UNIVERSIDADE FEDERAL DE SANTA MARIA
CENTRO DE CIÊNCIAS NATURAIS E EXATAS
PROGRAMA DE PÓS-GRADUAÇÃO EM CIÊNCIAS BIOLÓGICAS: BIOQUÍMICA TOXICOLÓGICA

AVALIAÇÃO DO SISTEMA PURINÉRGICO E DA ENZIMA ACETILCOLINESTERASE EM CÓRTEX CEREBRAL DE RATOS APÓS HIPÓXIA-ISQUEMIA NEONATAL

TESE DE DOUTORADO

Victor Camera Pimentel

Santa Maria, RS, Brasil
2013

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Victor Camera Pimentel

Tese apresentada ao Curso de Doutorado do Programa de Pós-Graduação em Ciências Biológicas: Bioquímica Toxicológica, da Universidade Federal de Santa Maria (UFSM, RS), como requisito parcial para a obtenção do grau de Doutor em Bioquímica Toxicológica

Orientadora: Maria Rosa Chitolina Schetinger

Santa Maria, RS, Brasil
2013

Ficha catalográfica elaborada através do Programa de Geração Automática
da Biblioteca Central da UFSM, com os dados fornecidos pelo(a) autor(a).

Camera Pimentel, Victor
AVALIAÇÃO DO SISTEMA PURINÉRGICO E DA ENZIMA ACETILCOLINESTERASE EM CÓRTEX CEREBRAL DE RATOS APÓS HIPÓXIA-ISQUEMIA NEONATAL / Victor Camera Pimentel.-2013.
128 p.; 30cm

Orientadora: Maria Rosa Chitolina Schetinger
Tese (doutorado) - Universidade Federal de Santa Maria, Centro de Ciências Naturais e Exatas, Programa de Pós-Graduação em Ciências Biológicas: Bioquímica Toxicológica, RS, 2013

1. Hipóxia-isquemia 2. Sistema Purinérgico 3. Acetilcolinesterase 4. Córtex cerebral 5. Citocinas I. Chitolina Schetinger, Maria Rosa II. Título.

Universidade Federal de Santa Maria
Centro de Ciências Naturais e Exatas
Programa de Pós-Graduação em Ciências Biológicas: Bioquímica Toxicológica

A Comissão Examinadora, abaixo assinada, aprova a

Tese de Doutorado

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elaborada por
Victor Camera Pimentel

como requisito parcial para a obtenção do grau de

Doutor em Bioquímica Toxicológica

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Profª. Drª. Paula Acosta Maldonado (FISMA)

Santa Maria, 15 de Março de 2013

Realmente ainda me faltam muitas conquistas, muitos objetivos a alcançar, muitos sonhos a se realizarem, porém não quero me prender ao que ainda não tenho, quero agradecer à Deus pelo que eu hoje já tenho, e o maior dos presentes são as pessoas que fazem parte da minha vida, minha família e amigos! Sou feliz por ter vocês!

(*Álvaro Carvalho*)

Aos meus queridos pais Moacir e Marina

Obrigado
Por terem me entendido enquanto eu crescia
e por terem aceitado minhas tão rápidas mudanças.

Por terem me ouvido e terem me dado claras e breves respostas
às dúvidas e perguntas que eu levava a vocês.

Por terem me aplaudido quando fui verdadeiro,
compreendido quando eu disse mentiras e
me provado que elas maculam nosso caráter.

Por terem me falado sobre os seus erros e sobre
as coisas que vocês aprenderam com eles.
Isso fez com que eu aceitasse meus próprios erros,
que também aprendesse e que me perdoasse.

Por prestarem atenção e gastarem
grande parte do seu tempo comigo.
Isso me levou a acreditar que sou importante
e que tenho muito valor.

Por terem considerado meus sentimentos e necessidades,
e terem me mostrado muitas vezes
que elas eram semelhantes às suas.

Pelos elogios e pelos incentivos.
Foi sempre por isso que eu me senti bom
e quis continuar sendo digno da sua fé em mim.

Por ajudarem a explorar os meus talentos e potenciais.
Por terem me ensinado que para ser feliz
eu tinha que ser eu mesmo e não como vocês
ou igual a outros que vocês admiravam.

Por me ensinarem a não desistir da felicidade.
Com isso eu aprendi a buscar uma vida feliz e bem sucedida.
Muito Obrigado

Agradecimento especial

Meu agradecimento especial a Prof. Dr (a) Maria Rosa Chitolina Schetinger, pelo exemplo de dedicação ao trabalho e orientação simples e clara.

AGRADECIMENTOS

Em primeiro lugar, agradeço a Deus pelo dom da vida.
À minha família; Moacir, Marina, Natália e Bruno, pelo amor, apoio e carinho em todos os momentos, esta conquista é pra vocês.
A minha namorada Jéssica, pelo carinho, ajuda e companheirismo. Não tenho palavras para dizer o quanto você é importante pra mim. Obrigado.
À professora Maria Rosa Chitolina Schetinger, minha orientadora, por todos os ensinamentos, atenção e dedicação; a minha gratidão.
À professora Ana Maria Sebastião e aos amigos que fiz em Lisboa pelos ensinamentos que me foram passados durante o período que estive em Portugal.
Aos amigos e colegas do Laboratório 2208 pelos momentos compartilhados e pela amizade durante o período de convívio.
Aos alunos de iniciação científica, pela dedicação e companheirismo durante este trabalho.
Aos amigos que passaram pelo laboratório
Aos meus vizinhos de laboratório pelo convívio e pela aptidão em ajudar nas mais diversas situações.
Aos professores da universidade que de alguma maneira contribuíram para a construção do meu conhecimento científico.
A UFSM e ao Programa de Pós-Graduação em Ciências Farmacêuticas pela oportunidade de realizar este curso.
Aos amigos e todas as pessoas que, de uma forma ou de outra, contribuíram para a realização deste trabalho, gostaria de expressar minha profunda gratidão.
Enfim, agradeço a todas as pessoas que a mim dedicaram seu tempo e esforço, pelas críticas que me ajudaram e certamente ainda ajudarão a crescer. Se fosse citá-las, correria o risco de omitir algumas, e por isso estendo estes agradecimentos no presente parágrafo.
Então, a todos que fazem parte de minha vida o meu muito obrigado.

RESUMO

Programa de Pós-Graduação em Ciências Biológicas: Bioquímica Toxicológica
Tese de Doutorado
Universidade Federal de Santa Maria, RS, Brasil

AVALIAÇÃO DO SISTEMA PURINÉRGICO E COLINÉRGICO EM CÓRTEX DE RATOS APÓS HIPÓXIA-ISQUEMIA NEONATAL

Autor: VICTOR CAMERA PIMENTEL
Orientadora: MARIA ROSA CHITOLINA SCHETINGER
Data e local de Defesa: Santa Maria, 15 de março de 2013.

A hipóxia-isquemia (HI) neonatal é uma das principais causas de morbidade e mortalidade durante o período perinatal, constituindo um fator de risco importante para o desenvolvimento de uma série de desordens neurológicas em humanos tais como, a paralisia cerebral, a epilepsia e déficits motores e de aprendizagem. A HI cerebral resulta em alterações hemodinâmicas, bioquímicas e neurofisiológicas como uma consequência direta da falta de oxigênio e glicose. O sistema nervoso central por possuir um consumo relativamente alto de oxigênio e glicose, dependendo quase que exclusivamente do processo de fosforilação oxidativa para a produção de energia, torna-se altamente suscetível ao insulto hipóxico-isquêmico. Durante o evento HI, a rápida supressão no processo de fosforilação oxidativa inicia uma série de eventos tóxicos que ocorrem simultaneamente e estão diretamente relacionados com a evolução da lesão cerebral. Assim, sabendo que a patogênese da HI neonatal é um evento multifatorial e altamente complexo, este trabalho teve como objetivo principal investigar, em diferentes tempos pós-insulto (imediatamente, 72 horas e 8 dias após a HI neonatal), as possíveis alterações nos sistemas purinérgico e colinérgico em córtex cerebral de ratos neonatos submetidos à HI. Além disso, foram realizadas análises para avaliar os níveis de peroxidação lipídica e marcadores periféricos de inflamação tais como o fator de necrose tumoral alfa (TNF-α), o interferon gama (IFN-γ) e as interleucinas 1β e 6 (IL-1β e IL-6, respectivamente). Os resultados demonstram que imediatamente após a HI a atividade da nucleotídeo trifosfato difosfoidrolase (NTPDase) e da 5`-nucleotidase (5`-NT) citosólicas aumentaram no córtex cerebral. Em sinaptossomas houve um aumento na atividade da ecto-adenosina desaminase (ecto-ADA), enquanto a atividade da Na^+/K^+ ATPase mostrou-se reduzida. Não foi observada nenhuma alteração na expressão da adenosina quinase (ADK). Interessantemente, a atividade da Na^+/K^+ ATPase correlacionou-se negativamente com a atividade da NTPDase citosólica e com os níveis de peroxidação lipídica. Nossos resultados demonstraram um aumento nos níveis de peroxidação lipídica imediatamente após o insulto, os quais foram mantidos 72 horas e 8 dias após a HI. A atividade da acetilcolinesterase (AChE) mostrou alterações tempo-dependente no córtex cerebral destes animais. O mesmo foi observado para a atividade da AChE e da ADA em eritrócitos. Quando os níveis das citocinas pró-inflamatórias (TNF-α; IFN-γ; IL-1β e IL-6) que foram investigadas, todas apresentaram seus níveis séricos aumentados. Imediatamente após a HI, a atividade da ADA apresentou uma forte correlação positivas com todas as citocinas analisadas. 8 dias após a HI observou-se um processo inflamatório com aumento da atividade da ADA, mieloperoxidase e N-acetil-β-D-glucosaminidase. Neste mesmo período, podemos evidenciar que a ADA1 é a isoenzima responsável pelo aumento da ativada da ADA neste momento pós-insulto. Interessantemente, observamos uma redução na expressão dos receptores de adenosina A1 (A1Rs) sem alteração na expressão da adenosina quinase (ADK). Assim, os resultados descritos aqui sugerem a HI neonatal altera a sinalização purinérgica e a atividade da acetilcolinesterase em córtex cerebral de ratos neonatos. Além disso, possibilitará uma melhor compreensão dos eventos que se inciam com o evento HI, e consequentemente auxiliaram na busca e desenvolvimento de novas terapias para a lesão cerebral hipóxico-isquêmica.

Palavras-chave: hipóxia-isquemia neonatal; inflamação; sistema purinérgico; córtex cerebral; acetilcolinesterase; citocinas.

ABSTRACT
Thesis of Doctor's Degree
Post-Graduate Program in Biological Sciences: Toxicological Biochemistry
Federal University of Santa Maria, RS, Brazil

EVALUATION OF PURINERGIC AND CHOLINERGIC SYSTEM IN THE CEREBRAL CORTEX OF RATS AFTER NEONATAL HYPOXIA-ISCHEMIA

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Date and place of the defense: Santa Maria, march, 15th, 2013.

Hypoxia-ischemia (HI) neonatal is a major cause of morbidity and mortality during the perinatal period and it is an important risk factor for the development of a number of human neurological disorders, such as cerebral palsy, epilepsy, and motor and learning deficits. Cerebral HI results in hemodynamic, biochemical and neurophysiological alterations as a direct consequence of the lack of oxygen and glucose. The central nervous system presents a relatively high consumption of oxygen and glucose which relies almost exclusively on the oxidative phosphorylation process for the production of energy thus it is highly susceptible to hypoxic-ischemic insult. During the HI event, the rapid suppression in the process of oxidative phosphorylation initiates a series of poisonings that occur simultaneously and are directly related to the evolution of brain injury. Thus, knowing that the pathogenesis of neonatal HI is a highly complex and multifactorial event, this study aimed to investigate possible changes in the purinergic and cholinergic systems in the cerebral cortex of newborn rats subjected to HI, at different post-insult (immediately, 72 h and 8 days after neonatal HI),. Furthermore, analyzes were performed to assess the levels of lipid peroxidation and some peripheral markers of inflammation such as tumor necrosis factor alpha – TNF-α; Interferon-gamma - IFN-γ; interleukins IL-1β and IL-6. Results demonstrated that immediately after HI, the activity of nucleotide triphosphate diphosphohydrolase (NTPDase) and 5 '-nucleotidase (5`-NT) cytosolic increased in the cerebral cortex. In synaptosomes, an increase in the activity of ecto-adenosine deaminase (ecto-ADA) was observed, while the activity of Na^+/K^+ ATPase was inhibited. There was no change in the expression of adenosine kinase (ADK). Interestingly, the Na^+/K^+ ATPase activity was correlated negatively with the cytosolic NTPDase activity and TBARS content. Our results showed an increase in lipid peroxidation levels immediately, 72 h and 8 days after HI. The activity of acetylcholinesterase (AChE) showed time-dependent changes in the cerebral cortex of these animals. The same was observed for AChE activity in erythrocytes and ADA. Regarding the levels of proinflammatory cytokines (TNF-α, IFN-γ, IL-1β and IL-6) investigated, all showed increased serum levels. Immediately after HI, the ADA activity showed a strong positive correlation with all cytokines analyzed. Eight days after HI there was an inflammatory process with increased activity of ADA, myeloperoxidase (MPO) and N-acetyl-glucosaminidase (NAG). In this same period, we observed that ADA1 isoenzyme was responsible for the increase in the ADA activity after HI insult. Interestingly, adenosine receptors A1 (A1Rs) and ADK protein expression showed a decrease 8 days after insult. Thus, the results described here suggest that neonatal HI alters the cholinergic and purinergic signaling in the cortex of newborn rats. However, the understanding of these events may help in the development of new therapies for hypoxic-ischemic brain injury.

Keywords: neonatal hypoxia-ischemia; inflammation; purinergic system; cerebral cortex; acetylcholinesterase; cytokines.

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**5`-NT** – 5`-nucleotidase
A1R – Receptor de adenosina A1
A2AR – Receptor de adenosina A2A
ACh – Acetilcolina
AChE – Acetilcolinesterase
ADA – Adenosina desaminase
Ado – Adenosina
ADK – Adenosina quinase
ADP – Adenosina difosfato
AMP – Adenosina monofosfato
AMPc – Adenosina monofosfato cíclico
ATP – Adenosina trifosfato
ChAT – Colina-acetiltransferase
ChT – Transportador de colina
CNT – Transportador de nucleosídeo concentrativo
d-Ado – 2`-desoxiadenosina
DPPIV – Dipeptidil peptidase IV
ENT – Transportador de nucleosídeo equilibrativo
EtN – Etanolamina
GABA – Ácido gama-aminobutírico
GPI – Glicosilfosfatidilinositol
HI – Hipóxia-isquemia
IFN-γ – Interferon gama
IL-1β – Interleucina 1β
IL-6 – Interleucina 6
NTPDase – Nucleotídeo trifosfato difosfoidrolase
mAChR – Receptores de acetilcolina muscarínicos
MPO – Mieloperoxidase
nAChR – Receptores de acetilcolina nicotínicos
NAG – N-acetil-glucosaminidase
NMDA – N-metil D-aspartato
SAH – S-adenosil homocisteína
SNC – Sistema nervoso central
TBARS – Substâncias reativas ao ácido tiobarbitúrico
TNF-α – Fator de necrose tumoral alfa
UTP – Uridina trifosfato
VAChT – Transportador de acetilcolina vesicular
PDE – Fosfodiesterase

SUMÁRIO

APRESENTAÇÃO

Este estudo consiste na tese de doutorado intitulada “Avaliação do sistema purinérgico e colinérgico em córtex de ratos após a hipóxia-isquemia neonatal”.

Os resultados dessa tese estão apresentados sob a forma de um artigo e dois manuscritos, os quais se encontram nos respectivos itens. As seções Materiais e Métodos, Resultados, Discussões e Referências Bibliográficas encontram-se nos próprios artigos e representam a íntegra deste estudo. Os itens Discussão e Conclusão, encontrados no final desta tese, apresentam interpretações e comentários gerais a respeito dos resultados demonstrados nos artigos contidos neste trabalho. As Referências Bibliográficas referem-se somente as citações que aparecem no item Introdução e Discussão desta tese.

1. INTRODUÇÃO

A taxa de mortalidade infantil é considerada um dos mais importantes indicadores epidemiológicos utilizados internacionalmente, pois está diretamente relacionada com os níveis de desenvolvimento socioeconômico e de saúde de um país (FILHO et al., 2007). Este indicador, além de informar sobre os níveis de saúde de uma população, sintetiza as condições de bem-estar social, político e ético de uma dada conformação social, pois ele representa a probabilidade de sobrevivência infantil no primeiro ano de vida e reflete não só as condições concretas de moradia, salário, alimentação, atenção à saúde, mas também, e talvez principalmente, o compromisso de uma dada sociedade com a sua reprodução social. Ou seja, avalia o quanto à sociedade protege a sua renovação geracional. Atualmente, podemos destacar entre as principais causas de mortalidade infantil: a prematuridade, as infecções, a asfixia perinatal e as malformações congênitas (Organização Mundial de Saúde – OMS, 2006).

Neste contexto, a asfixia perinatal ganha destaque, pois apesar do número limitante de dados disponíveis na literatura, estima-se que esta condição seja responsável pela morte de aproximadamente 920.000 neonatos a cada ano, além de estar diretamente associada com 1.1 milhões de natimortos intraparto. Entre as manifestações clínicas da asfixia perinatal, a encefalopatia hipóxico-isquêmica, considerada a mais grave dentre as manifestações clínicas da asfixia no período perinatal, é responsável por aproximadamente 23% de todas as mortes que ocorrem no período neonatal (entre 0 e 27 dias de vida), sendo que, 99% destas ocorrem nos países de baixo e médio rendimento, motivo da falta de informação e de recursos especializados para a tomada de decisões imediatas. Em relação aos neonatos que sobrevivem ao insulto, as estatísticas apontam que mais de 1 milhão destes irão desenvolver problemas neuropsicomotores em alguma etapa do seu desenvolvimento (BRYCE et al., 2005; LAWN et al., 2005; CHAO et al., 2006; OMS, 2006).

Quanto à etiologia, a asfixia pode ocorrer antes, durante ou após o parto; a maioria dos casos ocorre no período intra (35% dos casos) e anteparto (20% dos casos, por causas maternas e fetais), e ainda no período pós-parto em 10% dos

casos (são secundários a doenças pulmonares, cardiovasculares ou neurológicas) (CLOHERTY & STARK, 2000; PROCIANOY & SILVEIRA, 2001).

Durante o trabalho de parto normal as contrações uterinas produzem uma diminuição do fluxo sanguíneo para a placenta num momento em que o consumo de oxigênio da mãe e do feto está incrementado. A desidratação e a alcalose respiratória por hiperventilação, frequentes neste período, também podem propiciar a diminuição do fluxo sanguíneo placentário. A convergência destes fatores considerados normais leva a uma queda da saturação fetal de oxigênio, porém o feto saudável resiste bem a este estresse. No entanto, alterações crônicas no fluxo sanguíneo durante este período crítico podem levar à asfixia. Assim, a asfixia é definida como o agravo ao feto ou ao recém-nascido, ocasionado por uma falta de oxigênio (hipoxemia) e/ou uma falta de perfusão (isquemia) de vários órgãos, de magnitude suficiente para produzir alterações bioquímicas e/ou funcionais (VOLPE, 2000).

Como citado anteriormente, a encefalopatia hipóxico-isquêmica, considerada a mais grave dentre as manifestações clínicas da asfixia no período perinatal, ocorre quando a asfixia compromete a perfusão tecidual com significativa diminuição da oferta de oxigênio, mudando o metabolismo celular de aeróbico para anaeróbico, com consequente disfunção múltipla de órgãos e graves lesões cerebrais manifestadas por convulsões e outros sinais neurológicos (GILSTRAP III et al., 1989; ADAMS-CHAPMAN, STOLL, 2007). No sistema nervoso central (SNC), os distúrbios neurológicos decorrentes da asfixia estão estritamente relacionados com o período de desenvolvimento do feto ou recém-nascido, com a intensidade do insulto e a área afetada. Neste contexto, a interrupção momentânea ou permanente do fluxo sanguíneo e aporte nutricional ao encéfalo irão caracterizar a lesão cerebral hipóxico-isquêmica.

A lesão cerebral hipóxico-isquêmica neonatal representa uma das mais graves e limitantes complicações que afetam o recém-nascido (WANG et al., 2002). É responsável por causar lesões graves com comprometimento crônico das capacidades neurológicas como, paralisia cerebral, epilepsia, retardo mental e dificuldades de aprendizagem podendo levar o indivíduo a morte. A lesão cerebral decorrente da hipóxia-isquemia (HI) neonatal se processa já no início do insulto estendendo-se até o período de recuperação, que se dá após a ressuscitação,

também chamado de intervalo de reperfusão. A consequência imediata deste processo é uma série de eventos que culminam em morte celular. Como já citado anteriormente, é importante ressaltar aqui, que as lesões causadas pela hipóxia-isquemia neonatal não possuem um padrão único de evolução, estas irão se apresentar em diferentes graus de intensidade dependendo do período de desenvolvimento do recém-nascido e da intensidade do insulto (VANNUCCI, 1990, RUFO-CAMPOS et al., 2000, MACAIA, 2000). Com base nestas observações, nas últimas décadas os estudos sobre a patofisiologia da HI neonatal vêm despertando cada vez mais o interesse do meio científico e social. No entanto, apesar dos avanços na medicina neonatal, ainda não há medidas totalmente eficazes para a prevenção e/ou tratamento das sequelas decorrentes da mesma.

Tendo em vista as consequências da asfixia perinatal e a falta de um padrão único referente à evolução da lesão cerebral relacionada à mesma, nas últimas décadas modelos têm sido utilizados a fim de contribuir para o melhor entendimento da patofisiologia da HI neonatal. Um dos modelos que tem sido amplamente utilizado com a finalidade de reproduzir a lesão cerebral hipóxico-isquêmica observada em humanos é o modelo descrito por Levine (1960), que posteriormente foi modificado por Rice et al (1981) (modelo utilizado nesta tese). Este modelo consiste na oclusão unilateral da artéria carótida comum seguido por um período de hipóxia sistêmica produzido pela inalação de 8% de oxigênio balanceado com nitrogênio (Ilustrado na Figura 1). Durante o curso da exposição ao ambiente hipóxico, os animais demonstram hipoxemia combinada com hipocapnia, produzida pela hiperventilação; a pressão arterial sistêmica diminui de 25-30% durante a hipóxia, assim o fluxo sanguíneo cerebral no hemisfério ipsilateral a oclusão da carótida é reduzido a 40-60% em relação ao controle (VANNUCCI et al., 1988).

Este modelo mostra-se vantajoso, pois apresenta alta reprodutibilidade (WALTON et al., 1999), baixo custo, baixa mortalidade e permite a recuperação do fluxo sanguíneo apesar da oclusão da carótida. O dano da HI experimental referente a este modelo ocorre apenas no hemisfério ipsilateral à oclusão da carótida, produzindo lesões nas regiões do córtex cerebral, substância branca periventricular e subcortical, estriado e hipocampo (ARTENI et al., 2003; HOSSAIN, 2005; PEREIRA et al., 2007).

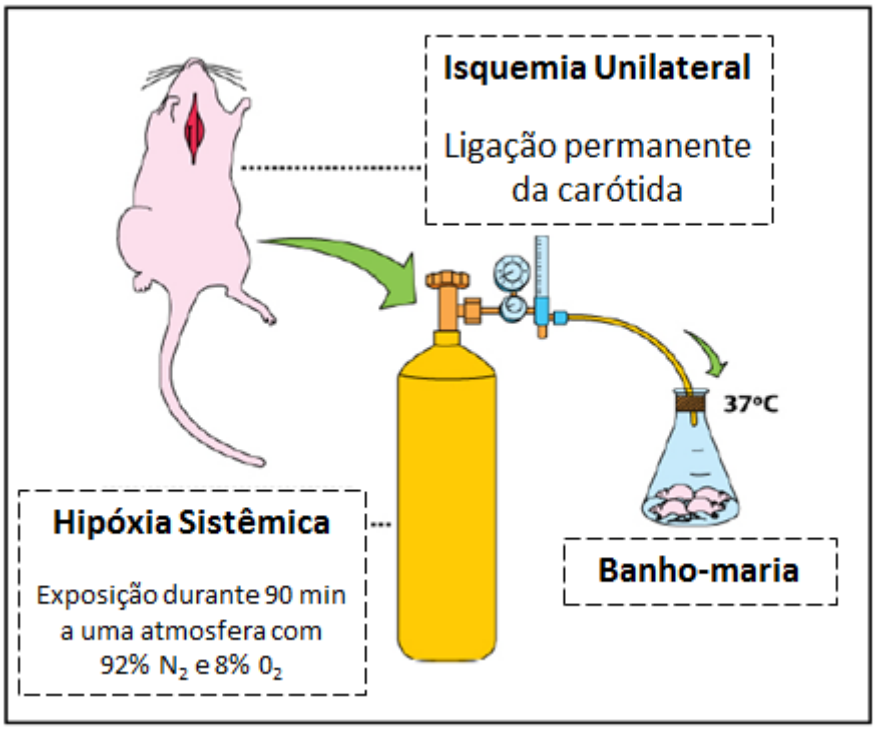

Figura 1. Desenho esquemático do procedimento experimental utilizado para reproduzir a HI neonatal em animais.

Assim, utilizando este modelo, o nosso e outros grupos de pesquisa buscam estudar os efeitos da HI neonatal sobre diferentes aspectos, a fim de buscar uma melhor compreensão dos mecanismos envolvidos na patofisiologia deste evento e as consequências patológicas subjacentes aos diferentes distúrbios neurológicos decorrentes da mesma.

Em situações fisiológicas, o tecido encefálico possui uma regulação do fluxo sanguíneo independente das variações de perfusão, graças a mecanismos de auto-regulação da circulação cerebral. Em uma situação de isquemia, estes mecanismos tornam-se falhos na zona afetada e a pressão de perfusão passa a ser dependente da tensão arterial (CASTILLO, 2000). Assim, o tecido encefálico por ser altamente dependente do fluxo sanguíneo contínuo para o suprimento de glicose e oxigênio, uma vez que seu metabolismo possui uma alta demanda energética, torna-se completamente vulnerável frente ao insulto hipóxico-isquêmico (MISHRA & DELIVORIA-PAPADOPOULOS, 1999). Destaca-se aqui, a suscetibilidade do SNC

de fetos e neonatos frente à injúria hipóxico-isquêmica, tendo em vista que neste período o SNC é imaturo e está em constante desenvolvimento, além de necessitar de um grande aporte de energia para a manutenção do seu metabolismo (YAGER et al., 2004; MCLEAN & FERRIERO, 2004). Portanto, o período perinatal, representa um período crítico, pois a janela de desenvolvimento do SNC ocorre somente uma vez não podendo ser revista ou repetida. As perturbações neste período do desenvolvimento provavelmente irão incidir negativamente sobre o desenvolvimento neuropsicomotor da criança, aumentando a probabilidade de alterações na aquisição de habilidades motoras, cognitivas e psicossociais.

Como já descrito, muitos fatores, incluindo a duração ou a gravidade do insulto, influenciam na progressão da lesão cerebral após a asfixia perinatal (PERLMAN, 2006). Desta maneira, diferentes mecanismos têm sido relacionados com a HI neonatal: menor perfusão sistêmica devido à hipovolemia, diminuição do aporte sanguíneo do parênquima cerebral com consequente déficit energético e peroxidação de macromoléculas biológicas devido à geração de radicais livres durante a reperfusão tecidual (processo que ocorre durante o período de ressuscitação do recém-nascido) (MISHRA & DELIVORIA-PAPADOPOULOS, 1999; MCLEAN & FERRIERO, 2004; HOSSAIN, 2005; PIMENTEL et al., 2011). Aqui, destacamos a importância do processo de reperfusão frente à evolução da lesão cerebral, pois durante o processo de reoxigenação do parênquima cerebral inicia-se uma série complexa de processos bioquímicos interligados entre si (LEVENE & EVANS, 2005).

Como já mencionado, o encéfalo é altamente dependente do consumo de oxigênio e glicose, dependendo quase que exclusivamente da fosforilação oxidativa (principal via para a síntese de ATP) para a produção de energia. Neste contexto, sabe-se que a patogênese da injúria hipóxico-isquêmica envolve uma série de eventos moleculares que se iniciam com a redução no aporte de oxigênio ao tecido encefálico e consequentemente supressão do processo de fosforilação oxidativa acarretando em falência energética (MAHURA, 2003; LAI & YANG, 2010). Este evento envolve mecanismos altamente complexos os quais se inter-relacionam com uma série de eventos tóxicos (liberação de glutamato, ativação de receptores glutamatérgicos, ativação da óxido nítrico sintase, influxo de cálcio entre outros) que ocorrem simultaneamente e contribuem para a disfunção e morte celular (MCLEAN

& FERRIERO, 2004; HU 2006; BUONOCORE & GROENENDAAL, 2007). Neste contexto, a HI neonatal, por causar alteração entre a demanda energética (depleção energética) e a ressíntese de ATP, implica em desequilíbrio na sinalização purinérgica (LATINI & PEDATA, 2001).

O sistema purinérgico é considerado um sistema primitivo, o qual é caracterizado por ser uma via de sinalização importante em diversos tecidos. Está envolvido em múltiplos efeitos celulares incluindo mecanismos neuronais e não neuronais, e em eventos de curta e longa duração, dos quais podemos citar a resposta imune, inflamação, dor, agregação plaquetária, vasodilatação mediada pelo endotélio, proliferação e morte celular (BURNSTOCK, 2013). No SNC a sinalização purinérgica tem sido descrita principalmente em eventos como neurotransmissão, neuromodulação, secreção, quimioatração e inflamação (ABBRACCHIO et al., 1998). Assim, devido ao envolvimento do sistema purinérgico nesta série de processos biológicos, atualmente este tem se tornado um importante alvo de estudos no meio científico.

O sistema purinérgico envolve três componentes principais: os nucleotídeos e nucleosídeos de adenina, os receptores através dos quais os nucleotídeos e nucleosídeos exercem seus efeitos e as enzimas responsáveis pela modulação dos níveis destas moléculas (YEGUTKIN, 2008).

Os nucleosídeos são moléculas resultantes da união de uma base púrica ou pirimídica à uma pentose. Exemplos destas moléculas incluem a citidina, a uridina, a guanosina, a timina, a inosina e a adenosina. Quando estes nucleosídeos são fosforilados por quinases específicas formam moléculas denominadas de nucleotídeos (ATKINSON et al., 2006). Extracelularmente os nucleotídeos e os nucleosídeos purínicos (como ATP, ADP, AMP e adenosina) atuam como importantes moléculas sinalizadoras que induzem uma multiplicidade de efeitos em diversos sistemas biológicos através de receptores purinérgicos localizados na superfície celular (RALEVIC & BURSTOCK, 1998; ILLES & RIBEIRO, 2004; YEGUTKIN, 2008). No SNC, os nucleotídeos e nucleosídeos purínicos podem ser liberados por células neuronais como gliais, onde desempenharam funções na neurotransmissão, na modulação de neurotransmissores e no desenvolvimento neuronal.

A liberação de nucleotídeos endógenos para o meio extracelular pode ocorrer por diversos mecanismos e representa um componente crítico para o início da cascata de sinalização (Figura 2) (YEGUTKIN, 2008). O extravasamento massivo de nucleotídeos por lise celular é um mecanismo não específico que está restrito à injúria de órgãos, estresse mecânico e algumas condições inflamatórias (BOURS et al., 2006; VOLONTÉ & D'AMBROSI, 2009). Além deste, mecanismos não-líticos de efluxo de nucleotídeos e nucleosídeos representam uma rota distinta e importante para o aparecimento destas moléculas no meio extracelular. No entanto, para que ocorra a modulação da sinalização mediada por estes nucleotídeos e nucleosídeos purínicos é necessário que haja mecanismos efetivos para a inativação destas moléculas no meio extracelular (ZIMMERMANN, 1996).

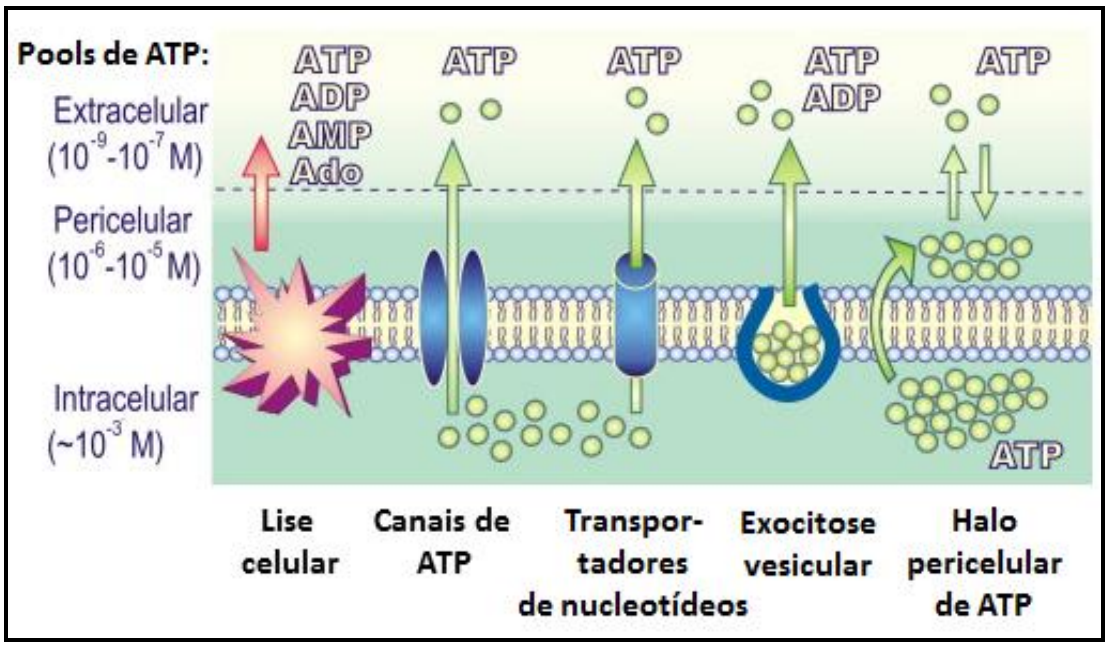

Figura 2. Vias de liberação das purinas para o espaço extracelular (Adaptado de Yegutkin, 2008).

O controle dos níveis extracelulares dos nucleotídeos e nucleosídeos de adenina e a sinalização purinérgica por eles induzida através dos receptores é realizada por uma série de enzimas ancoradas na membrana celular (ecto-enzimas) ou localizadas no meio intersticial (forma solúvel) (ZIMMERMANN et al., 2012). Dentre estas enzimas podem-se destacar as nucleotídeo trifosfato difosfoidrolases (NTPDases, EC 3.6.1.5), a 5`-nucleotidase (5`-NT, EC. 3.1.3.5) e a adenosina desaminase (ADA, EC 3.5.4.4) (YEGUTKIN, 2008). Estas enzimas atuam em

conjunto, formando uma cadeia enzimática que tem início com a ação da NTPDase, a qual catalisa a hidrólise de ATP e do ADP formando AMP (ZIMMERMANN et al., 2012), seguida da enzima 5'-NT que hidrolisa a molécula de AMP gerando a adenosina, a qual posteriormente é desaminada à inosina pela ação da ADA (YEGUTKIN, 2008). Assim, estas enzimas constituem uma cascata enzimática altamente organizada responsável por modular a sinalização mediada pelos nucleotídeos de adenina (ATP, ADP e AMP), controlando a taxa, a quantidade e o tempo de hidrólise destes nucleotídeos, desde a degradação do ATP até a formação do nucleosídeo adenosina (BIANCHI & SPYCHALA, 2003; WINK et al., 2006; IPATA, 2011). No meio extracelular, a "via das ectonucleotidases" e a ecto-ADA constituem um eficiente mecanismo de controle dos níveis de nucleotídeos e nucleosídeos purínicos, estando envolvidas assim, diretamente com a manutenção da sinalização purinérgica (Figura 3) (ZIMMERMANN, 1996; ZIMMERMANN, 2001; BONAN, 2012).

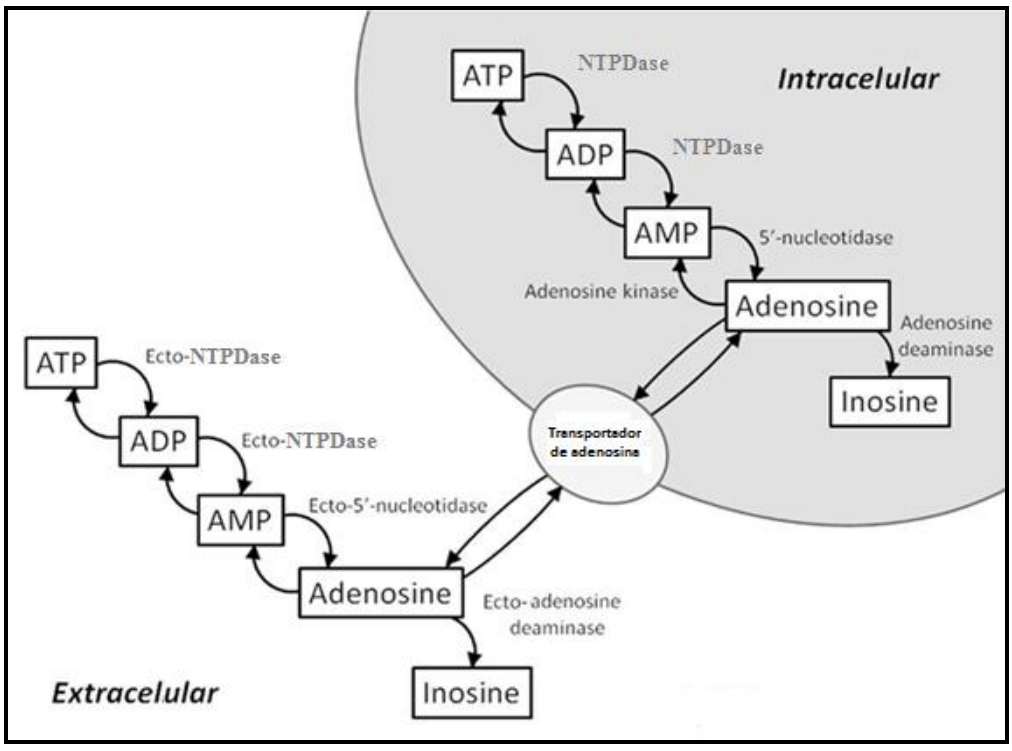

Figura 3. Metabolismo do ATP intra e extracelular. Intracelularmente, a adenosina é formada a partir do AMP pela ação da 5`-nucleotidase citosólica. A adenosina quinase e a adenosina desaminase convertem a adenosina em AMP e inosina, respectivamente. No meio extracelular, ATP, ADP e o AMP são convertidos à adenosina pelas ecto-nucleotidases. Adaptado de CHIKAHISA & SÉI, 2011.

As NTPdases, família de enzimas responsáveis pela hidrólise de nucleotídeos tri e difosfatados, são expressas em uma ampla variedade de sistemas incluindo, o vascular, o imune, o renal e o nervoso central e periférico (ZIMMERMANN et al., 2012). Atualmente, baseado em sua estrutura e propriedades catalíticas, particularmente na relação de hidrólise ATP/ADP, a família das NTPDases em mamíferos está constituída de oito membros clonados e funcionalmente caracterizados: NTPDase 1, NTPDase 2, NTPDase 3, NTPDase 4, NTPDase 5 e NTPDase 6, NTPDase 7 e NTPDase 8 (Figura 4) (ZIMMERMANN, 2001; GRINTHAL, 2004; KUKULSKI et al., 2005). Dos oito membros descritos até o momento, quatro (NTPDases 1, 2, 3 e 8) estão localizados na membrana celular com o sitio ativo voltado para o meio extracelular. As NTPDases 5 e 6 se localizam intracelularmente porém são secretadas após expressão heteróloga. As NTPDases 4 e 7 apresentam localização intracelular com o sítio ativo voltado para o lúmen de organelas citoplasmáticas (VORHOFF et al., 2005; ROBSON et al., 2006).

No SNC, o principal papel fisiológico proposto para as NTPDases é a participação em uma cadeia enzimática junto com uma 5`-NT levando à hidrólise completa do ATP até adenosina na fenda sináptica (SCHADECK et al., 1989).

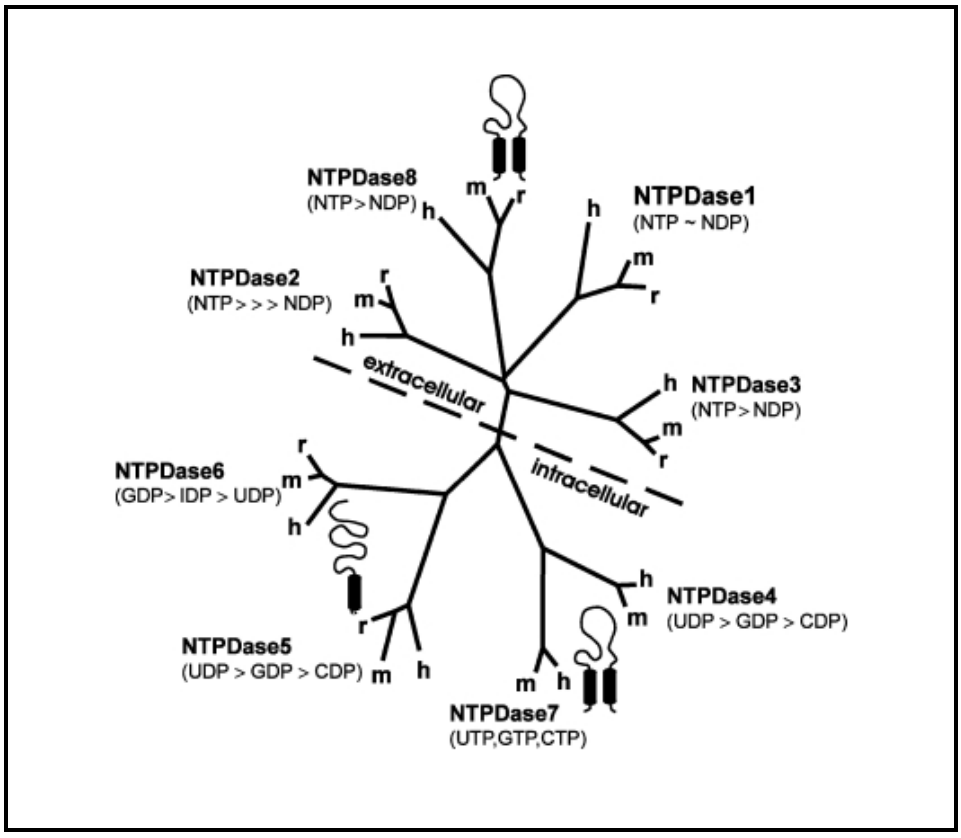

Figura 4. Árvore filogenética hipotética para os membros da família das NTPDases (NTPDase 1 a 8) de rato (r), humano (h) e camundongo (m). O comprimento das linhas indica as diferenças entre as seqüências de aminoácidos. O gráfico representa a separação entre NTPDases localizadas na superfície (superior)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

As 5'-NTs são enzimas encontradas na forma solúvel ou presente nas membranas (ecto-5`-NT) de parasitos, bactérias, células vegetais e animais, apresentando grande diferença nos substratos utilizados (ZIMMERMANN, 2012). A ecto-5`-NT é uma enzima que está ancorada à membrana plasmática através de uma âncora lipídica de glicosilfosfatidilinositol (GPI), com peso molecular aparente de 62 a 74 kDa e que possui forma estrutural de dímero com pontes dissulfeto entre as cadeias (ZIMMERMANN, 2001; POROWIŃSKA ET AL., 2011, ZIMMERMANN, 2012). O ancoramento por GPI pode ser clivado pela ação de um fosfolipase C específica dando origem assim as formas solúveis desta enzima (Figura 5) (BJELOBABA et al., 2007; LANGER et al., 2008).

Nos mamíferos, a ecto-5´-NT é parte da cascata para finalizar a ação de nucleotídeos como ATP e moléculas sinalizadoras que agem em receptores P2X e P2Y, sendo a principal enzima responsável pela hidrólise do AMP e consequentemente pela formação de adenosina, atuando assim como um importante modulador das ações dos nucleosídeos e nucleotídeos de adenina (ZIMMERMANN, 2012; BURNSTOCK, 2013). Tem sido descrita como uma enzima chave na regulação do metabolismo extracelular de ATP e do processo inflamatório, uma vez que esta desloca o equilíbrio ATP (pró-inflamatório) /adenosina (anti-inflamatório) (BRISEVAC et al., 2012). Assim, a função desta enzima associada à atividade da ADA está diretamente relacionada com a modulação dos níveis de adenosina e consequentemente com as funções fisiológicas e patológicas relacionadas com este nucleosídeo (DUNWIDDIE & MASINO, 2001; SCHETINGER et al., 2001).

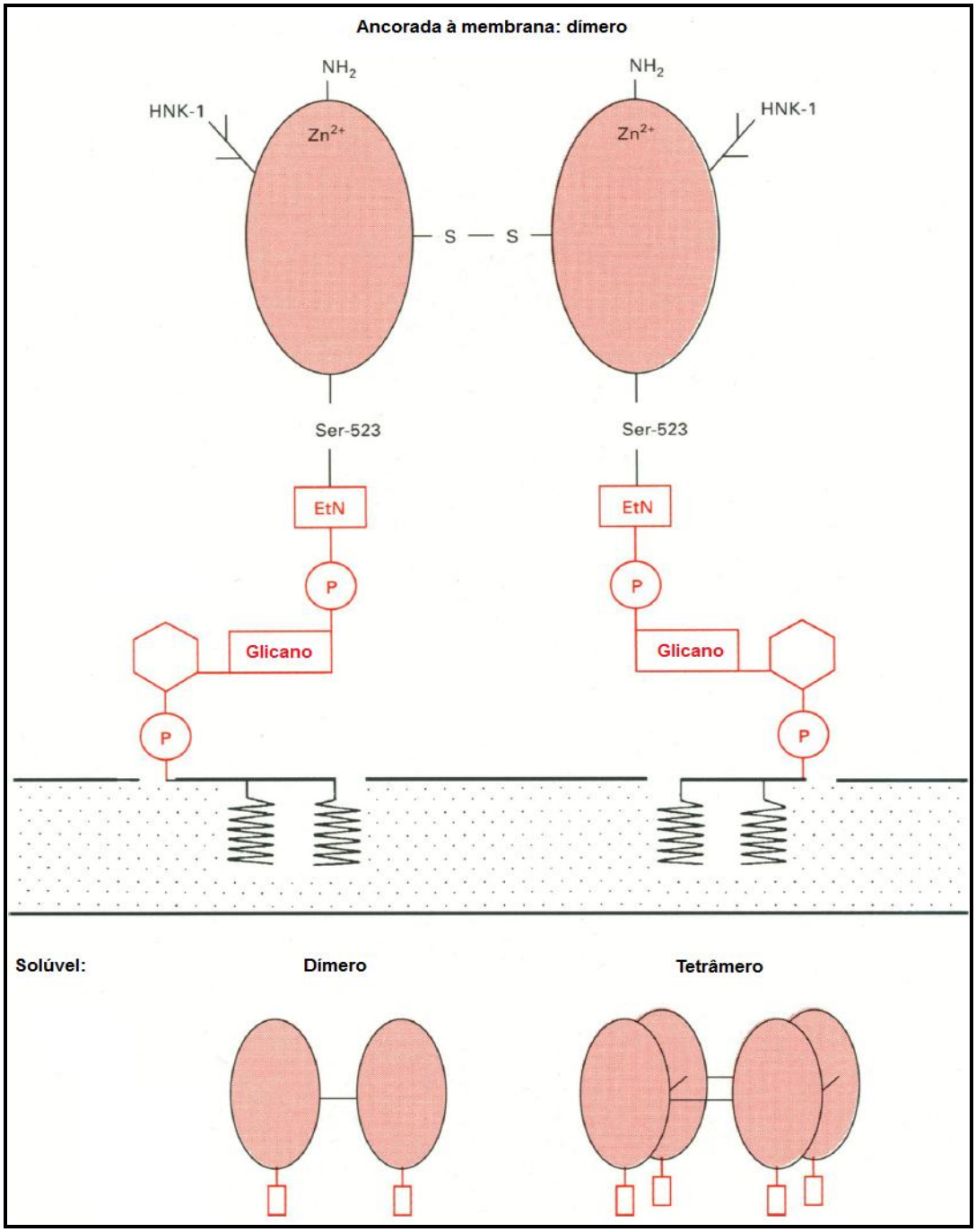

Figura 5. Modelo molecular da 5`-NT solúvel e da ecto-5`-NT. A forma ancorada a membrana plasmática por uma âncora lipídica de glicosilfosfatidilinositol (GPI) existe como um dímero onde suas duas subunidades estão unidas por uma ponte dissulfeto (S-S). A forma solúvel apresenta-se como um dímero ou tetrâmero Os glicanos (vermelho) permanecem anexados a proteína. Abreviação: EtN, etanolamina. (Adaptado de ZIMMERMANN, 1992).

Da mesma forma, a ADA também faz parte do conjunto de enzimas responsáveis pela degradação sequencial dos nucleotídeos e nucleosídeos de

adenina (YEGUTKIN, 2008). Na cascata purinérgica, a ADA, é responsável por promover a desaminação hidrolítica da adenosina e 2'-desoxiadenosina em inosina e 2'-desoxinosina, respectivamente (Figura 6). É encontrada principalmente no sistema linfóide (linfonodos, baço e timo), sendo menos expressa nos eritrócitos (CRISTALLI et al., 2001; SABOURY et al., 2003). A ADA possui um importante papel biológico no metabolismo das purinas, sendo essencial para a proliferação e diferenciação de células linfóides, particularmente células T, e maturação de monócitos (BOTA et al., 2001; ANTONIOLI et al., 2008). No SNC, a ADA, tem sido extensivamente estudada. Por muitos anos esta enzima foi considerada exclusivamente citosólica, no entanto, estudos já têm reportado a sua presença nas membranas de diversos tipos celulares incluindo linfócitos e neurônios (FRANCO et al., 1997).

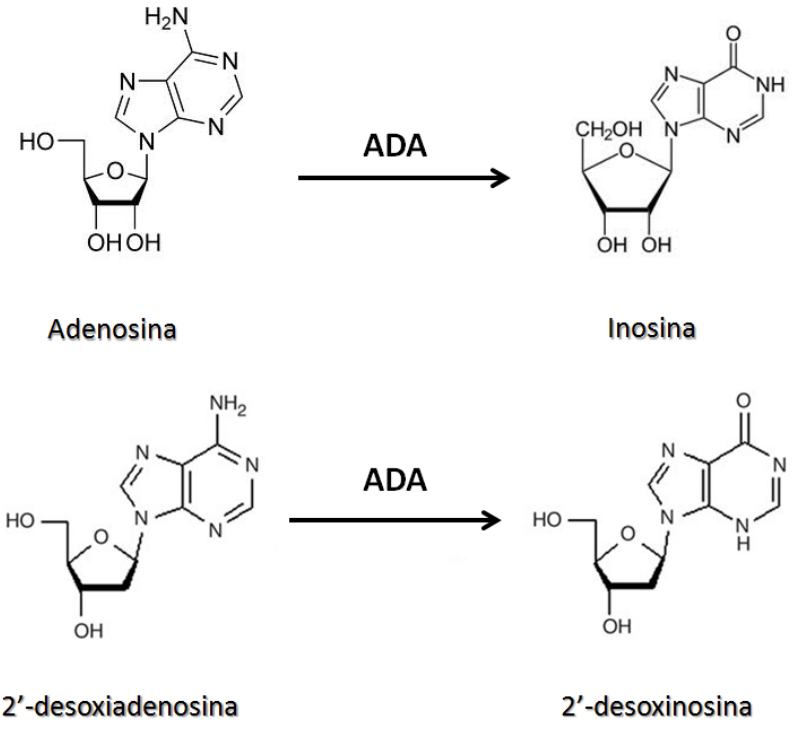

Figura 6. Reações catalisadas pela ADA.

A atividade desta enzima é o resultado da ação de suas duas isoformas: a ADA1 e a ADA2. Nas membranas celulares, duas proteínas que ancoram a ADA têm sido descritas: a dipeptidil peptidase IV (DPPIV) e os A1Rs. Assim, além de

metabolizar a adenosina extracelular, evidências têm demonstrado que a ecto-ADA possui um papel funcional extra-enzimático na sinalização mediada pela adenosina através dos A1R (CIRUELA et al., 1996; RUIZ et al., 2000).

A adenosina, produto final da ação das nucleotidases, é um nucleosídeo de grande importância dentro do sistema purinérgico, sendo encontrada em todos os tecidos, este nucleosídeo atua modulando diversos processos fisiológicos e patológicos (Figura 7) (WARDAS, 2002; CUNHA, 2005; ABBRACCHIO et al., 2009;BURNSTOCK, 2013). Ao contrário do ATP, a adenosina tem sido descrita como um potente agente anti-inflamatório (HASKO & CRONSTEIN, 2004; SITKOVSKY et al., 2004), estando envolvida em uma série de eventos como, a reatividade microglial (GYONEVA et al., 2009), controle da viabilidade neuronal, regulação da astrogliose (BRAMBILLA et al., 2003; HINDLEY et al., 1994) e da liberação de glutamato (LI et al., 2006) e mediadores pró-inflamatórios (FIEBICH et al., 1996; SCHWANINGER et al., 1997; ROMIO et al., 2011), participando assim de forma ativa nos processos de neuromodulação da transmissão sináptica e neuroproteção.

Este nucleosídeo está presente nos meios intra e extracelular sendo sua disponibilidade altamente controlada devido ao importante papel neuromodulador e homeostático que exerce (FREDHOLM et al., 2005). Em condições fisiológicas, as concentrações intracelulares de adenosina encontram-se na ordem de 10 a 50 nm, enquanto que as concentrações extracelulares encontradas na fenda sináptica são de aproximadamente 0,5 a 4 μM (CUNHA, 2001; CUNHA, 2005). No meio intracelular, a adenosina pode ter origem a partir de duas fontes: (1) formação de adenosina a partir da clivagem de S-adenosilhomocisteína (SAH) pela S-adenosilhomocisteína hidrolase (essa via não possui uma grande importância na produção deste nucleosídeo) (PAK et al, 1994); (2) a partir da degradação do AMP pela 5`-NT (BRUNDEGE & DUNWIDDIE, 1997). Como citado anteriormente, os níveis de adenosina no meio extracelular são modulados principalmente pela desfosforilação do seu precursor imediato, o AMP, o qual tem como precursores o ADP e o ATP. Assim, o ATP constitui uma das principais fontes para produção de adenosina no meio extracelular, e isto ocorre devido à ação das ectonucleotidases citadas anteriormente. Em condições de normóxia, a adenosina intracelular é fosforilada pela adenosina quinase (ADK) à AMP e subsequentemente à ATP. No

entanto, sob condições de estresse metabólico, os níveis de adenosina saturam a ADK e o excesso de adenosina intracelular pode ser então, metabolizado à inosina pela ADA ou sofrer difusão facilitada para o meio extracelular através do seu transportador bi-direcional. De uma maneira geral, os transportadores bidirecionais de nucleosídeos são divididos em transportadores equilibrativos (ENT) e concentrativos (CNT). Estes transportadores, constituídos por proteínas integrais de membrana, são responsáveis pelo transporte de adenosina, de outros nucleosídeos, de nucleobases e de drogas que sejam análogos de nucleosídeos (KONG et al., 2004; BONAN, 2012).

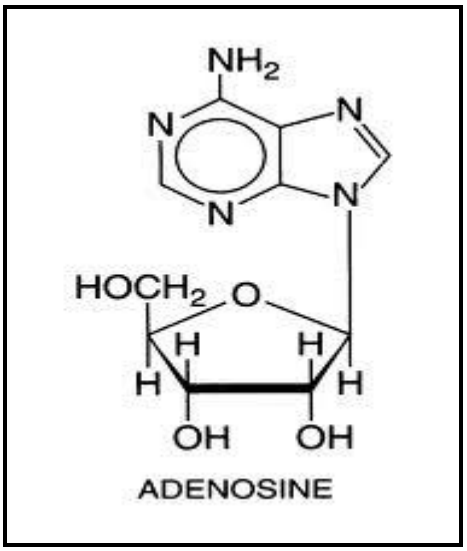

Figura 7. Estrutura química da adenosina.

Os transportadores equilibrativos realizam suas funções de acordo com os níveis de nucleosídeos nos meios intra e extracelulares. Já os transportadores concentrativos, como o nome já sugere, promovem o influxo de nucleosídeos contra o gradiente de concentração, usando para tal a energia derivada do gradiente de concentração de sódio existente nas membranas celulares (PODGORSKA et al., 2005; BONAN, 2012). É importante ressaltar que estes transportadores constituem uma das principais vias de regulação dos níveis de adenosina no SNC.

Já o processo de eliminação da adenosina nas células envolve reações catalisadas pela ADA e pela ADK, formando como produto a inosina ou AMP,

respectivamente (SHRYOCK & BELARDINELLI,1997). No meio extracelular, a remoção da adenosina ocorre em parte pela sua recaptação através dos transportadores bidirecionais, seguida por sua fosforilação à AMP pela enzima ADK (esta reação de fosforilação predomina quando a adenosina está em baixas concentrações (concentrações fisiológicas < 1 μM), e em parte por sua degradação à inosina pela ADA (a ADA é ativada quando os níveis de adenosina se encontram aumentados, ex: condições de hipóxia (> 10 μM) (LATINI & PEDATA, 2001; BOROWIEC et al., 2006). Após a adenosina ser metabolizada a inosina, esta última é degradada até ácido úrico através de uma série de reações enzimáticas. As vias de produção, metabolismo e transporte de adenosina são mostradas na figura 8.

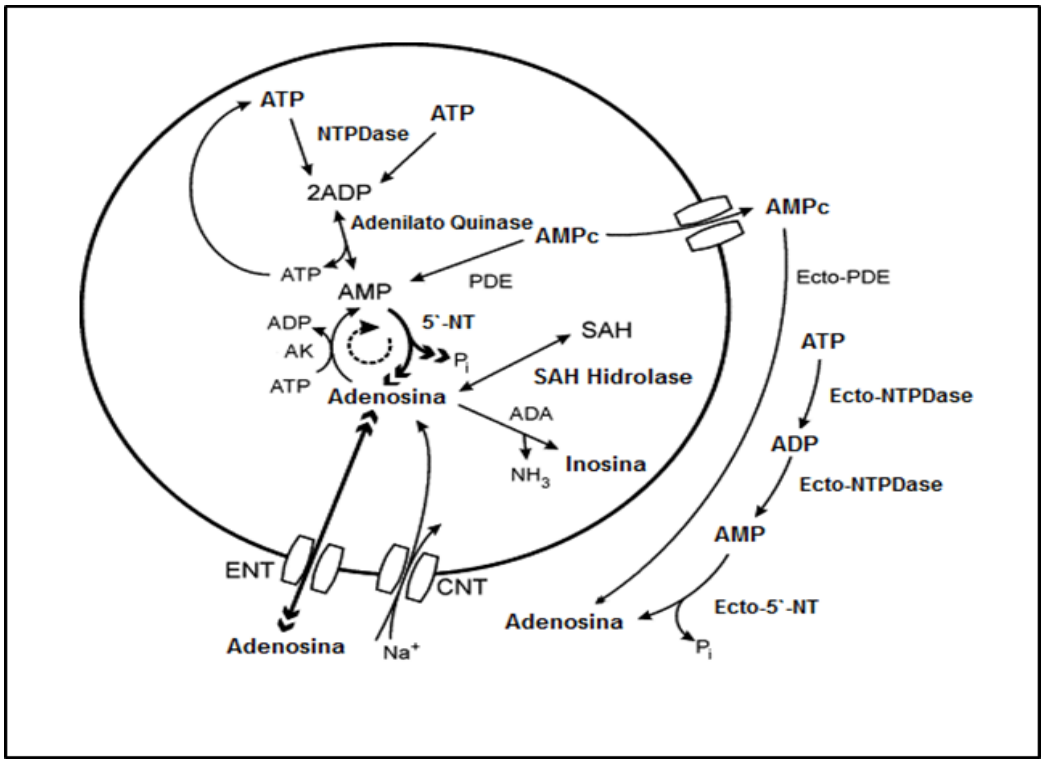

Figura 8. Vias de produção, metabolismo e transporte de adenosina (Latini & Pedata, 2001 para revisão). Abreviações: ADA, adenosina desaminase; AK, adenosina kinase, CNT, transportador concentrativo; ENT, transportador equilibrativo; PDE, fosfodiesterase; SAH, S-adenosil-homocisteína. (Adaptado de BUCK & SHIN, 2002).

No meio extracelular, os efeitos fisiológicos dos nucleotídeos purínicos são mediados via receptores purinérgicos específicos. Estes receptores estão presentes

na superfície de diversas células e exercem a função de reconhecimento do ATP extracelular e seus metabólitos. Dependendo de características como a estrutura molecular, a distribuição tecidual e a afinidade pelo seu ligante, estes são classificados dentro de duas famílias: P1 ativados por adenosina e P2 ativados por ATP, ADP, UTP e ADP (Figura 9) (WARDAS, 2002; PEDATA et al., 2007; BURNSTOCK, 2007).

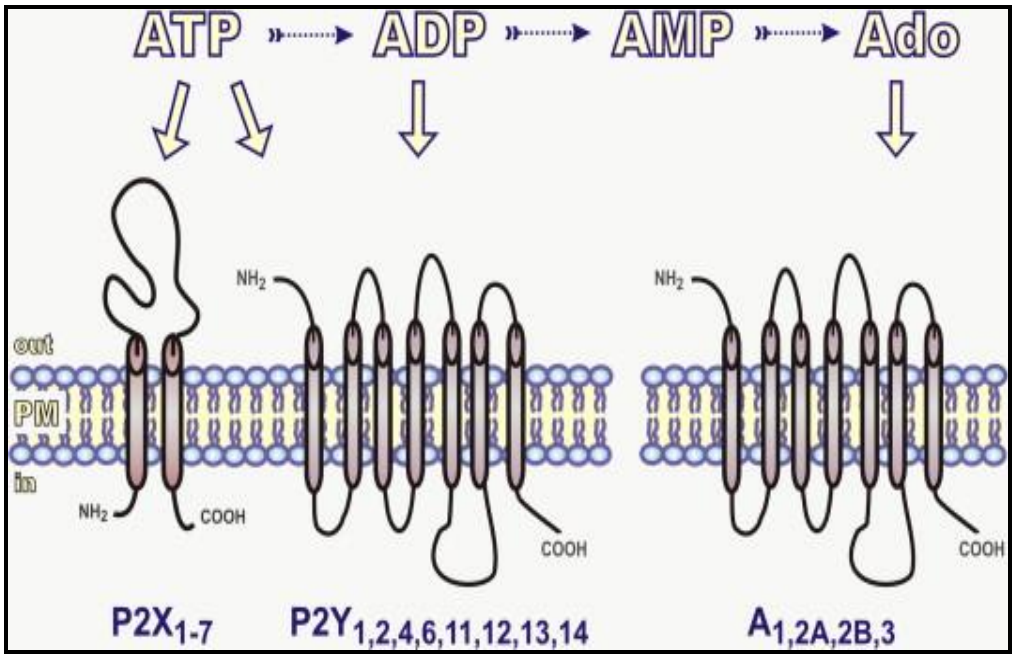

Figura 9. Vias de sinalização purinérgica. A sinalização mediada nucleotídeos ocorre através de receptores P2X (ionotrópicos) e P2Y (metabotrópicos), os quais são classificados de acordo com a afinidade dos mesmos pelo ATP e ADP. A adenosina atua sobre os receptores P1 (metabotrópicos). (Adaptado de YEGUTKIN, 2008).

A família P1 é composta por 4 subtipos (A1, A2A, A2B e A3), e representa a classe de receptores ativados pela adenosina. Todos apresentam sete domínios transmembrana formados por aminoácidos hidrofóbicos que presumivelmente se organizam formando sete alfa-hélices. A porção N-terminal do receptor está voltada para o meio extracelular e a porção C-terminal está voltada para o lado citosólico da membrana plasmática (RALEVIC & BURNSTOCK, 1998). Estes receptores estão acoplados às proteínas G e estão relacionados com vários mecanismos de sinalização (Tabela 1).

Tabela 1. Mecanismos de ação, sinalização e localização dos receptores de adenosina.

Subtipo	Mecanismo de Sinalização	Localização e algumas funções biológicas
A1	Inibe a adenilato ciclase. Estimula a guanilato ciclase. Estimula a fosfolipase A2 e C. Ativa canais de K^+ e inativa canais de Ca^{2+}.	Afinidade para a adenosina em concentrações nanomolares. Expressa em muitas áreas do cérebro (córtex, cerebelo, hipocampo e tálamo). Inibem a liberação de neurotransmossores, induz o sono, efeito antinociceptivo e neuroprotetor.
A2A	Estimula a adenilato ciclase.	A afinidade para a adenosina em concentrações nanomolares mais elevadas. É expresso principalmente no estriado. Facilitam a neurotransmissão. Antagonizam receptores dopaminérgicos.
A2B	Estimula a adenilato ciclase. Transdução de sinal pode ocorrer também através da fosfolipace C e/ou fluxo de íons.	Expresso principalmente no cólon, na bexiga, no cérebro, no fígado. Afinidade para adenosina em baixas concentrações micromolares. Favorece a degranulação de mastócitos.
A3	Negativamente acoplado a adeninato ciclase. Transdução de sinal pode ocorrer também através da fosfolipace C e/ou fluxo de íons.	Expresso em mastócitos, pulmão coração, rim, córtex, hipocampo, estriado, bulbo olfativo. Favorece a degranulação de mastócitos. Inativa a migração de eosinófilos.

Obtido de NYCE, 1999; GODING, 2000; RIBEIRO et al., 2001 e LOPES et al., 2003.

A família P1 é responsável por diversas ações da adenosina no SNC, participando de diversas funções neuronais, como a neuromodulação de neurotransmissores, indução do sono (PORKKA-HEISKANEN, 1999) e a modulação da plasticidade sináptica (DE MENDONÇA & RIBEIRO, 1997). Apesar de todos os receptores P1 estarem presentes no SNC, o receptor do tipo A1 (A1R) é o principal representante desta família neste sistema, sendo encontrado em neurônios, e em menor densidade em astrócitos (BIBER et al., 1997), micróglia (GEBICKE-HAERTER et al., 1996) e oligodendrócitos (OTHMAN et al., 2003; TSUTSUI et al, 2004) de hipocampo, cerebelo e córtex (BURNSTOCK, 2013). Estes receptores estão envolvidos em uma grande variedade de respostas, as quais podem ser causadas pelo seu acoplamento à diferentes proteínas G (proteína Gi/Go). A principal via de sinalização mediada por estes receptores é relacionada com a

inibição da adenilato ciclase e consequentemente redução nos níveis do segundo mensageiro AMPc (RALEVIC & BURNSTOCK, 1998; KULL et al., 2000).

No SNC, são os A1Rs os responsáveis pela maioria dos efeitos neuroprotetores da adenosina descritos na literatura. A neuroproteção mediada por estes receptores envolve uma série de mecanismos pré e pós-sinápticos, sendo o controle pré-sináptico da liberação de neurotransmissores, como o glutamato, o mais descrito. Assim, os A1R apresentam um papel importante em condições de injúria tecidual, pois diminuem a excitabilidade neuronal e a transmissão sináptica (THOMPSON et al., 1992; DE MENDONÇA et al., 1995; CUNHA, 2005). Além disso, a sua ativação tem sido descrita por inibir a condutância de K^+ a nível pós-sináptico levando à hiperpolarização neuronal. A maior densidade deste receptor nas membranas pós-sinápticas também antecipa um importante papel da adenosina no controle da integração de sinais a nível pós-sináptico, onde a ativação tônica dos mesmos teria um papel importante na modulação da plasticidade sináptica nos circuitos excitatórios (DE MENDONÇA & RIBEIRO, 1997). Isto poderia ser resultado da habilidade dos A1Rs em controlar eficientemente os receptores NMDA (N-metil D-Aspartato) e os canais de cálcio sensíveis à voltagem localizados nas membranas pós-sinápticas (MOGUL et al., 1993; DE MENDONÇA et al., 1995; KLISHIN et al., 1995; PASCUAL et al., 2005). Entretanto, a ativação do A1R e o efeito produzido por este dependem do tipo celular já que o mesmo controla a liberação de glutamato, acetilcolina (ACh) e serotonina mas não modula a liberação de ácido gama-aminobutírico (GABA) e noradrenalina (CUNHA, 2001).

Por outro lado, o receptor do tipo A2A (A2AR) pode ser encontrado em poucas regiões do cérebro, sendo encontrado em maior densidade no estriado. No entanto, também está presente em neurônios e células da glia de outras regiões cerebrais como, o cerebelo, o córtex e o hipocampo (LI et al., 2001). Nos gânglios da base estes receptores estão localizados predominantemente nas espinhas dendríticas e nas regiões pós-sinápticas (HETTINGER et al., 2001; RODRIGUES et al., 2005). Já no hipocampo, estão localizados principalmente nas sinapses, em particular nas regiões pré-sinapticas (REBOLA et al., 2005). Com uma resposta antagônica àquela dos A1Rs, a ativação dos A2ARs resulta em ativação da proteína G estimulatória (Gs), a qual aumenta os níveis intracelulares de AMPc (KESSEY & MOGUL, 1998). A adenosina, através da ativação destes receptores, parece ter uma

função crucial na regulação e na ativação de múltiplos receptores que afetam a liberação de neurotransmissores e/ou a transmissão sináptica em particular, receptores para neuropetídeos (receptores de peptídeos relacionados com o gene de calcitonina e o peptídeo vasoativo intestinal), receptores NMDA, receptores metabotrópicos de glutamato, receptores nicotínicos auto-facilitatórios, receptores dopaminérgicos e A1Rs.

Assim, a maneira pela qual estes receptores estão envolvidos com os receptores de outros neurotransmissores e neuromoduladores possibilita a busca de novas ações e interpretações para a funcionalidade deste nucleosídeo no SNC. De maneira geral, quer por modulação direta dos seus e de outros receptores ou através de mecanismo pós-receptor, a adenosina, na sua “obsessão” para proteger as células de diferentes insultos, utiliza de um complexo sistema de receptores para sincronizar a transmissão sináptica, a fim de exercer o que parece ser o “destino” deste nucleosídeo – proteger o sitema nervoso (SEBASTIÃO & RIBEIRO, 2009). Neste contexto, vários estudos têm demonstrado que o bloqueio do A2AR promove a inibição da liberação de neurotransmissores principalmente o glutamato. Esse fato se torna importante durante episódios de isquemia e hipóxia já que o bloqueio do A2AR inibe a liberação de glutamato e confere proteção às células (LATINI et al., 1999).

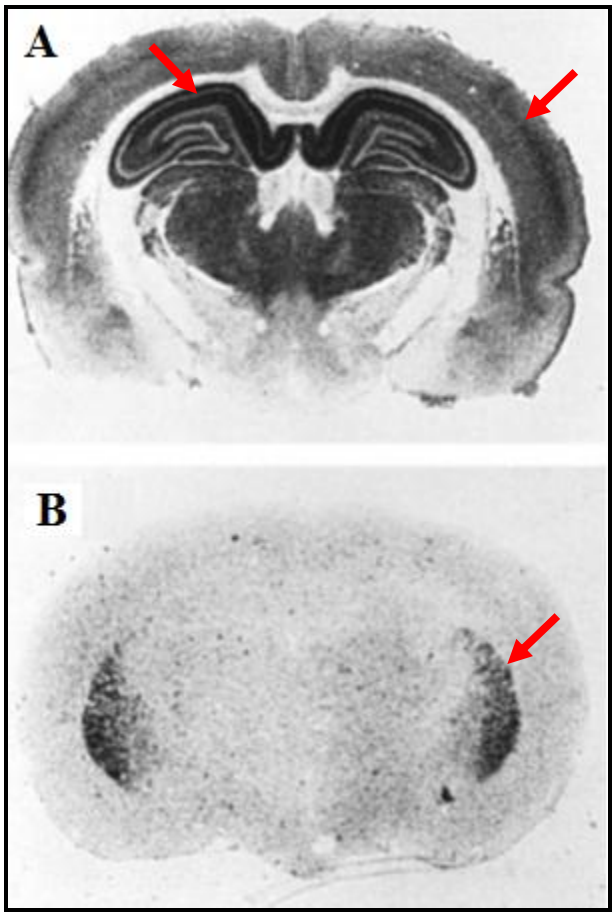

Figura 10. Distribuição dos receptores A1 e A2A em cérebro de ratos. Autoradiografia mostrando a presença do A1R no córtex e hipocampo (A) e do A2AR no estriado (B)(setas vermelhas). Fonte: http://www.ncbi.nlm.nih.gov/books/bv.fcgi?rid=bnchm.figgrp.1262

Ao contrário dos receptores A1 e A2A que são de elevada afinidade para a adenosina, os receptores A2B são de baixa afinidade e são pouco expressos no cérebro. O receptor A3 apesar de ter níveis de expressão baixo no cérebro, em humanos é um receptor de alta afinidade para a adenosina (RIBEIRO et al., 2003).

Neste contexto, os receptores de adenosina A1 e A2A destacam-se frente aos outros receptores adenosinérgicos, pelo fato de que, as ações mediadas pela a adenosina ocorrem principalmente devido a um balanço entre as ações inibitórias (A1R) e excitatórias (A2AR) exercida por estes (CUNHA, 2001). Assim, a presença dos receptores de adenosina A1 e A2A em todas as células do sistema nervoso (neurônios e glia), junto com a intensa liberação de adenosina após insultos, fazem da adenosina um elemento chave na coordenação das funções cerebrais. Na figura

10 são demostradas algumas das ações positivas e negativas dos receptores A1 e A2A frente às células neuronais.

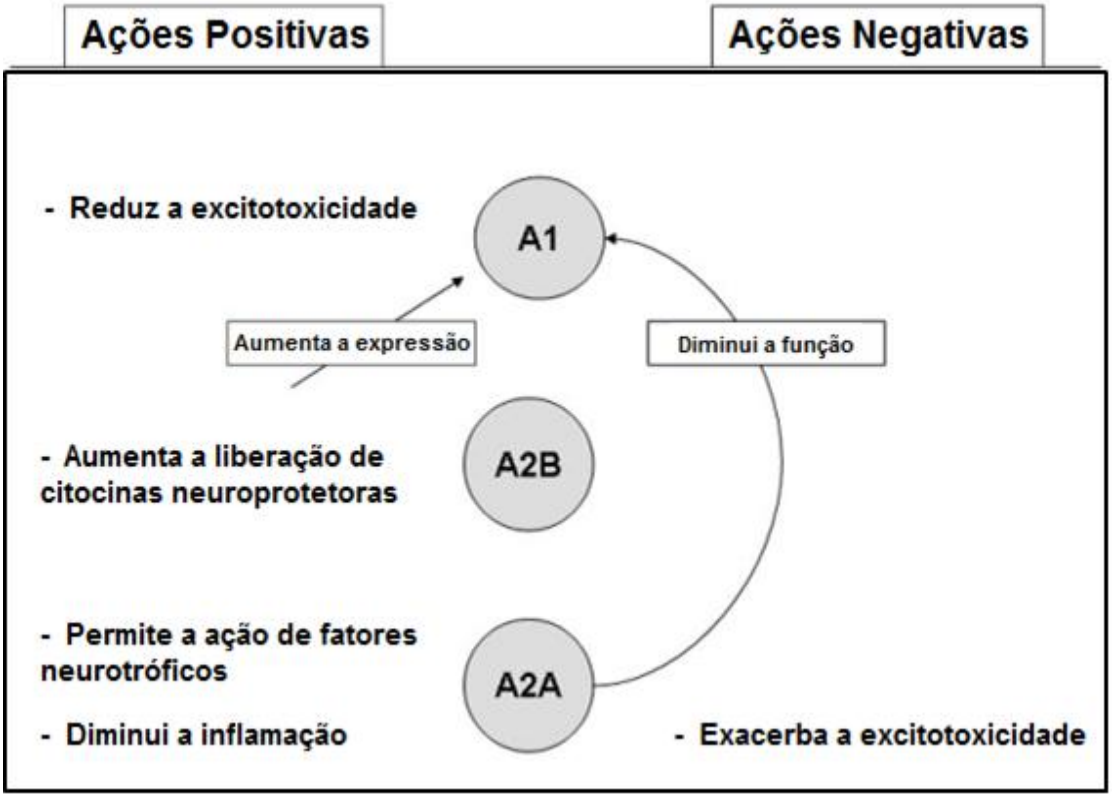

Figura 11. Ações positivas e negativas dos A1Rs e A2ARs para proteger as células neuronais. Enquanto que, para os A1Rs as ações são predominantemente neuroprotetoras, os A2ARs podem operar mecanismos que levam tanto ao dano como a proteção neuronal. (Adaptado de RIBEIRO, 1999).

Como citado anteriormente, no SNC em nível sináptico, o sistema purinérgico se mostra envolvido na modulação e interação com outros sistemas. No sistema colinérgico, a adenosina tem sido descrita principalmente nos eventos que modulam a liberação do neurotransmissor ACh. Estes achados tem sido demonstrados em córtex, hipocampo e estriado e são atribuídos principalmente aos A1Rs (os quais inibem a liberação de ACh) e aos A2ARs (os quais favorecem a liberação de ACh) (BROWN et al. 1990; KIRK & RICHARDSON 1994; KUROKAWA et al., 1994; CUNHA et al. 1994a, b; CUNHA, 1995; JIN & FREDHOLM, 1997; CUNHA; 2001).

O sistema colinérgico é caracterizado por ser uma das mais importantes vias de modulação do SNC desempenhando um papel fundamental em várias funções vitais, como aprendizado, memória, organização cortical do movimento e controle do

fluxo sanguíneo, o que faz deste sistema, alvo de muitas pesquisas (MESULAM et al., 2002).

Neste contexto é relevante destacar os principais componentes que constituem o sistema colinérgico: a acetilcolina; a colina-acetiltransferase (ChAT); o transportador de colina (CHT); o transportador de acetilcolina vesicular (VAChT); os receptores de acetilcolina muscarínicos (mAChR) e os nicotínicos (nAChR) e a acetilcolinesterase (AChE; E.C.3.1.1.7) os quais juntos são responsáveis por modular a neurotransmissão colinérgica (SARTER & PARIKH, 2005; DREVER et al., 2011).

Em particular, a AChE é uma importante enzima regulatória que controla a transmissão do impulso nervoso através da sinapse colinérgica pela rápida hidrólise e inativação da ACh modulando a concentração deste neurotransmissor nas sinapses (Figura 12) (GRISARU et al., 1999; SOREQ & SEIDMAN, 2001). É uma glicoproteína globular encontrada nos neurônios colinérgicos, nas proximidades das sinapses colinérgicas principalmente nas junções neuromusculares. Além de seu papel clássico na transmissão colinérgica, a AChE tem sido implicada em várias ações não colinérgicas como crescimento dos neuritos (DAY & GREENFIEL, 2002), regulação estrutural da diferenciação pós-sináptica, osteogênese (CHACÓN et al., 2003) e também tem sido proposto a atividade hematopoiética pela presença desta enzima em células progenitoras do sangue (SOREQ & SEIDMAN, 2001). A AChE também foi localizada e identificada em linfócitos e em eritrócitos (células sanguíneas que mais expressam a AChE) (WRIGHT & PLUMMER, 1973; KAWASHIMA & FUJII, 2000). No entanto, no sistema periférico, a função do sistema colinérgico presente nas células sanguíneas ainda não está claro. Contudo, recentemente estudos tem proposto que esta enzima possa estar envolvida nos processos de regulação de diversas funções biológicas tais como, proliferação, diferenciação, organização do citoesqueleto, contato célula-célula, ou funções imunes (FALUGI et al., 1983; SANTOS et al., 2007; PALEARI et al., 2008). Alterações na atividade da AChE tem sido demonstrado pelo nosso grupo de pesquisa em diversas condições patológicas e de intoxicação sugerindo que esta enzima poderia ser um importante parâmetro a ser avaliado em condições fisiológicas e patológicas (KAIZER et al., 2005; SCHMATZ et al., 2009; GONÇALVES et al., 2010; KAIZER et al., 2010). Assim uma inibição ou ativação

desta enzima pode ter consequências devastadoras no cérebro e outros órgãos (MESULAN et al., 2002).

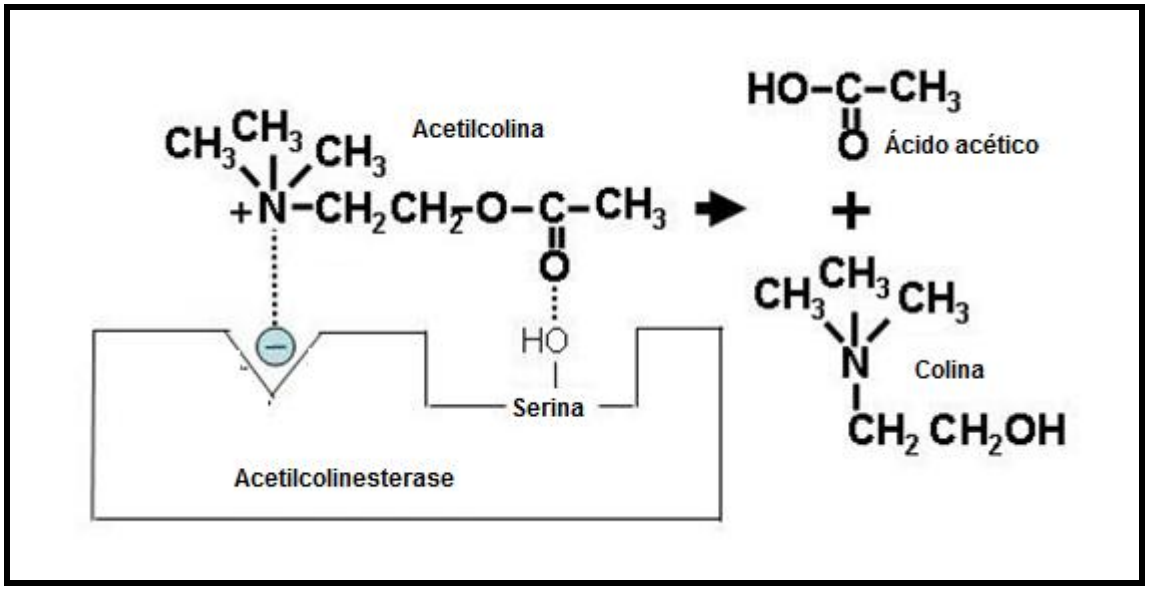

Figura 12. Acetilcolinesterase promove a hidrólise da acetilcolina em colina e ácido acético. Fonte: http://analytical.wikia.com/wiki/File:Acetylcholinesterase.png

Nos últimos anos, diversos estudos desenvolvidos por nosso grupo de pesquisa têm evidenciado a importância das ecto-enzimas NTPDase, 5'-nucleotidase e ADA em várias condições patológicas, como a hipóxia-isquemia neonatal (PIMENTEL et al., 2009; PIMENTEL et al., 2011), câncer (MALDONADO et al., 2010, ZANINI et al., 2013), esclerose múltipla (SPANEVELLO et al., 2010a; SPANEVELLO et al., 2010b), artrite reumatóide (BECKER et al., 2010), infarto agudo do miocárdio (BAGATINI et al., 2011) e diabetes (LUNKES et al., 2008; SCHMATZ et al., 2009). De particular interesse, no SNC de ratos neonatos submetidos à hipóxia-isquemia observou-se um aumento na atividade da ADA em relação ao grupo controle 8 dias após o insulto, neste período foi observado também um processo inflamatório crônico associado com a presença de infiltrados leucocitários (PIMENTEL et al., 2009; PIMENTEL et al., 2011). Interessantemente, neste mesmo estudo, não observamos alteração na atividade da 5`-NT no período em que a atividade da ADA mostrou-se alterada, o que nos leva a questionar e

investigar a fundo o comportamento do sistema purinérgico sobre a modulação dos níveis de nucleotídeos e nucleosídeos de adenina após a HI neonatal.

Assim, como abordado anteriormente, a asfixia perinatal constitui uma das principais causas de morbidade e mortalidade durante o período neonatal em países em desenvolvimento, e apesar dos esforços dedicados pela comunidade científica, os mecanismos que levam aos danos neuronais/óbito e as consequências patológicas subjacentes aos diferentes distúrbios neurológicos não estão completamente esclarecidos. Isto ocorre devido principalmente à complexidade dos eventos e mecanismos que se iniciam junto com o insulto hipóxico-isquêmico. Os nucleotídeos purínicos assim como a acetilcolina, exercem um papel importante durante eventos fisiológicos e patológicos no SNC. A presença em especial, da adenosina e da acetilcolina e seus receptores, assim como, as enzimas responsáveis pela modulação dos níveis destas moléculas se encontram presentes desde as fases iniciais do desenvolvimento cerebral, o que atribui a estas uma importância ainda maior frente ao desenvolvimento do SNC. Considerando os aspectos citados acima, torna-se importante avaliar o efeito da HI neonatal sobre os sistemas purinérgico e colinérgico no encéfalo de ratos. Além disso, a melhor compreensão do envolvimento destes sistemas na patofisiologia da HI neonatal possibilitará uma melhor manipulação dos mesmos a fim de promover a busca de novos alvos terapêuticos.

1. OBJETIVOS

2.1. Objetivo geral

Avaliar o efeito da HI neonatal sobre os sistemas purinérgico e colinérgico em córtex cerebral de ratos.

2.2. Objetivos específicos

I) Sabe-se que o primeiro evento que ocorre durante a HI neonatal é a rápida depleção energética seguida pelo aumento nos níveis de adenosina. No entanto, os mecanismos enzimáticos que promovem o aumento da adenosina em resposta ao insulto hipóxico-isquêmico não estão completamente esclarecidos. Neste contexto, um dos objetivos deste estudo foi investigar no córtex cerebral de ratos:

- A atividade das enzimas NTPDase, 5`-nucleotidase e adenosina desaminase nas frações sinaptosomal e citosólica imediatamente após a HI neonatal;
- A expressão da adenosina quinase;
- Os níveis de peroxidação lipídica;
- A atividade da enzima Na^+/K^+ ATPase.

II) Recentemente nosso grupo de pesquisa reportou que a HI neonatal causa um aumento na atividade da adenosina desaminase 8 dias após o insulto hipóxico-isquêmico. Para dar continuidade a este trabalho, o próximo objetivo do nosso grupo foi investigar em córtex cerebral destes animais, 8 dias pós-insulto:

- A isoenzima da adenosina desaminase responsável por sua atividade aumentada;

- A expressão da adenosina quinase;

- A atividade da mieloperoxidase e da N-acetil-glucosaminidase;

- A expressão dos receptores de adenosina A1.

III) Tem sido demonstrado que o cérebro do neonato é altamente suscetível à peroxidação lipídica. Sabe-se também que a acetilcolinesterase responde a vários insultos incluindo o estresse oxidativo e a inflamação, e de acordo com nossos resultados obtidos nos trabalhos anteriores, podemos observar que estes eventos estão diretamente relacionados com o insulto hipóxico-isquêmico. Neste contexto, o próximo objetivo do nosso trabalho foi investigar em diferentes tempos pós-insulto (imediatamente, 72 horas e 8 dias após HI neonatal):

- A atividade da acetilcolinesterase no córtex cerebral;

- Os níveis de peroxidação lipídica no córtex cerebral destes animais;

- A atividade da acetilcolinesterase e da adenosina desaminase eritrocitária;

- Os níveis séricos de citocinas pró-inflamatórias (fator de necrose tumoral alfa (TNF-α), o interferon gama (IFN-γ) e as interleucinas 1β e 6 (IL-1β e IL-6, respectivamente).

3. METODOLOGIA E RESULTADOS

Os resultados desta tese estão apresentados na forma de um artigo científico e dois manuscritos. Os itens materiais e métodos, resultados, discussão e referências bibliográficas encontram-se nos próprio artigo e manuscritos e representam a íntegra deste estudo. O artigo está estruturado de acordo com a versão publicada na revista Neurochemical Research, e os manuscritos estão nas normas das revistas Neurochemistry International e Molecular and Cellular Biochemistry para as quais foram submetidos.

Artigo I: Hypoxia-ischemia alters nucleotide and nucleoside catabolism and Na^+, K^+-ATPase activity in the cerebral cortex of newborn rats.

Manuscrito I: Neuroinflammation after neonatal hypoxia-ischemia is associated with alterations in the purinergic system: Adenosine deaminase 1 isoenzyme is the most predominant after insult.

Artigo II: Evaluation of acetylcholinesterase and adenosine deaminase activities in brain and erythrocytes and pro-inflammatory cytokines levels in rats submitted to neonatal hypoxia ischemia model.

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3.1. ARTIGO I

Hypoxia-ischemia alters nucleotide and nucleoside catabolism and Na^+,K^+-ATPase activity in the cerebral cortex of newborn rats

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Neurochem Res
DOI 10.1007/s11064-013-0994-3

ORIGINAL PAPER

Hypoxia–Ischemia Alters Nucleotide and Nucleoside Catabolism and Na^+,K^+-ATPase Activity in the Cerebral Cortex of Newborn Rats

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Received: 9 December 2012 / Revised: 22 January 2013 / Accepted: 29 January 2013

Abstract It is well known that the levels of adenosine in the brain increase dramatically during cerebral hypoxic-ischemic (HI) insults. Its levels are tightly regulated by physiological and pathophysiological changes that occur during the injury acute phase. The aim of the present study was to examine the effects of the neonatal HI event on cytosolic and ecto-enzymes of purinergic system—NTPDase, 5′-nucleotidase (5′-NT) and adenosine deaminase (ADA)—in cerebral cortex of rats immediately post insult. Furthermore, the Na^+/K^+-ATPase activity, adenosine kinase (ADK) expression and thiobarbituric acid reactive species (TBARS) levels were assessed. Immediately after the HI event the cytosolic NTPDase and 5′-NT activities were increased in the cerebral cortex. In synaptosomes there was an increase in the ecto-ADA activity while the Na^+/K^+ ATPase activity presented a decrease. The difference between ATP, ADP, AMP and adenosine degradation in synaptosomal and cytosolic fractions could indicate that NTPDase, 5′-NT and ADA were differently affected after insult. Interestingly, no alterations in the ADK expression were observed. Furthermore, the Na^+/K^+-ATPase activity was correlated negatively with the cytosolic NTPDase activity and TBARS content. The increased hydrolysis of nucleotides ATP, ADP and AMP in the cytosol could contribute to increased adenosine levels, which could be related to a possible innate neuroprotective mechanism aiming at potentiating the ambient levels of adenosine. Together, these results may help the understanding of the mechanism by which adenosine is produced following neonatal HI injury, therefore highlighting putative therapeutical targets to minimize ischemic injury and enhance recovery.

Keywords Hypoxia–ischemia · Adenosine · Nucleotidases · Adenosine deaminase · Adenosine kinase · Cortex

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Introduction

Hypoxic–ischemic (HI) brain damage in neonates is a major risk factor of a variety of serious human neurological disorders such as motor and learning disabilities, cerebral palsy, epilepsy and seizures of which 20–50 % die within the newborn period [1, 2]. It is appreciated that the brain tissue has a relatively high consumption of oxygen and

Published online: 09 February 2013

glucose, and depends almost exclusively on oxidative phosphorylation for energy production. Moreover, the pathogenesis of HI injury has profound molecular consequences that begin with energy failure [3, 4].

During the HI event the lack of sufficient oxygen suppresses oxidative phosphorylation, a major pathway of 5′-triphosphate (ATP) synthesis, resulting in mitochondrial dysfunction, formation of free radical species [5, 6], induction of oxidative stress, and failure of membrane ion pumps [7]. This mechanism highly complex is interrelated with toxic events (glutamate release, activation of glutamate receptors, coupled to nitric oxide synthase activation, calcium influx, and release of nitric oxide) that occur simultaneously and contribute to cellular dysfunction and death [8–10].

Nucleotides and nucleosides of adenine are ubiquitous signaling molecules that play crucial roles for brain functions. ATP is currently recognized as a neurotransmitter and a neuromodulator in the nervous system and may directly control neuronal activity either by activating P_2 receptors [11, 12] or indirectly by modulating neuronal excitability after its extracellular catabolism generating adenosine [13, 14]. Adenosine plays an important role in mediating hypoxic increases in cerebral blood flow by effective decreases in cerebrovascular resistance. During cerebral ischemia, the levels of adenosine increase up to 100-fold in the brain and exert a neuroprotective influence largely via the A1 receptor, which inhibits glutamate release and neuronal activity [15–17].

The presence of the hydrolases participating in the degradation of nucleotides and nucleosides of adenine has previously been reported in the central nervous system (CNS). Their ubiquitous abundance on the surface of the synaptic membranes, neurons and glial cells seems to be fully consistent with their substantial role in biological signaling in the brain [18].

The extracellular metabolism of ATP to adenosine is usually mediated by a variety of enzymes with an extracellularly oriented catalytic site. These enzymes act in sequence to achieve complete dephosphorylation of ATP to adenosine, where the ATP and ADP are hydrolyzed by ecto-NTPDase and the AMP is hydrolyzed to adenosine by ecto-5′-nucleotidase (5′-NT). The presence of these ectoenzymes has earlier been reported in the CNS and this presence on the surface of the cells seems to be consistent with their significant role in biological signaling in the brain through the modulation of ligand availability at nucleotide and nucleoside receptors [19, 20].

One of the most important enzymes metabolizing adenosine is adenosine deaminase (ADA; EC 3.5.4.4), which deaminates adenosine and 2′-deoxyadenosine to ammonia and inosine or 2′-deoxyinosine, respectively [21]. In mammalian brains, ADA activity may be found mainly in the cytosol, but the presence of ecto-ADA has been established also on the surface of synaptosomes and neurons. Because ecto-ADA is colocalized with adenosine A_1 and A_{2B} receptors, adenosine cleavage at synaptic cleft is crucial for controlling P1 signaling [22]. In view of this, our group has studied this enzyme after neonatal HI [23, 24].

It has been known that the first event that occurs during neonatal HI is a rapid energetic depletion followed by an increase in adenosine levels. However, the enzymatic mechanisms subserving the increase of adenosine in response to neonatal HI are not clear. Thus, the purpose of this study was to investigate the NTPDase, 5′-NT and ADA activities in synaptosomal and cytosolic fractions of cerebral cortex from rats in order to better understand the role of these enzymes in the modulation of adenosine levels in brain immediately after neonatal HI. Furthermore, the ADK expression was also evaluated due to the role of this enzyme to control cytosolic adenosine concentrations [25]. Na^+/K^+ ATPase activity and lipid peroxidation were also assessed in view of importance of energetic metabolism and oxidative stress on maintenance of Na^+/K^+ ATPase activity during HI insult.

Materials and Methods

Animal Protocol

The study was in accordance with the guidelines of the Ethics Committee for Animal Research of the Federal University of Santa Maria which approved the experimental protocol (No. 23081.007419/2007-10). Seven-day-old male Wistar rats, weighing 14–16 g obtained from our own breeding colony were fed ad libitum and maintained on 12 h light/12 h dark cycle, at room temperature.

Hypoxic-Ischemic (HI) Injury

The pups for this study were randomly divided into 2 groups: a control group (without ischemia and hypoxia; $n = 10$) and hypoxia/ischemia (HI; $n = 10$). The association of unilateral occlusion of the common carotid artery with exposure to a hypoxic atmosphere in order to produce unilateral damage in the rat brain was made as described by Moretto et al. [26]. Animals were anesthetized with halothane. The left common carotid artery was permanently occluded with surgical silk thread. After a 2 h recovery period, groups of four pups were placed into a 1500 ml chamber and exposed to an 8 oxygen-92 % nitrogen atmosphere delivered at 5 l/min for 1.5 h, with the chamber partially immersed in a 37 °C water bath to maintain a constant thermal environment [27]. The pups were killed

by decapitation immediately after the HI insult, their brains were promptly removed, and the cerebral cortex hemispheres were carefully separated for the analysis.

Cytosolic Fraction

Wistar rats were euthanized, their brains were promptly removed and their cerebral cortex was carefully separated. In brief, firstly the tissue was homogenized in 8 volumes of 50 mmol/l phosphate buffer pH 7.0, centrifuged (30 min, 14,000×*g*). The supernatant fraction was then isolated [28]. All the procedures described above were performed at 0–4 °C.

Synaptosomal Fraction

The cerebral cortex was homogenized in 10 volumes of an ice-cold medium (medium I), consisting of 320 mM sucrose, 0.1 mM EDTA and 5 mM HEPES, with a pH of 7.5, in a motor driven Teflon-glass homogenizer. Synaptosomes were isolated as described by Nagy and Delgado-Escueta [29] using a discontinuous Percoll gradient. The pellet was suspended in an isoosmotic solution and the final protein concentration was adjusted to 0.4–0.6 mg/ml. Synaptosomes were prepared fresh daily, maintained at 0–4 °C throughout the procedure, and used for assay.

Assay of NTPDase and 5′-NT Activities

The NTPDase enzymatic assay was carried out in a reaction medium containing 5 mM KCl, 1.5 mM $CaCl_2$, 0.1 mM EDTA, 10 mM glucose, 225 mM sucrose and 45 mMTris–HCl buffer, pH 8.0, in a final volume of 200 µl as described in a previous work from our laboratory [30]. Twenty microliters of enzyme preparation (8–12 µg of protein) was added to the reaction mixture and pre-incubated at 37 °C for 10 min. The reaction was initiated by the addition of ATP or ADP to obtain a final concentration of 1.0 mM and incubation proceed for 20 min in either case. 5′-NT activity was determined essentially by the method of Heymann et al. [31] in a reaction medium containing 10 mM $MgSO_4$ and 100 mM Tris–HCl buffer, pH 7.5, in a final volume of 200 µl. Twenty microliters of enzyme preparation (8–12 µg of protein) was added to the reaction mixture and pre-incubated at 37 °C for 10 min. The reaction was initiated by the addition of AMP to a final concentration of 2.0 mM and preceded for 20 min. In all cases, the reaction was stopped by the addition of 200 µl of 10 % trichloroacetic acid (TCA) to obtain a final concentration of 5 %. Following, the tubes were chilled on ice for 10 min. The released inorganic phosphate (Pi) was assayed by the method of Chan et al. [32], using malachite green as colorimetric reagent and KH_2PO_4 as standard. Controls were carried out by adding the synaptosomal fraction after TCA addition to correct for non-enzymatic nucleotide hydrolysis. All samples were run in triplicate. Enzyme activities are reported as nmol Pi released/min/mg of protein.

ADA Activity

ADA activities were estimated spectrophotometrically previously described by Giusti et al. [33] with modifications, which is based on the direct measurements of the formation of ammonia, produced when ADA acts in excess of adenosine. The sample was added to the reaction mixture containing 50 mM sodium phosphate buffer (pH 6.5). The samples were incubated at 37 °C for 1 h and the reaction was started by the addition of the substrate (adenosine). The reaction was stopped by adding phenol-nitroprusside. The reaction mixtures were immediately mixed to alkaline-hypochlorite and vortexed. Ammonium sulphate was used as ammonium standard. The values were expressed as U/mg of protein.

Na^+,K^+-ATPase Activity

Na^+,K^+-ATPase activity was measured as previously described [34] with minor modifications. Briefly, the assay medium consisted of (in mM) 30 Tris–HCl buffer (pH 7.4), 0.1 EDTA, 50 NaCl, 5 KCl, 6 $MgCl_2$ and 50 µg of protein in the presence or absence of ouabain (1 mM), in a final volume of 350 µl. The reaction was started by the addition of adenosine triphosphate to a final concentration of 3 mM. After 30 min at 37 °C, the reaction was stopped by the addition of 70 µl of 50 % (w/v) TCA. Saturating substrate concentrations were used, and the reaction was linear with protein and time. Appropriate controls were included in the assays for non-enzymatic hydrolysis of ATP. The amount of inorganic phosphate (Pi) released was quantified colorimetrically, as previously described [35], using NaH_2PO_4 as reference standard. Specific Na^+,K^+-ATPase activity was calculated by subtracting the ouabain-insensitive activity from the overall activity (in the absence of ouabain) and expressed in nmol of Pi/min/mg of protein.

ADK Expression by Western Blot Assay

Frozen tissue was placed in RadioImmunoprecipitation-Assay (RIPA) buffer (50 mM Tris, 1 mM EDTA, 150 mM NaCl 0, 1 % SDS, 1 % NP 40, pH 8) supplemented with protease inhibitors (ROCHE) and homogenized. The volume of the suspension was completed with 300 µl of RIPA solution and centrifuged at 1,000×*g* during 10 min at 4 °C.

After protein quantification the appropriate volume of each sample was diluted in four volumes of water and one volume of sample buffer (350 mM Tris pH 6.8, 30 % glycerol, 10 SDS, 600 mM DTT and 0,012 % Bromophenol blue). Prior to loading, the samples were denatured at 95 °C for 15 min. The samples and the molecular weight marker were separated by

SDS-PAGE (10 % according to the protein molecular weight and a 5 % stacking) in denaturing conditions and electro-transferred to PVDF membranes (Millipore). Membranes were blocked with 5 % non-fat dry milk for 1 h and a half, washed with TBS-T 0.1 % (Tris buffer saline solution, 200 nM Tris, 1.5 M NaCl with 0.1 % Tween-20), and incubated with primary antibody overnight at 4 °C. After washing again for 30 min, the membranes were incubated with secondary antibody for 1 h at room temperature. After 40 min of washing with TBS-T, chemoluminescent detection was performed with ECL-PLUS western blot detection reagent (GE Healthcare) using X-ray films (Fujifilm). Optical density was determined with Image-J software and normalized to the respective α-tubulin band density.

Lipid Peroxidation

The cerebral cortex was homogenized in 8 volumes of 10 mM Tris–HCl buffer solution pH 7.4. The homogenate was centrifuged at 1,000 × *g* for about 10 min. Thiobarbituric acid reactive species (TBARS) levels were determined by a modification of the method of Buege and Aust [36]. In brief, 250 ml of cerebral cortex homogenate was mixed thoroughly with 500 ml of a stock solution of 10 % (w/v) trichloroacetic acid and 750 ml of thiobarbituric acid. The mixture was heated for 15 min in a boiling water bath. After cooling, the red pigment produced was extracted with 1.5 ml of n-butanol and measured in absorbance at 535 nm.

Protein Determination

Protein was measured by the Coomassie blue method according to Bradford [37] using serum albumin as standard.

Statistical Analysis

The statistical analysis was performed using one-way ANOVA, followed by Duncan's multiple range tests. All data were expressed as mean ± SEM. The correlations were assessed by Pearson rank correlation coefficient. Differences were considered significant when the probability was $p \leq 0.05$. All the statistical analyses were conducted using GraphPad Prism version 5.00 for Windows (GraphPad Software, San Diego, CA, USA).

Results

Neonatal HI Alters Cytosolic NTPDase but not Ecto-NTPDase Activity

As shown in Fig. 1, neonatal HI led to selective and fast changes in nucleotidase activity in the brain. Thus, ATP and ADP hydrolysis increased only in the left hemisphere of the HI group when compared with the control group and contralateral hemisphere ($p < 0.05$ and $p < 0.01$, respectively) (Fig. 1a), allowing to conclude that cytosolic NTPDase activity was altered immediately after HI. No significant changes in ATP (control left = 79.57 ± 10.76; HI left = 90.64 ± 7.75) and ADP (control left = 33.65 ± 2.50; HI left = 39.74 ± 3.88) hydrolysis were observed in the synaptosomal fraction.

Effects of HI on Cytosolic 5′-NT and Ecto-5′-NT Activities

The cytosolic 5'-NT activity is shown in Fig. 1b. Post hoc analysis revealed that AMP hydrolysis was significantly increased in the left hemisphere of the HI group in cytosolic fraction when compared with the control group ($p < 0.05$). No significant changes in AMP hydrolysis in cerebral cortex synaptosomes were observed (control left = 17.19 ± 2.64; HI left = 15.65 ± 1.96).

Neonatal HI on Cytosolic ADA and Ecto-ADA Activities

ADA activity in synaptosomes (Fig. 2) was significantly increased in the left hemisphere of the HI group when

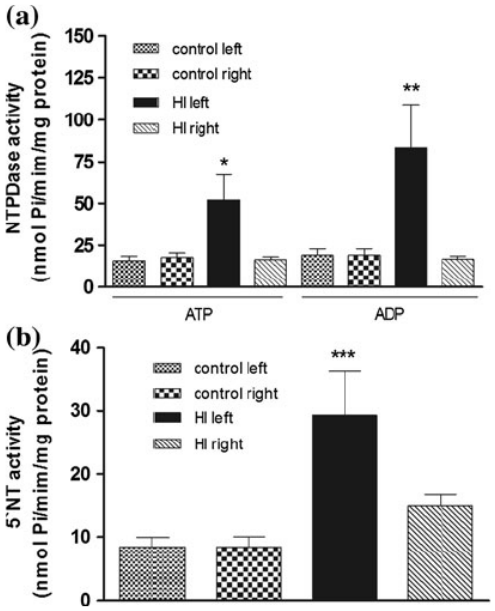

Fig. 1 The NTPDase and 5′-NT activities in cytosolic fraction of cerebral cortex immediately after neonatal HI. **a** NTPDase activity (ATP and ADP hydrolysis) and **b** 5′-NT activity (AMP hydrolysis). Statistically significant differences from controls, as determined by one-way ANOVA for multiple group comparison. Post hoc analysis was carried out by Duncan's multiple range test. *Bars* represent the mean ± SEM. The symbol (*) indicates significantly difference when compared to the control group (*$p < 0.05$; **$p < 0.01$; *** $p < 0.001$)

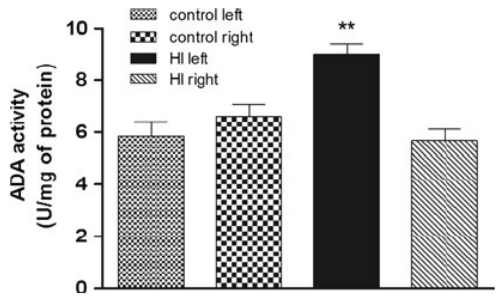

Fig. 2 The ecto-ADA activity in synaptosomes of cerebral cortex immediately after neonatal HI was evaluated by adenosine hydrolysis. Statistically significant differences from controls, as determined by one-way ANOVA for multiple group comparison. Post hoc analysis was carried out by Duncan's multiple range test. *Bars* represent the mean ± S.E.M. The symbol (*) indicates significantly difference when compared to the control group (** $p < 0.01$)

compared with the control group ($p < 0.01$). On the other hand, no significant changes in ADA activity were observed in the cytosolic fraction (control left = 13.67 ± 0.94; HI left = 13.69 ± 0.54).

Effects of HI on Na^+,K^+-ATPase Activity

In synaptosomes of cerebral cortex (Fig. 3), Na^+,K^+-ATPase activity was significantly reduced immediately after HI insult in the left hemisphere of HI group when compared with the control group ($p < 0.001$).

ADK Expression After Neonatal HI

ADK protein levels were quantified in the cerebral cortex of rats immediately after HI injury. We did not observe changes in the ADK protein levels immediately after HI (Fig. 4).

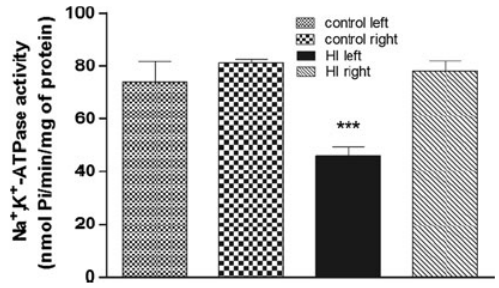

Fig. 3 Na^+, K^+–ATPase activity in synaptosomes of cerebral cortex of neonatal rats submitted to neonatal HI. Data are expressed as mean ± SEM. Statistically significant differences from controls, as determined by two-way ANOVA for multiple group comparison. Post hoc analysis was carried out by Duncan's multiple range test. *Bars* represent the mean ± SEM. The symbol (*) indicates significantly difference when compared to the control group (***$p < 0.001$)

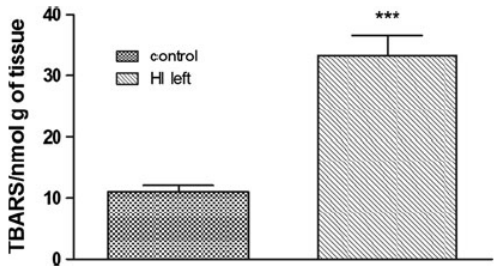

Fig. 4 TBARS levels in the cerebral cortex of rats immediately after neonatal HI. Statistically significant differences from controls, as determined by Student's *t* test. *Bars* represent the mean ± S.E.M. The symbol (*) indicates significantly difference when compared to the control group (***$p < 0.001$)

Lipid Peroxidation

To evaluate whether the decreased Na^+,K^+-ATPase activity immediately after HI coincided with the lipid peroxidation, we measured the TBARS levels in the cerebral cortex. The results obtained are presented in Fig. 4. Cerebral cortex homogenates of the HI group presented TBARS values that were significantly higher than the control group immediately after neonatal HI ($p < 0.001$).

Correlations between cytosolic NTPDase (ATP hydrolysis) vs Na^+,K^+-ATPase Activities and Cytosolic NTPDase (ATP hydrolysis) versus TBARS Levels

Experimental data demonstrated some correlations between the parameters analyzed in cerebral cortex immediately after neonatal HI. Interestingly, we observed a negative correlation between (Fig. 5a) NTPDase (ATP hydrolysis) and Na^+,K^+-ATPase activity ($p = 0.0016$; $r = -0.7401$), (Fig. 5b) TBARS levels and Na^+,K^+-ATPase activity in cerebral cortex ($p = 0.0001$; $r = -0.8282$). The data of Fig. 5A are derived from Fig. 1a (NTPDase—ATP hydrolysis) and Fig. 3. The data of Fig. 5b are derived from Figs. 3 4. Each individual point on the graph represents the relationship between two variables analyzed in the same animal.

Discussion

The cerebral response to HI can be acute or chronic, and it is characterized by rapid energetic depletion in which several mechanisms can contribute to the progression of the insult. Purine compounds such as ATP and adenosine are known to accumulate in the extracellular space and to elicit various cellular responses during HI. In order to evaluate the role of the enzymes responsible for modulating the levels of these nucleotides and nucleosides of adenine, we investigated the effect of HI on the NTPDase,

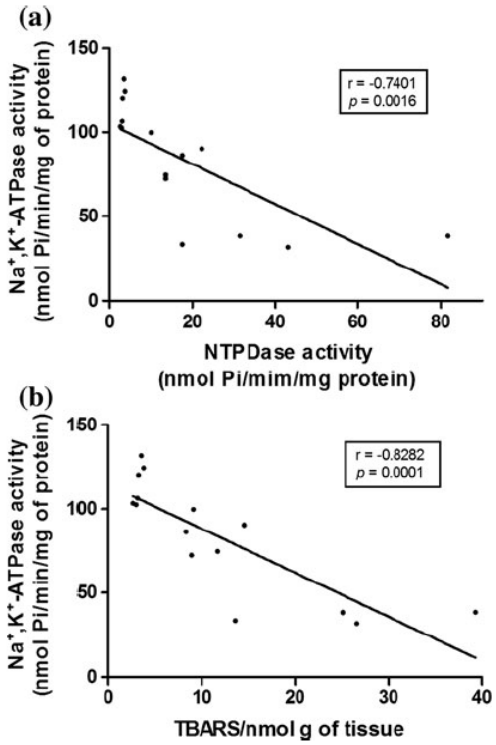

Fig. 5 Correlation between enzymatic activities and TBARS levels in the cerebral cortex after neonatal HI. Significant correlations between **a** cytosolic NTPDase (ATP hydrolysis) *vs* Na^+, K^+–ATPase activity ($p = 0.0016$; $r = -0.7401$) and **b** TBARS levels *vs* Na^+, K^+–ATPase activity ($p = 0.0001$; $r = -0.8282$). The Pearson's correlation coefficient was determined by using GraphPad Prism software. Differences were considered significant when the probability was $p \leq 0.05$

5′-NT and ADA activities in cytosolic and synaptosomal fractions from cerebral cortex of rats immediately after insult. Furthermore, Na^+,K^+-ATPase activity, ADK expression, and TBARS levels were evaluated. The data obtained in this study clearly indicate that immediately after HI there is a significant elevation of adenine nucleotide (ATP, ADP and AMP) hydrolysis but not of adenosine in the cytosolic fraction. This suggests that NTPDase and 5′-NT in the cytosol is involved in the degradation of ATP during HI, therefore promoting adenosine formation eventually as an adaptation to protect the tissue against excitotoxicity induced by neonatal HI.

In synaptosomes, no alterations were found in the ecto-nucleotidase activities immediately after insult, in line with previous indications that release of ATP may not contribute substantially to the adenosine concentration in the extracellular milieu during ischemia [38, 39]. Indeed, the increase in extracellular adenosine concentration during the early period after HI insult may be primarily caused by the impairment of energetic balance and consequently degradation of cytoplasmic ATP to adenosine [15, 38, 39]. Accordingly, a preferential release of adenosine *per se* instead of ATP has been described in several experimental preparations submitted to depolarizing stimuli (K^+, veratridine and electrical stimulation), glutamate, and ischemic conditions [40–42]. It is therefore not surprising that HI did not induce alterations in the ecto-nucleotidase activities. Since no measurable alterations in the cytosolic ADA activity or in the ADK expression were detected immediately after HI, the increase in ATP, ADP and AMP hydrolysis in the cytoplasm likely leads to accumulation of intracellular adenosine, therefore favouring its transport into the extracellular space through bi-directional transporters. To agree, several authors have described that the appearance of adenosine in the extracellular media is inhibited by the equilibrative nucleoside transport inhibitor, indicating that adenosine is formed intracellularly during these ATP-depleting conditions, and released from cells via a nucleoside transport system [43–45]. This suggests that modulation of the adenosine flux through nucleoside transporters could also be a potential target in the hypoxic-ischemic conditions.

Adenosine accumulates in the extracellular space during ischemia [15, 36], and by activating inhibitory adenosine A_1 receptors, protects from excessive neuronal excitation [46]. In the brain, the inactivation of extracellular adenosine is mediated by ecto-ADA, which catalyzes the deamination of adenosine to inosine. Indeed, Frenguelli et al. [47] have showed that most of the extracellular inosine accumulation after a hypoxic insult delivered to rat hippocampal slices was due to the extracellular degradation of adenosine by ecto-ADA. In addition, adenosine can be taken up by the cell membrane of neurones or neighbouring cells, and then be phosphorylated into AMP. As we now show, ecto-ADA activity is increased immediately after HI. The up-regulation of the ecto-ADA activity may therefore impair adenosine-mediated neuroprotection by promoting a decrease in adenosine availability at the synaptic cleft. So, our data suggest that inhibition of ecto-ADA may be beneficial in extending adenosine-mediated neuroprotection during the early moments after neonatal HI. Several authors have demonstrated that the use of inhibitors of ADA increases brain extracellular concentrations of adenosine after hypoxia or ischemia [48, 49]. Our study is the first to show that the ecto-ADA activity increases after HI condition, prompting a rational for its inhibition as a therapeutic strategy to maximize the endogenous self-protecting capability of the brain after an injury. Our results therefore open up a research avenue on the putative role of ecto-ADA and ecto-ADA inhibitors in the adenosine metabolism during pathological conditions.

It is however worthwhile to note that inosine, the major degradation product of adenosine, may accumulate during

ischemia and elicit protective effects [50]. Thus, the immediate increase of intracellular ATP, ADP and AMP hydrolysis after the HI insult is most probably the starting of a protective cascade leading to an increase in the extracellular adenosine levels; adenosine can be protective or be deaminated into inosine, a process favoured by the HI-induced upregulation of ecto-ADA activity. Further studies are required to figure out the relative importance of adenosine- *vs* inosine- mediated neuroprotection to predict the neuroprotective potential of ecto-ADA inhibitors after ischemic insults.

The increase in the NTPDase and 5′-NT activities could exacerbate the depletion of ATP leading to cellular dysfunctions such as perturbations in the active transport of sodium and potassium (as we now show) and, consequently, cellular death [51]. It is known that the brain tissue O_2 stores are so small that they sustain normal energy consumption for just a few seconds. During the HI insult, a reduction in the cerebral blood flow results in a precipitous decrease in tissue O_2 and glucose, which are absolutely essential for the maintenance of cellular ATP levels [52]. Na^+,K^+-ATPase is a crucial enzyme responsible for the homeostasis of osmotic pressure, cell volume, and the maintenance of electrochemical gradients, which are prerequisite for neuronal activity and survival. This enzyme uses the energy of ATP to maintain the transmembrane ionic gradient. Thus, the suppression of oxidative phosphorylation is largely linked to a rapid suppression of the Na^+,K^+-ATPase activity in neuronal cells. Furthermore, the hypoxia and/or ischemia may induce Na^+,K^+-ATPase inhibition through the suppression of oxidative phosphorylation and release of endogenous inhibitors [53]. The decrease in enzymatic activity does not appear to be related to the expression of the enzyme since studies in models of focal cerebral ischemia and HI have shown Na^+,K^+-ATPase inhibition without alterations in the expression of any of the enzyme isoforms [54, 55]. Furthermore, the decrease Na^+,K^+-ATPase here reported may be a result of oxidative damage, which has been extensively reported in several tissues during HI event [56–58]. Indeed, it is known that during oxidative stress the increase of lipid peroxidation can participate in the inhibition of Na^+,K^+-ATPase activity by modifying specific active sites [59]. Accordingly, we found a negative correlation between Na^+,K^+-ATPase activity and lipoperoxidation as well as between Na^+,K^+-ATPase and NTPDase activities, which suggests that increasing the production of free radicals associated with increases in the ATP hydrolysis immediately after reperfusion may be related to the currently reported reduction of Na^+,K^+-ATPase activity. Thus, increased lipid peroxidation observed here could be a contributing factor for neuronal injury leading to delayed neuronal death after neonatal HI.

Conclusion

This is the first study assessing the activity of enzymes that hydrolyze nucleotides and nucleosides of adenine in different fractions of cerebral cortex immediately after HI injury. We show that HI leads to a change in intracellular adenine nucleotide hydrolysis immediately after the insult, indicating that there is a coordinate alteration in enzymatic activities during early stages after neonatal HI in order to increase the adenosine levels. However, the increase of ecto-ADA activity could impair the adenosine signaling after the insult. Furthermore, this study demonstrates a rapid suppression of Na^+,K^+-ATPase correlating with increased lipid peroxidation and NTPDase alteration in the cerebral cortex after HI insult, which suggest that lipid peroxidation, in association with the increase in the NPTDase activity can contribute to the inhibition of Na^+,K^+-ATPase activity immediately after HI event.

Our findings provide a new insight about the mechanisms involved immediately after HI insult. The understanding of these early pathological processes is critical for the future identification of neuroprotection strategies that may be beneficial for newborns that have experienced HI episodes.

Acknowledgments This work was supported by the Fundação Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES), INCT for Excitotoxicity and Neuroprotection and FINEP research grant “Rede Instituto Brasileiro de Neurociência (IBN-Net). Work in Lisbon was supported by Fundação para a Ciência e Tecnologia.

Conflict of interest The authors have declared that there is no conflict of interest.

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3.2. MANUSCRITO I

Neuroinflammation after neonatal hypoxia-ischemia is associated with alterations in the purinergic system: Adenosine deaminase 1 isoenzyme is the most predominant after insult

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Abstract - Hypoxic-ischemic (HI) injury perinatal brain is a major contributor to morbidity and mortality to infants and children. Adenosine may play a role in the pathophysiology of HI, since it modulates the inflammatory process and the release of several neurotransmitters. Thus, the aim of this study was to identify the isoforms of ADA responsible for the enzymatic activity as well as the adenosine kinase (ADK) and A1 adenosine receptor (A1R) expression in the cerebral cortex eight days after HI. Myeloperoxidase (MPO) and N-acetyl-glucosaminidase (NAG) were assessed as inflammation markers. ADA activity was analyzed, in the presence or absence of a specific ADA1 inhibitor, erythro-9-(2-hydroxy-3-nonyl) adenine (EHNA). The ADA1 activity (92.6%) was significantly higher than ADA2 (7.4%) activity in the cerebral cortex eight days after HI. A1Rs and ADK protein expression showed decreased 8 days after insult. Interestingly, the ADA1, MPO and NAG activities were correlated positively. In view of this, we conclude that the inhibitor of ADA1, *in vitro* conditions, was effective in decreasing the ADA activity, and that mainly ADA1 isoform is responsible for the increase in the ADA activity eight days after HI insult. Therefore, HI neonatal was able to alter the ADK and A1R expression. Thus, due to the importance of adenosine signaling in the regulation of inflammatory and immune process and the crucial role of ADA in the postischemic homeostase of adenosine as well as during inflammatory process, we suggest that ADA1 inhibitors may play an important role in the regulation of events that follow the HI insult, favoring the increase in the adenosine in the sites of tissue injury. Together, these results may help the understanding of the purinergic signaling involvement in the pathophysiology of HI neonatal.

Keywords: Hypoxia ischemia; cerebral cortex; adenosine deaminase; isoenzymes; adenosine receptors; inflammation markers

Abbreviations

HI, hypoxia-ischemia; ADA, adenosine deaminase; ADK, adenosine kinase; A1R, adenosine A1 receptor; MPO, myeloperoxidase; NAG, N-acetyl-glucosaminidase; EHNA, erythro-9-(2-hydroxy-3-nonyl) adenine.

INTRODUCTION

Hypoxic-ischemic (HI) brain injury is a significant cause of mortality and morbidity during the perinatal period and causes long-term neurological disorders such as motor and learning disabilities, cerebral palsy, epilepsy and seizures even death (Vannucci and Hagberg, 2004; Ferriero, 2004; Nelson and Lynch, 2004; Derrick et al., 2007). The HI brain injury is not a static process that is complete at the time of insult. Several types of processes are involved at different stages of the injury, which include inflammation, oxidative stress, neuronal production of nitric oxide and excitotoxicity (Berger and Garnier, 2000; Moretto et al., 2005; Quiniou et al., 2008). However, despite the efforts to understand the mechanisms involved in neonatal HI, it is not yet clear.

Because of the rapid energy depletion in HI insult, the adenosine levels in the brain increase dramatically. The adenosine accumulates during HI conditions has several functions within the central nervous system (CNS). Adenosine may be formed intracellularly in the CNS from degradation of ATP by nucleotidases (Richardson et al., 1987; Latini and Pedata, 2008; Dale and Frenguelli, 2009). After its formation, adenosine may be released from cells via specific bi-directional transporters, which then acts on adenosine receptors to elicit responses as excitatory and inhibitory effects on neurotransmission. Furthermore, it is well-established that adenosine generated at sites of tissue injury serves also to regulate inflammation and damage by engaging cell surface adenosine receptors (A1R, A2AR, A2BR, and A3R). However, the effect of adenosine in the context of inflammatory events can be beneficial or harmful, depending on receptor expression, cell-signaling pathways activated by adenosine receptors, local adenosine concentrations, and the conditions studied (Fredholm et al., 2001; Hasko and

Cronstein, 2004; Jacobson and Gao, 2006; Abbracchio and Ceruti, 2007). Substantial experimental data suggest that adenosine serves mainly as an anti-inflammatory agent following injury; however, if inflammation progresses unresolved and becomes chronic in nature, excessive tissue damage and adenosine accumulation may activate exacerbation pathways (Blackburn, 2003).

In the brain, the inactivation of adenosine is mediated by adenosine deaminase (ADA, EC 3.5.4.4), which catalyzes the deamination of adenosine to inosine and 2-deoxyadenosine to 2`-deoxyinosine. This enzyme is widely distributed in vertebrate tissues and plays a critical role in a number of physiological systems (Sharoyan et al., 2006; Franco et al., 2007). ADA is mainly a cytosolic enzyme, however, its presence has been established also on the surface of synaptosomes and neurons. Because ADA is co-localized with adenosine A1 (Saura et al., 1998) and A2B (Herrera et al., 2001) receptors, adenosine cleavage at synaptic cleft is crucial for controlling P1 signaling. On the other hand, the extracellular adenosine might be uptake across the cell membrane of neurons or neighboring cells and then to be phosphorylated to adenosine 5'-monophosphate (AMP) by adenosine kinase (ADK, EC 2.7.1.20). In this way, by modulation of adenosine levels, the ADA and ADK play an important role in multiple processes such as inflammatory and immune response.

The myeloperoxidase (MPO) and the N-acetylglucosaminidase (NAG) are two enzymes that have been used as markers of neutrophil and macrophage accumulation, respectively (Xavier et al., 2010). MPO, one of the most abundant enzymes secreted by inflammatory cells (Bradley et al., 1982; Nagra et al., 1997), contributes to host defense via the production of the powerful oxidant hypochlorous

acid (Klebanoff, 1970). In addition, it has been reported that MPO enhances the binding of leukocytes including monocytes and neutrophils to the endothelium. Therefore, MPO participates in the recruitment of cells into an area of inflammation (Johansson et al., 1997). MPO can be used in conjunction with NAG as markers of neutrophil and macrophage accumulation, respectively (Xavier et al., 2010). In this line, the ADA activity has been also described as an inflammatory marker.

Recently, we have reported an increase in the ADA activity associated with strong inflammatory process (reactive gliosis and infiltration of leucocytes into the ischemic area) in the cerebral cortex 8 days after HI insult (Pimentel et al., 2009). Therefore, in view that the effect of adenosine in the context of inflammatory events can be beneficial or harmful, depending on adenosine receptor expression and local adenosine concentrations, this study goes forward in terms to examining in the cerebral cortex 8 days after neonatal HI: 1) the isoforms of ADA responsible for the enzymatic activity; 2) A1R and ADK protein expression; 3) the MPO and NAG activities.

EXPERIMENTAL PROCEDURES

Animals

Wistar dams and their pups were housed in animal facilities maintained at 22 °C and on a 12 h light/dark cycle with food and water accessible ad libitum. All procedures for the animal studies were approved by the Animal Care and Use Committee of the University of Santa Maria. The number of animals used and their suffering were minimized.

HI animal model

Seven-day-old male Wistar rats, weighing 14-16 g were randomly divided into 2 groups: a control group (without ischemia and hypoxia; n=8) and hypoxia/ischemia (HI; n=8). Brain HI was produced by a combination of unilateral occlusion of the common carotid artery with exposure to a hypoxic atmosphere (8% O_2). Briefly, the animals were anesthetized with halothane. The left common carotid artery was exposed and ligated permanently with surgical silk thread. The incision was sutured and the pups were returned to their dams. Following 2 h of recovery, the pups were placed into a 1500 ml chamber and exposed to an 8% oxygen-92% nitrogen atmosphere delivered at 5 l/min for 1.5 h, with the chamber partially immersed in a 37 °C water bath to maintain a constant thermal environment. After the period of HI, the pups were then returned to their dams. The cerebral cortex was collected 8 days after HI event.

Tissue processing

The pups were euthanized and their brains were promptly removed and the cerebral cortex of all rats was carefully separated. In brief, firstly the tissue was homogenized in 10 volumes of 50 mmol/l phosphate buffer pH 7.0, centrifuged (30 min, 14000xg), and then the supernatant fraction was isolated (Mustafa and Tewari, 1970). All the procedures described above were performed at 0–4 °C. Homogenate were prepared fresh daily, maintained at 0–4 °C throughout the procedure and used for enzymatic assays.

For Western blotting assays, the cerebral cortex tissue homogenate was placed in RadioImmunoprecipitation-Assay (RIPA) buffer (50 mM Tris, 1 mM EDTA, 150 mM NaCl 0,1% SDS, 1% NP 40, pH 8) supplemented with protease inhibitors

(ROCHE) and homogenized in a sonicator. The volume of the suspension was completed to 300 µl to 750 µl with RIPA solution and centrifuged at 1000xg during 10 minutes at 4ºC. The supernatant was collected and corresponds to the whole tissue homogenate. Protein was quantified according to Bradford (1976) using the BioRad Protein assay kit. This sample was used for relative quantification of all proteins.

Enzyme assays

ADA activity

ADA activities were estimated spectrophotometrically as previously described by Giusti et al. (1974) with modifications, which is based on the direct measurements of the formation of ammonia, produced when ADA acts in excess of adenosine. The sample was added to the reaction mixture containing 50 mM sodium phosphate buffer (pH 6.5). The samples were incubated at 37 °C for 1 h and the reaction was started by the addition of the substrate (adenosine). The reaction was stopped by adding phenol-nitroprusside. The reaction mixtures were immediately mixed to alkaline-hypochlorite and vortexed. Ammonium sulphate was used as ammonium standard.

To investigate the isoforms of ADA the samples were incubated directly with or without erythro-9-(2-hydroxy-3-nonyl) adenine (EHNA 100 µmol). EHNA was evaluated for their in vitro ability to inhibit the isoform 1 of ADA. ADA1 activity was determined by subtracting ADA2 from total ADA activity and expressed in % of inhibition. The values were expressed as U/mg of protein for ADA. All experiments were performed in triplicate and the mean was used for calculation.

MPO and NAG activities

The enzyme activity of tissue of NAG and MPO were assessed according Lloret and Moreno (1995) and Suzuki et al. (1983). Enzyme activity was determined using a Micro plate reader. The results were expressed as optical density (OD)/g of tissue.

Western blot assay

After protein quantification the appropriate volume of each sample was diluted in four volumes of water and one volume of sample buffer (350 mM Tris pH 6.8, 30% glycerol, 10% SDS, 600 mM DTT and 0,012% Bromophenol blue). Prior to loading, the samples were denatured either at 60ºC for 15 minutes. The samples and the molecular weight marker were separated by SDS-PAGE (10%) in denaturing conditions and electro-transferred to PVDF membranes (Millipore). The percentage of resolving gels and protein loading amounts are summarized in table 1. Membranes were blocked with 5% non-fat dry milk for 1 hour and a half, washed with TBS-T 0.1% (Tris buffer saline solution, 200 nM Tris, 1.5 M NaCl with 0.1% Tween-20) and incubated with primary antibody overnight at 4ºC (anti-α-tubulin -1:5000; anti-A1 adenosine receptor- 1:1000; anti-ADK – 1:7500). After washing again for 30 minutes, the membranes were incubated with secondary antibody for 1 hour at room temperature (primary and secondary antibody dilution are in table 1). After 40 minutes of washing with TBS-T, chemoluminescent detection was performed with ECL-PLUS western blot detection reagent (GE Healthcare) using X-Ray films (Fujifilm). Optical density was determined with Image-J software and normalized to the respective α-tubulin band density. All the antibodies were purchased from Santa Cruz Biotechnology.

Table 1: Primary and secondary antibodies and related conditions used in the Western blot experiments for individual proteins. All primary antibodies were diluted in 3% Bovine Serum Albumin with 0.1% NaN3 and secondary antibodies in 5% non-fat dry milk.

Protein	Protein loading (µg)	Resolving gel %	Animal	Primary antibody	Secondary antibody	Dilution
ADK	45	10	Rabbit	1:7500	Sta. Cruz Biotechnology (goat anti-rabbit)	1:10000
A1R	45	10	Rabbit	1:1000		1:7500
α-tubulin	45	10	Rabbit	1:5000		1:10000

Protein determination

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

Statistical analysis

The statistical analysis was performed using one-way ANOVA, followed by Duncan's multiple range tests. All data were expressed as mean ± SEM. The correlations were assessed by Pearson rank correlation coefficient. Differences were considered significant when the probability was $P \leq 0.05$. All the statistical analyses were conducted using GraphPad Prism version 5.00 for Windows (GraphPad Software, San Diego, CA, USA).

RESULTS

The ADA1 is the dominant isoenzyme responsible for the ADA activity after HI neonatal

Experimental data demonstrated an increase in ADA activity in the cerebral cortex in the HI group when compared to control group (Fig. 1A). However, when cerebral cortex was incubated with EHNA, we observed 7.4% of ADA2 activity. This result indicates that in the presence of EHNA, ADA1 activity was 92.6% (P<0.001)(Fig. 1B).

Effect of neonatal HI on A1R and ADK protein expression in cerebral cortex

The protein expression of A1R and ADK were detected in cerebral cortex 8 days after HI insult. Significant decrease in densitometry of the A1R immunoreactive band of 37 kDa was observed when compared the HI group with the control group (P<0.01) (Fig. 2A). Following, significant decrease in ADK immunoreactive band of 40 kDa was observed (P<0.05)(Fig. 2B).

MPO and NAG enzymatic activities

The measurement of NAG and MPO activity was shown to be significantly increasing in the left hemisphere when compared to the control and to other groups (Fig. 3) (P<0.001).

Correlations between enzymatic activities after neonatal HI

Experimental data demonstrated some correlations between the enzymatic activities analyzed in cerebral cortex immediately after neonatal HI. Interestingly, we observed a positive correlation between MPO *vs.* NAG activity (P = 0.0011; r = 0.8687) (Fig. 4). The same was observed between ADA *vs.* MPO activity (P = 0.006; r = 0.8401), ADA *vs.* NAG activity in cerebral cortex (P = 0.0001; r = 0.9429)(Table 2).

DISCUSSION

In the last years, the purinergic system has been emerging as an attractive therapeutic target for modulating brain injury in a variety of animal models of neurological disorders (Burnstock, 2007). Adenosine is a ubiquitous signaling nucleoside that is rapidly generated as a net result of ATP catabolism that occurs in situations of cellular stress or damage. Once generated, adenosine can influence a diverse array of cellular functions through the activation of the adenosine receptors, followed by either phosphorylation to AMP by ADK or deamination to inosine by ADA.

Adenosine levels are known to increase in tissues as a result of ischemia-reperfusion injury, hypoxia, inflammation and cellular stress (Pearson et al., 2003; Latini and Pedata, 2008; Dale and Frenguelli, 2009). However, the changes in the concentrations of adenosine and its metabolites inosine and hypoxanthine during complete ischemia depend on the ADA activity in each brain region (Kobayashi et al., 1998). Two ADA isoenzymes are known as ADA1 and ADA2. We have recently shown that ADA activity is increased 8 days after neonatal HI. We now identify that ADA1 is the dominant isoenzyme for the ADA activity after HI injury. The identification of isoenzymes in this model reveals the important role of ADA1 after HI insult since this isoform displays higher affinity for adenosine than ADA2 isoform (Ungerer et al., 1992; van Ede et al., 2002). Considering that the ADA1 is the major enzyme responsible for the degradation of adenosine, the inhibition of its activity should represent one of the best ways to increase accumulation of adenosine in tissues under stress conditions. In the same way, the inhibition of ADA activity not only may increase the levels of adenosine within ischemic regions, but also to prevent the xanthine oxidase-mediated radical generation (Xia et al., 1996; Gervitz et

al., 2001). From a clinical perspective, it is plausible to suggest that the use of ADA1 inhibitors could represent a legitimate strategy to increase the adenosine levels favoring the antinflammatory and immunosuppressive effects of this nucleoside after HI brain injury.

It is known that the action of ADA is opposed by ADK which converts adenosine to AMP. In order to investigate the role of ADK in this time after HI insult, the expression of ADK was assessed. Data obtained in this study indicate that 8 days after HI there is a significant decrease of ADK protein expression in the cerebral cortex. Thus, based on the results above mentioned, it is plausible to suggest that in this period after neonatal HI, adenosine would be more deaminated and less phosphorylated. However, the increased deamination and decreased phosphorylation of adenosine could serve as an innate adaptation to protect the organism from potentially deleterious effects or long-term elevation of adenosine. Taking this into account, the ADA1 induction could confer increased resistance to adenosine toxicity upon the immune cells, mainly lymphocytes.

Likely, a close correlation has been found between the severity of inflammation and a local increase in both expression and activity of ADA (Conlon and Law, 2004; Desrosiers et al., 2007). Several studies have demonstrated that the release of ADA from leukocytes at the site of injury reflects the activation of lymphocytes (Blackburn et al., 1998). Accordingly, our group has also found an increase in the ADA activity 8 days after insult associated with a strong inflammatory process in the brain (glial fibrillar protein acid positive reactive, astrocytosis, astrogliosis, lymphocytic infiltration in the ischemic area and neuronal death) (Pimentel et al., 2009). We now assessed the MPO and NAG activities, which are two enzymes that have been used as markers of neutrophil and macrophage

infiltration, respectively (Werner and Szelenyi, 1992; Barcelos et al., 2004; Xavier et al., 2010). In agreement with our previous results, in this study we found an increase in the MPO and NAG, which suggests the presence of inflammatory cells in the cerebral cortex. MPO has been described as one of the most abundant enzymes secreted by inflammatory cells (Bradley et al., 1982), including neutrophils, macrophages and microglia (Nagra et al., 1997), enhancing the binding of leucocytes in the endothelium and the recruitment of immune cells into an area of inflammation (Johansson et al., 1997). We have also recently reported the association of MPO and NAG activity with the presence of macrophages in the hippocampus of neonate rats 8 days after HI insult (Pimentel et al., 2011). However, MPO and their derivative acids, hypochlorous and hypobromous, are also important oxidant *in vivo* and their action has been well documented. Nonetheless, MPO is capable of generating free radicals and thus can contribute to the oxidative stress in this time after HI insult. Accordingly, in a previous study of our group we have observed in the same time the increased lipid peroxidation in cerebral cortex (Pimentel et al., 2009). Interestingly, we found a positive correlation between MPO, NAG and ADA1 activities in the cerebral cortex. This finding helps to support the evidence that MPO, NAG and ADA are markers of the inflammatory process (Blackburn et al., 1998; Winterbourn et al., 2000; Fröde and Medeiros, 2001; Iqbal et al., 2008; Østerholt et al., 2012). Taken together, these results provide new tools for a better understanding of the immunoregulatory pathways that occur in inflammation.

It is however worthwhile to note that inosine, the major degradation product of adenosine, may accumulate during ischemia and elicit antinflammatory effects (Hasko et al., 2000; Hasko et al., 2004; Shen et al., 2005). Inosine is degraded further to the stable end-product uric acid, which also has anti-inflammatory

properties (Spitsin et al., 2001). Thus, the increase in the ADA1 activity could favor the increase of those anti-inflammatory molecules. Thus, further studies are required to figure out the relative importance of ADA1 inhibitors on the neuroinflammation induced by HI insult.

In the brain, the effects of adenosine are mediated predominantly through specific cell surface receptors, of which four subtypes (A1, A2A, A2B and A3) have been described. However, because of neuroprotector effects mediate by A1Rs, the role of these receptors in ischemic conditions has received considerably more attention. Data obtained in this study indicate that 8 days after HI there is a decrease in the A1R protein expression. It is known that immediately following the onset of harmful stimuli, the activation of A1Rs by adenosine exerts a potent, presynaptic, feedback-inhibitory effect on the release of injurious excitatory neurotransmitters, mainly glutamate (Johansson et al., 2001; Sebastiao et al., 2001; Wardas, 2002; Arrigoni et al., 2005). However, a more delayed protective pathway involving the isolation of the damaged tissue by an astrocytic scar and the potentiation of the astrocytic support of neurons has been described (De Mendonca et al., 2000). In this way, the abnormally increased extracellular levels of adenosine in the micromolar range moderate the excessive astrocyte proliferation through inhibitory signals mediated by A1Rs (Ciccarelli et al., 1994). This control is lost when, following the massive rise of extracellular purine levels, most of these compounds are metabolised and subsequently cleared. Thus, the astrocytes are free to proliferate in response to signals generated by the tissue damage (Ciccarelli et al., 2001). According to the abovementioned, our group has recently demonstrated a strong astrogliosis and astrocytosis in the cerebral cortex 8 days after HI insult. Thus, knowing that adenosine has an inhibitory effect on the astrocyte proliferation and it is mediate by

A1Rs, it is plausible to suggest that the increase of ADA1 activity and decrease in the A1Rs protein expression could be a collaborative mechanism in order to favor the tissue repair in the ischemic area after HI insult. However, it is important to mention that contradictory data about the role of A1Rs have been described (Rudolphi et al., 1992; Hallea et al., 1997; Jacobson et al., 1996; Turner et al., 2002; Turner et al., 2003) and that the adenosine may have both proinflammatory and antinflammatory functions depending on the subtype of adenosine receptor it activates, on the type of inflammatory cell involved, on the duration of neuroinflammation and on the stage of development (Dunwiddie and Masino, 2001; Farber and Kettenmann, 2006).

In the same way, several studies with animal models and humans, where a longlasting enhanced release of adenosine occurs, have demonstrated a long-term down-regulation of A1Rs in different brain regions (Lewin and Bleck, 1981; During and Spencer, 1992; Cunha, 2005). Likewise, other studies have shown that short periods of brain ischemia, which also trigger a robust increase in the extracellular levels of adenosine (Kobayashi and Millhor, 1999), produce a long-lasting decrease in the density of A1Rs in several brain regions. Thus, it has been suggested that the activation of A1Rs is an endogenous neuroprotective system; however, its usefulness is limited to acute noxious brain conditions (i.e., to control the onset or enhance the threshold of neuronal damage). In the same way, other authors suggest that neuronal A1R desensitization occurs in large time frames of exposure to exogenously added A1R agonists *in vitro* as well as *in vivo* (Cunha, 2005). Thus, the understanding of time course of modification of adenosine receptors expression in the brain as well as adenosine metabolism becomes particularly critical to understand the adenosine effects in this period after HI insult.

In view of our results, we suggest that A1Rs density is altered after HI insult in order to favor the repair of tissue damage. However, to clear these findings further studies are necessary.

CONCLUSSION

Taken together, these results suggest that there is highly complex interconnection between adenosine signaling pathways and the mechanism of modulation of this nucleoside level in the cerebral cortex 8 days after HI neonatal. Our data showed that late phase following neonatal HI leads to inflammatory response with increase in the ADA, MPO and NAG activities in the cerebral cortex, where ADA1 is the dominant isoenzyme for the ADA activity after neonatal HI. In this context, the inhibition of ADA1 activity could represent one of the best ways to increase the adenosine levels in this period after insult. Furthermore, this study demonstrated that HI insult implicates in alterations of A1Rs and ADK expression. By focusing on the study, we can provide information about the events involved during the evolution of brain lesion after HI event and thus contribute to the future investigation of new neuroprotective strategies.

Acknowledgments

This study was funded by the Research Foundation of the State of Rio Grande do Sul (FAPERGS—Fundação de Amparo à Pesquisa do Rio Grande do Sul) and supported by the Research and Graduate Provost Office (PRPPG—Pró Reitoria de Pesquisa- UFSM), Brazil.

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TABLE

Table 2. Pearson correlation between ADA1 activity *vs.* MPO activity and ADA1 activity *vs.* NAG activity in the cerebral cortex of neonate rats 8 days after HI injury.

	Parameter	Correlation Coefficient (r)	*P* Value
ADA1 activity *vs*.	MPO	0.8401	< 0.001
	NAG	0.9429	< 0.0001

LEGENDS

Figure 1. Effect of EHNA (100 µMol in vitro) on ADA activity in the cerebral cortex of rats 8 days after HI (only the left hemisphere). Each column represents the mean ± S.E.M. Post hoc analysis was carried out by Duncan's multiple range test. Bars represent the mean ± S.E.M. The symbol (*) indicates significant difference when compared to the control group (*** P<0.001). The symbol ($^{\#}$) indicates significant difference when compared to the HI left + EHNA 100 µMol group ($^{\#\#\#}$ P<0.001). Statistical differences among groups were determined by one-way ANOVA followed by Duncan's test.

Figure 2. Western blotting of A1R and ADK protein expression in the cerebral cortex tissue supernatants of rats 8 days after neonatal HI. (A) A1R (37 kDa), (B) ADK (40 kDa) and α-tubulin (55 kDa) immunoreactive bands. Bar graphs show a significant change in the A1Rs and ADK protein expression. Each point is the mean ± S.E.M of eight experiments. The symbol (*) indicates significant difference when compared to the control group (*P<0.05 and ** P<0.01).

Figure 3. The enzyme activity of NAG and MPO in the cerebral cortex 8 days after HI. Statistically significant different from controls, as determined by one-way ANOVA for multiple group comparison. Post hoc analysis was carried out by Duncan's multiple range test. Bars represent the mean ± S.E.M. The symbol (*) indicates significant difference when compared to the control group (* P<0.001).

Figure 4. Correlation between MPO and NAG activities in the cerebral cortex after neonatal HI. Significant correlations between MPO and NAG activities (P = 0.0011; r = 0.8687). The Pearson's correlation coefficient was determined by using GraphPad Prism software. Differences were considered significant when the probability was P≤0.05.

FIGURES

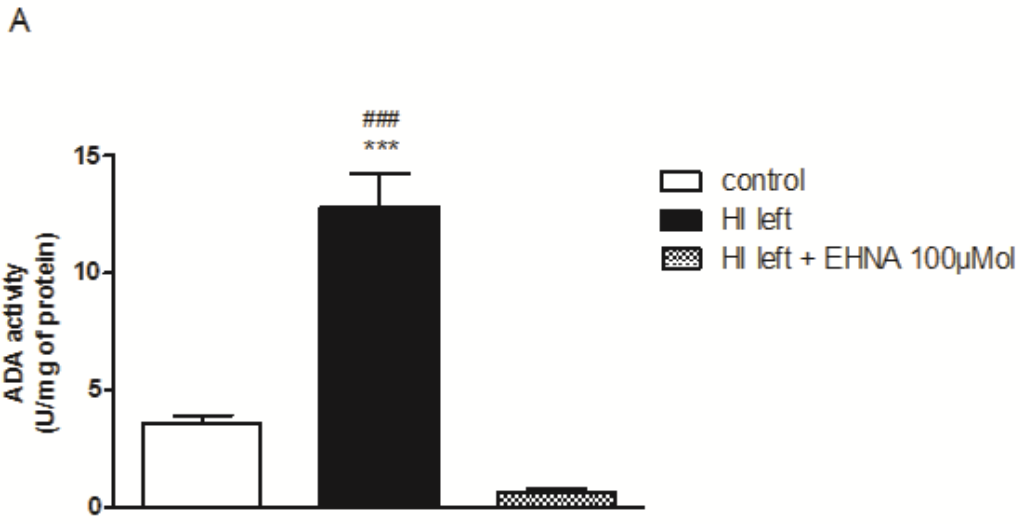

Fig 1.

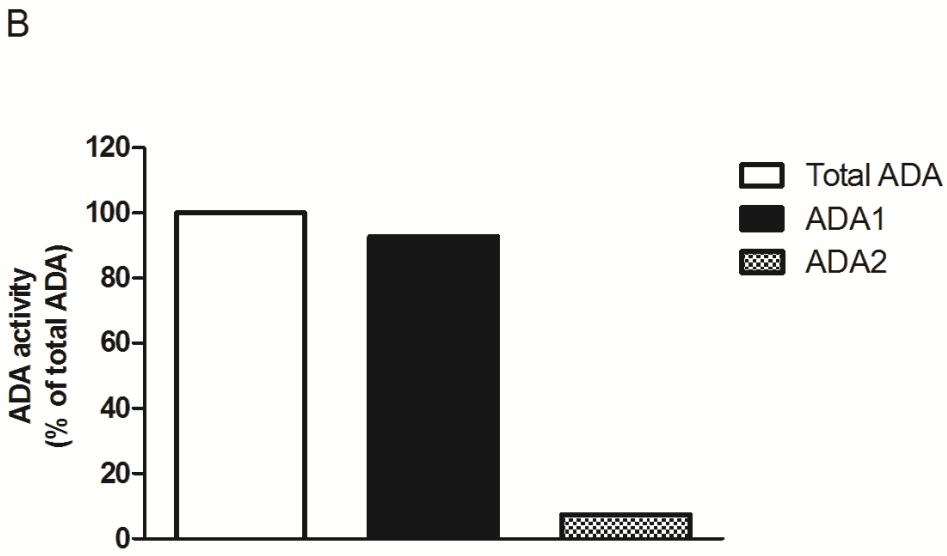

Fig 1.

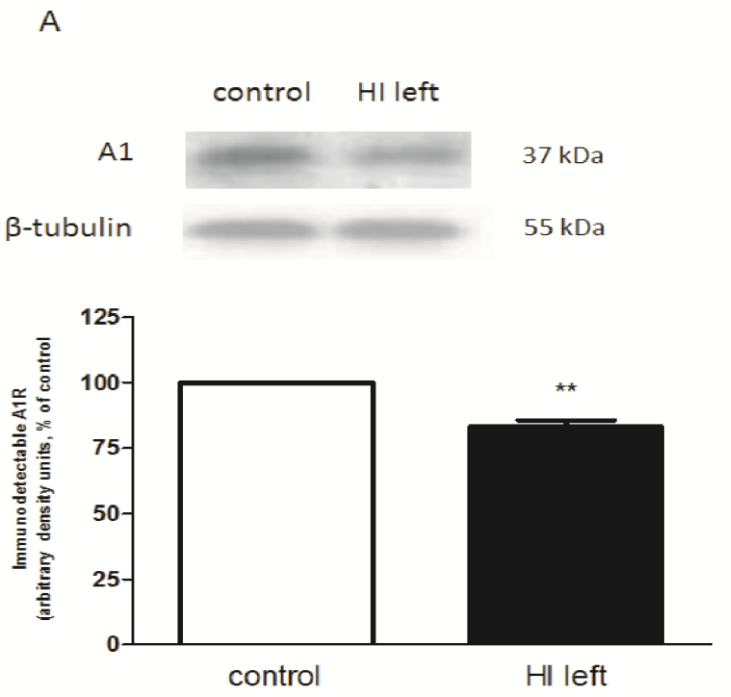
A
control
HI left
A1
37 kDa
β-tubulin
55 kDa
Immunodetectable A1R
(arbitrary density units, % of control)
125
100
75
50
25
0
**
control
HI left

Fig 2.

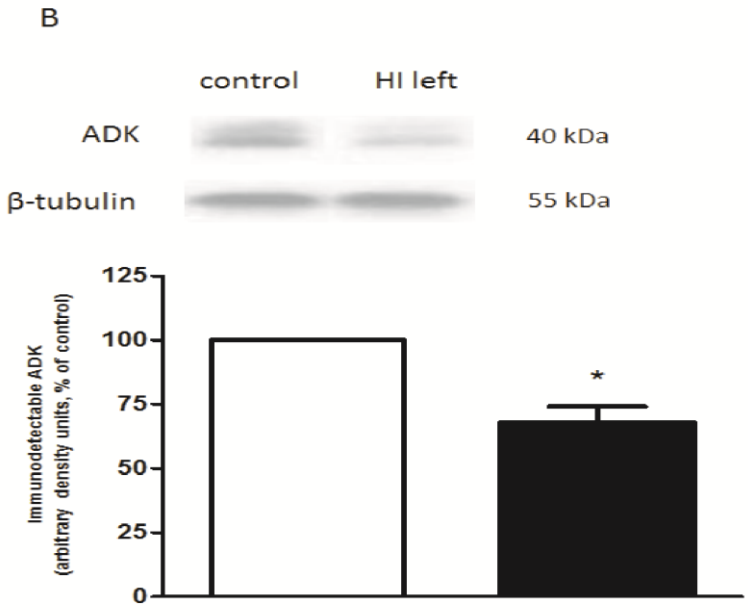
B
control
HI left
ADK
40 kDa
β-tubulin
55 kDa
Immunodetectable ADK
(arbitrary density units, % of control)
125
100
75
50
25
0
*

Fig 2.

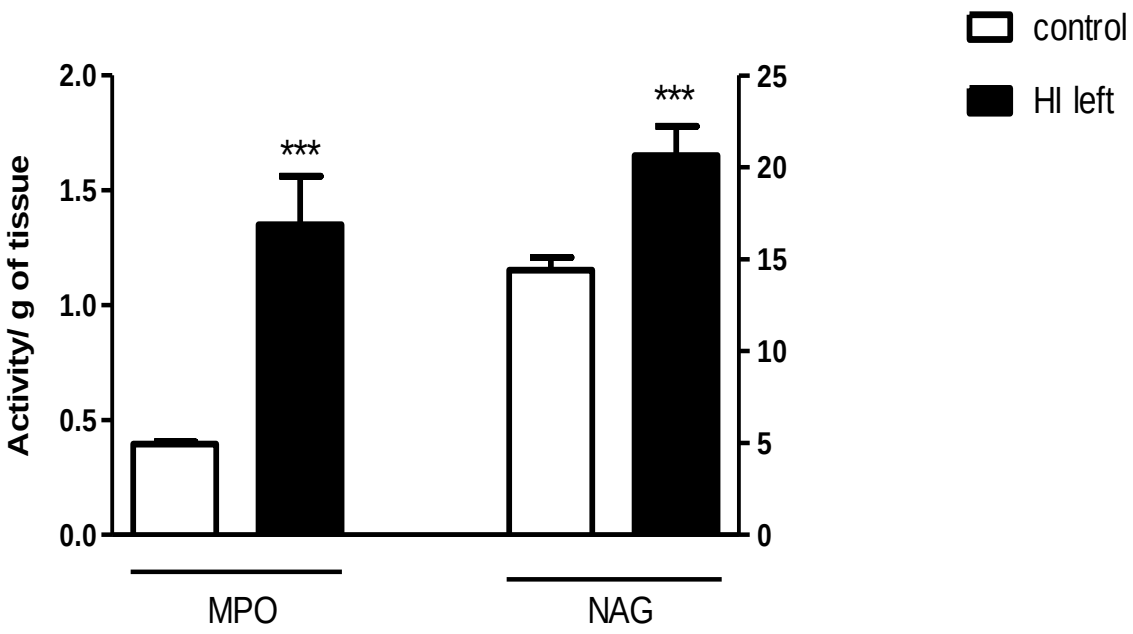
control
HI left
Activity/ g of tissue
2.0
1.5
1.0
0.5
0.0
25
20
15
10
5
0
***
***
MPO
NAG

Fig 3.

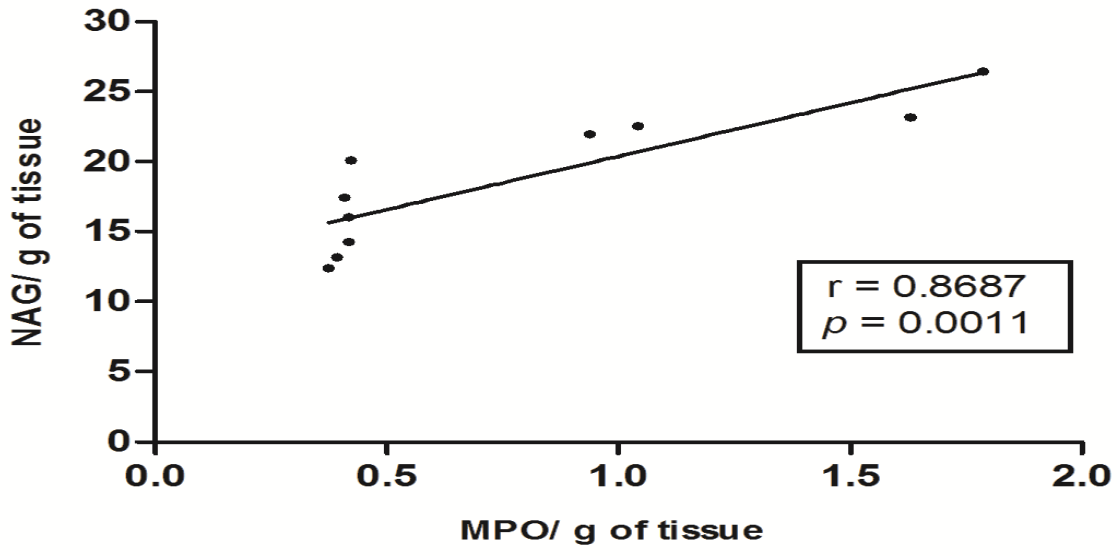
NAG/ g of tissue
30
25
20
15
10
5
0
0.0
0.5
1.0
1.5
2.0
MPO/ g of tissue
r = 0.8687
p = 0.0011

Fig 4.

3.2. ARTIGO II

Evaluation of acetylcholinesterase and adenosine deaminase activities in brain and erythrocytes and pro-inflammatory cytokines levels in rats submitted to neonatal hypoxia ischemia model

Victor Camera Pimentel; Jéssica Lopes Gomes; Daniela Zanini; Fátima Husein Abdalla; Pauline da Costa; Jamile Fabbrin Gonçalves; Marta Maria Medeiros Frescura Duarte; Maria Beatriz Moretto; Vera Maria Morsch; Maria Rosa Chitolina Schetinger

Mol Cell Biochem
DOI 10.1007/s11010-013-1615-9

Evaluation of acetylcholinesterase and adenosine deaminase activities in brain and erythrocytes and proinflammatory cytokine levels in rats submitted to neonatal hypoxia-ischemia model

Victor Camera Pimentel · Jéssica Lopes Gomes · Daniela Zanini · Fátima Husein Abdalla · Pauline da Costa · Jamile Fabbrin Gonçalves · Marta Maria Medeiros Frescura Duarte · Maria Beatriz Moretto · Vera Maria Morsch · Maria Rosa Chitolina Schetinger

Received: 17 November 2012 / Accepted: 2 March 2013

Abstract Perinatal hypoxic-ischemic (HI) brain injury is a common problem with severe neurologic sequelae. The definitive brain injury is a consequence of pathophysiological mechanisms that begin at the moment of HI insult and may extend for days or weeks. In this context, the inflammatory response and the formation of reactive oxygen species seem to play a key role during evolution of brain damage after injury. Thus, the aim of this study was to describe the chronological sequence of acetylcholinesterase (AChE) activity and the lipid peroxidation changes in the cerebral cortex using the classic model of neonatal HI. Furthermore, the erythrocyte AChE and adenosine deaminase (ADA) activities as well as the serum levels of proinflammatory cytokines were assessed. We observed that neonatal HI caused an increase of lipid peroxidation immediately after HI insult, which remained for several days afterward. There was a time-related change in the AChE activity in the cerebral cortex and the same was observed in erythrocyte AChE and ADA activities. In addition, immediately after HI, ADA activity showed a strong positive correlation with all proinflammatory cytokines assessed. Together, these findings may help the understanding of some mechanism related to the pathophysiology of neonatal HI, therefore highlighting the putative therapeutic targets to minimize brain injury and enhance recovery.

Keywords Hypoxic-ischemic brain injury · Inflammation · Erythrocytes · Acetylcholinesterase · Adenosine deaminase · Cytokines

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Published online: 21 March 2013

Abbreviations

HI	Hypoxia-ischemia
AChE	Acetylcholinesterase
ACh	Acetylcholine
ADA	Adenosine deaminase
TBARS	Thiobarbituric acid reactive species
TNF-α	Tumor necrosis factor alpha
IFN-γ	Interferon-gamma
IL	Interleukins
BChE	Butyrylcholinesterase

Introduction

Perinatal hypoxia-ischemia (HI) brain injury is a significant health problem and remains a major contributor to perinatal morbidity and mortality in neonates. Cerebral HI is an

emergency condition that needs to be treated quickly since it can rapidly cause severe brain damage which may lead to major neurologic deficits and impairment in the growth and development of survivors [1, 2]. Studies have reported that HI injury involves multiple pathways of oxidative stress, inflammation, and excitotoxicity which lead to early and late phases of cell damage and afterward to death [3–7].

Cholinergic neurons and their projections are widely distributed throughout the central nervous system (CNS) with an essential role in regulating several vital functions, such as learning, memory, cortical organization of movement, and the control of cerebral blood flow. In particular, the enzyme acetylcholinesterase (AChE; E.C.3.1.1.7) is one of the key enzymes of this system and is often used as a marker of its functioning. AChE is present in the organism in membrane-bound and soluble forms. It plays a central role in the cholinergic transmission regulating the concentration of acetylcholine (ACh). When ACh is released from growing axons, it regulates growth, differentiation, and plasticity of developing CNS neurons. In addition to intrinsic cholinergic neurons, the cerebral cortex and hippocampus receive extensive innervation from cholinergic neurons in the basal forebrain, beginning prenatally and continuing throughout the period of active growth and synaptogenesis [8–10].

Besides its pivotal role in neurotransmission, studies have revealed that AChE is also expressed in several types of hematopoietic cells including erythrocytes [8] and megakaryocytes [11]. However, in the peripheral system, the function of the cholinergic system present in blood has not yet been clarified. Recently, some studies have shown that this enzyme can be involved in the regulation of biologic functions such as proliferation, differentiation, organization of the cytoskeleton, cell–cell contact, or immune functions [12–14]. Alterations in the AChE activity have been demonstrated by our research group in various diseases and poisonings, suggesting that this enzyme could be an important physiologic and pathological parameter [15–18].

Adenosine deaminase (ADA; E.C.3.5.4.4) is a key enzyme in purine metabolism catalyzing the irreversible deamination of adenosine and 2′-deoxyadenosine to inosine and 2′-deoxyinosine, respectively. This enzyme is widely distributed in vertebrate tissues and plays a critical role in a number of physiologic systems to maintain strict control of adenosine levels [19, 20]. The main function of ADA is related to development and maintenance of the immune system [21–23]. This role has been suggested because congenital deficiency of this enzyme is associated with severe combined immunodeficiency disease (SCID), which is characterized by the absence of functional T and B lymphocytes due to the accumulation of adenosine [24, 25].

It has been demonstrated that the immature brain is highly susceptible to lipid peroxidation because it presents poorly developed scavenging systems and high availability of iron for the catalytic formation of free radicals [26]. It is also known that AChE responds to various insults including oxidative stress and inflammation [27–30], which are two important events that have been related to HI insult [6, 7, 31–33]. Based on this, the aim of our study was to investigate the AChE activity as well as the lipid peroxidation levels in the cerebral cortex in different chronological times after neonatal HI. Furthermore, the erythrocyte AChE and ADA activities as well as the serum levels of proinflammatory cytokines were assessed.

Materials and methods

Chemicals

Acetylthiocholine, Percoll, Tris (hydroxymethyl)-aminomethane GR, 5,5-dithiobisnitrobenzoic acid (DTNB), and Coomassie brilliant blue G were obtained from Sigma Chemical Co (St. Louis, MO, USA) and bovine serum albumin and K_2HPO_4 from Reagan. Adenosine was obtained from Merck (Darmstadt, Germany). All other chemicals were of analytical grade and obtained from standard commercial suppliers.

Animals

The animals utilized in this study were fed ad libitum and maintained on 12 h light/12 h dark cycle at room temperature. The study was in accordance with the guidelines of the Ethics Committee for Animal Research of this University, which approved the experimental protocol (No. 23081.007419/2007-10).

Surgical procedures

Seven-day-old Wistar male rats, weighing 14–16 g, were subjected to a neonatal HI model by unilateral common carotid artery ligation followed by hypoxia based on the Rice et al. [34] model. Briefly, the animals were anesthetized with halothane and the left common carotid artery of each pup was exposed, isolated from nerve and vein, and ligated with 4-0 surgical silk. After surgery, the rat pups were returned to their dams for 2 h. The pups were placed into a 1,500-mL chamber and exposed to an 8 % oxygen–92 % nitrogen atmosphere delivered at 5 L/min for 1.5 h. The temperature was maintained at 37 °C water bath to maintain a constant thermal environment. The pups were returned to their dams. Seven-day-old pups were euthanized according to the following protocols: (1)

immediately, 72 h, and 8 days after to verify the changes in the AChE activity in the cerebral cortex, erythrocyte ADA and AChE activities, and TBARS content; (2) immediately after HI injury to investigate the serum cytokines levels. The pups for this study were randomly divided into two groups: the control group ($n = 8$) and the HI group ($n = 8$), which was submitted to both the ligation of the left carotid artery and hypoxia conditions (8 % oxygen–92 % nitrogen atmosphere). Only the left hemisphere of both groups was used. In this neonatal HI model, the cerebral hemispheres of rats receiving hypoxia are differently affected: The left hemispheres will present neuronal death since it suffers ischemia (due to carotid ligation) associated with hypoxia, while the right hemisphere (receiving only hypoxia) suffers no overt morphological damage. The pups that suffer only the carotid ligation (ischemic group) did not demonstrate biochemical and morphological alterations according to Moretto et al. [35].

Tissue preparation

The brains of the experimental animals were quickly removed and placed on ice. The cerebral cortex was gently dissected. Then, the left hemisphere of the HI group (ipsilateral to ligation of carotid artery) and the left hemisphere of the control group were immediately frozen in liquid nitrogen and stored at −80 °C until the analyses.

AChE activity assay

Briefly, the tissues were homogenized in a glass potter in a solution of 10 mM Tris–HCl, pH 7.4, on ice, at a proportion of 1:10 (w/v). The homogenate was centrifuged at 3,000×*g* for 10 min and the resulting supernatant was utilized. Protein was determined previously and adjusted for cerebral cortex (0.6–0.8 mg/mL). The AChE enzymatic assay was determined by a modification of the spectrophotometric method of Ellman et al. [36]. The reaction mixture (2 mL final volume) contained 100 mM K^+-phosphate buffer, pH 7.5, and 1 mM 5,5′-dithio-bis-nitrobenzoic acid (DTNB). The method is based on the formation of the yellow anion, 5,5′-dithio-bis-acid nitrobenzoic, measured by absorbance at 412 nm during 2-min incubation at 25 °C. The enzyme was pre-incubated for 2 min. The reaction was initiated by adding 0.8 mM acetylthiocholine iodide (AcSCh). All samples were run in duplicate or triplicate and enzyme activity was expressed in µmol AcSCh/h/mg of protein.

Lipid peroxidation assay

The tissues were homogenized in a glass potter in a solution of 10 mM Tris–HCl, pH 7.4, on ice, at a proportion of 1:8 (w/v). The homogenates were centrifuged at 1,000×*g* for about 10 min. Thiobarbituric acid reactive species (TBARS) levels were determined by a modification of the method of Buege and Aust [37]. In brief, 250 µL of cerebral cortex homogenate was mixed thoroughly with 500 µL of a stock solution of 10 % (w/v) trichloroacetic acid and 750 µL of thiobarbituric acid. The mixture was heated for 15 min in a boiling water bath. After cooling, the red pigment produced was extracted with 1.5 mL of n-butanol and measured in absorbance at 535 nm. The TBARS content was expressed in nmol/g of tissue.

Preparation of erythrocytes

Whole blood was collected in tubes with EDTA, centrifuged for 10 min at 1,000×*g*, and then the plasma was discarded. Erythrocyte sediment was washed three times with tenfold isotonic NaCl solution. After each procedure, the erythrocyte–saline mixture was centrifuged at 1,000×*g* for 10 min, and finally the erythrocytes obtained were used to measure ADA and AChE activities.

Erythrocyte AChE activity

AChE activity was determined by the method of Ellman et al. [36] modified by Worek et al. [38]. To achieve temperature equilibration and complete reaction of sample matrix sulfhydryl groups with DTNB, the mixture was incubated for 10 min prior to the addition of substrate. Enzyme activity was corrected for spontaneous hydrolysis of the substrate and DTNB degradation. The activity of butyrylcholinesterase (BChE; EC 3.1.1.8) was inhibited by ethopropazine. The AChE activity was measured at 436 nm and calculated from the quotient between the AChE activity and the hemoglobin content (Hb). Hb was determined using the Zijlstra-modified solution. Results were expressed as mU/umol Hb.

Erythrocyte ADA activity

ADA was determined according to Guisti and Galanti [39]. The reaction was started by addition of the substrate (adenosine) to a final concentration of 21 mmol/L and incubations were carried out for 1 h at 37 °C. The reaction was stopped by adding phenol-nitroprusside solution. The reaction mixtures were immediately mixed with alkaline hypochlorite (sodium hypochlorite) and vortexed. Ammonium sulfate, 75 µmol/L, was used as the ammonium standard. The ammonia concentration is directly proportional to the absorption of indophenol at 620 nm. All experiments were performed in triplicate and the mean was used for calculation. The specific activity was reported as U/L/g of hemoglobin.

Cytokines measurements

Serum concentrations of cytokines (tumor necrosis factor alpha—TNF-α; Interferon-gamma—IFN-γ; interleukins IL-1β and IL-6) were determined with commercially available enzyme-linked immunosorbent assay (ELISA) kits (eBIOSCIENCE, San Diego, USA). The concentration of cytokine was measured by optical densitometry at 450 nm and expressed in Pg/mL. All of the analyses were performed in triplicate.

Protein determination

Protein was determined by the Coomassie blue method according to Bradford [40] using bovine serum albumin as the standard.

Statistical analyses

The statistical analyses were performed using two-way ANOVA, followed by Duncan's multiple range tests. All data were expressed as mean ± SEM. The correlations were assessed by Pearson rank correlation coefficient. Differences were considered significant when the probability was $P < 0.05$. All the statistical analyses were conducted using GraphPad Prism version 5.0 for Windows (GraphPad Software, San Diego, CA, USA).

Results

Changes in the AChE activity in the cerebral cortex after neonatal HI

The effect of neonatal HI on the AChE activity was observed in different stages of development. Immediately after HI, the AChE activity decreased when compared with the control group ($P < 0.05$). However, 72 h post insult, the AChE activity increased when compared to the control ($P < 0.05$), returning to the normal values (baseline activity) 8 days after HI (Fig. 1).

Lipid peroxidation remains increased for several days after neonatal HI

Results obtained for oxidative stress in the cerebral cortex at different times after neonatal HI are presented in Fig. 2. Cerebral cortex homogenates of the experimental group presented TBARS values that were significantly higher than the control group immediately, 72 h, and 8 days after neonatal HI ($P < 0.001$, $P < 0.05$, and $P < 0.05$, respectively).

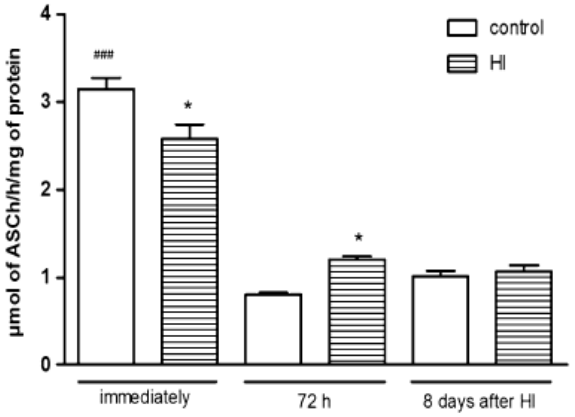

Fig. 1 AChE activity in the cerebral cortex of rats at different times after neonatal HI. $^{*}P < 0.05$ statistically significant from its respective control. $^{###}P < 0.001$ significantly different when compared with all other control groups. Statistical differences among groups were determined by two-way ANOVA followed by Duncan's test. Data are expressed as mean ± SEM ($n = 8$)

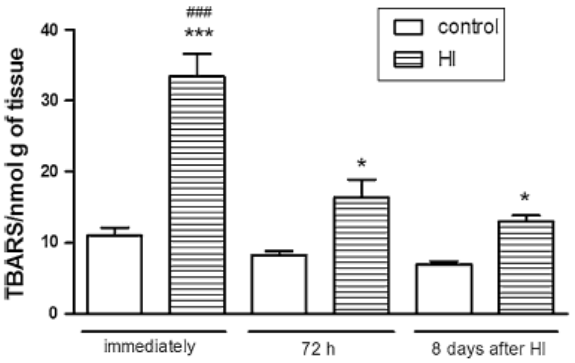

Fig. 2 TBARS levels in the cerebral cortex of rats at different times after neonatal HI. $^{*}P < 0.05$, $^{***}P < 0.001$ statistically significant compared with its respective control. $^{###}P < 0.001$ significantly different when compared with all other HI groups. Statistical differences among groups were determined by two-way ANOVA followed by Duncan's test. Data are expressed as mean ± SEM ($n = 8$)

Effect of neonatal HI on the erythrocyte AChE and ADA activities

Results obtained for the AChE activity in erythrocytes are presented in Fig. 3. The HI group showed significantly increased erythrocyte AChE activity when compared to the control group only 72 h after HI ($P < 0.001$). In relation to ADA, increased activity was observed immediately after HI ($P < 0.001$) (Fig. 4).

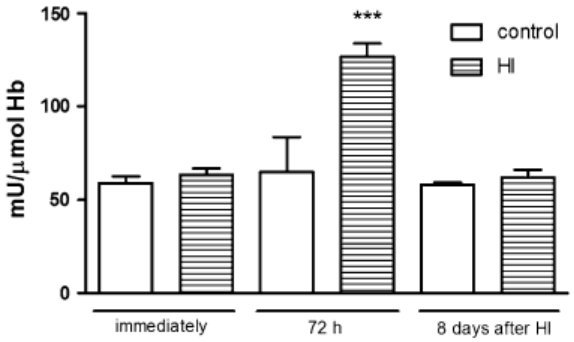

Fig. 3 Erythrocyte AChE activity at different times after neonatal HI. $^{***}P < 0.001$ statistically significant comparing with its respective control group. Statistical differences among groups were determined by two-way ANOVA followed by Duncan's test. Data are expressed as mean ± SEM ($n = 8$)

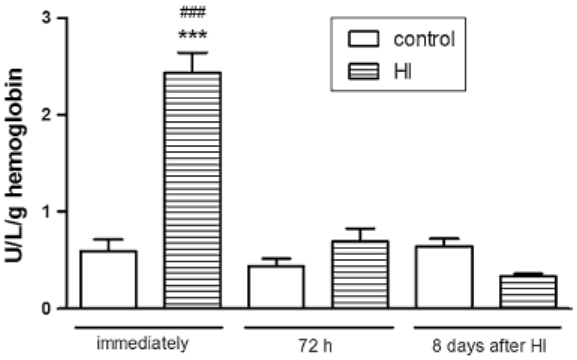

Fig. 4 Erythrocyte ADA activity at different times after neonatal HI. $^{***}P < 0.001$ statistically significant comparing with its respective control. $^{\#\#\#}P < 0.001$ significantly different when compared with all other HI groups. Statistical differences among groups were determined by two-way ANOVA followed by Duncan's test. Data are expressed as mean ± SEM ($n = 8$)

Changes in serum levels of IL-1β, IL-6, TNF-α, and INF-γ after neonatal HI

Figure 5 shows that the serum concentrations of IL-1β, IL-6, TNF-α, and INF-γ in the HI group were significantly higher than in the control group immediately after HI insult ($P < 0.001$).

Correlations between erythrocyte ADA activity and serum cytokine concentration

As shown in Table 1, a significant correlation between erythrocyte ADA activity and the IL-1β ($r = 0.9512$; $P < 0.0001$), IL-6 ($r = 0.9678$; $P < 0.0001$), TNF-α ($r = 0.8993$; $P < 0.0001$), and INF-γ ($r = 0.7411$; $P < 0.0007$) was found.

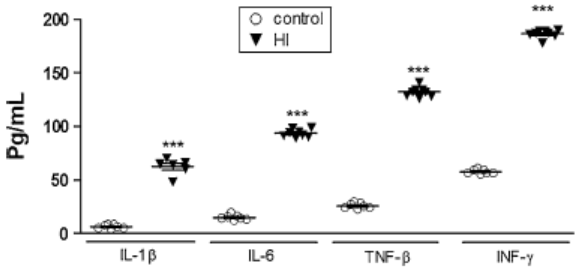

Fig. 5 Cytokines level in serum of neonatal rats immediately after HI injury. $^{***}P < 0.001$ statistically significant comparing with its respective control. Statistical differences among groups were determined by two-way ANOVA followed by Duncan's test. Data are expressed as mean ± SEM ($n = 8$)

Table 1 Pearson correlation between erythrocyte ADA activity and cytokines levels in serum of neonate rats immediately after HI injury

	Parameter	Correlation coefficient (r)	P value
ADA activity vs.	IL-1β	0.9512	<0.0001
	IL-6	0.9678	<0.0001
	TNF-β	0.8993	<0.0001
	INF-γ	0.7411	<0.0007

Discussion

HI brain damage in neonates is a major risk factor for a variety of serious human neurologic disorders such as motor and learning disabilities, cerebral palsy, epilepsy, seizures, or even death. Individuals affected by these disorders can experience substantial and lifelong disabilities for which there is still no promising therapy [41]. It is well known that the progression and extension of injured tissue evolve over time, so that the results of a study performed hours or days after hypoxic-ischemic insult can differ significantly from those of a study performed several weeks later. Likewise, the brain lesion has been related to several mechanisms, many of them involving an inflammatory and oxidative response [2, 42]. In this way, this study was performed immediately, 72 h and 8 days after HI insult.

To our knowledge, this is the first work that reports the AChE activity in different chronological times in this model of HI injury. Here, we evaluated the time-dependent changes in the AChE activity in the cerebral cortex after neonatal HI. In the present study, we observed a decrease in the AChE activity immediately post insult, followed by an increase at 72 h. Further, this study describes the AChE activity alterations of the brain in developing rats subjected to HI. According to others studies, the profile of the development pattern of AChE activity in the cerebral cortex in the control group decreased with advancing age [43–46]. The AChE activity has shown a decrease in the cerebral cortex with advancing age. We now observed that

there was a significant difference between the control groups; thus, it is possible to suggest that there are AChE activity alterations during the development of rats. In the same way, our group has demonstrated similar results in the ADA activity in the hippocampus and cerebral cortex [6, 7]. However, it is important to mention that to confirm this effect, more studies with more point times should be performed. Likewise, an abnormal lipid peroxidation immediately after reperfusion was observed and maintained 72 h and 8 days after insult. The abnormal production and/or impaired clearance of reactive oxygen species has been seen as a key mechanism in the process of tissue and functional damage in HI brain injury [33]; the main reason is that the immature brain has a poorly developed scavenging system associated with high levels of iron for the catalytic formation of free radicals [26, 47]. Furthermore, some lipid peroxidation products may stimulate leukocyte recruitment to the site of injury contributing to the pathophysiology of inflammation [48]. In this line, previous results of our group have shown the strong inflammatory response in the brain associated with progressive destruction, vascular endothelial proliferation, astrocytosis/astrogliosis, and invasion of leukocytes (neutrophils, macrophages, and lymphocytes) into the ischemic areas [6, 7]. Thus, the increased lipid peroxidation observed here could be a contributing factor for neuronal injury, leading to delayed neuronal death after neonatal HI. From a clinical perspective, early suppressing oxidative response could present a legitimate strategy to reduce HI brain injury.

It is well known that lipid peroxidation can change the physiologic functions of cell membranes modifying properties of membrane bilayer such as membrane potential, fluidity, or permeability to different substances and/or by post-translational chemical modifications [47, 49]. Alterations in the lipid membrane by oxidative stress could be a decisive factor in the modification of the conformational state of several molecules, including AChE [17, 18]. Thus, the rise in lipid peroxidation found immediately after neonatal HI could explain the early decrease of AChE activity in the present study. Another plausible possibility for the rapid decrease in the AChE activity observed immediately after insult could be a compensatory mechanism to increase the ACh levels in order to preserve the blood flow in the ischemic areas during the HI insult and enhance the suppressive anti-inflammatory effect of ACh. In accordance with our results, Beley et al. [50] have shown the depression of the cholinergic system during hypoxic insult. On the other hand, an increase in the AChE activity was observed 72 h after HI. This could lead to fast ACh degradation and a subsequent down stimulation of ACh receptors reducing the cholinergic neurotransmission efficiency in the cerebral cortex in this period. Further studies are necessary to better understand the involvement of AChE in the pathophysiology of neonatal HI since there is a time-dependent behavioral of this enzyme after HI insult.

This study describes for the first time the erythrocyte ADA and AChE activities in neonate rats subjected to HI insult. ADA is one of the key enzymes of the purine catabolic pathway and it catalyzes the hydrolytic deamination of adenosine to inosine and ammonia [19, 20]. In the circulating system, the adenosine has a very short half-life and it is a consequence of the rapid catabolism into inosine by erythrocytes which possess high activities of ADA. Thus, the disappearance rate of adenosine in whole blood is highly dependent on the amount of ADA present in the red cells. Here, we observed that immediately after insult, there was an increase in the erythrocyte ADA activity, which could reflect in lower adenosine levels in the circulation after HI insult. Recently, our group reported a higher level of uric acid, a product of adenosine catabolism, in the serum and urine of rats immediately after neonatal HI, which presents a good correlation with the results cited above [51].

In addition, it is known that ADA plays an important role in the mechanism of the immune system, participating actively in the immunoregulation of inflammatory processes [23, 52, 53]. In order to investigate if the increase in ADA activity could be related to the activation of the immune system, the serum levels of main cytokines were assessed. Our results clearly demonstrated that neonatal HI injury produces a condition with increased serum levels of proinflammatory cytokines, corroborating other studies [54–57]. Furthermore, we can emphasize the strong positive correlation of ADA with all cytokines evaluated shortly after the HI event. These results lead us to suggest that erythrocyte ADA could constitute part of the fine immunoregulatory mechanism of adenosine-mediated signaling in the pathophysiology of HI since the changes in the concentrations of adenosine can modulate the release of proinflammatory cytokines and the expression of adhesion molecules [58–61]. Other reports have likely described the importance of other ectoenzymes in the immune system through the modulation of adenosine levels [62, 63]. In a simplistic extrapolation, due to the strong adenosine-mediated immune suppression, the increase of erythrocyte ADA activity could behave as an immune adjuvant to favor the immune response during the beginning of the inflammatory process after neonatal HI. Recently, we demonstrated the overstimulation of immune cells associated with the increase of ADA activity in the brain [6, 7]. Thus, based on the above-mentioned point, it is possible to suggest that the inhibitors of ADA activity could contribute to the treatment of the HI neonatal. In particular, the pharmacological modulation of ADA through specific

inhibitors has already been used to attenuate inflammation in several pathological conditions [23, 64]. In the same way, peripherally derived cytokines have been described in the elaboration and exacerbation of inflammatory response in the brain through several routes, such as the destruction of the blood brain barrier (BBB) and the favoring of leukocytes rolling into the brain [65–68]. Taking this into account, erythrocyte ADA, by degrading adenosine, is likely to be capable of enhancing immune cell functions and consequently increasing the production of proinflammatory cytokine levels in the serum, promoting inflammatory reactions contributing to brain damage after HI.

It is known that erythrocytes are the peripheral blood elements with more AChE content in the membranes [69]; however, the function of AChE in erythrocytes is still unknown. Recently, several works have demonstrated that the inhibition of the AChE has anti-inflammatory effects by modulating the release of cytokines through the activation of the α7 nicotinic ACh receptor in immune cells [70–72]. In accordance with the above-mentioned information, neonatal HI leads to a rapid activation of the immune system, which was demonstrated by increasing cytokine levels in the peripheral blood; however, the erythrocyte AChE activity was not altered immediately after HI insult, but showed an increase only 72 h after HI.

However, another important aspect observed in this study was that the enzymatic activities had different behaviors after neonatal HI. This was observed for both ADA and AChE activities in the cerebral cortex and erythrocytes. The results show that there is a mixed pattern of enzyme induction/inhibition in the specific times after the HI event. In this context, it is well known that there are several molecular mechanisms involved in the evolution of HI brain damage [2], and that the sequence of events following the HI insult is complex and multifactorial and develops over the days after the insult. In this context, to establish definitively the exact mechanism involved in the enzymatic alterations and the consequence of this, more detailed studies will be necessary.

Conclusion

In conclusion, our data demonstrate that the early phase following HI in the developing rat brain leads to oxidative stress which is sustained in the days after insult. Furthermore, the neonatal HI implicates in alterations of cerebral AChE activity as well as erythrocyte ADA and AChE activities at different times after HI insult. By focusing on the study, we can provide information on the biochemical and immunologic mechanisms involved during the evolution of peripheral and central alterations caused by neonatal HI. The evolution of brain lesions after HI is a response to the combination of central and peripheral mechanisms that start with insult; thus, a thorough understanding of these events can help in the search for new therapeutic intervention. The present findings show a promising route to be explored.

Acknowledgments This work was supported by the Fundação Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES), the INCT for Excitotoxicity and Neuroprotection, and the FINEP research grant "Rede Instituto Brasileiro de Neurociência (IBN-Net)."

Conflict of interest The authors have declared that there is no conflict of interest.

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4. DISCUSSÃO

Atualmente a HI neonatal representa uma das principais causas de morbidade e mortalidade durante o período perinatal. No entanto, apesar dos avanços no meio científico ainda não há uma terapêutica eficaz para o tratamento das sequelas decorrentes da lesão cerebral, e isto ocorre devido a multifatoriedade e complexidade dos eventos envolvidos na patofisiologia da HI neonatal. Neste contexto, para buscar uma melhor compreensão destes mecanismos este trabalho teve como objetivo principal investigar as possíveis alterações nos sistemas purinérgico e colinérgico em córtex cerebral de ratos neonatos expostos a HI neonatal. Como demonstrado anteriormente esta tese foi subdividida em três capítulos (um artigo e dois manuscritos), assim a discussão deste trabalho será baseada nos resultados descritos nestes trabalhos.

O capítulo 1 desta tese teve por objetivo principal investigar o efeito da HI neonatal sobre a atividade de enzimas envolvidas no catabolismo de nucleotídeos e nucleosideos de adenina imediatamente após o insulto hipóxico-isquêmico, com o intuito de possibilitar um melhor entendimento sobre a participação da cascata enzimática purinérgica nos momentos iniciais após a HI neonatal. Neste contexto, foram investigadas a atividade da NTPDase, 5`-NT e ADA nas frações sinaptossomal e citosólica assim como a expressão da ADK, a atividade da Na^+, K^+–ATPase e os níveis de peroxidação lipídica no córtex cerebral destes animais.

Os resultados encontrados neste estudo indicam que imediatamente após a HI neonatal há um aumento na hidrólise dos nucleotídeos de adenina (ATP, ADP e AMP), mas não da adenosina na fração citosólica. Estes dados sugerem que a NTPDase e a 5`-NT citosólicas estão envolvidas na degradação do ATP durante a HI, promovendo um aumento nos níveis de adenosina provavelmente como um resposta de adaptação para proteger o SNC contra a excitotoxicidade induzida pela evento hipóxico-isquêmico. Em contrapartida, imediatamente após o insulto, não foram observadas alterações na atividade das ecto-nucleotidases, indicando que nos estágios inicias após o insulto hipóxico-isquêmico, o ATP extracelular pode não contribuir substancialmente para a o aumento dos níveis de adenosina no meio extracelular. Estes dados estão de acordo com outros estudos que têm demonstrado que o aumento nos níveis de adenosina no meio extracelular durante os primeiros

momentos após a HI é causado principalmente pela degradação do ATP citoplasmático seguido da liberação da adenosina formada para o meio extracelular através de transportadores de nucleosídeo específicos (PEARSON et al., 2003; LATINI & PEDATA, 2008; DALE & FRENGUELLI 2009; PONS et al., 1980). Corroborando com o citado acima, não foram observadas alterações na atividade da ADA citosólica e na expressão da ADK, o que reforça que o aumento na hidrólise de ATP, ADP e AMP facilmente levaria ao acúmulo de adenosina intracelular, o que favoreceria a sua difusão para o meio extracelular.

Além disso, um aumento na atividade da ecto-ADA também foi observado neste estudo. Tendo em vista que durante episódios de hipóxia e/ou isquemia a adenosina extracelular apresenta um importante papel neuroprotetor, principalmente através da ativação dos A1Rs, o aumento na atividade da ecto-ADA poderia contribuir para remoção da adenosina da fenda sináptica e assim prejudicar a neuroproteção mediada por este nucleosídeo. Por outro lado, a inosina, produto da degradação da adenosina pela ADA, também tem sido descrita como neuroprotetora durante eventos de isquemia (SHEN et al., 2005). Neste contexto, estudos ainda serão necessários para definirmos os efeitos neuroprotetores mediados pela adenosina/inosina e o potencial uso terapêutico de inibidores da ADA frente às lesões decorrentes da HI neonatal.

Sabe-se que dois tipos de lesões compõem o modelo experimental da HI em neonatos: a isquemia e a hipóxia. Durante a isquemia, a interrupção do fluxo sanguíneo leva à diminuição do suprimento de glicose e oxigênio para as estruturas afetadas. Como consequência, ocorre uma diminuição geral do metabolismo energético a fim de manter a homeostase celular, evento que também ocorre durante a hipóxia sistêmica. Entretanto, no período imediatamente após o final da hipóxia, ou seja, durante o período de reoxigenação, o encéfalo dos neonatos experimenta uma situação de hiperoxigenação, responsável pela grande produção de radicais livres através da cadeia transportadora de elétrons. Os radicais livres formados reagem com os lipídios das membranas celulares, causando a desnaturação da bicamada lipídica e consequentemente a inibição da atividade da Na^+, K^+–ATPase (KAKO et al., 1988; RAZDAN et al., 1993; RAUCHOVÁ et al., 1999; MATTÉ et al., 2006). Como observado no presente estudo, houve um aumento na hidrólise de ATP (aumento na atividade da NTPDase), evento este que poderia

exacerbar a depleção energética nos primeiros momentos após a HI. Neste contexto, e com base nos dados citados anteriormente nós decidimos investigar a atividade da Na^+, K^+–ATPase e os níveis de peroxidação lipídica no mesmo período em que foi observado um aumento na hidrólise de ATP (aumento na atividade da NTPDase). Nossos resultados demonstraram um aumento na lipoperoxidação e uma inibição na atividade da Na^+, K^+–ATPase imediatamente após a HI neonatal. Além disso, quando a análise correlacional foi realizada entre estes parâmetros, uma correlação negativa entre a atividade da Na^+, K^+–ATPase e os níveis de peroxidação lipídica bem como entre a atividade da Na^+, K^+–ATPase e a atividade da NTPDase (hidrólise do ATP) foram observadas. Estes dados sugerem que o aumento na produção de radicais livres associado com o aumento na hidrólise de ATP imediatamente após a reperfusão pode estar relacionado com a redução na atividade da Na^+, K^+–ATPase observada no presente estudo. Os resultados aqui observados estão de acordo com outros trabalhos que demonstram que a rápida depleção energética assim como o dano oxidativo podem inibir a atividade da Na^+, K^+–ATPase (RAUCHOVÁ et al., 1999). Assim, pode-se sugerir que a associação destes eventos nas fases iniciais após a reperfusão pode ser um fator importante e contribuinte para a evolução da lesão cerebral decorrente do evento hipóxico-isquêmico.

Nos últimos anos, a modulação do sistema purinérgico tem emergido como um potente alvo terapêutico em condições de injúria cerebral em uma série de desordens neurológicas. Motivo este, que é atribuído principalmente aos efeitos mediados pela adenosina em nível de SNC. A adenosina, como citado anteriormente, é um nucleosídeo de adenina que é rapidamente gerado do catabolismo do ATP em condições de estresse ou dano celular. Uma vez gerada, a adenosina pode influenciar uma série de eventos celulares através da ativação de seus receptores, sendo em seguida fosforilada à AMP pela ADK ou desaminada à inosina pela ADA. Neste contexto, estas duas enzimas possuem um papel importante na modulação dos níveis de adenosina e consequentemente participam indiretamente da modulação dos mecanismos relacionados com a ativação dos receptores de adenosina.

Em relação à ADA, a sua atividade origina-se de duas principais isoenzimas: a ADA1 e a ADA2. Recentemente, em um estudo realizado por nosso grupo de

pesquisa, foi observado um aumento na atividade da ADA em córtex e hipocampo de ratos neonatos 8 dias após a HI. No entanto, a atividade da ADA observada correspondia a atividade associada das suas duas isoenzimas. Como demostrado agora no capítulo 2 desta tese, nós buscamos identificar a isoenzima da ADA responsável pelo aumento na sua atividade após a HI, além de investigar a expressão da ADK e dos A1Rs. Para caracterizarmos o processo inflamatório as atividades da MPO e NAG foram analisadas. Neste estudo foi utilizado o mesmo modelo experimental citado anteriormente, no entanto, os animais foram eutanasiados 8 dias após o insulto hipóxico-isquêmico. Nossos resultados claramente indicam que a ADA1 é a principal isoenzima responsável pelo aumento da atividade da ADA após a HI neonatal. A identificação das isoenzimas neste modelo revela o importante papel da ADA1 após a HI neonatal desde que esta possui maior afinidade pela adenosina do que a ADA2 (Km = 5,2 x 10^{-5} M e 200 x $l0^{-5}$ M, respectivamente) (UNGERER et al., 1992; VAN EDE et al., 2002). Contudo, considerando que a ADA1 é a principal enzima responsável pelo aumento da atividade da ADA 8 dias após a HI neonatal e que esta possui uma alta afinidade pela adenosina, é possível sugerir que a utilização de inibidores específicos para esta isoenzima poderia representar uma das melhores vias para proporcionar um aumento os níveis de adenosina em tecidos sob condições de estresse. Além disso, a inibição da atividade da ADA1 não somente poderia contribuir com o aumento dos níveis de adenosina nas áreas isquêmicas, como também prevenir a geração de espécies reativas de oxigênio pela via da xantina oxidase (XIA et al., 1996; GERVITZ et al., 2001). Portanto, num contexto clínico, é possível sugerir que o uso de inibidores específicos da ADA1 poderia representar uma estratégia para aumentar os níveis de adenosina após a HI neonatal, favorecendo assim os efeitos anti-inflamatório e imunossupressor mediados por este nucleosideo.

Associado ao aumento da atividade da ADA1 nós observamos uma redução na expressão da ADK. Assim, com base nos dados obtidos, é plausível sugerir que 8 dias após a HI neonatal a adenosina é preferencialmente desaminada à inosina do que fosforilada à AMP, o que poderia representar um mecanismo inato de proteção do organismo contra os efeitos deletérios da acumulação da adenosina ao longo da evolução da lesão cerebral. Por outro lado, a inosina, produto da desaminação da adenosina pela ADA, tem sido descrita com propriedades anti-inflamatórias durante

condições de isquemia (HASKO et al., 2000; HASKO et al., 2004; SHEN et al., 2005). Neste contexto, o aumento na atividade da ADA1 também poderia favorecer o aumento nos níveis de inosina a qual poderia desempenhar efeitos anti-inflamatórios frente o insulto HI. Assim, podemos observar uma dualidade do aumento da atividade da ADA1 frente à HI neonatal. Portanto, para que tenhamos um melhor entendimento e possamos realmente configurar a importância da utilização de inibidores da ADA1 frente às lesões causadas pela HI outros estudos serão necessários.

Como citado anteriormente os efeitos da adenosina sao mediados pela ativação de receptores de adenosina, os quais podem ser sub-divididos em receptores A1, A2A, A2B e A3. No entanto, devido aos efeitos neuroprotetores mediados pelos A1Rs, estes tem recebido uma maior atenção em condições de hipóxia e/ou isquemia. Assim, para investigarmos o comportamento deste receptor neste período após a HI neonatal, a expressão dos A1Rs foi realizada. Os dados obtidos neste estudo indicam que 8 dias após o insulto hipóxico-isquêmico há uma redução na expressão deste receptor no córtex destes animais. Sabe-se que imediatamente após a HI, a ativação dos A1Rs exerce um potente efeito neuroprotetor o qual é mediado principalmente pela diminuição na liberação de neurotransmissores excitatórios (principalmente o glutamato) (WARDAS, 2002; DE MENDONÇA et al., 2005; ARRIGONI et al., 2005). No entanto, um mecanismo de neuroproteção que ocorre em uma fase mais tardia após o insulto é o isolamento das áreas isquêmicas pelos astrócitos (formação da cicatriz glial) (DE MENDONÇA et al., 2000). Neste contexto, o aumento exacerbado nos níveis de adenosina atua como um modulador negativo sobre a proliferação astrocítica, mecanismo este que é mediado pela ativação dos A1Rs. No entanto, este efeito modulatório mediado pela adenosina é perdido quando a adenosina é metabolizada, e assim o processo de astrogliose e astrocitose pode ocorrer em resposta à lesão tecidual (CICCARELLI et al., 2001). É interessante destacarmos que recentemente nosso grupo tem demonstrado uma forte resposta astrocitária no córtex 8 dias após a HI neonatal (PIMENTEL et al., 2009). Assim, sabendo que a adenosina possui um potente efeito inibitório sobre a proliferação e diferenciação dos astrócitos, e que este mecanismo é mediado pela ativação dos A1Rs, é possível sugerir que o aumento na atividade da ADA1 e a redução na expressão dos A1Rs poderiam ser um mecanismo

colaborativo com o objetivo de promover o reparo tecidual nas áreas isquêmicas. No entanto, é importante ressaltarmos que efeitos contraditórios sobre o papel dos A1Rs têm sido descritos (RUDOLPHI et al., 1992; TURNER et al., 2002; TURNER et al., 2003) e que a adenosina pode atuar tanto como uma molécula anti-inflamatória como pró-inflamatória dependendo sob qual sub-tipo de receptor esta estará atuando, qual tipo celular estará envolvido e o estágio de desenvolvimento e duração da neuroinflamação (DUNWIDDIE & MASINO, 2001; FARBER & KETTENMANN, 2006).

Neste mesmo contexto, diversos estudos nos quais utilizam modelos que favorecem um aumento nos níveis de adenosina (LEWIN & BLECK, 1981; DURING AND SPENCER, 1992; BERMAN et al., 2000), têm demonstrado uma redução na expressão dos A1Rs ao longo do tempo em diversas regiões cerebrais (CUNHA, 2005). Da mesma forma, estudos têm demonstrado que curtos períodos de isquemia, os quais também são descritos por aumentar os níveis extracelulares de adenosina, produzem uma redução na densidade destes receptores. Assim, tem se sugerido que a ativação dos A1Rs é um sistema de neuroproteção, no entanto, acredita-se que seja um mecanismo limitado aos estágios iniciais após o insulto (CUNHA, 2005). Portanto, as modificações na expressão dos A1Rs bem como as alterações enolvidas com o metabolismo da adenosina que ocorrem ao longo do tempo de evolução da lesão cerebral tornam-se particularmente críticos para buscarmos uma melhor compreensão dos efeitos deste nucleosideo nos períodos que suscedem a HI neonatal.

Similiarmente aos resultados obtidos em um estudo recente do nosso grupo (PIMENTEL et al., 2011), neste trabalho também foi observado um aumento na atividade da MPO e NAG no córtex dos animais expostos a HI neonatal, o que indica a presença de células inflamatórias (PIMENTEL et al., 2009). Além disso, quando a análise de correlação foi realizada, podemos observar uma correlação positiva entre a atividade da ADA1 e as atividades da MPO e NAG. Estes dados reforçam o papel destas enzimas como marcadores do processo inflamatório (BLACKBURN et al., 1998; GROOTEMAN et al., 2000; WINTERBOURN et al., 2000; FRÖDE & MEDEIROS, 2001; IQBAL et al., 2008) e proporcionam novas ferramentas para melhor compreender as vias imunoregulatórias que ocorrem na inflamação decorrente da HI neonatal.

Como demonstrado nos capítulos anteriores, a HI neonatal promove uma série de alterações relacionadas à sinalização purinérgica. No entanto, pouco se tem descrito sobre os efeitos da HI sobre o sistema colinérgico. Neste contexto, sabendo que uma série de eventos estão relacionados com evolução e progressão da lesão cerebral decorrentes da HI, das quais podemos destacar o estresse oxidativo e a inflamação (VEXLER & FERRIERO, 2001; FERRIERO, 2004), e que a AChE responde a estes estímulos, no capítulo 3, nosso objetivo principal foi investigar a atividade da AChE em córtex cerebral de ratos neonatos submetidos a HI neonatal. Em um segundo momento, os níveis de peroxidação lipídica, a atividade da AChE e da ADA eritrocitária e os níveis séricos de marcadores pró-inflamatórios (citocinas pró-inflamatórias) foram analisados. Para a realização deste trabalho, o mesmo modelo experimental foi utilizado. Os animais foram eutanasiados imediatamente, 72 horas ou 8 dias após o insulto hipóxico-isquêmico.

No presente estudo nós observamos alterações tempo-dependentes na atividade da AChE após a HI neonatal. A atividade da AChE mostrou-se diminuída imediatamente após HI, por outro lado, a atividade da enzima aumentou 72 horas após o insulto. Do mesmo modo, observamos um aumento nos níveis de peroxidação lipídica imediatamente após o processo de reperfusão, o qual se manteve aumentado 72 horas e 8 dias após o insulto. A produção anormal e/ou alterações no processo de remoção das espécies reativas de oxigênio tem sido descritas na literatura como um dos mecanismos chave no processo de dano funcional e tecidual após a HI neonatal (VASILJEVIĆ et al., 2012), e a principal razão para que isto ocorra é a imaturidade do SNC durante o período neonatal (LAFEMINA et al., 2006; RAUCHOVÁ et al., 2012).

Diversos estudos na literatura têm demonstrado que a peroxidação lipídica é capaz de alterar diversas funções fisiológicas da membrana plasmática celular, alterando propriedades da bicamada lipídica tais como, o potencial de membrana, fluidez e permeabilidade a diferentes substâncias e/ou modificações químicas pós-translacionais (RAUCHOVÁ et al., 2012; SCHMIDT-KASTNER & FREUND, 1991). Neste contexto, alterações na membrana lipídica causadas pelo estresse oxidativo também têm sido descritas como um fator decisivo na modificação conformacional de diversas moléculas, incluindo a AChE (GONÇALVES et al., 2010; KAIZER et al., 2010). Com base no descrito acima, o aumento na peroxidação lipídica observado

imediatamente após a HI poderia explicar a inibição da atividade da AChE observada neste mesmo período. De acordo com os nossos resultados, Beley et al. (BELEY et al., 1991) tem demostrado a depressão no sistema colinérgico durante eventos de hipóxia.

Além do efeito direto das espécies reativas de oxigênio frente às membranas celulares, alguns produtos da peroxidação lipídica têm sido descritos por participar indiretamente da injúria tecidual, estimulando o recrutamento de leucócitos para as regiões isquêmicas contribuindo assim com a exacerbação do processo inflamatório (GUÉRAUD et al., 2010). Neste contexto, estudos recentes do nosso grupo de pesquisa têm demonstrado uma acentuada resposta neuroinflamatória associada com proliferação do endotélio vascular, astrogliose/astrocitose e presença de leucócitos (neutrófilos, macrófagos e linfócitos) na área isquêmica (PIMENTEL et al., 2009; PIMENTEL et al., 2011). Com base nos dados obtidos, chegamos a conclusão que o aumento na peroxidação lipídica observada neste estudo poderia ser um importante fator contribuinte para o dano neuronal e consequentemente progressão da lesão cerebral decorrente da HI neonatal. Assim, abordando estes resultados de um ponto de vista clínico, o uso de terapias antioxidantes nos primeiros momentos após o processo de reperfusão poderia representar uma estratégia concreta para reduzir a injúria cerebral causada pela HI neonatal.

Neste trabalho, também foi avaliado a atividade da ADA e da AChE eritrocitária. Para o nosso conhecimento, este é o primeiro trabalho na literatura que descreve a atividade destas duas enzimas em eritrócitos de ratos neonatos submetidos à HI. No presente estudo, nós observamos que houve um aumento na atividade da ADA imediatamente após o evento hipóxico-isquêmico, o que poderia refletir em um aumento no catabolismo da adenosina e consequentemente em uma redução dos níveis deste nucleosídeo na circulação. Corroborando com estes dados, recentemente nós observamos um aumento nos níveis de ácido úrico (hiperuricosúria e hiperuricemia) em ratos neonatos submetidos à HI (PIMENTEL et al., 2010). Interessantemente, a hiperuricosúria e a hiperuricemia ocorreram no mesmo período em que a atividade da ADA se mostrou aumentada no estudo agora abordado.

Seguindo o que foi discutido em relação aos resultados observados para a atividade da ADA, nós decidimos verificar se neste mesmo período em que sua

atividade se mostrou aumentada haveria uma possível ativação do sistema imune. Para isso, nós realizamos a quantificação de citocinas pró-inflamatórias no soro dos animais submetidos à HI neonatal. Os animais foram eutanaziados imediatamente após o insulto hipóxico-isquêmico e o soro foi coletado e utilizado para as análises. Com base nos resultados destas análises e corroborando com outros estudos, nos observamos que a HI neonatal produz uma condição com elevados níveis séricos de citocinas pró-inflamatórias nos momentos iniciais após a reperfusão (CHIESA et al., 2003; ALY et al., 2006; BOSKABADI et al., 2010; KHALIMBETOV, 2012). Além disso, foi encontrada uma forte correlação positiva entre a atividade da ADA e todas as citocinas avaliadas. Neste contexto, sabe-se que a ADA possui um importante papel na modulação e ativação do sistema imune, participando ativamente da imunoregulação do processo inflamatório (CRONSTEIN, 1994; ANTONIOLI et al., 2007; ANTONIOLI et al., 2012; GHAEMI et al., 2011; MARTINEZ-NAVIO et al., 2011), isto ocorre principalmente, mas não exclusivamente, devido ao papel desta enzima frente a modulação dos níveis de adenosina. Com base nestes resultados nós sugerimos que o aumento observado na atividade da ADA poderia ser um constituinte do mecanismo imunoregulatório mediado pela adenosina na patofisiologia da HI, tendo em vista que, a adenosina pode atuar modulando a liberação de citocinas pró-inflamatórias e moléculas de adesão e expressão (BOUMA et al., 1996; HUANG et al., 1997; CORDERO et al., 2001; LAPPAS et al., 2005). Recentemente nós demonstramos que a atividade da ADA está relacionada com a ativação do sistema imune no SNC de ratos expostos a HI neontal (PIMENTEL et al., 2009; PIMENTEL et al., 2011). Assim, de acordo com o citado acima, é possível sugerir que o uso de inibidores da atividade da ADA poderia contribuir para o tratamento da HI neonatal. Em particular, a modulação da atividade da ADA utilizando inibidores específicos já vem sendo utilizada para atenuar o processo inflamatório em diversas condições patológicas (ANTONIOLI et al., 2007; KUNO et al., 2006).

Finalmente, os resultados obtidos neste trabalho demonstram que a HI neonatal altera a atividade das enzimas e a expressão de receptores envolidos na sinalização purinérgica bem como no sistema colinérgico. Estes dados claramente indicam a participação do estresse oxidativo e do processo inflamatório no decorrer do desenvolvimento da lesão cerebral. Além disso, evidencia-se que estas

alterações ocorrem de maneira distinta durante o processo de evolução da lesão cerebral que é iniciada após o evento hipóxico-isquêmico. Em conjunto, estes resultados ganham um papel importante do ponto de vista clínico, sendo que os mesmos possibilitam uma melhor compreensão dos eventos envolvidos na patofisiologia da HI neonatal. Desta forma, o melhor entendimento destes eventos facilitará a busca de novos alvos terapêuticos relacionados com o sistema purinergico e colinérgico afim de, evitar ou reduzir as sequelas decorrentes da HI neonatal.

5. CONCLUSÕES

- A HI neonatal altera a atividade da NTPDase e 5`-NT citosólicas imediatamente após o insulto, proporcionando assim, um aumento nos níveis de adenosina. No entanto, um aumento na atividade da ecto-ADA foi observado neste mesmo período. Nenhuma alteração foi observada na atividade da ADA citosólica e na expressão da ADK, o que reforça o papel das nucleotidases citosólicas na contribuição do aumento dos níveis de adenosina nos momentos inciais após o evento hipóxico-isquêmico.

- Um correlação negativa foi observada entre a atividade da NTPDase (ATP) e a Na^+, K^+–ATPase bem como nos níveis de TBARS e na atividade da Na^+, K^+–ATPase, sugerindo que o aumento na peroxidacao lipídica assim como o aumento na hidrólise de ATP podem estar relacionados com a inibição/perda de função da Na^+, K^+–ATPase.

- A ADA1 é a isoenzima da ADA responsável pelo aumento na sua atividade 8 dias após a HI neonatal. O aumento da atividade da ADA1 associado a uma redução na expressão da ADK observado neste mesmo estudo sugeri que 8 dias após a HI neonatal a adenosina é preferencialmente desaminada à inosina do que fosforilada à AMP.

- Uma alteração tempo-dependente foi observada em relação à atividade da AChE em cortex. Além disso, um persistente aumento na peroxidação lipídica foi observado, o qual teve seus níveis aumentados logo no início do insulto e persistiram até os 8 dias após a HI. Além disso, a atividade da ADA em eritrócitos mostrou uma correlação positiva com os marcadores inflamatórios séricos analisados (IL-1β, IL-6, TNF-α e INF-γ). Este dado reforça o papel imunoregulatório da ADA durante condições de injúria através da modulação dos níveis de adenosina.

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