gel, we used Tris-Acetate PAGE systems [19]. After running the gel, the proteins were transferred to PVDF membranes and viewed by immunoblotting, as described elsewhere [17]. Band intensities were analyzed with a gel documentation system (LAS-3000 Fujifilm). Protein levels were standardized with respect to mTOR or Ran levels and expressed as a percentage of controls.

Confocal microscopy- MCF-7 cells were fixed with 4% paraformaldehyde for 20 min at room temperature (RT). The cells were blocked and permeabilized with 10% fetal bovine serum and 0.1% Triton X-100 in PBS for 2h. The primary antibodies, anti-PT180/Y182-p38 (1:200), anti-P-S380-RSK (1:50), anti-P-ERK1/2 (1:200) and anti-P-H2AX (γH2AX) (1:500), were incubated at 4ºC overnight; and the secondary antibodies, at RT for 2h. Nuclei were stained with TO-PRO-3 and the cells were examined by laser confocal microscopy.

Immunoprecipitations and kinase assay- Lysates from MCF7 cells were immunoprecipitated with anti-RSK or anti-MSK antibodies. Lysis and immunoprecipitation were carried out in a buffer containing 40 mM Hepes, pH 7.5, 120 mM NaCl, 50 mM NaF, 0.3% CHAPS and the above protease inhibitors. Immunoprecipitates were washed three times with lysis buffer and once with kinase

buffer (30 mM Tris-HCl, pH 7.5, 10 mM $MgCl_2$, 1 mM DTT). The kinase assay was performed as previously described [17] in kinase buffer using GST-S6 as substrate (3 μg substrate per assay) in the presence or absence of ATP (500 μM), during 30 min at 30°C. Reactions were stopped in ice with sample buffer and analyzed by immunoblot with anti-P-S235/S236-S6 antibody to detect the incorporation of phosphate. Band intensities were analyzed with a gel documentation system (LAS-3000 Fujifilm).

Statistical analysis- The results are expressed as mean ± SEM. Data were analyzed by one-way ANOVA followed by Dunn's post-hoc test.

Results

Phosphorylation of p85 and S6 ribosomal proteins in response to H_2O_2

To study S6K1 regulation in response to oxidative stress, we analyzed by Western blot the phosphorylation of endogenous S6K1 by H_2O_2, using a commercial monoclonal anti-P-T389-S6K1 antibody (1A5, Cell Signaling Technology). MCF7 cells were incubated with H_2O_2 for 30 min. We used these human cells because they have been extensively used to study the response to oxidative stress. As a positive control of S6K1 activation, MCF7 cells were stimulated with insulin after overnight serum deprivation. As shown in Fig. 1A, phosphorylation of endogenous S6K1 p70 isoform was detected by Western blot with anti-P-T389-S6K1 antibody after 30 min of treatment with insulin. No variation in p85 isoform phosphorylation was detected in this cell line with insulin treatment. Unexpectedly, after treatment with H_2O_2, we detected an increase in a band with similar mobility to S6K1 p85 isoform that we named p85 protein. No increase was seen in p70 isoform phosphorylation. With both treatments, an increase in the phosphorylation of the ribosomal S6 protein was observed. These treatments did not modify endogenous levels of S6K1 or S6 proteins (Fig. 1A). Levels of other proteins involved in the S6K1 signaling pathway such as mTOR were also unaltered (Fig. 1A). These results were confirmed in other human cell types such as U2OS or H1299 (Fig. 1B), indicating that the response to H_2O_2 is not restricted to one cell type.

Experiments of time and dose course confirmed the previous data. Thus, rapid (5 min) and specific phosphorylation of endogenous p85 protein was seen on incubation with various concentrations of H_2O_2 (Fig. 1C/1D). Maximum effects were observed after 15-30 min incubation.

The mTOR/S6K1 signaling pathway is not activated in response to the oxidative stress produced by H_2O_2

To analyze the phosphorylation regulation of p85 protein, we performed experiments with H_2O_2 in the presence of known inhibitors of S6K1 activation (Fig. 2). We observed that the phosphorylation of p85 protein was not significantly modified by rapamycin or wortmannin, inhibitors of mTORC1 and PI3K and mTOR kinases, respectively (see also Fig. 6). S6 phosphorylation correlated with the increase in p85 phosphorylation and was not significantly modified by rapamycin and wortmannin. As control, in parallel experiments, cell stimulation with insulin confirmed the previously reported inhibition of S6K1 phosphorylation and S6 phosphorylation by rapamycin and wortmannin (Fig. 2).

It was shown that rapamycin inhibits the phosphorylation of S6K1 isoforms by mTORC1 [10]. The above data seem to indicate that, in response to H_2O_2, this phosphorylation is not regulated by rapamycin (Fig. 2) and suggest that another kinase might be involved in this regulation. To discard a role of mTOR protein, knockdown experiments were performed. As shown in Fig. 3, in response to H_2O_2, the phosphorylation of endogenous p85 protein was not altered by the absence of mTOR, indicating that p85 protein is not a substrate of mTOR complexes.

We had previously reported that, in response to amino acids, anti-P-T389-S6K1 antibody recognized a phosphorylated p85 protein that was not S6K1 [17]. The similarity of these results with what we obtained with H_2O_2 led us to analyze whether the phosphorylated p85 protein was S6K1. To this end, knockdown experiments of S6K1 were performed. As shown in Fig. 3, the phosphorylation of endogenous p85 protein was not altered by the absence of S6K1, indicating that the phosphorylated protein detected by the anti-P-T389-S6K1 antibody is not S6K1.

Activation of the MAPK signaling pathways in response to the oxidative stress produced by H_2O_2

The previous data indicated that the phosphorylation of a p85 protein detected with anti-P-T389-S6K1 antibody in response to the oxidative stress mediated by H_2O_2 was independent of the mTOR/S6K1 signaling pathway. Thus, other pathways and proteins must be involved in this response. The MAPK signaling pathways are activated by incubation with H_2O_2. This activation is mediated by ERK and p38 kinases [7,20]. We had previously reported that these kinases also regulate amino acid signaling [17]. Under these conditions, ERK and p38 phosphorylate and activate RSK and MSK proteins in response to amino acids. RSK and MSK proteins are members of the family of serine /threonine kinases named AGC. S6K1 is also a member of this family. RSK and MSK proteins have a high degree of homology with the hydrophobic motif of S6K1, where T389 is located [16,17]. These structural similarities, together with the electrophoretic mobility of RSK and MSK proteins around 85-90 kDa and the previously shown cross-reaction of anti-P-T389-S6K1 antibody with phosphorylated RSK and MSK proteins, led us to check whether these proteins were phosphorylated in response to oxidative stress by H_2O_2. We had used antibodies against phosphorylated residues of RSK and MSK equivalents to T389 in S6K1, concretely anti-P-S380-RSK and anti-P-S376-MSK antibodies. We observed that RSK and MSK proteins were phosphorylated after incubation with H_2O_2 (Fig. 4A/4B). The time and dose course was similar to that found with the anti-P-T389-S6K1 antibody (Fig. 1). These effects were also observed in other human cells such as H1299 and, in less extension, in U2OS cells (Fig. 4C). As positive control of the response to H_2O_2, we analyzed the activation of

ERK and p38. As shown in Fig. 4, phosphorylation of ERK and p38 correlated with the phosphorylation of RSK and MSK proteins.

Functional activation of the MAPK signaling pathways included the translocation to the nucleus of phosphorylated p38 and ERK. We analyzed these translocations in response to H_2O_2. As shown in Fig. 5, a rapid nuclear translocation of phosphorylated p38 and ERK was observed at 1-5 min of incubation. Phosphorylation and translocation of RSK was also detected at 1-5 min of incubation. Phosphorylation of H2AX (γH2AX) in response to DNA damage was used as a positive control of treatment with H_2O_2. After 30 min, foci of γH2AX were clearly detected.

Phosphorylation of p85 and S6 ribosomal proteins by H_2O_2 correlated with phosphorylation of RSK and MSK and were sensitive to inhibitors of the MAPK signaling pathways

The previous data suggested that the phosphorylation of p85 protein detected with anti-P-T389-S6K1 antibody was the phosphorylation of RSK and MSK. Thus, it is would be expected that inhibition of the MAPK signaling pathway must inhibit phosphorylation of p85 protein in a similar manner to RSK and MSK. We checked this possibility. We performed experiments with H_2O_2 in the presence of known inhibitors of the MAPK signaling pathways (Fig. 6). We observed that the phosphorylation of p85 protein was dependent on U0126, a specific inhibitor of ERK phosphorylation, and SB 203580, a specific inhibitor of p38 activity. Interestingly, when both inhibitors were simultaneously used, phosphorylation of p85 protein was almost completely inhibited, suggesting crosstalk between ERK and p38 signaling (Fig. 6). Similar results were obtained with the phosphorylation of RSK and MSK. As a positive control, phosphorylation of ERK and p38 was analyzed. Phosphorylation of S6 ribosomal

protein was specifically inhibited by the ERK inhibitor, suggesting a specific role of this kinase or of a downstream kinase in this regulation. Under these conditions, the presence of rapamycin or wortmannin did not significantly affect the phosphorylation of ERK, p38, RSK or MSK proteins. None of these treatments modified endogenous levels of S6K1, RSK, MSK or S6 proteins (Fig. 6). Levels of other proteins such as mTOR were not altered (Fig. 6).

To provide some evidence that p85 was likely RSK or MSK, knockdown experiments were performed. We used siRNA against the most abundant isoforms of RSK (RSK1 and RSK2) and MSK (MSK1/MSK2). The mix of siRNA against RSK or MSK isoforms decreased the p85 detection with anti-P-T389-S6K1 antibody (Fig. 7). This decrease was more evident with simultaneous mix of siRNA against RSK and MSK isoforms. These results show that the anti-P-T389-S6K1 antibody was detecting RSK and MSK phosphorylated.

Activation of RSK and MSK by H_2O_2

The previous data suggest that the kinase activity of RSK and/or MSK is regulated by H_2O_2. To show this point, kinase assays were performed using purified kinases and GST-S6 fusion protein as substrate. As it is shown in Fig. 8, the activities of RSK and MSK were stimulated by H_2O_2.

p38α did not regulate phosphorylation of p70 S6K1 in response to the oxidative stress produced by H_2O_2

It has been reported that loss of p38α impairs mTOR/p70 S6K1 activation in response to H_2O_2 through Akt-independent mechanisms [12]. These experiments were performed in wild-type (WT) and p38α-deficient mouse embryonic fibroblasts (MEFs).

Bearing in mind the above data, we analyzed S6K1 and MAPK signaling pathways in WT and p38α-deficient MEFs. To analyze the phosphorylation of mouse S6K1 p70 isoform, we used the well-shown activation and phosphorylation of p70 S6K1 by insulin as positive control. We observed an increase in the phosphorylation of p70 S6K1 after 30 min treatment with insulin (Fig. 9). No significant changes were observed in the phosphorylation of RSK, MSK and p38 proteins due to insulin treatment. In parallel experiments, we compared the response of WT and p38α-deficient MEFs to treatment with H_2O_2 (Fig. 9). Under these conditions and in line with the above data in human cells, increased phosphorylation of mouse p85 protein was observed in WT MEFs. Phosphorylation of mouse p70 S6K1 was not regulated in WT and p38α-deficient MEFs. Incubation with H_2O_2 activated the MAPK signaling pathway. Thus, phosphorylation of RSK, MSK and p38 proteins was seen in WT MEFs. In p38α-deficient cells, we observed a marked reduction in phosphorylation of RSK and MSK proteins (Fig. 9). Phosphorylation of p85 protein was also reduced in p38α-deficient cells, suggesting that the anti-P-T389-S6K1 antibody detected phosphorylated RSK and MSK in mouse cells. Altogether, these results confirm our previous observations of human cells in response to the oxidative stress produced by H_2O_2.

Discussion

The mTOR signaling pathway has an essential role in the regulation of mammalian growth and development. Hormones such as insulin and nutrients such as amino acids mediate their cellular effects through this pathway [21]. Several studies have analyzed the mTOR pathway in response to oxidative stress by H_2O_2. Most of these studies analyzed the activity of the mTOR complex 1 (mTORC1) through the analysis of S6K1 phosphorylation at T389 using anti-P-T389 antibodies. In some cases inhibition of mTORC1 activity was reported [11,14,15], whereas in others an increase was described [12,13,22]. These apparently contradictory results have been justified by the complexity of the mechanisms involved, cell type, H_2O_2 concentration and duration of the stress signal [12].

Stress conditions that produce DNA damage activate cell repair mechanisms where p53 activation is involved [1,23,24]. During p53 activation, inhibition of mTOR signaling has been observed [25]. Exposure of the cells to high H_2O_2 concentrations and/or during long time periods produces DNA damage and p53 activation. Thus, in these conditions, inhibition of the mTOR signaling pathway would be expected [11,14,15]. For low H_2O_2 concentrations or during shorter time periods, we showed that mTOR signaling was not involved and explained previous observations by the use of the anti-P-T389-S6K1 antibody from Cell Signaling. We showed that this antibody recognized a phosphorylated protein of 85 kDa in response to H_2O_2. Knockdown experiments together with the use of specific inhibitors let us show that this phosphorylated protein of 85 kDa was not regulated by mTOR and was not S6K1. A similar situation had been previously reported in signaling by amino acids, for which authors showed that the phosphorylated p85 protein recognized by the anti-P-T389-S6K1 antibody was phosphorylated RSK and MSK proteins [17]. To avoid

misinterpretations in future experiments, we recommend that researchers using this anti-P-T389-S6K1 antibody check the correct size of the band detected and confirm their results with knockdown experiments. Simultaneous analysis with another well-known stimulus such as insulin also helps to detect the involvement of S6K1. We would also like to highlight that, since the S6 ribosomal protein may be a substrate of different kinases such as S6K1 or RSK [10,17], results using anti-P-S6 antibody to analyze mTOR/S6K1 signaling can be misinterpreted under conditions of activation of the MAPK signaling pathway.

Members of the MAPK family such as ERK, JNK or p38 are phosphorylated and activated in response to oxidative stress [1,7,20]. We confirmed the phosphorylation and activation (nuclear translocation) of ERK and p38 proteins by H_2O_2. Moreover, we reported the phosphorylation and activation of their substrates RSK and MSK. Interestingly, in human cells phosphorylation of both RSK and MSK proteins was sensitive to ERK and p38 activities, indicating that both kinases were necessary to phosphorylate RSK and MSK proteins completely in response to H_2O_2. In contrast, in mouse cells, phosphorylation of MSK by H_2O_2 seemed exclusively dependent on p38 since, in the absence of p38α, MSK phosphorylation was completely abolished. In this context, regulation was reported of mTORC1/p70 S6K1 by p38 in *Drosophila melanogaster* cells [13] and in MEF knockdown for p38α [12]. Using this last model, we showed that p70 S6K1 phosphorylation was stimulated by insulin, but not by H_2O_2. As expected, insulin did not activate the MAPK signaling pathways. Instead, oxidative stress activated phosphorylation of p38/ ERK/MSK in WT MEFs and of RSK in p38α-deficient MEFs. Anti-P-T389-S6K1 antibody detected the phosphorylated p85 protein and its regulation correlated with phosphorylated RSK and MSK proteins. All these observations suggest that, at least in mouse and in human cells, the fast response to

oxidative stress caused by low concentrations of H_2O_2 is mediated by MAPK signaling pathways and not by the mTORC1/p70 S6K1 signaling pathway. In summary, we believe that this report helps to explain previous controversial results and to clarify the cellular signaling activated in response to oxidative stress.

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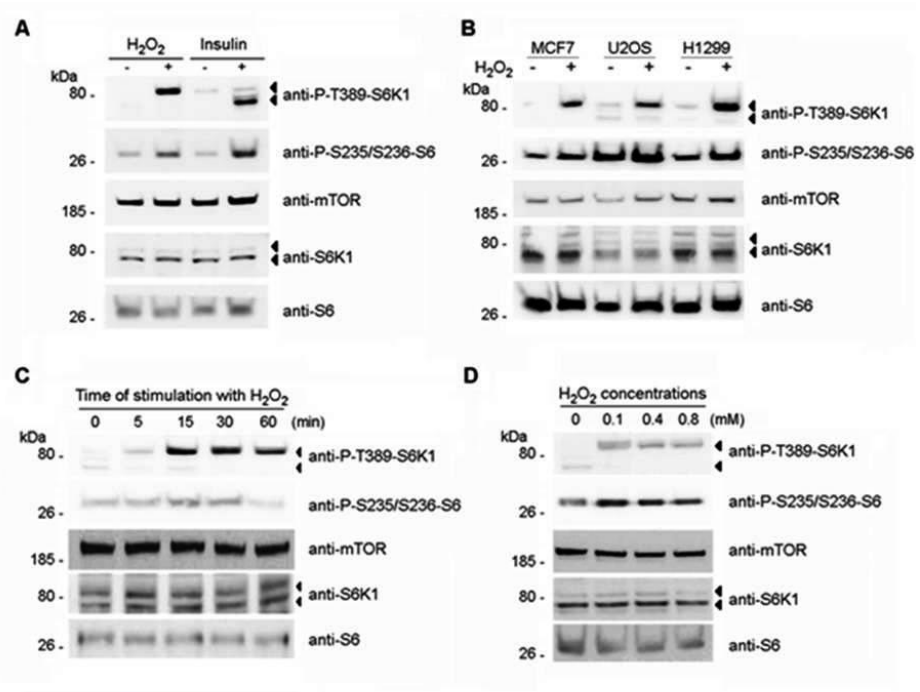

Fig. 1. Phosphorylation of p85 and S6 ribosomal proteins in response to H_2O_2.

Human cells were treated with 0.4 mM H_2O_2 for 30 min (A,B) or deprived of serum overnight and then stimulated with 200 nM insulin for 30 min (A). Experiments of time and dose course were performed in MCF7 cells with 0.4 mM H_2O_2 (C) or for 30 min (D), respectively. Cell lysates were analyzed by Western blot with the indicated antibodies. Molecular weight markers are indicated on the left.

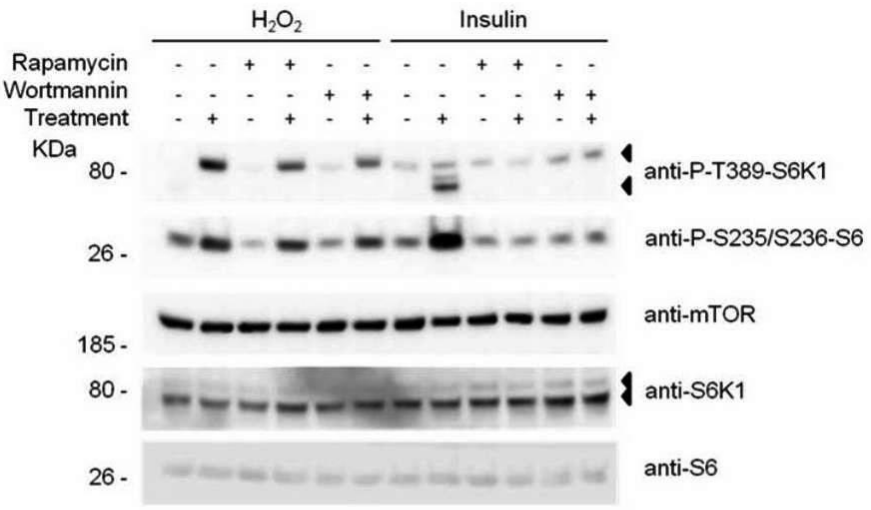

Fig. 2. Effect of rapamycin and wortmannin on phosphorylation of p85 protein by H_2O_2.

MCF7 cells were treated with 0.4 mM H_2O_2 for 30 min. Where indicated, MCF7 cells were pre-incubated with 100 nM wortmannin or 20 nM rapamycin for 60 min before treatment with H_2O_2. Cell lysates were analyzed by Western blot with the indicated antibodies. Molecular weight markers are indicated on the left.

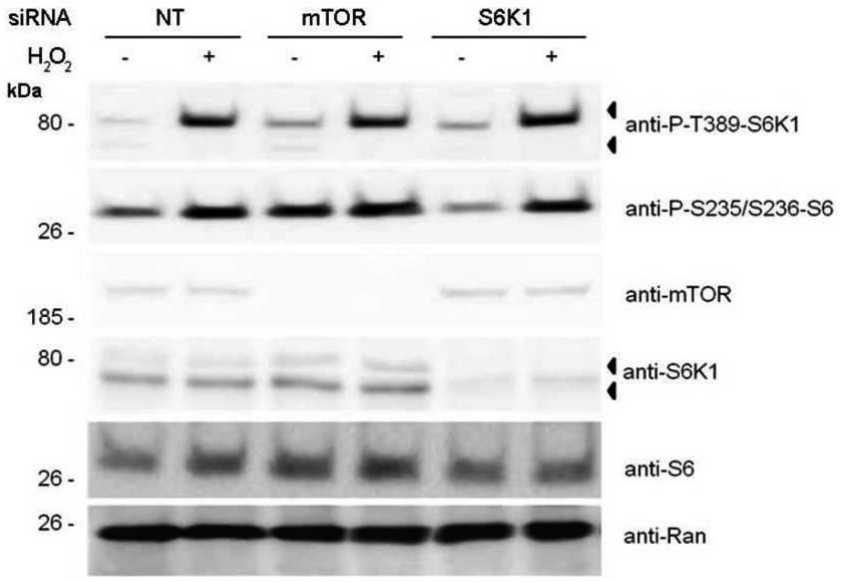

Fig. 3. The mTOR/S6K1 signaling pathway is not activated in response to the oxidative stress produced by H_2O_2.

MCF7 cells were transfected with the indicated siRNAs 72 h before treatment with 0.4 mM H_2O_2 for 30 min. Cell lysates were analyzed by Western blot with the indicated antibodies. Molecular weight markers are indicated on the left. NT means non-targeting control.

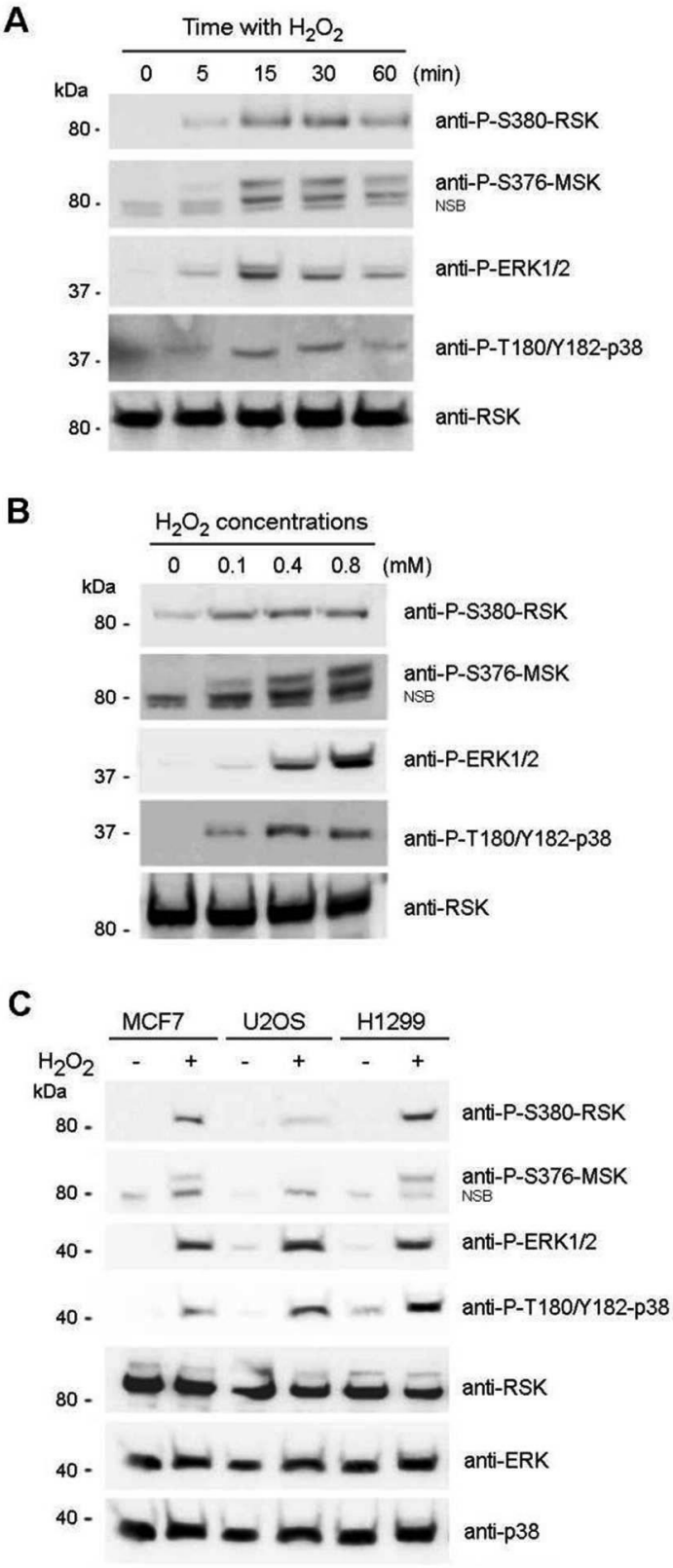

Fig. 4. Activation of the MAPK signaling pathways in response to H_2O_2.

Experiments of time and dose course were performed in MCF7 cells with 0.4 mM H_2O_2 (A) or for 30 min (B), respectively. Human cells were treated with 0.4 mM H_2O_2 for 30 min (C). Cell lysates were analyzed by Western blot with the indicated antibodies. NSB means non-specific band recognized by the antibody. Molecular weight markers are indicated on the left.

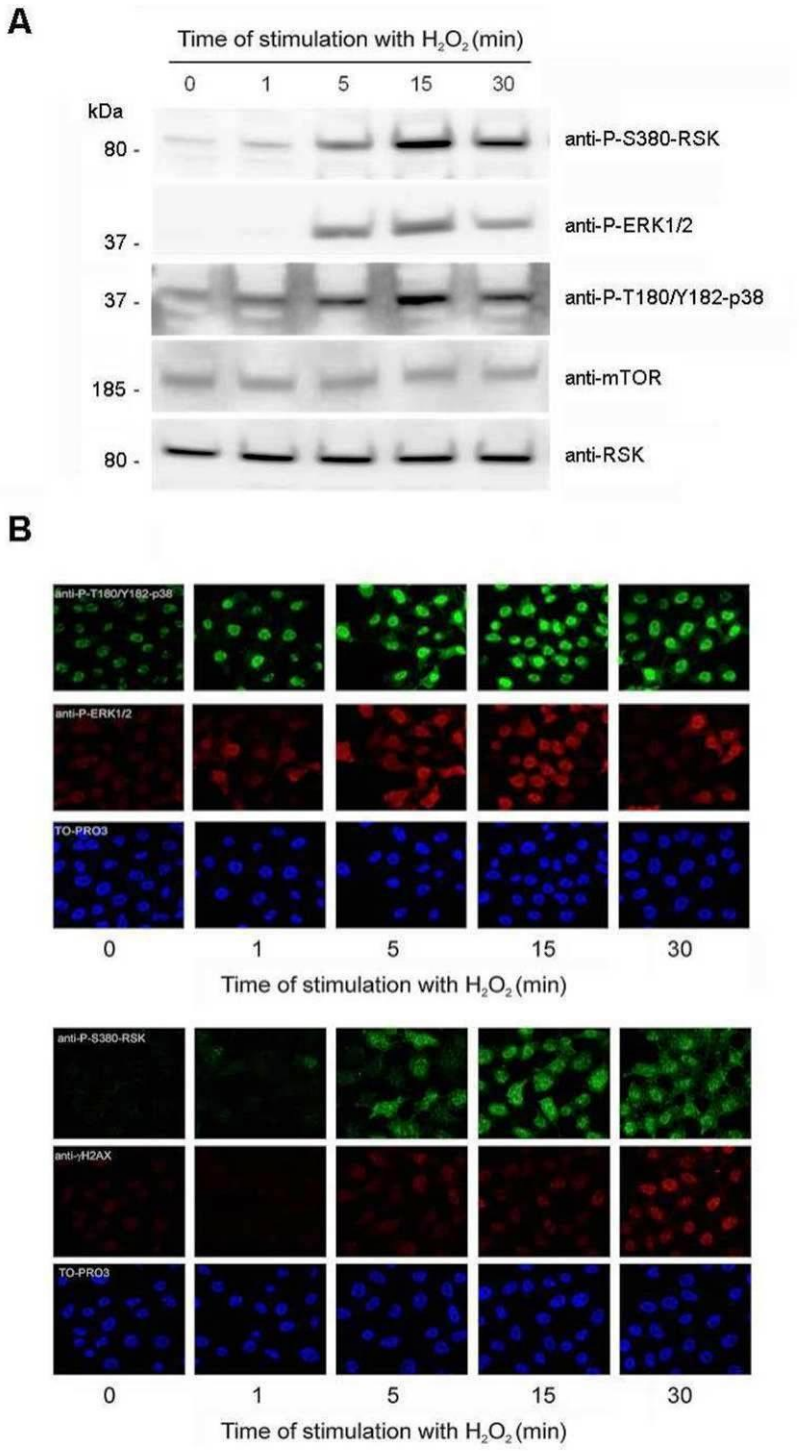

Fig. 5. Nuclear translocation of phosphorylated ERK, p38 and RSK proteins.

MCF7 cells were treated with 0.4 mM H_2O_2 for indicated times and analyzed by Western blot (A), as described in Fig. 1, or by immunofluorescence (B) and using antibodies, as indicated in Experimental Procedures. Nuclear staining was detected with TO-PRO3.

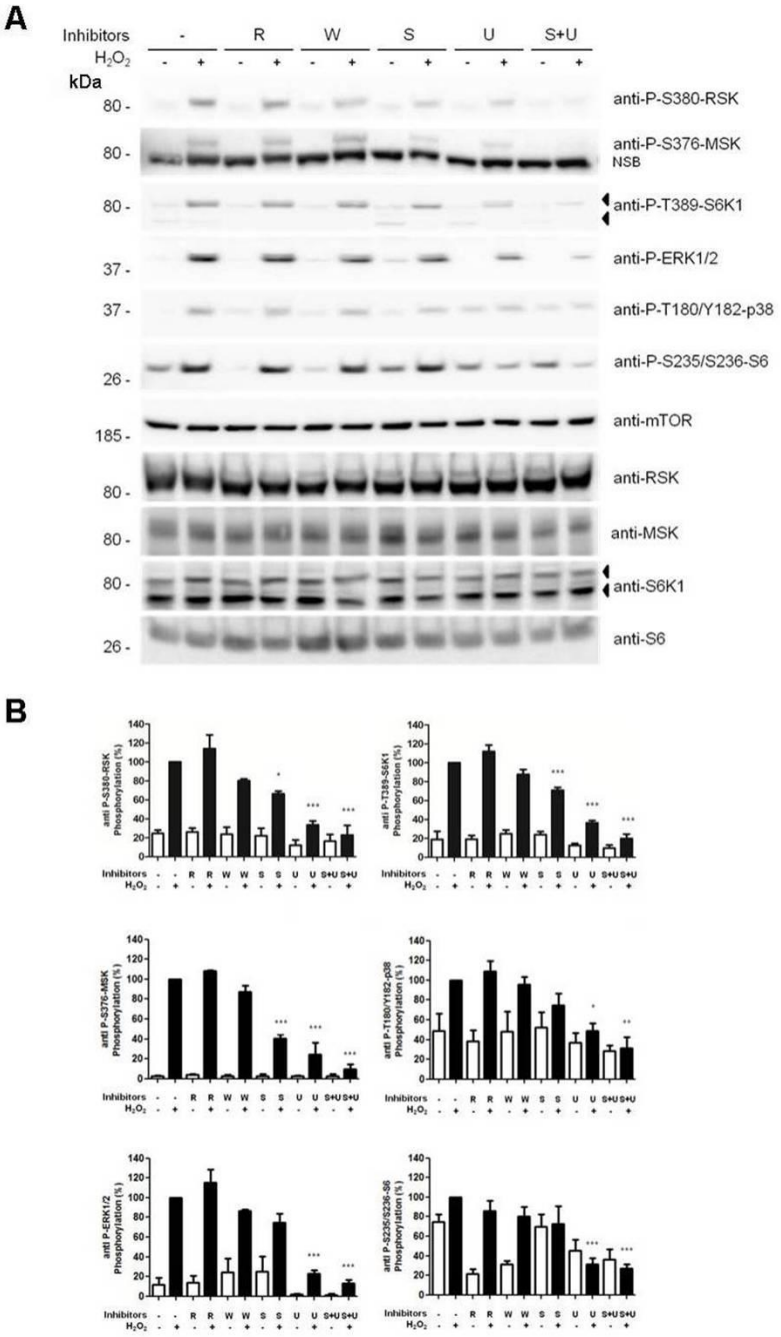

Fig. 6. Phosphorylation of p85, RSK and MSK proteins was sensitive to inhibitors of the MAPK signaling pathways.

(A) MCF7 cells were treated with 0.4 mM H_2O_2 for 30 min. Where indicated, cells were pre-incubated with 5 µM SB 203580 (S), 5 µM U0126 (U), 100 nM wortmannin (W) or 20 nM rapamycin (R) for 60 min before treatment with H_2O_2. Cell lysates were analyzed by Western blot with the indicated antibodies. NSB means non-specific band recognized by the antibody. Molecular weight markers are indicated on the left. (B) Histograms represent the phosphorylation ratio of the indicated proteins. All bands were standardized with respect to mTOR levels. Values are the means ± SEM of the percentage of respective control for at least three independent experiments. Asterisks indicate values that are significantly different (*, $p<0.05$; **, $p<0.01$; ***, $p<0.001$) from the corresponding control value.

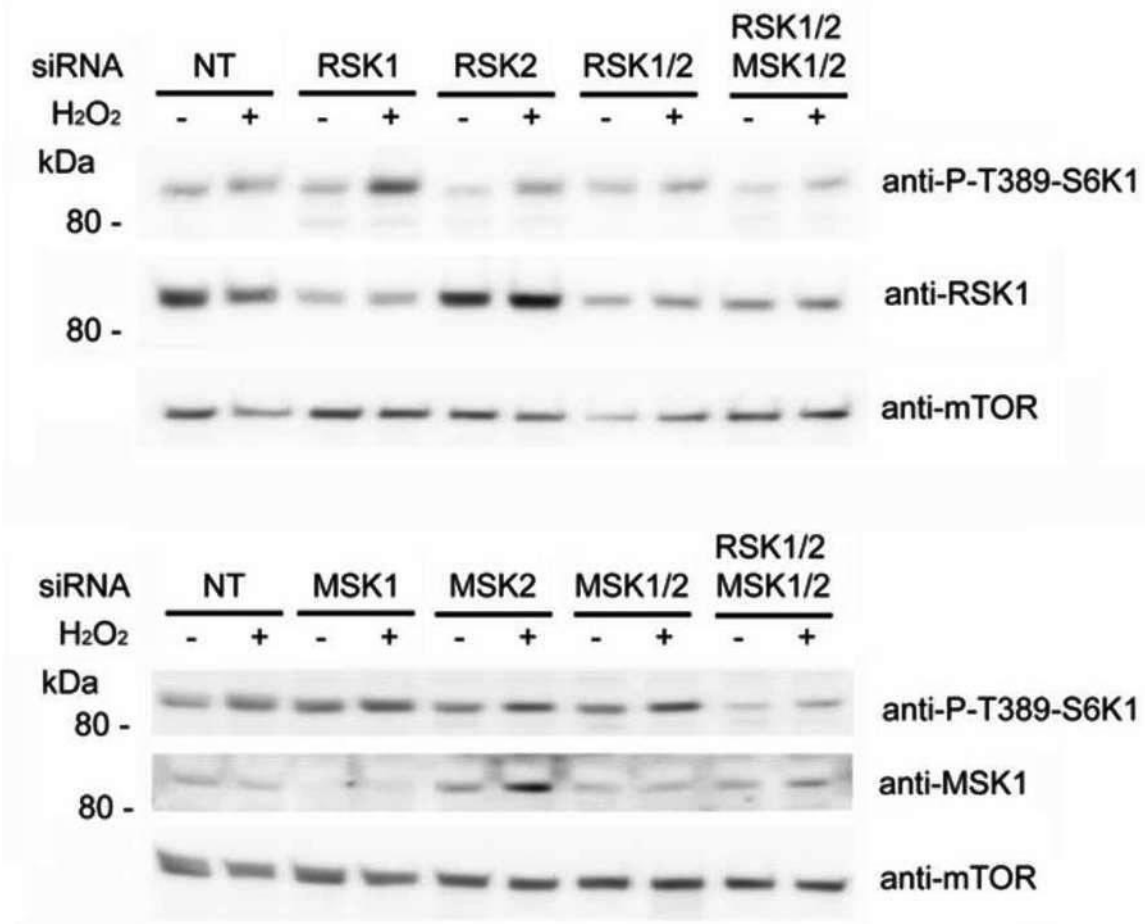

Fig. 7. Anti-P-T389-S6K1 antibody detected RSK and MSK phosphorylated.

MCF7 cells were transfected with the indicated siRNA 72 h before treatment with 0.4 mM H_2O_2 for 30 min. Cell lysates were analyzed by Western blot with the indicated antibodies. Molecular weight markers are indicated on the left. NT means non-targeting control.

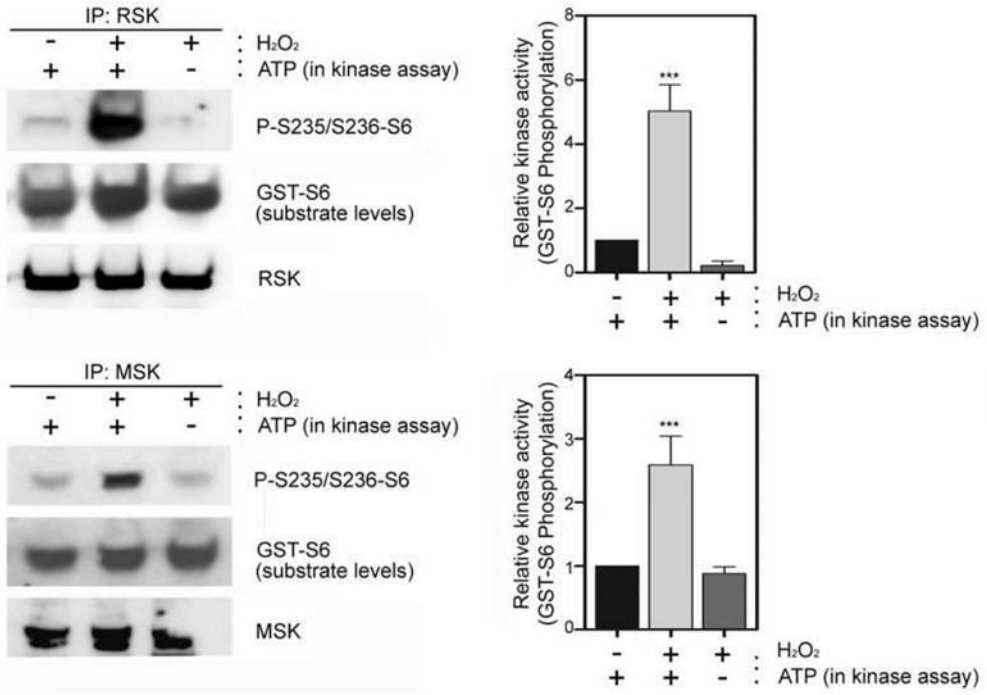

Fig. 8. Activation of RSK and MSK by H_2O_2.

In vitro kinase assay using RSK or MSK immunoprecipitates and purified GST-S6 as substrate. Lysates from MCF7 cells were immunoprecipitated with anti-RSK or anti-MSK antibodies (IP). Immunocomplexes were suspended in kinase buffer and incubated with purified GST-S6 in the presence or absence of ATP during 30 min at 30 °C. Reactions were stopped and the incorporation of phosphate was analyzed by immunoblotting using the anti-P-S235/S236-S6 antibody. Histograms: bands were normalized with respect to GST-S6 substrate levels detected with anti-GST antibody. Data represent the ratio of P-S235/S236-GST-S6 phosphorylation and are expressed as mean±SEM of percentage of respective control. Statistical analysis was carried out as indicated in Materials and methods.

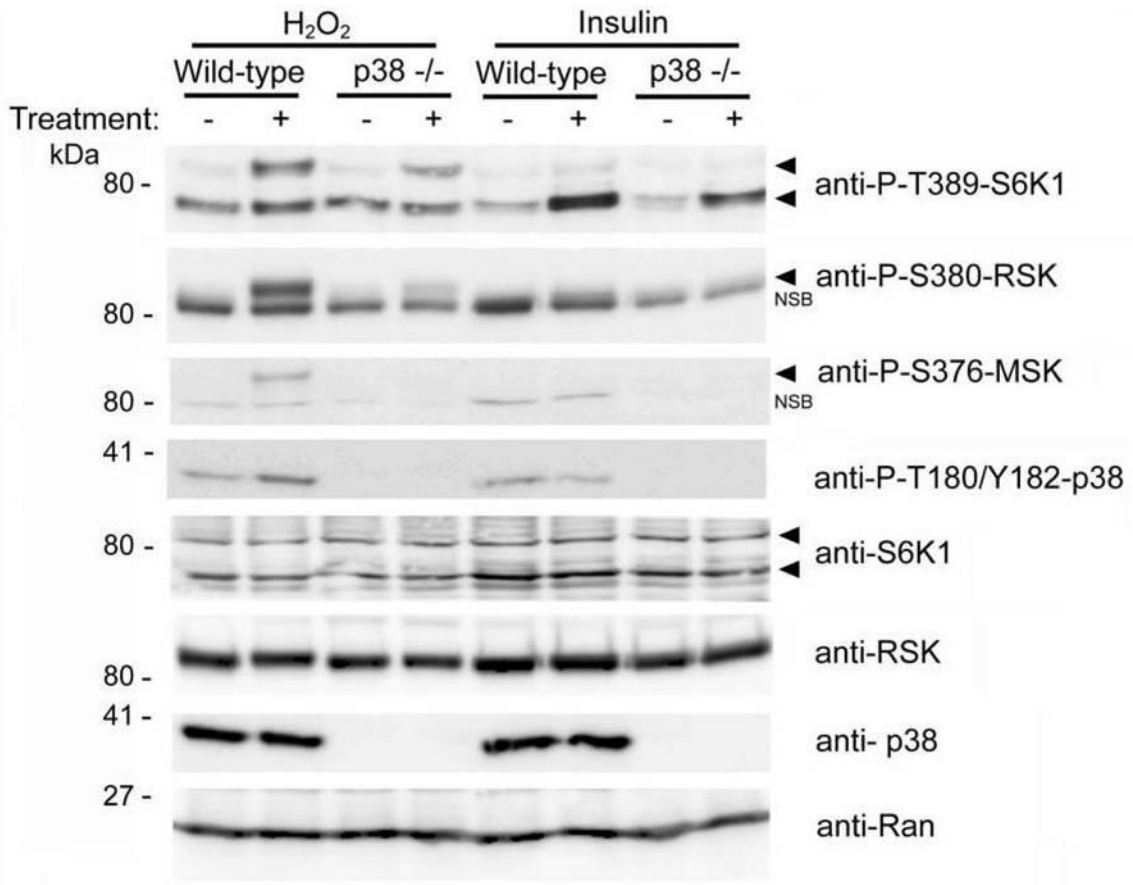

Fig.9. p38α did not regulate phosphorylation of p70 S6K1 in response to the oxidative stress caused by H_2O_2

WT and p38α-deficient MEFs were treated with 0.4 mM H_2O_2 for 30 min or deprived of serum overnight and then stimulated with 200 nM insulin for 30 min. Cell lysates were analyzed by Western blot with the indicated antibodies. NSB means non-specific band recognized by the antibody. Molecular weight markers are indicated on the left.

4. Discussão

A epilepsia é uma das principais doenças crônicas do SNC, atingindo em torno de 1% da população mundial (Pitkanen, 2010). Os pacientes com epilepsia apresentam predisposição crônica à ocorrência de crises convulsivas, sendo necessário o tratamento contínuo com fármacos antiepilépticos (Pitkanen, 2010). Estes fármacos constituem a principal opção no tratamento da epilepsia e têm como objetivo impedir a ocorrência de crises convulsivas, porém não levam à cura e não impedem o agravamento da doença (Bialer & White, 2010). Os mecanismos que envolvem a ocorrência das descargas neuronais súbitas, excessivas e sincrônicas características das crises convulsivas não estão completamente esclarecidos (Badawy et al., 2012). Sabe-se que ocorre o aumento súbito de sinapses excitatórias e falhas nos mecanismos inibitórios, que podem envolver alterações em propriedades neuronais intrínsecas, como instabilidade da membrana, disfunções nos canais iônicos, e capacidade neuronal de manter e propagar o estímulo (Elger & Schmidt, 2008).

Atualmente, existem diversos fármacos antiepilépticos disponíveis, porém muitos compostos atuam por meio de um mesmo mecanismo (Bialer & White, 2010). Estes fármacos atuam principalmente reduzindo a excitabilidade elétrica das membranas celulares, controlando canais iônicos e também por meio da potencialização da neurotransmissão gabaérgica e redução da neurotransmissão glutamatérgica (Rogawski & Löscher 2004a,b). Mesmo com o avanço no desenvolvimento de novos fármacos e a grande variedade de fármacos antiepilépticos disponíveis, em torno de 30% dos pacientes com epilepsia não respondem de maneira satisfatória aos tratamentos farmacológicos disponíveis, não alcançando a total supressão das crises convulsivas (Stefan & Hopfengartner, 2009). Além disso, apenas 10% dos pacientes com epilepsia decorrente de lesões no SNC respondem de maneira positiva ao tratamento farmacológico. Nestes casos, a principal alternativa é a cirurgia para ressecção do foco epileptogênico (Wiebe et al., 2001; Duncan et al., 2006). Para que se possa obter o efetivo controle das crises convulsivas em pacientes epilépticos, é necessária a melhor caracterização das diferentes vias

de sinalização envolvidas na ocorrência de crises convulsivas. A identificação dos diferentes mecanismos envolvidos permitirá buscar tratamentos que atuem em outras vias de sinalização distintas daquelas moduladas por fármacos antiepilépticos clássicos, o que poderá aumentar as opções terapêuticas para a epilepsia.

A adenosina, claramente identificada como um neuromodulador endógeno da excitabilidade neuronal, teve sua ação neuroprotetora identificada há mais de 20 anos, quando estudos mostraram que seus níveis aumentavam rapidamente durante crises convulsivas e contribuíam para o término da convulsão (Dragunow et al.,1985; Dragunow, 1991; During & Spencer,1992). Em crises convulsivas, além do aumento nos níveis extracelulares de adenosina, também ocorre o aumento nos níveis de ATP (Pascual et al., 2005). Diferentemente da adenosina, o ATP pode apresentar efeitos pró-convulsivantes, porém sua rápida degradação à adenosina pode inibir a generalização do foco epiléptico e levar à diminuição da duração da crise convulsiva (Klaft et al., 2012). Assim, o ATP tem propriedades excitatórias, mas é também substrato para a produção de um modulador com efeitos inibitórios (Klaft et al., 2012). As ectonucleotidases, família de enzimas que inclui as NTPDases e a ecto-5'-nucleotidase, são responsáveis pela degradação do ATP em adenosina. Sendo assim, a modulação dos níveis de ATP e adenosina através das ectonucleotidases constitui um importante mecanismo no controle da epilepsia e pode ser um alvo para terapias farmacológicas (Bonan, 2012; Cognato & Bonan, 2010).

Durante nossos estudos, verificamos que a indução de um único episódio de crise convulsiva em peixe–zebra não provoca alterações na hidrólise de ATP, ADP e AMP pelas ectonucleotidases (Siebel et al., 2013, Capítulo I). Em estudo anterior, também não foi verificada alteração na atividade enzimática das ectonucleotidases imediatamente após sucessivas crises convulsivas em peixe–zebra (Siebel et al., 2011). Nossos resultados estão de acordo com estudos prévios em roedores, que mostram não haver alterações na atividade das ectonucleotidases após uma única exposição ao PTZ (Bonan et al., 2000b). Entretanto, em modelo *kindling* induzido por PTZ foi verificada uma maior atividade das ectonucleotidases em animais que eram

mais resistentes à ocorrência de crises convulsivas (Bonan et al., 2000a). Estudos anteriores demonstraram que roedores com epilepsia de lobo temporal induzida por pilocarpina e ácido caínico apresentaram um aumento na atividade das ectonucleotidases na fase crônica da doença (Bonan et al., 2000b; Vianna et al., 2005). Além disso, ratos submetidos a um único episódio de crise convulsiva aos 7 dias de vida apresentaram um aumento na hidrólise extracelular de ATP em sinaptossomas hipocampais na vida adulta (Cognato et al., 2011). Estes resultados sugerem que episódios agudos de crises convulsivas não provocam alterações imediatas na atividade das ectonucleotidases. Portanto, estes achados indicam que as ectonucleotidases são alteradas de maneira tardia e prolongada na fase crônica da doença, como um possível mecanismo de adaptação resultante da plasticidade sináptica induzida por esta condição.

Durante crises convulsivas, nas quais ocorre o aumento nos níveis de adenosina, a atividade da ADA torna-se um mecanismo importante no controle dos níveis deste nucleosídeo (Latini & Pedata, 2001; Boison, 2006). Em nosso estudo, identificamos um aumento significativo na atividade da ADA logo após um único episódio de crise convulsiva induzida por PTZ (Siebel et al., 2013, Capítulo I). Esse aumento na atividade da ADA foi prevenido pelo pré-tratamento dos animais com fármacos antiepilépticos. A supressão do aumento da atividade da ADA coincidiu com o efeito anticonvulsivante dos fármacos antiepilépticos em peixe–zebra. Animais tratados com fármacos antiepilépticos antes da indução da crise convulsiva não tiveram aumento na atividade da ADA e levaram mais tempo para atingir o estágio tônico-clônico da crise convulsiva quando comparados aos animais diretamente expostos ao PTZ. Outro estudo do nosso grupo mostrou uma diminuição na atividade da ADA após episódios sucessivos de crises convulsivas (Siebel et al., 2011). Estes resultados mostram que a ADA tem diferentes respostas a crises convulsivas, estando ativada imediatamente após o primeiro episódio e inibida ao final de várias convulsões recorrentes. Além disso, mostramos que a ADA é sensível à modulação por fármacos antiepilépticos. Estes resultados mostram que a atividade de desaminação da adenosina pela ADA pode ter importante participação no desenvolvimento de crises convulsivas.

Devido ao importante efeito da adenosina na atividade neuronal, estudamos o papel dos diferentes mecanismos envolvidos na sinalização adenosinérgica em crises convulsivas em peixe-zebra. Portanto, neste estudo analisamos o efeito de agonistas e antagonistas seletivos e não-seletivos dos receptores de adenosina em parâmetros comportamentais de crises convulsivas induzidas por PTZ. Estudos prévios mostram que a ação anticonvulsivante da adenosina ocorre principalmente através da ativação de receptores de adenosina A_1, que induz a inibição pré-sináptica por meio da redução do influxo de Ca^{+2}, e reduz a excitabilidade da membrana pós-sináptica, aumentando a liberação de K^+ (Fredholm et al., 2005). Diferentemente das características bem esclarecidas dos receptores A_1, estudos já demonstraram diferentes propriedades, anticonvulsivantes e pró-convulsivantes dos receptores A_{2A} (Boison, 2007). Enquanto os receptores A_1 e A_{2A} têm sido frequentemente estudados, os receptores A_{2B} e A_3, de menor afinidade e abundância, não têm sido considerados alvos terapêuticos na epilepsia (Boison, 2005, 2007).

Alguns estudos mostram que a cafeína, um antagonista não-seletivo de receptores de adenosina, pode diminuir o limiar convulsivo em pacientes com epilepsia (Kaufman & Sachdeo 2003). Além disso, a cafeína diminuiu o efeito protetor de fármacos antiepilépticos clássicos em modelo de epilepsia induzida por eletrochoque em camundongos (Chrooeciñska-Krawczyk et al., 2011). Em zebrafish, o tratamento agudo com cafeína induziu crises convulsivas (Wong et al., 2010). Nossos resultados mostraram que o pré-tratamento com cafeína apresenta efeitos pró-convulsivantes em crises convulsivas induzidas por PTZ em peixe-zebra, acelerando o desenvolvimento da crise convulsiva. Animais que receberam cafeína antes da exposição ao PTZ atingiram o estágio tônico-clônico da crise convulsiva mais rapidamente que os animais que não receberam tratamento. Este efeito pró-convulsivante da cafeína ocorreu possivelmente através do bloqueio de receptores A_1. A inibição de receptores A_1 por metilxantinas pode contribuir diretamente para a ictogênese e para a generalização do foco epiléptico (Boison, 2011).

Nossos resultados reforçam o papel da adenosina no controle de crises convulsivas, principalmente através de receptores A_1. Estudos mostram que

agonistas de receptores A_1 promovem o aumento da latência para o início de crises convulsivas, diminuição na incidência de convulsões, menor duração das convulsões e diminuição da mortalidade em diferentes modelos animais de crises convulsivas (Li et al., 2013). No nosso estudo, o agonista de receptores A_1 (CPA) apresentou efeitos anticonvulsivantes, uma vez que aumentou a latência para o início do estágio tônico-clônico das crises convulsivas. Já o antagonista A_1 (DPCPX) diminuiu a latência para o estágio tônico-clônico, mostrando efeito pró-convulsivante. Estes resultados confirmam a ação anticonvulsivante da adenosina através de receptores A_1 em peixe-zebra.

A participação dos receptores A_{2A} nas crises convulsivas não está devidamente esclarecida. Hosseinmardi et al. (2007) demonstrou que o pré-tratamento com antagonista de receptores A_{2A} (ZM 241385) não teve efeito na ocorrência de crises convulsivas em roedores. Porém, o pré-tratamento com o agonista A_{2A} (CGS 21680) aumentou a descarga neuronal durante as convulsões (Hosseinmardi et al., 2007). Estes resultados são contrários a outro estudo, que mostrou o efeito anticonvulsivante do uso de agonista A_{2A} (Huber et al., 2002). Em nosso estudo, o agonista (CGS 21680) e o antagonista (ZM 241385) de receptores A_{2A} não provocaram alterações nos parâmetros comportamentais das crises convulsivas. Portanto, nossos resultados reforçam a ideia de que a modulação de crises convulsivas por adenosina ocorre necessariamente por meio da ativação de receptores A_1.

O aumento nos níveis de adenosina durante crises convulsivas pode ocorrer através da degradação de nucleotídeos pelas ectonucleotidases ou através de sua liberação por transportadores de nucleosídeos (King et al., 2006; Bonan, 2012). Apesar da importante ação neuromoduladora da adenosina, os mecanismos envolvidos no aumento dos níveis deste nucleosídeo em crises convulsivas não estão devidamente identificados. Estudo prévio mostrou que a deleção genética e a inibição farmacológica da ecto-5'-nucleotidase não tiveram efeito na ativação de receptores A_1, sugerindo que a adenosina extracelular não é gerada a partir da hidrólise de ATP e sim liberada pelos neurônios diretamente no espaço extracelular (Klyuch et al., 2012). Porém, outro estudo mostrou a supressão da atividade sináptica através da liberação, por astrócitos, de ATP e sua consequente degradação em

adenosina (Pascual et al., 2005). Considerando o importante papel dos transportadores de nucleosídeos e das ectonucleotidases na sinalização adenosinérgica, nós avaliamos o efeito de moduladores destes mecanismos em parâmetros comportamentais de crises convulsivas induzidas por PTZ. Nossos resultados mostraram que a inibição da produção de adenosina a partir da hidrólise de nucleotídeos apresenta efeito pró-convulsivante. O tratamento com inibidor da ecto-5´-nucleotidase (AMPCP) antes da exposição ao PTZ fez com que os animais atingissem o estágio tônico-clônico da crise convulsiva mais rapidamente do que os animais que não receberam este pré-tratamento. Estes resultados reforçam a teoria de que a hidrólise extracelular de nucleotídeos pelas ectonucleotidases é um mecanismo importante no controle dos níveis de adenosina e, consequentemente, no desenvolvimento de crises convulsivas.

Outro mecanismo importante no controle nos níveis extracelulares de adenosina envolve a ação dos transportadores de nucleosídeos. Nossos resultados mostraram que a inibição de transportadores de nucleosídeos através do pré-tratamento com dipiridamol provocou o aumento na latência para o início do estágio tônico-clônico da crise convulsiva. Nossos resultados sugerem a ação anticonvulsivante da inibição de transportadores de nucleosídeos em peixe–zebra. O inibidor de transportador de nucleosídeos retarda a eliminação da adenosina do espaço extracelular ao bloquear a recaptação da adenosina, provocando um efeito neuroprotetor contra crises convulsivas (George & Kulkarni, 1997).

Em situações de aumento nos níveis de adenosina, como o que ocorre em crises convulsivas, outro mecanismo essencial na sinalização adenosinérgica é o controle dos níveis de adenosina através de sua degradação pela ADA (Latini & Pedata, 2001). Nossos estudos anteriores mostraram que a atividade da ADA é regulada de diferentes maneiras logo após uma única convulsão ou após vários episódios sucessivos de crises convulsivas. Porém, existem poucos estudos mostrando o efeito da modulação farmacológica da ADA em crises convulsivas. Nossos resultados mostraram que a inibição da ADA promove a supressão de crises convulsivas em peixe-zebra. Animais pré-tratados com EHNA demoraram mais tempo para atingir o

estágio tônico-clônico da crise convulsiva quando comparados a animais que não receberam este pré-tratamento. Um estudo anterior havia mostrado um efeito neuroprotetor da inibição da ADA através do tratamento com EHNA em modelo de epilepsia genética e em crises convulsivas induzidas por PTZ (Southam et al., 2002).

De forma conjunta, nossos resultados mostraram a importância das enzimas ectonucleotidases e ADA, além dos transportadores de nucleosídeos, no controle dos níveis de adenosina e, consequentemente, no controle do desenvolvimento de crises convulsivas em peixe-zebra. Além disso, mostramos que o efeito anticonvulsivante da adenosina em peixe-zebra ocorre, necessariamente, através de receptores A_1.

O segundo objetivo geral deste estudo foi esclarecer os diferentes resultados encontrados em análises da via de sinalização TOR, visando futuros estudos envolvendo esta via de sinalização em peixe-zebra. Diversos estudos mostraram o envolvimento da sinalização por mTOR/S6K na resposta celular ao estresse oxidativo. Porém, enquanto alguns estudos mostraram a inibição desta via em situações de insulto oxidativo, outros mostraram sua ativação (Jin et al., 2009; Alexander et al., 2010; Cully et al., 2010; Gutierrez-Uzquita et al., 2012). Nós investigamos a resposta das proteínas S6K1 e S6 ao estresse oxidativo induzido por H_2O_2, em diferentes tipos celulares. Para analisar a ativação de S6K1, utilizamos um anticorpo anti-phospho-Thr389-S6K1, frequentemente utilizado em diferentes estudos da via mTORC1. Nós verificamos que este anticorpo reconhece uma proteína de 80-90 KDa, que é rapidamente fosforilada em resposta ao estresse oxidativo. Porém, este aumento na fosforilação de S6K1 não apresentou alterações quando as células foram pré-tratadas com inibidores da via mTORC1. Este resultado de ativação também permaneceu inalterado após *knockdown* da proteína S6K1, mostrando que a proteína que estava sendo reconhecida pelo anticorpo não era a S6K1. Além disso, a fosforilação da proteína S6 em S235/S236 não foi diminuída quando foram utilizados inibidores de mTORC1 ou quando foi feito o *knockdown* da proteína S6K1. Devido à grande homologia entre as proteínas S6K1, RSK e MSK, o anticorpo poderia estar reconhecendo a fosforilação de proteínas RSK e MSK. Nós demonstramos que o estresse oxidativo induzia a

fosforilação de RSK e MSK em resíduos homólogos ao Thr389 de S6K1. Nossos resultados também mostraram que esta fosforilação era dependente da atividade das proteínas MAPK, p38 e ERK. Experimentos com fibroblastos *knockout* para p38 confirmaram esta hipótese. Portanto, nossos resultados mostraram que a via de sinalização S6K1 não é ativada em condições de estresse oxidativo, conforme alguns estudos prévios haviam sugerido. Estes resultados contraditórios podem ser atribuídos a interpretação de resultados obtidos a partir da detecção inespecífica de proteínas RSK e MSK pelo anticorpo phospho-Thr389-S6K1 e por resultados mostrando a fosforilação de S6, que responde a via de sinalização MAPK, além da via mTORC1. Além disso, nossos resultados evidenciaram que o estresse oxidativo induzido por H_2O_2 provoca, especificamente, a ativação da via MAPK, através das proteínas ERK, p38, RSK e MSK.

De forma conjunta, nossos resultados podem contribuir para um maior entendimento das vias de sinalização envolvidas nos mecanismos de controle de crises convulsivas e representar uma alternativa para o desenvolvimento de fármacos antiepilépticos, aumentando as opções terapêuticas em epilepsia. Nossos resultados também podem contribuir para futuros estudos referentes à caracterização e modulação da via de sinalização TOR em peixe-zebra.

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