
**UFSM**

**Dissertação de Mestrado**

# ENZIMAS QUE HIDROLISAM NUCLEOTÍDEOS DE ADENINA EM SINAPTOSSOMAS DE CÓRTEX CEREBRAL E PLAQUETAS DE RATOS DESMIELINIZADOS PELO BROMETO DE ETÍDIO E TRATADOS COM INTERFERON-β

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**Roselia Maria Spanevello**

**PPGBT**

**Santa Maria, RS, Brasil**

**2006**

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por

**Roselia Maria Spanevello**

Dissertação apresentada ao Curso de Mestrado do Programa de Pós-Graduação em Bioquímica Toxicológica, da Universidade Federal de Santa Maria (UFSM, RS), como requisito parcial para a obtenção do grau de

**Mestre em Bioquímica Toxicológica**

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**Universidade Federal de Santa Maria**
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**Programa de Pós-Graduação em Bioquímica Toxicológica**

A Comissão Examinadora, abaixo assinada, aprova a Dissertação de Mestrado

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elaborada por

**Roselia Maria Spanevello**

como requisito parcial para a obtenção do grau de
**Mestre em Bioquímica Toxicológica**

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Santa Maria, 23 de fevereiro de 2006

**“A vida só pode ser compreendida olhando para trás, mas só pode ser vivida olhando-se para frente”**

Sören kierkegaard

***Dedico este trabalho aos meus pais por serem simplesmente assim tão.... MARAVILHOSOS!***

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A todos os amigos e pessoas que de alguma forma me ajudaram, meu sincero agradecimento a todos.

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## MANUSCRITO I (ARTIGO):

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# LISTA DE ABREVIATURAS

ADP - adenosina difosfato

AMP - adenosina monofosfato

ATP - adenosina trifosfato

BE - brometo de etídio

$CaCl_2$ - cloreto de cálcio

CMP - citidina monofosfato

EDTA - ácido etilenodiamino tetraacético

EM - esclerose múltipla

FDA - food and drug administration

GMP - monofosfato de guanosina

HEPES - ácido N-2-hidroxietilpiperazina

IFN-$\alpha$ - interferon alfa

IFN-$\beta$ - interferon beta

IFN-$\gamma$ - interferon gama

IFNs - interferons

IMP - ionosina monofosfato

KCl - cloreto de potássio

NaCl - cloreto de sódio

OPCs - células progenitoras de oligodendrócitos

Pi - fosfato inorgânico

PP - forma progressiva primária da esclerose múltipla

PRP - plasma rico em plaquetas

PS - forma progressiva secundária da esclerose múltipla

SNC - sistema nervoso central

SNP - sistema nervoso periférico

SR - forma surto/remissão da esclerose múltipla

TCA - ácido tricloroácetico

UMP - uridina monofosfato

## RESUMO

Dissertação de Mestrado

Programa de Pós-Graduação em Bioquímica Toxicológica

Universidade Federal de Santa Maria

# ENZIMAS QUE HIDROLISAM NUCLEOTÍDEOS DE ADENINA EM SINAPTOSSOMAS DE CÓRTEX CEREBRAL E PLAQUETAS DE RATOS DESMIELINIZADOS PELO BROMETO DE ETÍDIO E TRATADOS COM INTERFERON-β

Autora: Roselia Maria Spanevello

Orientador: Vera Maria Morsch

Data e local de Defesa: Santa Maria, 23 de Fevereiro de 2006.

A atividade das enzimas NTPDase (E.C. 3.6.1.5, apirase, CD39) e 5'-nucleotidase (E.C. 3.1.3.5, CD73) foram avaliadas em sinaptossomas de córtex cerebral e plaquetas de ratos submetidos a desmielinização experimental pelo brometo de etídio (BE) associado ao tratamento com interferon beta (IFN-β). Os seguintes grupos foram estudados: I - controle (salina), II - (salina e IFN-β), III - (BE) e IV - (BE e IFN-β). Após 7, 15 e 30 dias do procedimento cirúrgico, os animais (n=5) foram sacrificados e as amostras coletadas para os ensaios enzimáticos. Os resultados demonstraram que a atividade da NTPDase e 5'-nucleotidase em sinaptossomas aumentou no grupo III após 7 e 15 dias em relação ao grupo controle. O tratamento com IFN-β aumentou a hidrólise do ATP em sinaptossomas nos grupos II e IV após 7 dias e no grupo IV aos quinze dias enquanto que não foram observadas alterações na hidrólise do ADP em relação ao grupo I. A atividade da 5'-nucleotidase em sinaptossomas também aumentou no grupo II e IV aos quinze dias quando comparado ao grupo controle. Em relação as ectonucleotidases de plaquetas, os resultados demonstraram que a atividade da NTPDase está reduzida no grupo III após 7 e 15 dias e o tratamento com IFN-β aumentou a hidrólise dos nucleotídeos no grupo IV em relação ao grupo III. No grupo II foi observado uma redução na hidrólise do ATP e ADP após 7 dias e um aumento após 15 dias em relação ao grupo I. A atividade da 5'-nucleotidase em plaquetas aumentou no grupo IV após 7 dias e reduziu nos grupos III e IV após 15 dias quando comparado ao grupo controle. Aos 30 dias nenhuma alteração foi observada na atividade das enzimas em sinaptossomas e plaquetas dos respectivos grupos estudados. Estes resultados demonstraram que a atividade das enzimas NTPDase e 5'-nucleotidase está alterada em sinaptossomas e plaquetas de ratos após um evento de desmielinização tóxica no sistema nervoso central e que IFN-β interferiu com a hidrólise dos nucleotídeos de adenina.

## ABSTRACT

Master Dissertation

Toxicological Biochemistry Post-Graduation

Universidade Federal de Santa Maria

**ENZYMES THAT HYDROLYZE ADENINE NUCLEOTIDES IN SYNAPTOSOMES FROM THE CEREBRAL CORTEX AND PLATELETS OF THE RATS DEMYELINATED BY ETHIDIUM BROMIDE AND TREATED WITH INTERFERON - β.**

Author: Roselia Maria Spanevello

Oriented by: Vera Maria Morsch

Place and date: Santa Maria, February 23, 2003.

The activities of the enzymes NTPDase (E.C. 3.6.1.5, apyrase, CD39) and 5'-nucleotidase (E.C. 3.1.3.5, CD73) were analyzed in synaptosomes from the cerebral cortex and platelets of rats submitted to demyelination by ethidium bromide (EB) associated with interferon-β (IFN-β) treatment. The following groups were studied: I - control (saline), II - (saline and IFN-β), III - (EB) and IV - (EB and IFN-β). After 7, 15 and 30 days of the surgical procedure, the animals (n=5) were sacrificed and samples was collected for enzymatic assays. The results showed that NTPDase and 5'-nucleotidase activities was increased in synaptosomes of the group III after 7 and 15 days in relation in the control group. The treatment with IFN-β increased the ATP hydrolysis in the groups II e IV after seven days and in the group IV in fifteen days, whereas no alteration was observed in ADP hydrolysis in relation the group I. The 5'-nucleotidase activity in synaptosomes also increased in the group II e IV in fifteen days, when compared with the control group. In relation the ectonucleotidases in platelets, the results demonstrated that the NTPDase activity was reduced in the group III after seven and fifteen days and the IFN-β treatment increased nucleotides hydrolysis in the group IV with relation the group III. In the group II was observed a reduction in the ATP and ADP hydrolysis after 7 days and an increase after 15 days with relation the group I. The 5'-nucleotidase activity in platelets increased in the group IV after 7days and reduced in the groups III and IV after 15 days when compared with the control. In 30 days, no significant alteration was observed in the enzymes activities in synaptosomes and platelets of the groups studied. These results demonstrated that the NTPDase and 5'-nucleotidase activities are altered in synaptosomes and platelets the rats after an event the toxic demyelination in the central nervous system and that IFN-β interfered with the adenine nucleotide hydrolysis.

# 1. INTRODUÇÃO

Os nucleotídeos de adenina ATP e ADP, e o nucleosídeo correspondente adenosina, representam uma importante classe de moléculas extracelulares que, ao interagirem com receptores específicos, sinalizam vias que são importantes para o funcionamento celular (Soslau & Youngprapakorn, 1997; Rathbone et al., 1999).

A sinalização induzida por estas moléculas correlaciona-se diretamente à atividade das enzimas NTPDase (ATP difosfoidrolase, apirase, Ecto/CD 39, E.C. 3.6.1.5) e 5'-nucleotidase (E.C. 3.1.3.5) (Zimmermann, 1999; 2001). A NTPDase hidrolisa ATP e ADP produzindo AMP, sendo este posteriormente hidrolisado pela 5'-nucleotidase gerando adenosina (Ziganshin et al., 1994; Zimmermann, 1996). Estas enzimas são as principais responsáveis por manter os níveis extracelulares destas moléculas, exercendo várias funções de acordo com sua localização tecidual e interação de seus produtos de hidrólise com receptores específicos (Battastini et al., 1991; Sarkis et al., 1995; Pilla et al., 1996; Leal et al., 2005).

Os nucleotídeos e os nucleosídeos de adenina possuem papéis fisiológicos relevantes nos sistemas nervoso e vascular. Eles estão envolvidos na neurotransmissão (Gib & Halliday, 1996), sinaptogênese (Rathbone et al.,1999), neuromodulação (Dunwiddie & Masino, 2001) e neuroproteção do sistema nervoso (Ciccarelli et al., 2001), além de serem responsáveis pela modulação do tônus vascular e das funções plaquetárias (Birk et al., 2002; Rozalcki et al., 2005). Por outro lado, tem sido relatado o envolvimento destas

moléculas em processos patológicos incluindo os eventos relacionados com desmielinização e remielinização do sistema nervoso, destacando assim sua importância em muitas patologias desmielinizantes, principalmente a Esclerose Múltipla (EM) (Agresti et al., 2005).

As doenças desmielinizantes são graves e diferem quanto à etiologia e localização no sistema nervoso (Siegel, 1999). São doenças que afetam a bainha de mielina, prejudicando assim a transmissão do impulso nervoso e levando ao aparecimento de distúrbios neurológicos severos (Bennet & Plum, 1997). A EM é considerada a patologia desmielinizante mais comum, e desde a sua descoberta vem sendo incansavelmente estudada (Poser & Brinar, 2004). No entanto, as causas que levam a destruição das bainhas de mielina e as formas de intervenção terapêutica ainda não foram totalmente elucidadas (Moreira et al., 2000; Fassas, 2004).

A remielinização, nestas situações patológicas, é de grande importância, pois a reconstrução das bainhas de mielina perdidas pode restabelecer a função neurofisiológica (Franklin, 2002; Zhao et al., 2005). Em relação a este processo, o ATP, o ADP e a adenosina podem ter papéis relevantes, pois estudos têm demonstrado que eles promovem a proliferação, migração e diferenciação de células progenitoras de oligodendrócitos (OPCs) em oligodendrócitos maduros, os quais podem atuar na remielinização das áreas lesadas (Stevens et al., 2002; Agresti et al., 2005). Além disso, o papel destes nucleotídeos em patologias desmielinizantes não tem se limitado somente a eventos de remielinização. Alterações na função plaquetária e uma maior sensibilidade das plaquetas ao ADP têm sido observadas em pacientes com

EM, o que pode nos indicar que estes nucleotídeos também estão envolvidos em processos de tromboregulação nesta condição (Neu et al., 1982; Cananzi et al., 1987).

Devido aos mecanismos ainda pouco compreendidos em relação a desmielinização e remielinização do sistema nervoso, numerosos estudos experimentais mimetizando estas situações têm sido desenvolvidos (Gold et al., 2000; Altmann & Boyton, 2004). O modelo experimental que utiliza o brometo de etídio (BE) como droga indutora de desmielinização tem sido amplamente utilizado (Reynolds et al., 1996; Graça et al., 2001; Guazzo, 2005), sendo considerado um dos principais modelos para avaliar a capacidade reparativa do sistema nervoso após a perda das bainhas de mielina (Stangel & Hartung, 2002).

Desde a descoberta do envolvimento do sistema imune na patogênese da EM, muitos agentes imunossupressores e imunomoduladores têm sido utilizados no tratamento desta doença, os quais também vêm sendo empregados em muitos modelos experimentais de desmielinização (Bondan et al., 2000; Smith & Franklin, 2001; Floris et al., 2002 ; Neuhaus et al., 2003). O interferon beta (IFN-$\beta$) é um agente imunomodulador atualmente empregado no tratamento da EM, porém os mecanismos celulares e moleculares envolvendo seus efeitos benéficos ainda permanecem pouco compreendidos (Revel, 2003).

Muitos mecanismos em relação a patologias desmielinizantes ainda precisam ser elucidados. Considerando que moléculas como o ATP, o ADP e a adenosina participam nestas situações, torna-se relevante estudar o envolvimento de enzimas que degradam estes nucleotídeos em um modelo de

desmielinização, para que se tenha uma melhor compreensão do papel destas em patologias graves como a EM.

Dessa forma os objetivos do presente trabalho serão descritos a seguir.

## 1.1 Objetivos

### 1.1.1 - Objetivo Geral

Avaliar a atividade das enzimas NTPDase e 5'-nucleotidase em sinaptossomas de córtex cerebral e plaquetas de ratos experimentalmente desmielinizados e tratados com IFN - β.

### 1.1.2 - Objetivos Específicos

- Analisar, *ex vivo,* a atividade das enzimas NTPDase e 5'-nucleotidase em sinaptossomas de córtex cerebral de ratos aos 7, 15 e 30 dias após a indução de desmielinização com BE em associação ao tratamento com IFN-β;

- Avaliar, *ex vivo*, a atividade das enzimas NTPDase e 5'-nucleotidase em plaquetas de ratos aos 7, 15 e 30 dias após a indução de desmielinização com BE em associação ao tratamento com IFN-β.

# 2. REVISÃO DE LITERATURA

## 2.1 Mielina

A mielina é uma estrutura membranosa característica do tecido nervoso, sendo sintetizada por oligodendrócitos no sistema nervoso central (SNC) e por células de Schwann no sistema nervoso periférico (SNP) (Purves et al., 2005). A mielina envolve os axônios funcionando como um isolante (Fig.1), aumentando a velocidade de transmissão do impulso nervoso entre neurônios e seus alvos (Rummler et al., 2004).

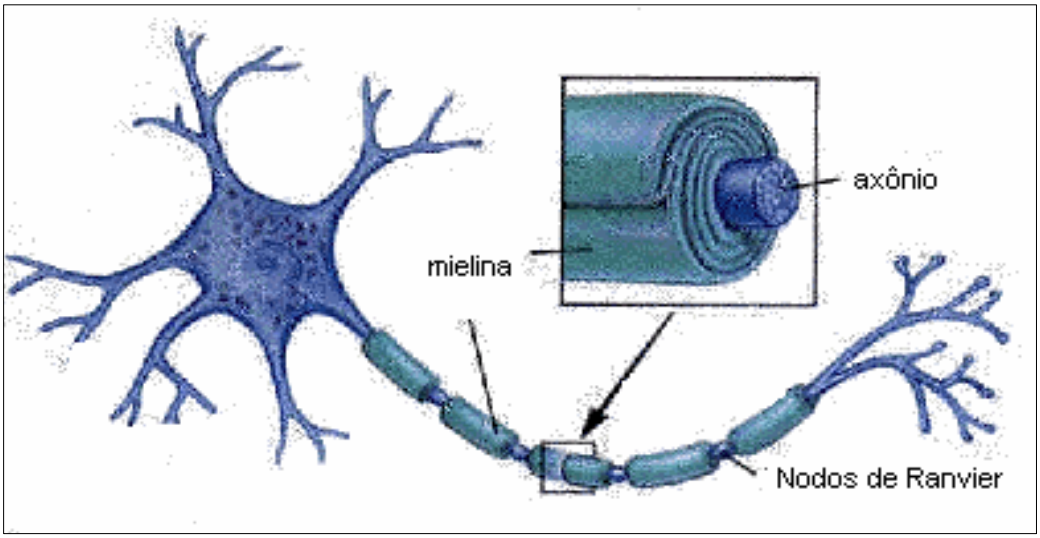



Figura 1 - Bainha de mielina envolvendo o axônio (http://www.pmdfoundation.org/neuron_and_myelin_damage.htm).

Quanto a sua composição bioquímica, a mielina é constituída por lipídios e proteínas, sendo que os lipídios perfazem 70% da estrutura e incluem os cerebrosídios, fosfolipídios e colesterol (Lazzarini, 2004). A fração protéica corresponde a 30% consistindo basicamente de dois constituintes principais: proteína mielínica básica e proteínas proteolíticas (Lazzarini, 2004; Rummler et al., 2004).

A síntese mais ativa de mielina se inicia intra-uterinamente e continua durante os dois primeiros anos de vida, entretanto, ela continua como parte do crescimento do cérebro e da medula até atingir o peso adulto do sistema nervoso (Bennet & Plum,1997).

O processo de mielinização é considerado um marco significante na evolução dos vertebrados por permitir a transmissão do impulso nervoso de forma rápida e eficiente (Lazzarini, 2004). Este processo promoveu o desenvolvimento de um sistema nervoso capaz de integrar funções sensoriais, motoras e cognitivas (Purves et al., 2005).

## 2.2 Desmielinização

A integridade das bainhas de mielina é dependente do funcionamento normal das células mielinogênicas (oligodendrócitos e células de Schwann) bem como da viabilidade dos axônios que elas revestem (Siegel, 1999).

Desmielinização refere-se ao processo de remoção das bainhas de mielina previamente formadas pelas células mielinogênicas, podendo ser classificada em dois tipos principais: primária e secundária (Lazzarini, 2004). Denomina-se desmielinização primária quando a lesão na mielina ocorre por instabilidade ou dano na célula mielinogênica, porém com preservação dos axônios, enquanto que desmielinização secundária ocorre devido primeiramente a uma lesão neuronal levando subseqüentemente a degeneração da mielina (Siegel, 1999; Lazzarini, 2004).

A alteração estrutural das bainhas de mielina é uma conseqüência comum após uma grande variedade de eventos patológicos incluindo reações

auto-imunes, intoxicações, desordens metabólicas, infecções virais e trauma mecânico, os quais podem afetar tanto as células formadoras de mielina quanto os próprios neurônios mielinizados (Siegel, 1999).

Várias doenças caracterizadas por desmielinização primária têm sido relatadas tanto em humanos como em animais, onde pode-se destacar a cinomose em cães e a esclerose múltipla em humanos (Bennet & Plum, 1997; Lazzarini, 2004), sendo os mecanismos patogênicos de muitas destas condições ainda são pouco compreendidos.

### 2.2.1 Esclerose múltipla

A esclerose múltipla (EM) é a doença desmielinizante mais comum do SNC afetando aproximadamente mais de 2 milhões de pessoas em todo o mundo (Javed & Reder, 2005), sendo a incidência maior em mulheres na faixa etária de 20 a 40 anos (Reipert, 2004).

O termo EM foi designado devido ao fato de múltiplas áreas de desmielinização ocorrerem no sistema nervoso, tanto em nível medular quanto cerebral (Minguetti, 2001). Os astrócitos proliferam e preenchem grande parte do espaço vago deixado pela mielina e oligodendrócitos, formando uma cicatriz que recebeu o nome de esclerose (Bennet & Plum, 1997).

Embora esta doença tenha sido reconhecida e descrita pela primeira vez há mais de 100 anos, sua causa ainda continua desconhecida (Poser & Brinar, 2004). A hipótese patogênica mais aceita é que a EM seja fruto de uma predisposição genética associada a um fator ambiental desconhecido que, ao se apresentarem num mesmo indivíduo originariam uma disfunção no sistema

imune (Fassas, 2004; Sotgiu et al., 2004). Esta disfunção mantém células T ativadas permitindo que elas atravessem a barreira hematoencefálica e desenvolvam uma ação lesiva contra a mielina e os oligodendrócitos (Fig. 2) ocasionando assim, um déficit na condução do impulso nervoso levando ao aparecimento de sintomas (Lassman, 2005; Purves et al., 2005).

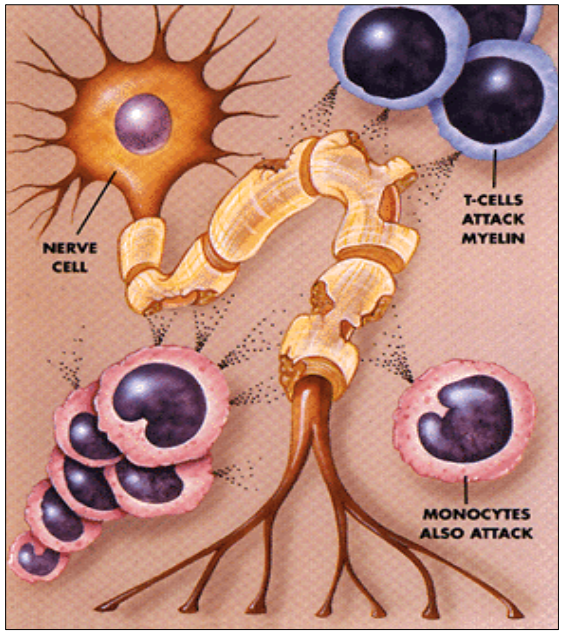



Figura 2 - Lesão na bainha de mielina pela ativação de células T.

(http://apu.sfn.org/content/publications/brainbriefings/ms.html)

Inicialmente a maioria dos pacientes apresenta a forma surto-remissão (SR) da EM, caracterizada por episódios de desmielinização com conseqüentes deficiências neurológicas, seguido por episódios de remielinização com desaparecimento de sintomas (Reipert, 2004). Com o passar do tempo, a remielinização torna-se menos freqüente e a forma SR transforma-se na forma progressiva secundária (PS), onde ocorre o comprometimento axonal levando a um aumento irreversível dos déficits neurológicos (Moreira et al., 2000; Bjartmar et al., 2003). Alguns pacientes, no

entanto, desde o início da doença manifestam sintomas e sinais neurológicos progressivos, caracterizando a forma progressiva primária (PP) da EM (Reipert, 2004).

Muitas pesquisas vêm sendo realizadas com o objetivo de descobrir mecanismos que estimulem o reparo mielínico (Franklin, 2002; Zhao et al., 2005). Este reparo na bainha de mielina, requer a proliferação e subseqüente diferenciação de células progenitoras de oligodendrócitos (OPCs) em oligodendrócitos maduros, os quais são responsáveis pela remielinização da área lesada, sendo este um fator crucial para evitar um processo de degeneração (Stangel & Hartung, 2002). No entanto, devido aos mecanismos complexos que envolvem a remielinização, a identificação de fatores que possam estimular a diferenciação destas células representará um importante achado para a intervenção terapêutica na EM (Levine & Reynolds, 1999; Blakemore et al., 2003; Penderis et al., 2003).

Alterações vasculares também têm sido descritas em portadores de EM (Wakefield et al., 1994; Vandenberghe et al., 2003). Estudos têm relatado que plaquetas de pacientes com esta doença apresentam maior sensibilidade a agonistas de agregação plaquetária como ADP, além de uma grande tendência a agregação espontânea quando comparado com pacientes normais (Millar et al., 1966; Neu et al., 1982). Os mecanismos envolvidos na alteração das funções plaquetárias nesta patologia não foi ainda elucidado, porém a hipótese mais aceita é que a destruição da bainha de mielina liberaria substâncias neurais, como a proteína básica de mielina que poderiam causar alterações na membrana da plaqueta (Chiang et al., 1982).

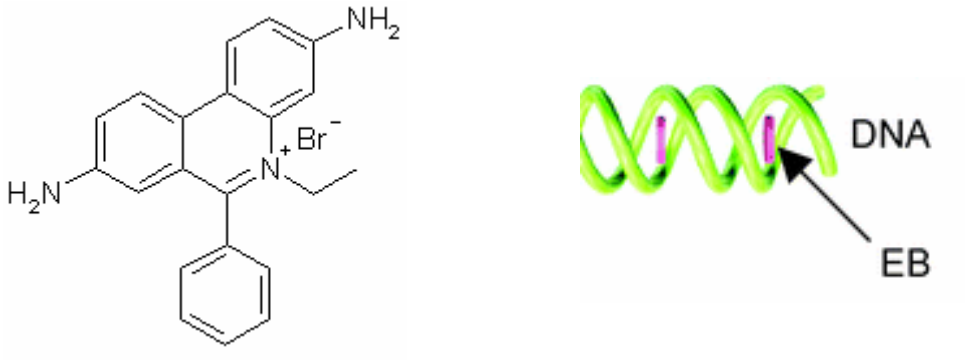
## 2.3 Modelos Experimentais de Desmielinização

Devido aos mecanismos complexos e ainda pouco conhecidos em relação aos processos de perda e reparo das bainhas de mielina, numerosos modelos experimentais que mimetizam estas situações têm sido desenvolvidos (Gold et al., 2000; Altmann & Boyton, 2004; Hart et al., 2004). Tais modelos vêm sendo utilizados com o objetivo de permitir o conhecimento dos eventos celulares envolvidos, bem como sugerir formas de intervenção terapêutica para restaurar a função neurofisiológica, especialmente no caso da EM (Steinman, 1999).

Os principais modelos empregados para o estudo de eventos desmielinizantes do sistema nervoso incluem a indução de reações imunológicas (Reynolds et al., 1996; Gold et al., 2000; Buddeberg et al., 2004) e administração de substâncias tóxicas como o cuprizone (Stangel & Hartung, 2002; Yu et al., 2004), a lisolecitina (Lovas et al., 2000) e o brometo de etídio (BE) (Bondan et al., 2000; Riet-Correa et al., 2002; Guazzo, 2005).

### 2.3.1 Modelo Experimental de Desmielinização pelo Brometo de Etídio (BE)

O BE (3,8-diamino-5-etil-6-fenil-fenatridina) é um composto púrpura fluorescente, cuja molécula possui propriedades que permitem sua intercalação entre os pares de bases do DNA, causando desta forma uma alteração conformacional da dupla hélice e, assim, impedindo sua replicação e transcrição (Fig. 3) (Luedtke & Tor, 2003; Guazzo, 2005).




(A) (B)

Figura 3 - Estrutura do brometo de etídio (3,8-diamino-5-etil-6-fenil-fenatridina) (A); intercalação entre os pares de bases do DNA (B). Adaptado de Luedtke & Tor, (2003).

O BE tem sido originalmente utilizado como agente tripanocida (Newston, 1957) e atualmente usado como corante de ácidos nucléicos submetidos à eletroforese em gel (Vardevanyan et al., 2001; Luedtke & Tor, 2003). Além disso, é considerado também uma droga indutora de desmielinização primária por destruir seletivamente células gliais - astrócitos e oligodendrócitos (Bondan, 1997; Graça et al., 2001).

Este modelo experimental vem sendo estudado desde a década de 80, e é considerado atualmente um dos modelos mais utilizados para explorar a capacidade reparativa do sistema nervoso em relação às lesões na bainha de mielina (Graça & Blakemore, 1986; Stangel & Hartung, 2002). O BE tem sido empregado para avaliar os eventos relacionados a desmielinização e remielinização na medula espinhal (Fushini & Shirabe, 2002), pedúnculo cerebral (Penderis et al., 2003), nervo óptico (Guazzo, 2005) e tronco encefálico (Reynolds et al., 1996; Bondan et al., 2000; 2002).

A desmielinização causada pelo BE no tronco encefálico de ratos tem sido bem estabelecida (Bondan, 1997; Graça et al., 2001; Bondan et al., 2004). O tronco encefálico é responsável por conduzir a informação entre o cérebro e a medula espinhal, controlando importantes funções vitais como, por exemplo, a respiração e a freqüência cardíaca (Ekman, 2000; Purves et al., 2005). Injeções de BE na cisterna basal induzem lesões que estendem-se desde o mesencéfalo até o corpo trapezóide, porém com maior gravidade na ponte, onde chega a comprometer de 1/3 a 1/2 desta estrutura (Bondan, 1997).

Análises histológicas têm demonstrado que a injeção intracisternal de BE causa mudanças degenerativas nos oligodendrócitos e astrócitos após 72 horas da indução. Axônios desmielinizados aparecem após o sexto dia da injeção e os primeiros sinais de remielinização são evidentes a partir dos 12 dias, apresentando-se num estágio mais avançado nos 30 dias pós-injeção (Reynolds et al., 1996; Bondan, 1997; Bondan et al., 2000; Graça et al., 2001).

Embora este modelo seja de natureza tóxica e, portanto não mimetize uma reação auto-imune como ocorre na EM, ele tem sido amplamente utilizado por apresentar boa reprodutibilidade de desmielinização e remielinização permitindo assim, o conhecimento de muitos fatores celulares envolvidos nestes eventos (Stangel & Hartung, 2002).

## 2.4 Imunomoduladores e Imunossupressores

Desde a descoberta do envolvimento do sistema imune na patogênese da EM, muitas drogas imunossupressoras e imunomoduladoras têm sido utilizadas tanto no tratamento desta doença (Neuhaus et al., 2003), quanto em modelos

experimentais (Bondan et al., 2000; Smith & Franklin, 2001; Floris et al., 2002). Essas drogas têm como objetivo principal controlar ou modular as funções de células T, as quais parecem desempenhar um papel importante na fisiopatologia do processo desmielinizante (Neuhaus et al., 2003).

O tratamento com imunossupressores na EM começou na década de 60, sendo que as primeiras drogas utilizadas foram a ciclosfosfamida, azatiotropina e metotrexate, e mais recentemente, a ciclosporina e mitoxantrone (Faulds et al., 1993; Kappos, 1998; Neuhaus et al., 2003; Edan et al., 2004; Weiner, 2004). No entanto, a maioria destes medicamentos não tem apresentado resultados eficazes e parecem não evitar a progressão da doença (Souza & Oliveira, 1999; Callegaro et al., 2002).

Atualmente o foco da terapia na EM está concentrado no uso de agentes imunomoduladores, os quais têm mostrado efeitos satisfatórios, reduzindo o número de surtos e minimizando a progressão da incapacidade neurológica que ocorre nesta patologia (Tilbery et al., 2000; Revel, 2003). Os agentes imunomoduladores utilizados atualmente são: acetato de glatiramer e IFN-$\beta$ (Neuhaus et al., 2003).

### 2.4.1 Interferons

Os interferons (IFNs) são proteínas sintetizadas e secretadas por muitos tipos celulares em resposta à infecção viral (Stites, 1991). Foram descritos pela primeira vez em 1957 como substâncias capazes de “interferir“ no processo de replicação viral (Calich & Vaz, 1988). Além desta atividade antiviral, experiências sucessivas colocaram em evidência outras ações biológicas dos

IFNs, como seus efeitos antiproliferativos e imunomodulatórios (Alam, 1995; Dafny & Yang, 2005).

Os IFNs são divididos em 3 classes principais: IFN - $\alpha$ , IFN - $\beta$ e IFN-$\gamma$ (Sharon, 1998). O IFN - $\alpha$ e o IFN - $\beta$ constituem os IFNs do tipo I, os quais são sintetizados de forma mais proeminente pelos leucócitos e fibroblastos, respectivamente. Ambos podem ligar-se ao mesmo receptor nas células alvo (Stites, 1991; Sharon, 1998). O IFN-$\gamma$ é denominado IFN tipo II, sendo sintetizado e secretado por linfócitos T ligando-se a um receptor distinto dos demais IFNs (Stites, 1991). Os IFNs do tipo I são atualmente comercializados por terem várias aplicações clínicas.

#### 2.4.1.1 Interferon beta (IFN-$\beta$)

O IFN-$\beta$ é clinicamente usado no tratamento da EM, bem como em casos de hepatite viral e certas formas de câncer (Alam, 1995; Javed & Reder, 2005). As tentativas iniciais no uso do IFN-$\beta$ na EM foram baseadas em suas propriedades antivirais e na hipótese de uma infecção viral persistente como causa da doença (Souza & Oliveira, 1999). Vários testes clínicos foram realizados até que, em 1993, o FDA (Food and Drug Administration) americano aprovou e autorizou o uso do IFN-$\beta$ em portadores da forma SR da EM (The IFN-$\beta$ Multiple Sclerosis Study Group, 1993).

Atualmente existem três formas de IFN-$\beta$ que podem ser usadas na terapia clínica: o natural (nIFN-$\beta$) obtido de fibroblastos humanos, o interferon recombinante-1b (IFN-$\beta$-1b) obtido de bactérias e o interferon recombinate-1a (IFN-$\beta$-1a) obtido de células de ovário de hamster (Alam, 1995). A molécula do

IFN-β-1a é idêntica ao IFN natural, enquanto que a molécula do IFN-β-1b difere pela substituição de um único resíduo de aminoácido e pelo fato de não ser glicosilada (Fig. 4) (Neuhaus et al., 2003; Revel, 2003).

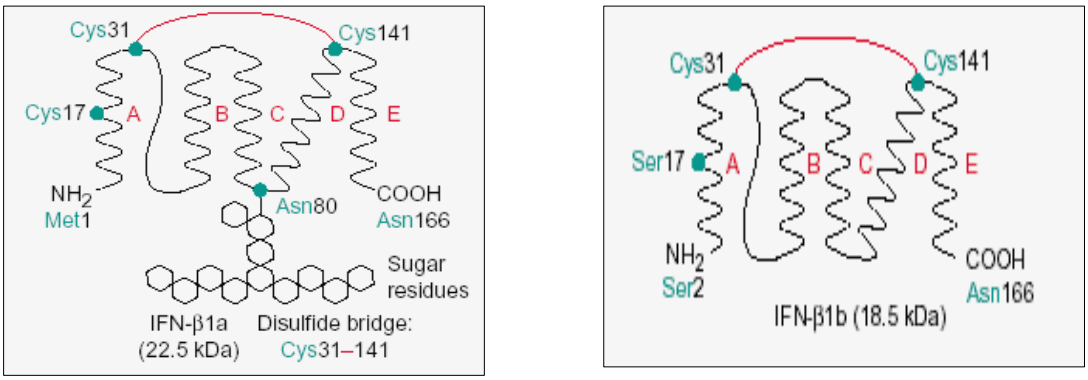



Figura 4 - Estrutura dos interferons recombinantes. Adaptado de Neuhaus et al. (2003).

A síntese de IFNs recombinantes foi de grande importância, pois propiciou uma maior produção a custos mais razoáveis, permitindo assim o desenvolvimento de ensaios clínicos com grupos maiores de pacientes (Tilbery et al., 2000). Em portadores de EM, os dois tipos de IFNs recombinantes têm mostrado resultados satisfatórios por reduzir o número de surtos, diminuir as lesões e melhorar os déficits neurológicos (Alam,1995; Souza & Oliveira, 1999; Tilbery et al., 2000; Barak & Achiron, 2002; Revel, 2003).

Muitas das questões envolvendo os efeitos benéficos do IFN-β na EM ainda não são bem compreendidas. Porém, acredita-se que a ação inibitória deste agente na ativação de células T e produção de citocinas inflamatórias seja um fator relevante para evitar a progressão da doença (Hall et al., 1997; Floris et al., 2002; Revel, 2003). Além disso, outras evidências têm indicado

que o IFN-β possui efeitos não imunes, exercendo importantes funções no controle da proliferação de astrócitos (Malik et al.,1998). Este achado pode ser de grande importância no tratamento da EM, já que a cicatriz astrocitária representa uma barreira substancial para os eventos de remielinização (Barca et al., 2003).

## 2.5 Nucleotídeos de adenina

Os nucleotídeos extracelulares de adenina ATP e ADP, e o nucleosídeo adenosina (Fig.5) são considerados, atualmente, importantes moléculas sinalizadoras, mediando seus efeitos através de receptores purinérgicos localizados na superfície celular (Illes & Ribeiro, 2004).

As concentrações destas moléculas nos fluídos extracelulares dependem de vários fatores como a quantidade liberada, os mecanismos de recaptação, situações de lise celular e a presença de enzimas como as ectonucleotidases (Rathbone et al., 1999).

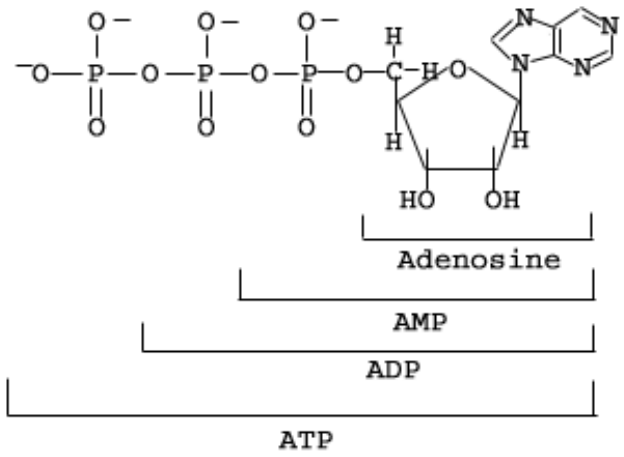



Figura 5 - Estrutura dos nucleotídeos e do nucleosídeo de adenina.

(http:/textbookofbacteriology.net/metabolism.html)

### 2.5.1 Nucleotídeos de adenina e o sistema nervoso

O papel dos nucleotídeos e do nucleosídeo de adenina na neurotransmissão e neuromodulação do sistema nervoso tem sido bem estabelecido (Cunha & Ribeiro, 2000; Dunwiddie & Masino, 2001). O ATP é um importante neurotransmissor excitatório nas sinapses nervosas purinérgicas, podendo ser também co-liberado juntamente com outros neurotransmissores como a acetilcolina e a noradrenalina (Gibb & Halliday, 1996; Sperlág & Vizi, 1996; Zimmermann, 1996; Illes & Ribeiro, 2004).

A adenosina tem um papel relevante na neuromodulação regulando a liberação de vários neurotransmissores, agindo tanto pré quanto pós-sinapticamente (Cunha, 2001; Dunwiddie & Masino, 2001; Ribeiro, et al., 2003). A regulação da liberação de neurotransmissores excitatórios por esta molécula tem se tornado importante em muitos processos patológicos, pois a adenosina pode limitar o dano causado pela excitotoxicidade destes neurotransmissores, exercendo assim uma ação protetora no sistema nervoso (Zimmermann et al., 1998; Dunwiddie & Masino, 2001; Purves et al., 2005).

Além das propriedades neurotransmissoras e neuromoduladoras, estudos colocaram em evidência muitas outras ações dos nucleotídeos e do nucleosídeo de adenina no sistema nervoso. Atualmente são reconhecidos por estarem envolvidos também na formação e regulação da sinaptogênese, plasticidade neuronal, proliferação de células gliais, e na diferenciação de células progenitoras de oligodendrócitos (OPCs) (Rathbone et al., 1999; Fiels & Stevens, 2000, Cicarelli et al., 2001; Stevens et al., 2002; Wink et al., 2003, Agresti et al., 2005).

### 2.5.2 Nucleotídeos de adenina e o sistema vascular

A sinalização purinérgica no sistema vascular tem o potencial de influenciar funções cardíacas, participar de respostas vasomotoras e controlar muitas funções plaquetárias (Burnstock, 2002).

As plaquetas são células anucleadas que desempenham importantes funções em processos hemostáticos, através de mecanismos de adesão, agregação e subseqüente formação de trombos em locais de injúria vascular (Harker, 1997; Marcus et al., 2002; Wagner & Burger, 2003).

Os nucleotídeos de adenina constituem 90% dos nucleotídeos plaquetários e encontram-se distribuídos em dois pools distintos: o pool metabólico, utilizado na manutenção das funções celulares, constituído principalmente por ATP, e o pool de armazenamento, que contém ATP e ADP para a liberação durante a secreção plaquetária (Lee et al., 1998).

O ADP constitui-se no principal agonista envolvido no recrutamento e agregação das plaquetas em locais de injúria vascular, enquanto que a adenosina possui efeitos inibitórios sobre esta agregação (Anfonsi et al., 2002; Rozalski et al., 2005). O ATP possui um papel complexo nos mecanismos de regulação de agregação plaquetária, sendo que em baixas concentrações ativa e em altas concentrações inibe a agregação de plaquetas induzida pelo ADP (Soslau & Younprapakon, 1997; Birk et al., 2002; Remijin et al., 2002; Rozalski et al., 2005).

## 2.6 Enzimas que degradam nucleotídeos de adenina

Os nucleotídeos de adenina extracelulares são hidrolisados por enzimas conhecidas como ecto-nucleotidases. Dentre estas destaca-se a NTPDase (apirase, CD39, ATP difosfoidrolase) e a 5'-nucleotidase, duas enzimas capazes de controlar a disponibilidade de ligantes como ATP, ADP e AMP à seus receptores específicos (Zimmermann, 2001).

### 2.6.1 NTPDase (ATP difosfoidrolase, Apirase, Ecto/CD39, E.C. 3.6.1.5)

E-NTPDases (Ecto – nucleosídeo trifosfato difosfoidrolases) é o termo genérico para designar uma família de enzimas responsáveis pela hidrólise de nucleotídeos tri e difosfatados (Zimmermann, 2001). Oito membros desta família já foram identificados e diferem quanto à especificidade de substratos, distribuição tecidual e localização celular (Shi et al., 2001; Zimmermann, 2001; Biogennese et al., 2004). Estas enzimas apresentam um alto grau de similaridade na sua seqüência de aminoácidos, particularmente dentro de cinco regiões que são conhecidas como "regiões conservadas da apirase" (ACRs), as quais são de extrema importância para a atividade catalítica (Zimmermann, 1999).

A NTPDase (ATP difosfoidrolase, Apirase, Ecto/CD 39) foi a primeira enzima da família E-NTPDase a ser descrita, e está ancorada na superfície celular através de duas regiões transmembranas próximas ao grupamento amino e carboxi terminal, com o seu sítio catalítico voltado para o meio extracelular (Fig.6) (Zimmermann, 2001). Esta enzima hidrolisa tanto ATP

como ADP formando AMP na presença de íons $Ca^{2+}$ e $Mg^{2+}$ (Ziganshin et al., 1994).

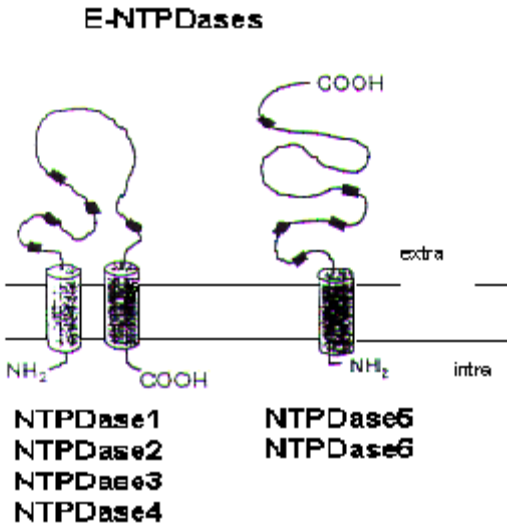



Figura 6 - Estrutura das enzimas da família NTPDase. Adaptado de Zimmermann (2001).

A presença da NTPDase (apirase) já foi relatada em plantas, parasitas, insetos e em vários tecidos e células de mamíferos, como por exemplo em córtex cerebral, linfócitos, células endoteliais e plaquetas (Battastini et al., 1991; Sarkis et al., 1995; Pilla et al., 1996, Wang & Guidotti, 1998; Leal et al., 2005).

Esta enzima vem sendo amplamente estudada nos últimos anos tanto em condições patológicas quanto em modelos experimentais (Bonan et al., 2000; Lunkes et al., 2003; Araújo et al., 2005). A função geral da apirase têm sido atribuída à hidrólise extracelular dos nucleotídeos ATP e ADP e portanto, dependendo da localização tecidual a atividade enzimática possui diferentes papéis fisiológicos (Sarkis et al., 1995; Zimmermann, 1999; Bonan et al., 2001).

### 2.6.2 5’-Nucleotidase (CD73, E.C. 3.1.3.5)

A ecto-5’-nucleotidase é uma enzima ancorada a membrana plasmática via uma molécula de GPI (glicofosfatidil inositol) com seu sítio catalítico voltado para o meio extracelular (Fig.7). Entretanto, formas solúveis e clivadas desta enzima também já foram descritas (Zimmermann, 2001; Hunsucker et al., 2005).

Esta enzima catalisa a desfosforilação de vários nucleotídeos 5’-monofosfatados como CMP, IMP, UMP, GMP e AMP à seus respectivos nucleosídeos (Zimmermann, 1996). No entanto, foi demonstrado que a 5’-nucleotidase hidrolisa mais eficientemente o AMP, sendo por isto considerada a principal enzima responsável pela formação de adenosina (Zimmermann, 1996; Zimmermann et al., 1998; Zimmermann, 2001).

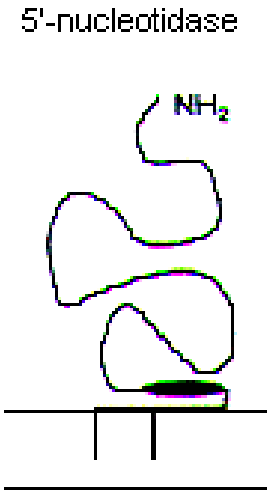



Figura 7 - Estrutura da 5’-nucleotidase ancorada a membrana plasmática via uma molécula de GPI. Adaptado de Zimmermann (2001).

As funções da 5’-nucleotidase correlacionam-se diretamente ao seu papel na produção de adenosina. Assim, de acordo com a sua localização tecidual, ela desempenha importantes funções como por exemplo no controle da agregação plaquetária, na regulação do tônus vascular e também na neuromodulação e neuroproteção do sistema nervoso (Zimmermann et al., 1998; Kawashima et al., 2000; Dunwiddie & Masino, 2001).

# 3. MANUSCRITOS

## 3.1 Manuscrito I (Artigo)

**Apyrase and 5'-nucleotidase activities in synaptosomes from the cerebral cortex of rats experimentally demyelinated with ethidium bromide and treated with Interferon-β**

R. M. Spanevello, C. M. Mazzanti, R. Kaizer, R. Zanin, D. Cargnelluti, L. Hannel, M. Côrrea, A. Mazzanti, R. Festugato, D. Graça, M. R. C. Schetinger, V. M. Morsch.

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ORIGINAL PAPER

# Apyrase and 5′-nucleotidase Activities in Synaptosomes from the Cerebral Cortex of Rats Experimentally Demyelinated with Ethidium Bromide and Treated with Interferon-β

**R. M. Spanevello · C. M. Mazzanti · R. Kaizer · R. Zanin · D. Cargnelluti · L. Hannel · M. Côrrea · A. Mazzanti · R. Festugato · D. Graça · M. R. C. Schetinger · V. M. Morsch**

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**Abstract** Apyrase and 5′-nucleotidase activities were analyzed in an ethidium bromide (EB) demyelinating model associated with interferon-β (IFN-β). The animals were divided in groups: I, control (saline); II, saline and IFN-β; III, EB and IV, EB and IFN-β. After 7, 15 and 30 days the animals ($n$=5) were sacrificed and the cerebral cortex was removed for synaptosome preparation and enzymatic assays. Apyrase activity using ATP as substrate increased in groups II, III and IV ($P<0.001$) after 7 days and in groups III and IV ($P<0.001$) after 15 days. Using ADP as substrate, an activation of this enzyme was observed in group III ($P<0.05$) after seven and 15 days. The 5′-nucleotidase activity increased in group III ($P<0.05$) after 7 days and in groups II, III and IV ($P<0.001$) after 15 days. After 30 days treatment, no significant alteration was observed in enzyme activities. Results showed that apyrase and 5′-nucleotidase activities are altered in demyelination events and that IFN-β was able to regulate the adenine nucleotide hydrolysis.

**Keywords** Apyrase · Rat · 5′-Nucleotidase · Synaptosome · IFN-β · Ethidium bromide

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## Introduction

The signaling action induced by extracellular ATP in the central nervous system (CNS) is directly correlated to ectonucleotidase enzyme activity [1, 2]. ATP is hydrolyzed to adenosine by a conjugated action of two enzymes: apyrase (ATP diphosphohydrolase, NTPDase, EC 3.6.1.5) and 5′-nucleotidase (EC 3.1.3.5) [3]. Apyrase is a non-covalent tetrameric protein that degrades ATP and ADP directly to AMP and, in the sequence, by action of 5′-nucleotidase AMP is then hydrolyzed to adenosine [4, 5]. These enzymes present a wide distribution in the CNS and have been characterized in synaptosomal fractions of the cerebral cortex as ecto-enzymes [6–8]. Their main physiological function is probably related to the metabolism of ATP and ADP to AMP and adenosine at the synaptic cleft [9–12].

ATP plays important roles in the cell to cell signaling via the activation of specific ionotropic P2X and G protein coupled P2Y receptors. It acts as a fast excitatory neurotransmitter [13] as a presynaptic neuromodulator [14], and it is also involved in neuron–glial interactions [15], with a

role in neuronal development and plasticity [16]. Furthermore, its breakdown product, adenosine, is considered an important endogenous neuromodulator in the nervous system and its actions are mediated by the activation of P1-purinoreceptors [17, 18].

In addition, ATP and adenosine also play a significant role in the pathophysiology of numerous acute and chronic disorders that include events related with CNS demyelination and remyelination [19, 20]. Demyelination is the pathological hallmark of multiple sclerosis (MS) lesions [21]. MS is a chronic inflammatory demyelinating disease of the CNS of unknown etiology, which represents the major disabling neurological illness of young adults. [22]. Many animal models have been utilized for mimicking demyelination and remyelination events of MS [23, 24], as for example, the toxic model induced by ethidium bromide (EB). EB is a substance that produces demyelination in the CNS by destroying, mainly, neuroglial cells, astrocytes and oligodendrocytes [25, 26].

Immunosuppressive and immunomodulatory agents have been widely used in toxic and immune-mediated demyelinating models, as well as in some demyelinating diseases [27, 28]. IFN-$\beta$, an immunomodulatory agent, has shown beneficial effects on relapsing-remitting MS by diminishing the severity and frequency of attacks [29, 30]. Although the cellular and molecular mechanisms that underlie the beneficial effects of IFN-$\beta$ remain little known, evidence indicates that non-immune effects may also contribute to its efficacy. Recent studies have demonstrated that IFN-$\beta$ has a protective effect on astrocytes against apoptotic cell death, a finding that may be of relevance in the treatment of MS [31, 32].

Considering that demyelination is a major cause of neurological disability in the human population and that molecules, such as ATP and its breakdown product adenosine, are involved in this pathological event, it is relevant to study apyrase and 5′-nucleotidase activities in the EB demyelinating model in normal and IFN-$\beta$ treated rats, in order to obtain more information about the role of these ectonucleotidases in diseases such as MS.

## Experimental procedure

### Chemicals

Nucleotides (ATP, adenosine 5′-triphosphate, disodium salt; ADP, adenosine 5′-diphosphate, sodium salt and AMP, adenosine 5′-monophosphate, sodium salt), Trizma base, Percoll, Ethidium bromide and Coomassie brilliant blue G were obtained from Sigma Chemical Co (St. Louis, MO, USA) and bovine serum albumin, $K_2HPO_4$, from Reagen. IFN-$\beta$ (Rebif®, 44 mcg, 12 MUI/0.5 ml) was purchased from Serono Bari (Italy). All the other chemicals used in this experiment were of the highest purity.

### Animals

Adult male Wistar rats (70–90 days, 220–300 g) were used in this experiment. The animals were maintained at a constant temperature (23 ± 1°C) on a 12 h light/dark cycle with free access to food and water. All animal procedures were approved by the Institutional Commission of the Federal University of Santa Maria and were in agreement with the International Council.

### Surgical procedure

The animals were randomly divided into four groups: I, control (injected with saline); II, (injected with saline and treated with IFN-$\beta$); III, (injected with 0.1% EB) and IV, (injected with 0.1% EB and treated with IFN-$\beta$). For the surgical procedure, the animals were anesthetized with ketamine chlorhydrate and xylazine (5:1, 0.1 ml/100 g) and after shaving the fronto-parietal-occipital area, antisepsis with 2% iodine solution was carried out. With the aid of an orthodontic roof motor and a number 2 drill, a hole was made 0.85 cm to the right of the bregma until the duramater was exposured. With the use of a Hamilton syringe with a removable needle of caliber 26s, the solutions were injected in the cistern pontis (basal), an enlargement of the subarachnoid space on the ventral surface of the pons. Ten microliters of EB were injected in the animals of groups III and IV, whereas the same volume of 0.9% of saline solution was injected in the animals of groups I and II. The duramater was left open and the skin, together with the remainder of subcutaneous tissue, was sutured with a 4.0 nylon thread. To confirm the good reproducibility of demyelination in this model, three rats injected with saline and three rats injected with EB were used for histological analysis of the lesion. The rats were perfused under deep anesthesia with 10% buffered formaline via the left ventricle at 7, 15, 21 and 30 days after injection (a.i). Brain stem coronal slices with the lesion were embedded in paraffin for routine processing and 5 μm sections were produced and stained with hematoxilin and eosin (H & E).

### Treatment with IFN-$\beta$

After 4 h of the surgical procedure, the animals belonging to groups II and IV subcutaneously received IFN-$\beta$ (500,000 U/animal per day) until the end of the experiment. Five animals of each group were sacrificed seven, 15 and 30 days after the surgical procedure, and the cerebral cortex was removed for enzyme assays.

### Synaptosome preparation

The synaptosomes were isolated as described by Nagy and Delgado-Escueta [33], using a discontinuous Percoll gradient. The cerebral cortex was gently 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 and then centrifuged at $1000 \times g$ for 10 min. An aliquot of 0.5 ml of the crude mitochondrial pellet was mixed with 4.0 ml of an 8.5% Percoll solution and layered into an isoosmotic discontinuous Percoll/sucrose gradient (10%/16%). The synaptosomes that banded at the 10 and 16% Percoll interface were collected with a wide-tip disposable plastic transfer pipette. The synaptosomal fraction was washed twice with isoosmotic solution by centrifugation at $15{,}000 \times g$ for 20 min to remove any contaminating Percoll. The pellet from the second centrifugation 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 apyrase and 5′-nucleotidase assays.

### Assay of apyrase and 5́-nucleotidase activities

The apyrase 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 mM Tris–HCl buffer, pH 8.0, in a final volume of 200 μl as described in a previous work from our laboratory [10]. Twenty microliters of enzyme preparation (8–12 μg of protein) were 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. 5́-Nucleotidase activity was determined essentially by the method of Heymann et al. [34] 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) were 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 proceeded for 20 min. In all cases, 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. [35], 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.

### In vitro assays

Synaptosomes obtained from the cerebral cortex of adult animals were used in this experiment. EB solutions were added to achieve 0.00625, 0.0125, 0.025, 0.05 and 0.1 mM final concentration. The synaptosomes were pre-incubated for 10 min with the EB solutions and apyrase and 5′-Nucleotidase activities were determined as described above.

### Protein determination

Protein was measured by the Coomassie blue method according to Bradford [36] using serum albumin as standard.

### Statistical analysis

The statistical analysis was performed using one way ANOVA, followed by Duncan multiple range test, and $P<0.05$ was considered to represent a significant difference in the analysis used. All data were expressed as mean ± SEM.

## Results

Concerning the model used in this study, the histological analysis of the ventral area of the pons showed alterations caused by the EB injection. After 7 days loss of myelin sheaths was seen as status spongiosus of the tissue and many macrophages were observed in the lesion area (Fig. 1a). After 15 days EB injection, more cellular activity and some new blood vessels were the alterations observed within the lesion area undergoing remyelination of the lost myelin sheaths (Fig. 1b). After 21 days many neuroglial cells processes and some myelinated axons were detected in the area where status spongiosus was formely seen (Fig. 1c). Resolution of the lesions occurred through remyelination of the axons demyelinated by EB at 30 days (data not shown).

In vivo apyrase and 5′-nucleotidase activities were altered according to EB injection time. The results showed a significant increase in the apyrase activity after 7 days when ATP was used as substrate ($F(3,8) = 40.17$, $P<0.001$), and post-hoc comparisons by Duncańs test disclosed that ATP hydrolysis was significantly higher in groups II, III and IV compared to the control group (Fig. 2a). Similarly, an activation of this enzyme was ob-

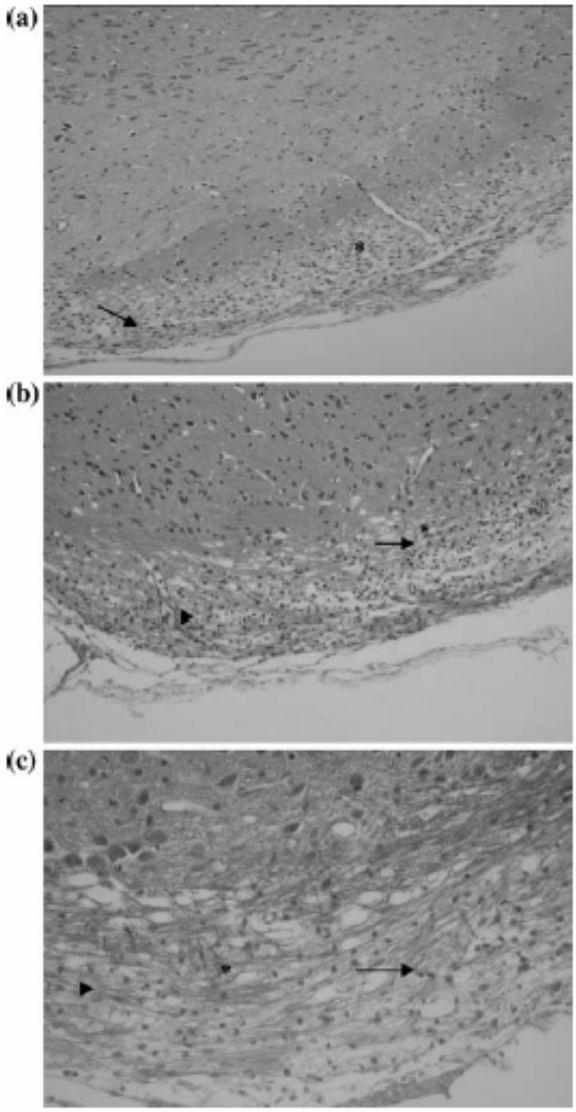


**Fig. 1** Demyelinating lesion in the ventral area of the pons caused by the injection of EB in the brainstem of Wistar rats. After 7 days (**a**) spongiosis of the tissue (*) and many macrophages with round dark nuclei (arrow) were seen (H&E, 100×). At 15 days (**b**) more cellular activity (arrow) and new blood vessels were detected (►) (H&E, 100×), while after 21 days many cells processes (*) neuroglial cells (arrow) and some myelinated axons (►) could be observed (H&E 200×)

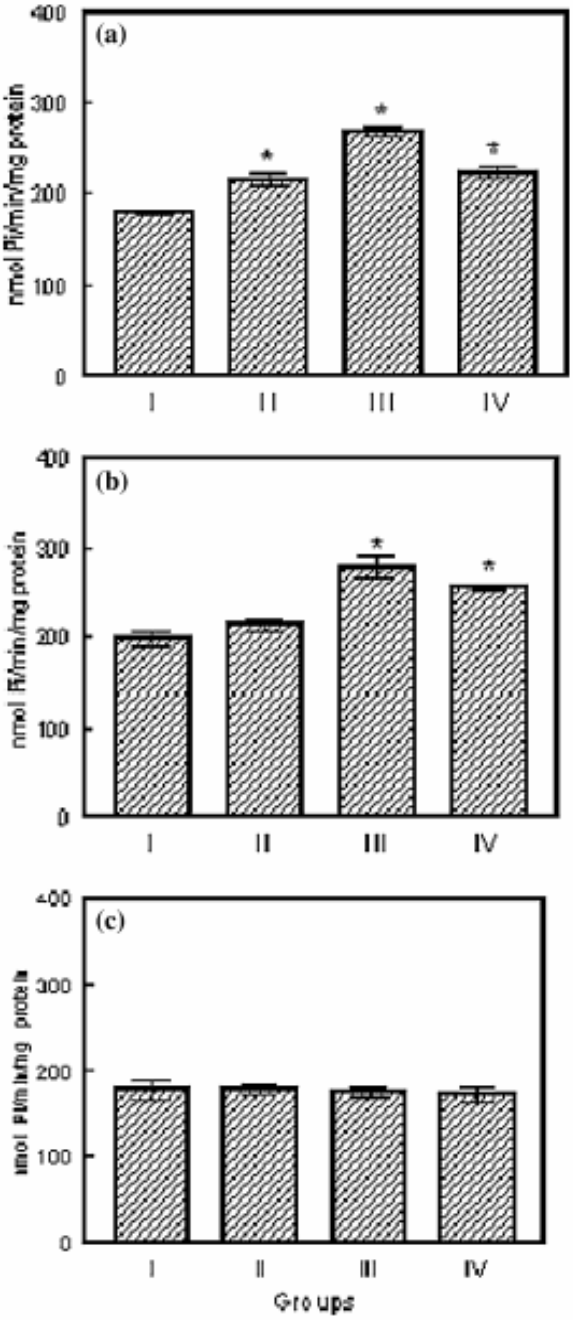


**Fig. 2** Apyrase activity in cerebral cortex synaptosomes using ATP as substrate seven (**a**); 15 (**b**) and 30 (**c**) days after EB or saline injection. Group I, saline; group II, saline+IFN-$\beta$; group III, EB and group IV, EB + IFN-$\beta$. Bars represent mean ± SEM. Different from control *$P < 0.05$. ($n$=5, all samples were run in triplicate)

served after 15 days ($F(3,8) = 11.81$, $P$=0.003), and post-hoc comparisons by Duncańs test disclosed that ATP hydrolysis was significantly higher in groups III and IV when compared to group I (Fig. 2b), while no significant difference was observed in ATP hydrolysis after 30 days ($F(3,12)$=0.125, $P$=0.944) (Fig. 2c).

In addition, apyrase activity increased significantly when using ADP as substrate ($F(3,8) = 3.80$, $P < 0.05$) 7 days after the injection of EB. Comparisons by Duncańs test revealed that ADP hydrolysis was significantly higher in group III when compared group I (Fig. 3a). After 15 days ADP hydrolysis was increased ($F(3,8) = 11.81$, $P$=0.003), and post-hoc comparisons by Duncańs test revealed that apyrase activity was significantly higher in group III when compared to group I (Fig. 3b). No significant difference in the hydrolysis of this nucleotide ($F(3,12) = 1.17$, $P = 0.360$) was observed after 30 days (Fig. 3c).

Finally, the same effect was observed in 5′-nucleotidase activity, which was significantly increased after 7 days

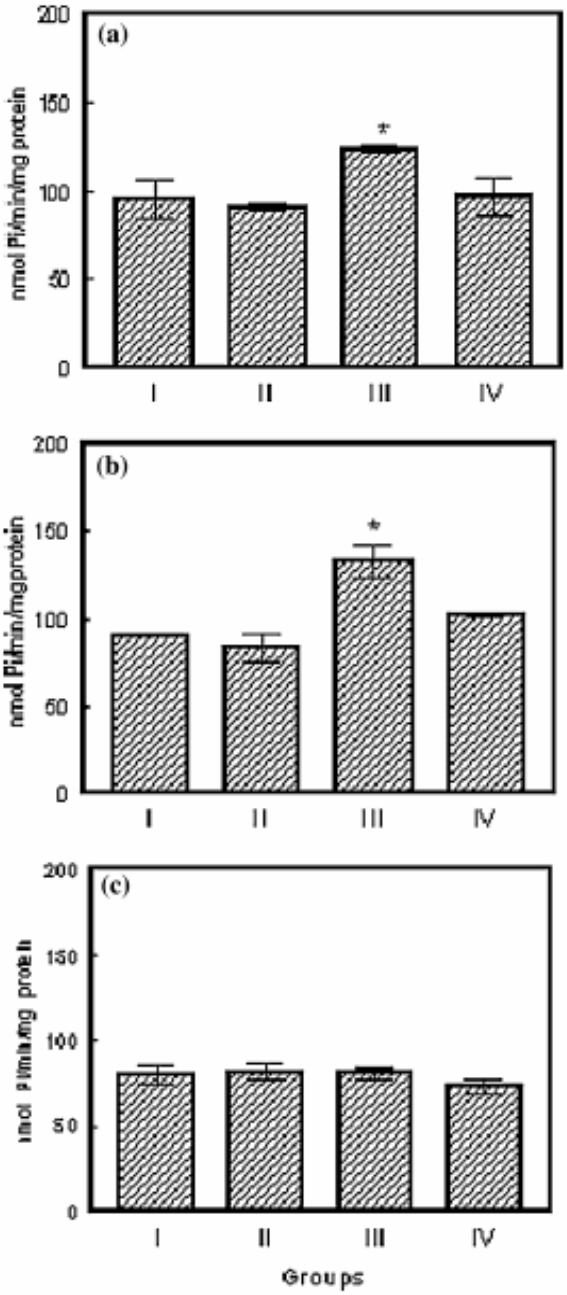


**Fig. 3** Apyrase activity in cerebral cortex synaptosomes using ADP as substrate seven (**a**); 15 (**b**) and 30 (**c**) days after EB or saline injection. Group I, saline; group II, saline+IFN-$\beta$; group III, EB and group IV, EB + IFN-$\beta$. Bars represent mean ± SEM. Different from control $^*P<0.05$. ($n = 5$, all samples were run in triplicate)

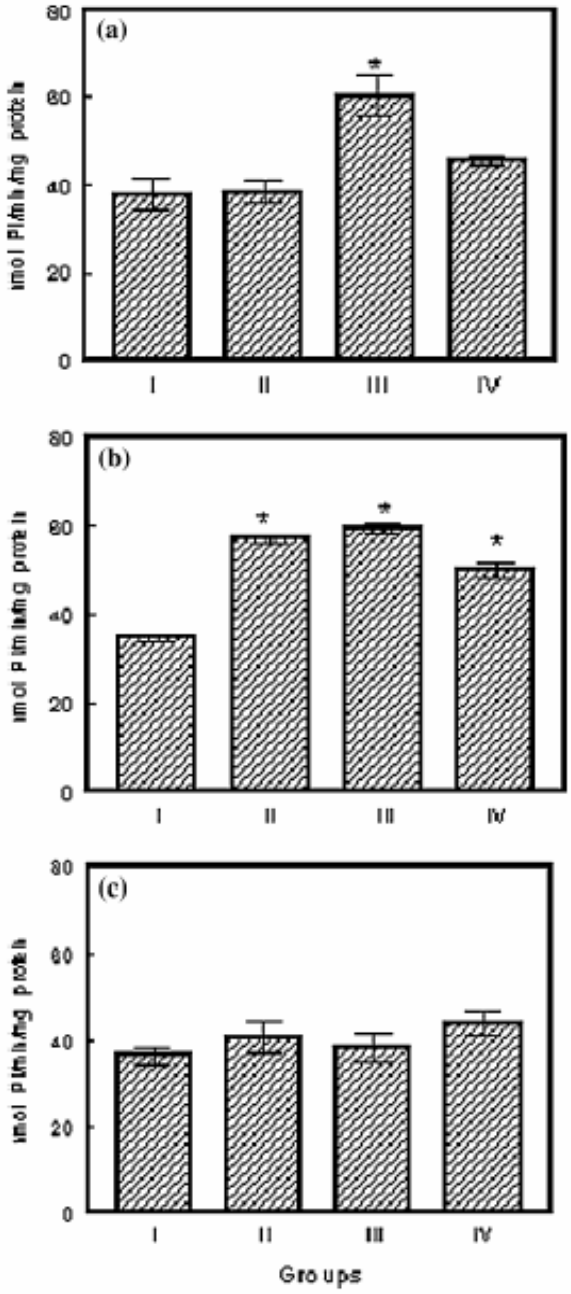


**Fig. 4** 5′-Nucleotidase activity in cerebral cortex synaptosomes using AMP as substrate seven (**a**); 15 (**b**) and 30 (**c**) days after EB or saline injection. Group I, saline; group II, saline + IFN-$\beta$; group III, EB and group IV, EB + IFN-$\beta$. Bars represent mean ± SEM. Different from control $^*P<0.05$. ($n = 5$, all samples were run in triplicate)

when using AMP as substrate ($F(3,8) = 10.25$, $P<0.05$). Comparisons by Duncańs test disclosed that AMP hydrolysis was significantly higher in group III when compared to group I (Fig. 4a). After 15 days, the activity of this enzyme continued to increase ($F(3,8) = 96.64$, $P<0.001$), and post-hoc comparisons by Duncańs test revealed that AMP hydrolysis was significantly higher in groups II, III and IV when compared to group I (Fig. 4b). In addition, no significant alteration was observed in this enzyme activity after 30 days ($F(3,12)=1.21$, $P=0.346$) (Fig. 4c).

In relation to the in vitro results, there were no significant differences in cerebral cortex synaptosomes when using ATP, ADP (apyrase) and AMP (5′-Nucleotidase) as substrates in the different EB concentrations tested in vitro. The control values (0 mM EB) were 181 ± 3.20, 89 ± 1.83 and 30 ± 1.53 for ATP, ADP and AMP hydrolysis,

respectively. For the highest EB concentration tested (0.1 mM) these values were 167 ± 7.81, 82 ± 2.51 and 32 ± 3.23 for the three nucleotides, respectively.

## Discussion

Studies have related the involvement of ectonucleotidases in many pathological conditions in the CNS [10, 37–39]. However, a study that correlates apyrase and 5′-nucleotidase activities with an experimental model of demyelination was not found in the literature. In the present study, we used the gliotoxic model induced by EB, which has been extensively studied, showing good reproducibility of demyelination, and is considered a commonly used model to explore the reparative capacity of the CNS [21, 25–27, 40].

This work was carried out in order to investigate possible changes in apyrase and 5′-nucleotidase activities in synaptosomes at different time periods after the injection of EB in the rat's brainstem. In fact, these periods are very important for evaluating the role of the enzymes in demyelination and remyelination events. Histological analysis in this investigation showed 7 days after EB injection a demyelinating lesion characterized by the loss of most myelin sheaths within the lesion area, whereas alterations detected at 15 and 30 days corresponded to remyelination of the lost myelin sheaths. Reynolds et al. [23] also showed that following the intracisternal injection of EB in rats maximum demyelination characterized by spongiosis and macrophages presence is observed at about 10 days. The first signs of remyelination in this experimental model are observed at 11–15 days after EB injection and remyelination is essentially complete at 25–30 days after the injection of this gliotoxic agent.

Our results obtained after in vivo exposition have shown that the ectonucleotidase activities are modified at the different times evaluated. A significant increase in apyrase, for both substrates, and 5′-nucleotidase activities was observed in group III (Figs. 2–4) after 7 and 15 days. We can suggest that the activation of these enzymes in the cerebral cortex is due to an increase in extracellular ATP levels caused by demyelination in the brainstem after EB injection. Confirming this hypothesis, our findings also have demonstrated that no alteration occurred in the enzymes' activities after 30 days, the period characterized by complete remyelination [23]. Moreover, in relation to the results obtained in vitro, it was observed that EB did not cause an alteration in apyrase and 5′-nucleotidase activities in any of the concentrations tested, suggesting that the increase in nucleotide hydrolysis is induced by a demyelinating event.

ATP is considered an important molecule for CNS remyelination because it regulates the differentiation of oligodendrocyte precursor cells (OPCs) [20]. However, in addition to the important role of ATP, a vigorous release of this molecule can contribute to neurodegeneration in many diseases, as for example, in MS [41]. Our findings lead us to the hypothesis that an increase in ectonucleotidase activities can contribute to extracellular adenosine production in this demyelination model.

In addition, studies have also demonstrated the role of adenosine in the proliferation and differentiation of OPCs in mature oligodendrocytes, underlining the importance of this molecule in regulating remyelination processes in MS [19, 42]. An important aspect to be discussed here is that at 7 and 15 days, the activity of both enzymes was increased in the group which received EB, and adenosine produced in this condition can be an important molecule for remyelination events in this experimental model. These findings raise questions about the importance of ectonucleotidases in demyelinating pathologies.

IFN-$\beta$ is widely used to treat the relapsing–remitting form of MS, however, the action mechanisms underlying its beneficial effects remain unknown [29, 30]. In the present investigation, we evaluated apyrase and 5′-nucleotidase activities in rats demyelinated by EB after treatment with IFN-$\beta$. It was observed that this immunomodulatory agent increased apyrase activity for ATP hydrolysis for both groups II and IV (Fig. 2a) after 7 days. 5′-Nucleotidase activity also increased after 15 days in groups II and IV (Fig. 4b). We suggest that IFN-$\beta$ can contribute to adenosine production in demyelination events, since no alteration was observed after 30 days.

On the other hand, IFN-$\beta$ decreased ectonucleotidase activities in group IV in relation to group III (Figs. 2–4), after 7 and 15 days. One possible hypothesis is that these alterations in purinergic signaling can be due, at least in part, to the indirect effect of IFN-$\beta$ on astrocytes [31]. This finding is very important since, in the experimental model used in our study, astrocytes are the first cells destructed after EB injection, and in this context IFN-$\beta$ can protect astrocytes against apoptotic cell death, hindering the development of new lesions [32].

In conclusion, an important adaptive plasticity of ectonucleotidases in the cerebral cortex pathway could occur, decreasing ATP levels and increasing adenosine levels during CNS demyelination. In this experimental model, IFN-$\beta$ interferes in nucleotide adenine hydrolysis and adenosine production, a finding relevant that can be useful to support the diagnosis for demyelinating diseases such as MS.

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## 3.2 Manuscrito II

**Activities of enzymes that hydrolyze adenine nucleotides in platelets from rats experimentally demyelinated with ethidium bromide and treated with Interferon-β**

Roselia M. Spanevello, Cínthia M. Mazzanti, Paula A. Maldonado, Rafael Zanin, André Morsch, Laura Hannel, Alexandre Mazzanti , Rafael Festugatto, Dominguita Graça, Maria Rosa C. Schetinger, Vera M. Morsch.

(Submetido a Revista Life Sciences)

# Activities of enzymes that hydrolyze adenine nucleotides in platelets from rats experimentally demyelinated with ethidium bromide and treated with Interferon-β

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**Abstract**

The activities of the enzymes NTPDase (E.C. 3.6.1.5, apyrase, CD39) and 5'-nucleotidase (E.C. 3.1.3.5, CD73) were analyzed in platelets from rats submitted to demyelination by ethidium bromide (EB) and treated with interferon-β (IFN-β). The following groups were studied: I - control (saline), II - (saline and IFN-β), III - (EB) and IV - (EB and IFN-β). After seven, fifteen and thirty days, the animals (n=5) were sacrificed and blood was collected and the platelets were isolated for the enzymatic assays. The NTPDase activity for ATP and ADP substrates was significantly lower in groups II and III after seven days, when compared to control ($p<0.001$). At fifteen days, ATP hydrolysis was significantly lower in group III and IV and higher in group II ($p<0.001$), while there was an increase of ADP hydrolysis in group II ($p<0.05$), when compared with the control. The 5'-nucleotidase activity was significantly higher in group IV ($p<0.05$) after seven days, and lower in the groups III and IV ($p<0.001$) after fifteen days in relation to the control. No significant differences were observed in NTPDase and 5'-nucleotidase activities after thirty days. In conclusion, our study demonstrated that the hydrolysis of adenine nucleotides is modified in platelets of rats demyelinated and of the rats treated with IFN - β.

**Keywords:** NTPDase, 5'-nucleotidase, platelets, demyelination, Interferon-β

## 1- Introduction

Platelets are small anuclear cells with an important role in homeostasis via adhesion, aggregation, and subsequent thrombus formation at the site of vascular injury (Harker et al., 1997; Pelagalli et al., 2003; Andrews and Berndt, 2004). However, platelet hyperactivity can lead to pathological thrombus formation and vascular occlusion (Remijin et al., 2002; Wagner and Burger, 2003; Keating et al., 2004).

Extracellular adenine nucleotides such as ATP, ADP and adenosine are known to regulate the vascular response to endothelial damage by exerting a variety of effects on platelets (Birk et al., 2002). It is recognized that ADP is the main promoter of platelet aggregation, whereas adenosine is a potent inhibitor of this aggregation and an important modulator of vascular tone (Anfossi et al., 2002; Remijin et al., 2002; Rozalski et al., 2005). Moreover, studies have suggested a complex role of ATP in the regulation of platelet aggregation (Soslau and Youngprapakorn, 1997; Birk et al., 2002).

The enzymes NTPDase (E.C. 3.6.1.5, CD39, ectoapyrase, ATP diphosphohydrolase) and 5'-nucleotidase (E.C. 3.1.3.5) make up an essential mechanism for the maintenance of blood fluidity (Kaczmarek et al., 1996; Gayle et al., 1998; Kawashima et al., 2000). NTPDase is a membrane-bound enzyme that hydrolyzes ATP and ADP to AMP, which is subsequently converted to adenosine by 5'-nucleotidase (Ziganshin et al., 1994; Zimmermann, 2001). Both enzymes are present in the platelet membrane and play an important role in the maintenance of normal hemostasis and in the prevention of excessive platelet aggregation (Pilla et al., 1996; Lunkes et al., 2004; Araújo et al., 2005).

Multiple sclerosis (MS) is a chronic demyelinating disease of the central nervous system of unknown etiology, characterized by various degrees of reactive astrogliosis, oligodendroglial loss and axonal pathology (Bjatmar et al., 2003; Reipert, 2004; Lassman, 2005). The medical literature has reported the occurrence of anomalous platelet behavior in this pathological condition (Millar et al., 1966; Neu et al., 1982; Khan et al.,1985; Cananzi et al., 1987). Platelets of MS patients exhibit a greater tendency to spontaneous aggregation and higher sensitivity to ADP induced aggregation (Neu et al., 1982). Besides, thrombus development in the demyelinating plaques has suggested that platelets may play a role in the demyelinaton of white matter (Cananzi et al., 1987).

Interferon -β (IFN-β) is a drug widely used to treat MS (Alam, 1995; Hall et al., 1997; Revel, 2003). To our knowledge, the majority of studies regarding the hematological effects of IFNs to date have focused on platelet number (Wandenvik et al., 1991; Panasiuk et al., 2004). Tsiara et al. (2000) reported that IFN-β has beneficial effects on thrombocytosis by reducing the platelet number to within a normal range, however the role of IFN-β in the mechanisms of platelet aggregation associated with demyelination has not been elucidated.

Many aspects of the relation between the demyelinating pathology and platelet function still need to be clarified; for example, previous studies have not conclusively answered whether the platelet alterations in MS patients are due to some abnormality or to some factor in the plasma. In concern to this question, we have examined the potential role of enzymes that participate in the hydrolysis of ATP, ADP and AMP in platelets of rats experimentally

demyelinated with ethidium bromide (EB) and the possible interference of IFN-β in adenine nucleotide hydrolysis in this condition.

## 2- Material and Methods

### *2.1 Chemicals*

Nucleotides, trizma base, HEPES, ethidium bromide (EB) and coomassie brilliant blue G were obtained from Sigma Chemical Co (St. Louis, MO, USA) and bovine serum albumin, $K_2HPO_4$, from Reagen. IFN-β (Rebif$^R$ 44mcg 12MUI/ 0,5ml) was purchased from SERONO BARI (Italy). All the other chemicals used in this experiment were of the highest purity.

### *2.2 Animals*

Adult male Wistar rats (70 - 90 days; 220 - 300g) were used in this experiment. The animals were maintained at constant temperature (23±1ºC) on a 12h light/dark cycle with free access to food and water. All animal procedures were approved by the Animal Ethics Committee from the Federal University of Santa Maria (protocol under number: 23081.016394-2005-83).

### *2.3 Surgical procedure*

The animals were randomly divided into four groups: I - control (injected with saline), II - (injected with saline and treated with IFN-β), III - (injected with 0.1% EB) and IV - (injected with 0.1% EB and treated with IFN-β). For the surgical procedure, the animals were anesthetized with ketamine chloridate and xylazine (5:1; 0.1 ml/100g) and after shaving the fronto-parietal-occipital area,

antisepsis with 2% iodine solution was carried out. With the aid of a roof motor of orthodontic use and a drill number 2, a hole was made 0.85cm to the right of the bregma until exposing the duramater. With the use of a Hamilton syringe with a removable needle of caliber 26s, the solutions were injected in the cisterna pontis (basal), an enlargement of the subarachnoid space on the ventral surface of the pons. Ten microliters of EB were injected in the animals of groups III and IV, whereas the same volume of 0.9% of saline solution were injected in the animals of groups I and II. The duramater was left open and the skin, together with the remainder of subcutaneous tissue, was sutured with a nylon thread 4.0.

*2.4 Treatment with IFN-β*

The administration of the immunomodulatory drug began four hours after the surgical procedure. The animals belonging to group II and IV received IFN-β (500,000 U/animal/per day) subcutaneously until the end of the experiment. Five animals from each group were sacrificed at seven, fifteen and thirty days after the surgical procedure, and the blood was collected for enzyme assays.

*2.5 Platelet –rich plasma preparation*

Platelet – rich plasma (PRP) was prepared by the method of Lunkes et al. (2004), with the following minor modifications. Total blood was collected from a cardiac punction with 0.120 M sodium citrate as anticoagulant. The total blood-citrate system was centrifuged at 160 × g during 15 min. The PRP was centrifuged at 1400 × g for 30 min and washed twice by centrifugation at 1400 ×

g for 10 min with 3.5 mM HEPES isosmolar buffer containing 142 mM NaCl, 2.5 mM KCl and 5.5 mM glucose. The washed platelets were resuspended in HEPES isosmolar buffer and adjusted to 0.4 – 0.6 mg of protein per milliliter.

*2.6 NTPDase and 5´- Nucleotidase assays*

The NTPDase enzymatic assay was carried out in a reaction medium containing 5 mM $CaCl_2$, 100 mM NaCl, 4 mM KCl, 50 mM glucose and 50 mM Tris-HCl buffer, pH 7.4, at a final volume of 200 µl as described by Lunkes et al. (2004). Twenty microliters of the enzyme preparation (8-12 µg of protein) were added to the reaction mixture and the pre-incubation proceeded for 10 min at $37^0$ C. The reaction was initiated by the addition of ATP or ADP at a final concentration of 1.0 mM, and the time of incubation was 60 minutes.

5´-Nucleotidase activity was determined essentially as described by Heymann et al. (1984) in a reaction medium containing 10 mM $MgSO_4$ and 100 mM Tris-HCl buffer, pH 7.5 at a final volume of 200 µl. Twenty microliters of enzyme preparation (8-12 µg of protein) was added to the reaction mixture and the pre-incubation proceeded for 10 min at $37^0$ C. The reaction was initiated by the addition of AMP at a final concentration of 2.0 mM. The time of incubation was 60 minutes.

Both enzyme assays were stopped by the addition of 200 µl of 10% tricloroacetic acid (TCA) to provide a final concentration of 5%. Subsequently, the tubes were chilled on ice for 10 min. Released inorganic phosphate (Pi) was assayed by the method of Chan et al. (1986) using malachite green as the colorimetric reagent and $KH_2$ $PO_4$ as standard. Controls were carried out to

correct for non-enzymatic hydrolyses of nucleotides by adding platelets after TCA addition. All samples were run in triplicate. Enzyme specific activities are reported as nmol Pi released/min/mg of protein.

*2.7 Protein Determination*

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

*2.8 Statistical Analysis*

The statistical analysis used was one-way ANOVA, followed by Duncan's multiple range test. $P < 0.05$ was considered to represent a significant difference in both analysis used. All data were expressed as mean ± SEM.

**3- Results**

NTPDase and 5'-nucleotidase activities in platelets were altered in relation to the time periods studied in this work. The results showed a significant alteration in NTPDase activity after seven days when ATP was used as substrate ($F(3,10)= 25.59$; $p<0.001$) and post-hoc comparisons by Duncan´s test revealed that ATP hydrolysis was significantly reduced in group II and III, while in group IV the hydrolysis of this nucleotide was significantly higher than control group (Figure 1). At fifteen days, a significant alteration was also observed in the activity of this enzyme ($F(3,10)=19.42$; $p<0.001$) and post-hoc comparisons by Duncan´s test revealed that ATP hydrolysis was significantly lower in groups III and IV,

while in group II the hydrolysis of this nucleotide was significantly higher when compared to the control group (Figure1). No significant difference was observed in NTPDase activity after thirty days ($F(3,10)=0.380$; $p=0.770$) (Figure 1).

Similarly, a decrease in ADP hydrolysis was observed after seven days ($F(3,10)=17.16$; $p<0.001$) and post-hoc comparisons by Duncan´s test revealed that ADP hydrolysis was significantly lower in groups II and III when compared with group I (Figure 2). After fifteen days, a significant increase in enzyme activity ($F(3,8)$: 7.96; $p=0.009$) was observed, and comparisons by Duncan´s test revealed that ADP hydrolysis was significantly higher in group II when compared to group I (Figure 2). No significant difference was observed in ADP hydrolysis ($F(3,10)=1.68$; $p=0.232$) after thirty days (Figure 2).

In relation to the 5'-nucleotidase enzyme, an increase in the activity of this enzyme was observed after seven days ($F(3,9)=3.91$; $p<0.05$), and comparisons by Duncan´s test revealed that AMP hydrolysis was significantly higher in group IV when compared to group I (Figure 3). After fifteen days, the activity of this enzyme decreased ($F(3,9)=15.51$; $p<0.001$), and post-hoc comparisons by Duncan´s test revealed that AMP hydrolysis was significantly decreased in groups III and IV when compared to group I (Figure 3). In addition, no significant alteration was observed in its activity after thirty days ($F(3,10)=0,482$; $p=0.701$) (Figure 3).

## 4- Discussion

Demyelination is the pathological hallmark of multiple sclerosis (MS) (Bjatmar et al., 2003; Reipert, 2004; Lassman, 2005). Considering that alteration of platelet function has been observed in this condition (Millar et al., 1966; Neu et al., 1982; Khan et al., 1985; Cananzi et al., 1987), an experimental demyelination model was used in this study to evaluate possible changes in NTPDase and 5'-nucleotidase activity in platelets, since as these are two important enzymes involved in the thromboregulation process (kaczmarek et al., 1996; Gayle et al., 1998; Kawashima et al., 2000).

In the present study, the demyelination model induced by EB was applied, having been extensively studied and showing good reproductibility of demyelination (Graça et al., 2001; Stangel and Hartung, 2002). Injection of EB in the rat's brainstem led to the loss of myelin sheaths after seven days, whereas histological alterations observed at fifteen and thirty days after injection corresponded to the remyelination process (Reynolds et al., 1996; Spanevello et al., 2006).

Concerning to the model used, we studied NTPDase and 5'-nucleotidase activities in platelets at different time periods to evaluate the role of these enzymes in demyelination and remyelination events. Our results show that NTPDase activity is reduced in group III after seven and fifteen days. Based on these results, it is possible to suggest that there may be a relation between ATP and ADP hydrolysis and demyelination,

since no alteration occurred in enzyme activity after thirty days, the period characterized by remyelination.

Studies have suggested that alterations in platelet function of patients with MS may be due to the breakdown of neural tissue (Millar et al. 1966). This breakdown could give rise to liberation of basic protein in the blood stream, which may alter the surface membrane of the platelets (Chiang et al., 1982; Neu et al., 1982; Khan et al., 1985; Cananzi et al. 1987). In regard to this, alterations in the membrane of platelets (Remijin et al., 2002; Wagner and Burger, 2003; Keating et al., 2004) could be a decisive factor in changing the conformational state of the NTPDase molecule, which would explain the reduced activity observed in present study in the period between seven and fifteen days.

NTPDase is accepted to be a potent antithrombotic agent because this enzyme rapidly metabolizes ADP, terminating further platelet recruitment and aggregation (Ziganshin et al., 1994; Pilla et al., 1996; Gayle et al., 1998; Lunkes et al., 2004; Araújo et al., 2005). The inhibition of this enzyme observed after seven days in ADP hydrolysis could lead to the extracellular increase of this nucleotide, which is recognized to induce platelet aggregation. Conversely, the inhibition of this enzyme in ATP hydrolysis should be seen as a compensatory mechanism in thromboregulation, because ATP is an inhibitor of ADP-induced platelet aggregation (Soslau and Youngprapakorn, 1997; Birk et al., 2002; Rozalski et al, 2005).

Another important aspect to be discussed is that 5'-nucleotidase activity is also reduced in group III after fifteen days. The alteration of this enzyme can indicate a reduced production of adenosine, which is an important molecule for the modulation of vascular tone and a well-known inhibitor of platelet aggregation (Harker et al., 1997; Kawashima et al., 2000; Anfonssi et al., 2002; Pelagalli et al., 2003; Andrews and Berndt, 2004).

IFN-β is now widely used to treat the relapsing-remitting form of MS (Alam, 1995; Hall et al., 1997; Revel, 2003). In this work, the treatment with IFN-β also altered nucleotide hydrolysis at the different time periods evaluated. An important fact observed in this study was that IFN-β *per se* decreased NTPDase activity at seven days, suggesting that this agent could be dangerous in that it promotes an increase in ADP extracellular levels. On the other hand, increased activity of this enzyme was observed after fifteen days for both substrates, which should be considered a compensatory mechanism of IFN- β in relation to nucleotide hydrolysis.

When IFN-β was associated with EB, there was an increase in NTPDase and 5'-nucleotidase activities in comparison with group III after seven days. We can suggest that activation of these enzymes by IFN-β has a benefical effect by protecting platelets from excessive aggregation and contributing in the control of adequate hemostasis in the demyelinating events of the disease.

In conclusion, the results of the present study demonstrated alterations in NTPDase and 5'-nucleotidase activities in platelets after a

demyelination event in the central nervous system. We suggest that treatment with IFN-β may be important in controlling the platelet coagulant status in the demyelinating process, but additional studies will be necessary to confirm this premise.

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**Legends**

**Figure 1:** NTPDase activity in platelets using ATP as substrate, seven, fifteen and thirty days after EB or saline injection in brainstem of rats. Group I: saline; group II: saline+IFN-β; group III: EB and group IV: EB+IFN-β. Bars represent mean ± SEM. [a,b] indicate a significant difference at $P < 0.05$ from columns not labeled with these letters.

**Figure 2:** NTPDase activity in platelets using ADP as substrate, seven, fifteen, and thirty days after EB or saline injection in brainstem of rats. Group I: saline; group II: saline+IFN-β; group III: EB and group IV: EB+IFN-β. Bars represent mean ± SEM. [a,b] indicate a significant difference at $P < 0.05$ from columns not labeled with these letters.

**Figure 3:** 5'-Nucleotidase activity in platelets using AMP as substrate, seven, fifteen and thirty days after EB or saline injection in brainstem of rats. Group I: saline; group II: saline+IFN-β; group III: EB and group IV: EB+IFN-β. Bars represent mean ± SEM. [a,b] indicate a significant difference at $P < 0.05$ from columns not labeled with these letters.

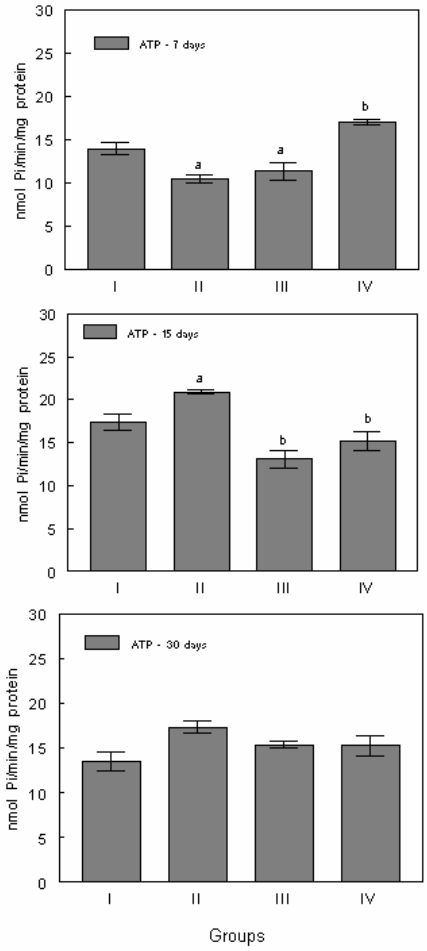
ATP - 7 days
ATP - 15 days
ATP - 30 days
nmol Pi/min/mg protein
Groups
I
II
III
IV
a
b

**Figure 1**

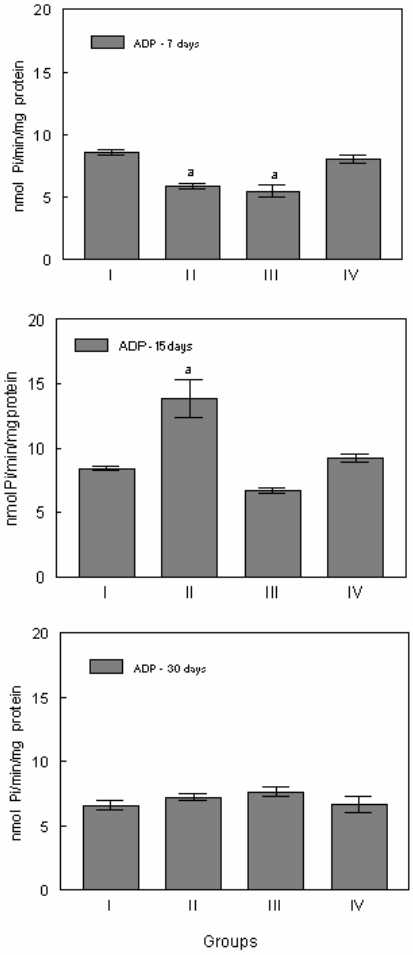
ADP - 7 days
nmol Pi/min/mg protein
20
15
10
5
0
a
a
I
II
III
IV
ADP - 15 days
nmol Pi/min/mg protein
a
ADP - 30 days
nmol Pi/min/mg protein
Groups

**Figure 2**

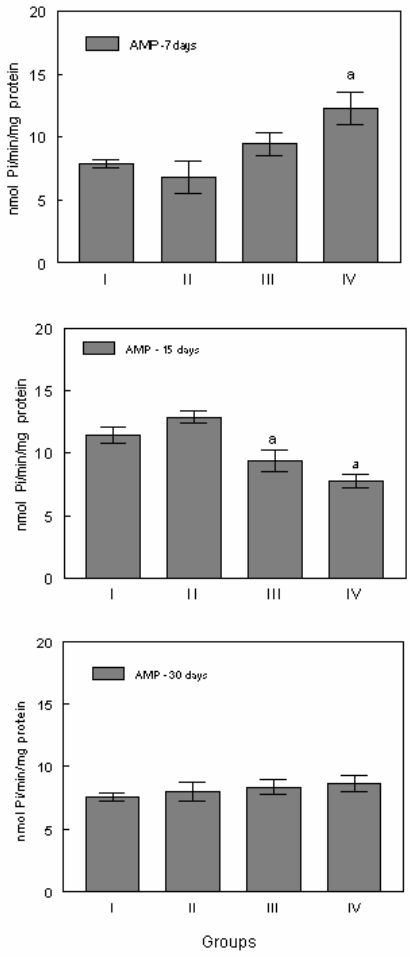
AMP-7 days
nmol Pi/min/mg protein
20
15
10
5
0
a
I
II
III
IV
AMP - 15 days
nmol Pi/min/mg protein
a
a
AMP - 30 days
nmol Pi/min/mg protein
Groups

**Figure 3**

# 4. DISCUSSÃO

As enzimas NTPDase e 5'-nucleotidase vêm sendo estudadas em muitas condições patológicas (Bonan et al., 2001; Lunkes et al., 2003; Araújo et al., 2005), no entanto não foi encontrado na literatura um estudo que relacionasse a atividade destas enzimas com o modelo de desmielinização induzida pelo BE.

As alterações histológicas observadas na ponte dos animais após 7 dias da injeção do BE confirmam uma lesão de desmielinização, enquanto que as alterações referentes aos 15, 21 e 30 dias pós-injeção correspondem a remielinização da área afetada (Manuscrito I, fig.1). Resultados semelhantes aos obtidos neste estudo também foram descritos por Reynolds et al. (1996) e Bondan (1997) após a injeção de 20 µl (0,15%) e 10 µl (0,1%) de solução de BE, respectivamente, no tronco encefálico de ratos.

Com relação a NTPDase e 5'-nucleotidase, nossos resultados demonstraram que a atividade destas ectonucleotidases em sinaptossomas e plaquetas estão alteradas nos tempos avaliados. Estas alterações ocorreram aos 7 e 15 dias, onde pode-se sugerir que estejam relacionadas com os eventos de desmielinização, já que aos 30 dias nenhuma mudança na atividade destas enzimas foi constatada.

O aumento na atividade da NTPDase e 5'-nucleotidase em sinaptossomas de ratos injetados com BE ( grupo III ) após 7 e 15 dias (Manuscrito I, fig. 2-4), nos sugere que a neurotransmissão purinérgica pode estar alterada após um episódio de desmielinização no SNC. O ATP tem sido considerado uma importante molécula em eventos de remielinização do

sistema nervoso por regular a diferenciação de células progenitoras de oligodendrócitos (Agresti et al., 2005). Entretanto, em adição ao importante papel do ATP, tem sido relatado que altos níveis extracelulares desta molécula podem contribuir para a neurodegeneração (Le Feuvre et al., 2002). Sendo assim, o aumento na atividade da NTPDase e 5'-nucleotidase como foi observado neste estudo, pode levar a uma eficiente remoção do ATP extracelular e um aumento na produção de adenosina. A adenosina exerce funções neuromoduladoras e neuroprotetoras no SNC, com papel importante também em eventos de remielinização (Cunha, 2001; Dunwiddie & Masino, 2001; Stevens et al., 2002; Ribeiro et al., 2003).

Foi demonstrado neste trabalho, que o tratamento com IFN-β foi capaz de alterar a hidrólise dos nucleotídeos em sinaptossomas dos diferentes grupos avaliados (Manuscrito I, fig. 2-4). Observou-se uma diferente sensibilidade da enzima NTPDase em relação a hidrólise do ATP e ADP nos grupos tratados com IFN-β (II e IV). O aumento na hidrólise do ATP após 7 e 15 dias de tratamento com IFN-β, pode ser importante em eventos de desmielinização, pois mais ADP pode ser formado, o qual também é considerado uma importante molécula em processos remielinizantes.

Um outro importante aspecto a ser discutido neste estudo é o efeito que o do IFN-β exerce sobre os astrócitos. Estudos têm demonstrado que o IFN-β exerce uma ação protetora contra a morte destas células (Malik et al., 1998; Barca et al., 2003; Okada et al., 2005), um achado importante pois neste modelo experimental os astrócitos são destruídos pela injeção de BE (Bondan, 1997; Graça et al., 2001; Bondan et al., 2002).

Os astrócitos possuem inúmeras funções relevantes tais como a manutenção da barreira hematoencefálica, suporte mecânico para oligodendrócitos durante a mielinização e reparo após agressões do tecido nervoso (Cicarelli et al., 2001; Newman, 2003). Neste contexto um efeito protetor do IFN-β sobre estas células poderia amenizar o desenvolvimento de novas lesões e contribuir para a remielinização.

Em relação à avaliação da atividade da NTPDase e 5'-nucleotidase em plaquetas nossos resultados demonstraram uma redução na atividade da NTPDase no grupo III (Manuscrito II, figs. 1,2,3). Uma diminuição na hidrólise do ADP aumenta os níveis extracelulares desta molécula, o que pode levar a um aumento na agregação plaquetária (Remijn et al., 2002). Por outro lado, a redução na hidrólise do ATP pode ser visto como um mecanismo compensatório na tromboregulação, já que o ATP é um inibidor da agregação plaquetária induzida pelo ADP (Soslau & Youngprapakorn, 1997).

Outro aspecto a ser discutido é que a atividade da 5'-nucleotidase está diminuída no grupo III após 15 dias da indução (Manuscrito II, fig. 3). Esta alteração pode levar a uma produção reduzida de adenosina o que pode contribuir para a agregação excessiva de plaquetas, pois a adenosina possui um importante papel na modulação do tônus vascular e na inibição da agregação plaquetária (Kawashima et al., 2000; Anfossi et al., 2002).

A associação entre alterações na função plaquetária e patologias desmielinizantes já foi descrita na literatura, mas ainda é pouco compreendida (Millar et al., 1966; Khan et al., 1985; Cananzi et al., 1987). A hipótese mais aceita é que devido ao processo de desmielinização muitas substâncias como

lipídios e a proteína básica mielínica podem ser liberadas para o sangue e alterar a superfície da membrana das plaquetas (Chiang et al.,1982; Neu et al., 1982). Considerando esta questão, uma alteração na membrana da plaqueta pode ser um fator decisivo em mudar o estado conformacional da molécula da NTPDase e 5'-nucleotidase, já que são enzimas ligadas a membrana, o que pode explicar as alterações que ocorreram neste estudo na atividade destas ectonucleotidases.

O tratamento com IFN-β também alterou a hidrólise dos nucleotídeos em plaquetas de ratos nos diferentes grupos avaliados. Com exceção da atividade da 5'-nucleotidase aos 15 dias, o IFN-β aumentou a atividade da NTPDase e 5'-nucleotidase no grupo IV em relação ao grupo III (Manuscrito II, figs. 1,2,3). De acordo com nossos resultados, podemos sugerir que a ação do IFN-β na atividade destas enzimas, pode ter um efeito benéfico, protegendo as plaquetas da agregação excessiva e contribuindo, desta forma, para a adequada hemostase em doenças desmielinizantes.

Neste estudo foi demonstrado que a atividade da NTPDase e da 5'-nucleotidase em sinaptossomas e plaquetas tem um papel fundamental na hidrólise do ATP, ADP e AMP nestes diferentes compartimentos celulares, sendo que alterações na hidrólise de nucleotídeos podem causar respostas celulares distintas mediante um evento de desmielinização tóxica no SNC.

## 5. CONCLUSÕES

- Um aumento na atividade das enzimas NTPDase e 5'-nucleotidase foi observado em sinaptossomas de córtex cerebral de ratos após 7 e 15 dias da injeção de BE, o que nos indica que uma importante plasticidade na atividade das ectonucleotidases pode ocorrer para diminuir os níveis extracelulares de ATP e aumentar os níveis de adenosina durante um evento de desmielinização tóxica no SNC;

- O tratamento com IFN-β alterou a hidrólise do ATP após 7 e 15 dias, e a hidrólise do AMP aos 15 dias, em sinaptossomas de ratos desmielinizados pelo BE, onde pode-se concluir que este agente interfere com a atividade das ectonucleotidases o que pode levar a alterações nos níveis extracelulares destes nucleotídeos;

- A atividade das enzimas NTPDase e 5'-nucleotidase também foi alterada em plaquetas de ratos injetados com BE no período de 7 e/ou 15 dias indicando que um evento de desmielinização tóxica no SNC pode interferir em processos de tromboregulação;

- O tratamento com IFN-β interferiu com a hidrólise de nucleotídeos em plaquetas de ratos desmielinizados pelo BE no período de 7 e/ou 15 dias; sugerindo que a ação do IFN-β na atividade destas enzimas pode ser importante na hemostase em doenças desmielinizantes;

- Nenhuma alteração foi observada na atividade das enzimas NTPDase e 5'-nucleotidase no período de 30 dias, tanto em sinaptossomas quanto em plaquetas de todos os grupos avaliados.

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