

UNIVERSIDADE FEDERAL DO RIO GRANDE DO SUL

**EFEITO DO INTERFERON BETA, DA CICLOSPORINA A, DO EBSELEN E
DA VITAMINA E NO SISTEMA COLINÉRGICO E PURINÉRGICO DE RATOS
NORMAIS E SUBMETIDOS À DESMIELINIZAÇÃO PELO BROMETO DE
ETÍDIO**

CINTHIA MELAZZO DE ANDRADE MAZZANTI

ORIENTADORA: Prof^a. Dr^a. Maria Rosa Chitolina Schetinger

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DEDICATÓRIA

Dedico esta tese ao meu mestre de vida, **Daisaku Ikeda**, pacifista, filósofo, poeta laureado e escritor com obras traduzidas para mais de vinte línguas, é sócio correspondente da Academia de Letras desde 1992. É atualmente presidente da Soka Gakkai Internacional (SGI), uma das maiores organizações não-governamentais das Nações Unidas que luta pela paz, cultura e educação.

O sol e a lua brilham por que acreditam em si mesmos! A convicção é a fonte de uma brilhante glória! Se desejar mudar algo é preciso ter determinação, vontade e coragem para agir, assim cria-se a oportunidade na vida!

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RESUMO

A esclerose múltipla é a principal doença desmielinizante do sistema nervoso central (SNC). É considerada a principal causa de incapacidade neurológica em adultos jovens. O comprometimento cognitivo é muito comum nessa doença, envolvendo o aprendizado, a memória e a organização cortical do movimento, funções vitais que são reguladas pelo sistema colinérgico. O modelo de desmielinização tóxica induzida pelo brometo de etídio (BE) foi utilizado neste estudo, para avaliar a atividade da enzima acetilcolinesterase (AChE) no estriado (ST), hipocampo (HP), córtex cerebral (CC), hipotálamo (HY) e ponte (PN) associado ao tratamento com interferon beta (IFN- β), ciclosporina A (CsA), vitamina E (vit E) e ebselen (Ebs). Além disso, também foi investigado o efeito *in vitro* do BE na atividade da AChE, juntamente com os parâmetros cinéticos dessa enzima no ST, HP, CC e CB de ratos adultos. Os resultados demonstraram que o BE inibiu significativamente a atividade da AChE no ST, HP, CC e CB nas concentrações de 0,00625, 0,0125, 0,025, 0,05 e 0,1mM e a análise dos dados cinéticos mostraram uma inibição do tipo incompetitiva no ST, HP e CC, enquanto no CB a inibição foi do tipo mista. No estudo *in vivo*, foi observado uma inibição na atividade da AChE no CC, ST, HP, HY, PN e CB nos diferentes períodos avaliados pós-injeção do BE (3, 7, 15, 21 e 30 dias). Quando ratos desmielinizados com BE foram tratados com IFN- β e CsA, não houve alteração na atividade dessa enzima. Por outro lado, o tratamento com Vit E e Ebs foram capazes de aumentar a atividade da AChE no ST, CC e HP. Estudos imuno-histoquímicos demonstraram que nos ratos tratados com Vit E e Ebs as lesões induzidas pelo BE foram menores, sugerindo que estes compostos interferem no desenvolvimento de lesões desmielinizantes. Foi também avaliado o efeito *per se* do IFN- β , da CsA, da Vit E e do Ebs na atividade da AChE, os quais demonstraram um efeito inibitório sobre a atividade dessa enzima. Em plaquetas de ratos desmielinizados, foi observado uma diminuição na atividade da NTPDase e o tratamento com a Vit E e o Ebs modularam a hidrólise dos nucleotídeos de adenina. O presente trabalho demonstrou que o BE é um potente inibidor da atividade da AChE *in vitro* e os resultados *in vivo* demonstraram que a atividade dessa enzima está alterada após um evento de desmielinização tóxica no SNC. Os resultados deste estudo ajudaram a confirmar que drogas utilizadas no tratamento de pacientes com esclerose múltipla tais como o IFN- β e a CsA causam efeitos na atividade da AChE. A Vit E e o Ebs, além de demonstrarem uma interação com a neurotransmissão colinérgica, também modularam a hidrólise dos nucleotídeos de adenina em plaquetas de ratos, contribuindo no controle da coagulação plaquetária em processos desmielinizantes. Neste contexto, sugere-se que o IFN- β , a CsA, a vitamina E e o ebselen podem ser investigados em estudos futuros com a intenção de encontrar uma melhor terapia para beneficiar pacientes com patologias desmielinizantes.

Palavras chave: brometo de etídio; acetilcolinesterase, NTPDase; desmielinização; análise cinética; rato

ABSTRACT

Multiple sclerosis is the main demyelinating disease of the central nervous system (CNS). It is the most common cause of neurological disability among young adults. The cognitive impairment is very common in this illness, involving learning, memory and cortical organization of the movement, vital functions regulated by the cholinergic system. The model of toxic demyelination induced by ethidium bromide (EB) was used to evaluate brain acetylcholinesterase (AChE) activity in the striatum (ST), hippocampus (HP), cerebral cortex (CC), cerebellum (CB), hypothalamus (HY) and pons (PN), associated with treatment with interferon beta (IFN- β), ciclosporine A (CsA), vitamin E (Vit E) and ebselen (Ebs). In addition, the *per se* effect of EB on AChE activity was studied *in vitro* together with the kinetic parameters of this enzyme in the ST, HP, CC and CB of adult rats. The results showed that EB *in vitro* significantly inhibited AChE activity in the ST, HP, CC and CB at concentrations of 0.00625, 0.0125, 0.025, 0.05 and 0.1mM. The kinetic analysis demonstrated an uncompetitive inhibition in the ST, HP and CC, whereas in the CB the inhibition type was mixed. In relation to the *in vivo* results, AChE activity was inhibited after demyelination by EB in the CC, ST, HP, HY, PN and CB at the post-injection points of time evaluated (3-7-15-21 and 30 days). When demyelinated rats were submitted to the treatment with IFN- β and CsA, the results demonstrated that these compounds did not alter AChE activity. However, Vit E and Ebs were able to increase AChE activity in the ST, CC and HP. In addition, immunohistochemistry studies demonstrated that in Vit E and Ebs treated rats, the lesions induced by EB were smaller suggesting that these compounds somehow interfered in the development of the lesions. The *per se* effect of IFN- β , CsA, Vit E and Ebs were also evaluated, demonstrating that these compounds have an inhibitory effect on AChE activity. Platelets of the demyelinated rats demonstrated a reduction in NTPDase activity and the treatments with Ebs and Vit E modulated adenine nucleotide hydrolysis. The present investigation demonstrated that EB is a strong inhibitor of AChE activity *in vitro* and the results *in vivo* showed that the activity of this enzyme is altered after an event of toxic demyelination in the CNS. The results this study help to confirm that drugs used in the treatment of the patients with multiple sclerosis such as IFN- β and CsA cause effects in the AChE activity. The Vit E and Ebs besides of interaction with the cholinergic neurotransmission also modulated adenine nucleotide hydrolysis in platelets of the rats, contributing to the control of the platelet coagulant status in the demyelinating process. In this context, we can suggest that IFN- β , CsA, Vit E and Ebs may be investigated in future studies with the intention of finding a better therapy for to improve patients with demyelinating pathologies.

Key words: ethidium bromide; acetylcholinesterase, NTPDase; demyelination; kinetic analysis; rat

Lista de abreviaturas

- ACh** - acetilcolina
AChBP - proteína de ligação da acetilcolina
AChE - acetilcolinesterase
ACRs - regiões conservadas da apirase
ADP - adenosina difosfato
AMP - adenosina monofosfato
ATP - adenosina trifosfato
BE - brometo de etídio
BuChE - butirilcolinesterase
CB - cerebelo
CC - córtex cerebral
ChAT - colina acetiltransferase
CHT - transportador de colina
CMP - citidina monofosfato
CsA - ciclosporina
EAE - encefalomielite auto – imune experimental
Ebs - ebselen
EI – complexo enzima - inibidor
EM - esclerose múltipla
E-NTPDases - ecto – nucleosídeo trifosfato difosfohidrolases
ESI - complexo enzima – substrato - inibidor
GFAP - proteína glial fibrilar ácida
GMP - monofosfato de guanosina
GPI - glicosilfosfatidil inositol
HP - hipocampo
HY - hipotálamo
IACHes - inibidores da acetilcolinesterase
IFN - interferon
IFN- β - 1a - interferon recombinante - 1a
IFN- β - 1b - interferon recombinante – 1b
IFN- β - interferon beta

IMP - inosina monofosfato

Ms - núcleos do septo medial

n IFN- β - interferon natural

Nb - núcleos basais

PAS - sítio aniônico periférico da acetilcolinesterase

PKC - proteína quinase C

PN - ponte

PNN - pedúnculo pontino

PP - forma progressiva primária da esclerose múltipla

PS - forma progressiva secundária da esclerose múltipla

SNC - sistema nervoso central

SNP - sistema nervoso periférico

SR - forma surto remissão da esclerose

ST- estriado

UMP - uridina monofosfato

VAcHT - transportador de acetilcolina vesicular

VIM - vimentina

Vit E - vitamina E

Introdução

1. Sistema colinérgico

O sistema colinérgico é um dos mais importantes caminhos modulatórios do sistema nervoso central (SNC) (Descarries et al., 1997; Perry et al., 1999). Os neurônios colinérgicos e suas projeções estão amplamente distribuídos através do encéfalo rostral (*forebrain*), região que incluem os hemisférios cerebrais, núcleos da base, diencéfalo e porção rostral do mesencéfalo (Bagley, 2005). Entre essas regiões é importante ressaltar os núcleos do septo medial (ms) e núcleos basais (nb), os quais representam o mais significativo grupo de neurônios colinérgicos com projeção para a região do hipocampo, amígdala e córtex cerebral. Já os neurônios colinérgicos localizados no núcleo pedúnculo pontino (PNN) se projetam para o tálamo enquanto os interneurônios estriatais apresentam um circuito colinérgico intrínscio (Mesulam, 1995; Perry et al., 1999) (Figura 1). Essas regiões representam um papel essencial na regulação de muitas funções vitais relacionadas com o aprendizado, a memória e com a organização cortical do movimento, o que faz do sistema colinérgico um importante alvo de pesquisa (Mesulam et al., 2002).

Neste contexto é relevante destacar os principais componentes que constituem o sistema colinérgico: a acetilcolina (ACh), a colinaacetiltransferase (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), os quais juntos são responsáveis por modular a neurotransmissão colinérgica (Kawashima & Fujii, 2003; Sarter & Parikh, 2005).

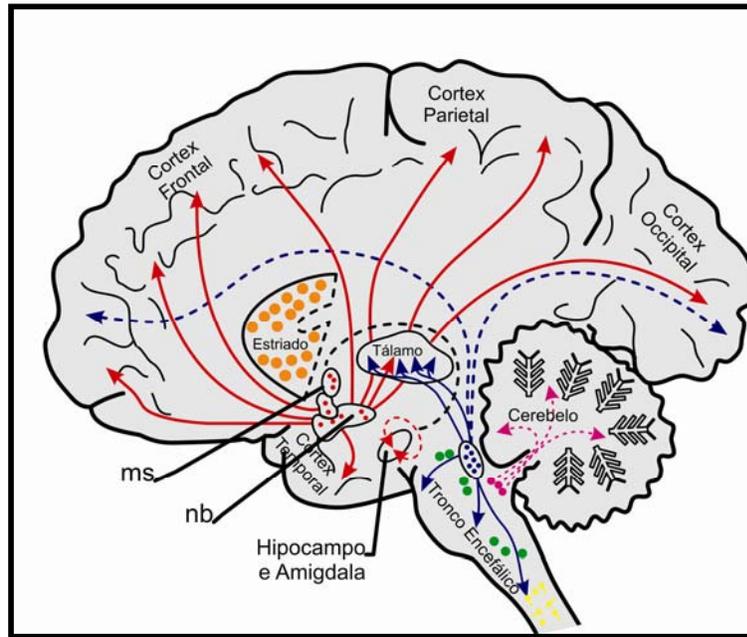


Figura 1. O sistema colinérgico no encéfalo humano. As localizações dos principais grupos de corpos celulares e tratos de fibras colinérgicas são mostrados em vermelho. Os núcleos septo medial (ms) e o núcleo basal (nb) estão em vermelho, o núcleo pedúnculo pontino em azul e os interneurônios estriatais em laranja. Adaptado de Perry et al. (1999).

1.1. Acetilcolina

Desde o início da história evolucionária das espécies e antes do surgimento do sistema nervoso em animais, a acetilcolina (ACh) é considerada uma importante molécula sinalizadora encontrada em bactérias, protozoários, algas e plantas (Gotti & Clementi, 2004; Kawashima & Fuji, 2003). A neurotransmissão química foi investigada por Otto Loewi em 1921 onde identificou a ACh como uma substância cardioativa liberada pelo nervo vago nomeando-a inicialmente como “vagusstoff” (vagoessência) (Van Der Zee & Luiten, 1999). Desde então, a ACh foi o primeiro composto a ser identificado como um neurotransmissor e passou a ser amplamente estudado nas sinapses do SNC e sistema nervoso periférico (SNP) (Descarries et al., 1997).

1.2 Sinapse colinérgica

A ACh é sintetizada pela enzima colina acetiltransferase (ChAT; E.C. 2.3.1.6) a partir de uma molécula de colina e acetil-coenzima A ou acetil-CoA. Posteriormente, este neurotransmissor é armazenado dentro de vesículas sinápticas pelo transportador de ACh vesicular (VAChT) (Prado et al., 2002). Com a chegada do potencial de ação a ACh é liberada na fenda sináptica e exerce seus efeitos mediados pela ativação de receptores nicotínicos e muscarínicos (Descarries et al., 1997). Até o momento cinco subtipos de receptores muscarínicos foram identificados (M1-M5) e agem via ativação de proteína G, sendo que os receptores M1 e M2 estão presentes em neurônios do SNC e SNP além de outros tecidos ganglionares (Van Der Zee & Luiten, 1999). Os receptores nicotínicos são estruturas pentaméricas que atuam como canais iônicos regulados por ligante e localizam-se nas sinapses ganglionares e nas junções neuromusculares. São compostos por cinco subunidades conhecidas por α_1 , α_2 , β , γ , δ (Arias, 1998).

A ACh que permanece na fenda sináptica é hidrolisada por uma colinesterase específica em ácido acético e colina. A maioria da colina resultante é captada pelo terminal do axônio colinérgico por um transportador de colina (CHT) e reutilizada na síntese de nova ACh (Mesulam et al., 2002) (Figura 2).

1.3 Colinesterases

Existem dois tipos de colinesterases: a acetilcolinesterase (AChE; E. C. 3.1.1.7) ou colinesterase verdadeira, que hidrolisa preferencialmente ésteres com grupamento acetil (como a ACh) e a butirilcolinesterase ou

pseudocolinesterase (BuChE; E.C. 3.1.8) que hidrolisa outros tipos de ésteres como a butirilcolina. A AChE é predominantemente encontrada no cérebro (10 vezes mais abundante que a BuChE), junção neuromuscular e eritrócitos (Cokugras, 2003). Já a BuChE é principalmente encontrada no plasma, rins, fígado, intestino, coração, pulmão e têm uma distribuição neuronal muito mais restrita do que a AChE (Mesulam et al., 2002).

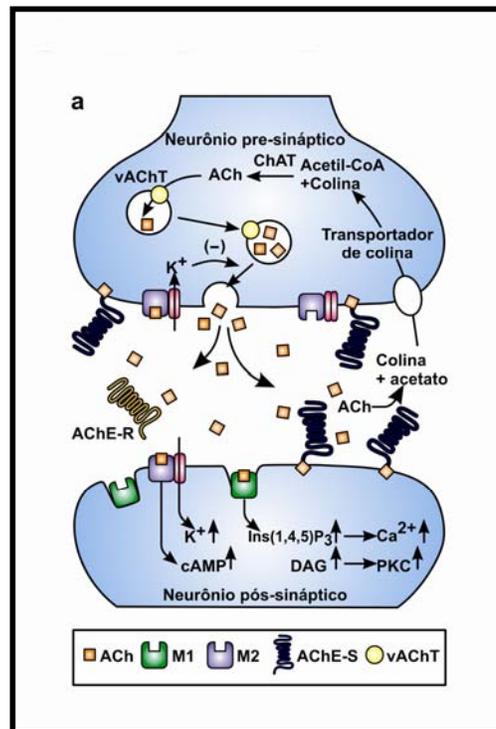


Figura 2. Sinapse colinérgica: Acetilcolina (ACh), acetilcolinesterase sináptica (AChE-S), receptor muscarínico do tipo 1 (M1), receptor muscarínico do tipo 2 (M2), transportador de ACh vesicular (vAChT). Adaptado de Soreq & Seidman, (2001).

1.3.1 Acetilcolinesterase

A AChE é uma serina hidrolase que hidrolisa rapidamente o neurotransmissor ACh tanto na sinapse colinérgica quanto na junção neuromuscular, finalizando assim a transmissão do impulso nervoso (Grisaru et al., 1999). Além de seu papel clássico na transmissão colinérgica a AChE tem

um potente efeito na adesão celular (Jonhson & Moore, 1999), no crescimento dos neuritos (Day & Greenfiel, 2002), participa na regulação estrutural da diferenciação pós-sináptica, na osteogênese e também foi proposto a atividade hematopoiética pela presença desta enzima em células progenitoras do sangue (Soreq & Seidman, 2001). A AChE também foi localizada e identificada nos linfócitos onde provavelmente representa um papel importante na regulação de funções imunes (Kawashima & Fujii, 2000). Assim, uma inibição desta enzima pode ter conseqüências devastadoras no cérebro e em outros órgãos (Mesulan et al., 2002).

1.3.1.1 Estrutura e Distribuição da Acetilcolinesterase

A AChE existe nas formas globular e assimétrica. A forma globular é composta por monômeros (G1), dímeros (G2) e tetrâmeros (G4) da subunidade catalítica. A forma G1 é citosólica e a G4 é ligada à membrana, sendo esta última a mais encontrada no tecido nervoso (Das et al., 2001; Aldunate et al., 2004) (Figura 3). Em sangue humano, a AChE é encontrada tanto nos eritrócitos quanto no plasma, onde predominam as formas G2 e G4, respectivamente (Skau, 1985).

Já a forma assimétrica consiste de um (A4), dois (A8) e três (A12), tetrâmeros catalíticos ligados covalentemente a uma subunidade estrutural colagênica chamada Q (ColQ). Essas formas estão associadas com a lâmina basal e são abundantes na junção neuromuscular (Aldunate et al., 2004) (Figura 3).

No SNC a secreção de AChE têm sido encontrada no hipotálamo, substância nigra, estriado, hipocampo, cerebelo e também no fluido

cerebroespinal (Paxinos, 1985). Esta enzima é secretada de ambos os terminais axonais dos neurônios, sendo esta taxa de secreção modulada pela estimulação neuronal, nível de neurotransmissor na fenda sináptica, bem como o tratamento com drogas (Descarries, 1997).

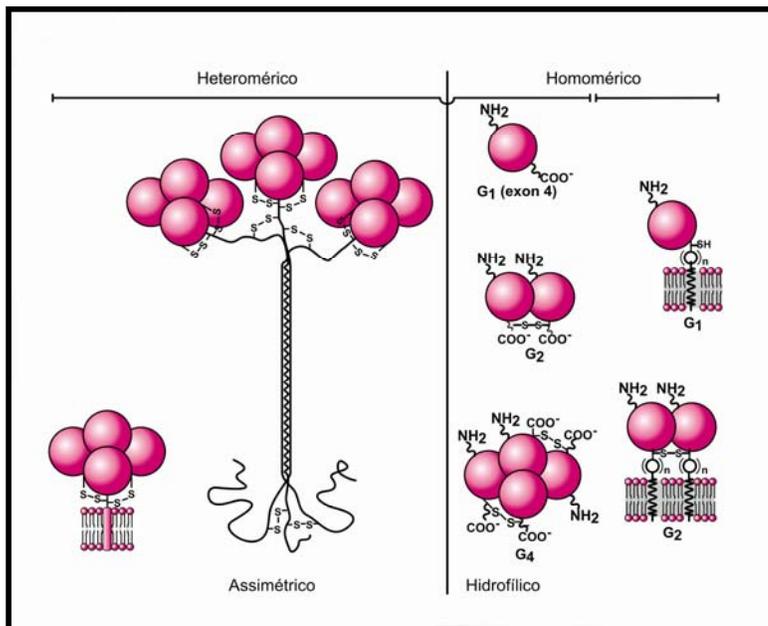


Figura 3. Estruturas molecular (G1, G2 e G4) e assimétrica (A12) da AChE.

(http://www.chemistry.emory.edu/.../ach_inactivation.htm)

1.3.1.2 Mecanismo de ação da Acetilcolinesterase

Vários estudos sobre as propriedades catalíticas da AChE possibilitaram o conhecimento detalhado do sítio ativo desta enzima. A estrutura tridimensional dimérica da AChE do *Torpedo californica* obtida por cristalografia de raio X foi um grande passo na compreensão do mecanismo catalítico da AChE, bem como o modo de ação de inibidores (Sussman, 1991). Mais tarde, a estrutura da AChE de ratos, *Drosophila* e do homem foram obtidas e mostraram ser similares (Legay, 2000).

O sítio ativo da AChE situa-se na parte inferior de um estreitamento semelhante a uma garganta (gorge) a 20 Å de profundidade, alinhado com resíduos hidrofóbicos, os quais parecem ser importantes na orientação do substrato ao sítio ativo (Johnson & Moore, 1999). Este sítio ativo é formado por um sítio esterásico que contém uma tríade catalítica formada pela serina, histidina e glutamato ou aspartato e um sítio aniônico ou carregado negativamente a qual a cadeia de nitrogênio quaternário da ACh carregada positivamente se liga (Silman et al., 1994) (Figura 4A). Na borda ou superfície do gorge, cerca de 14 Å do sítio ativo, situa-se um segundo sítio aniônico que se tornou conhecido como sítio aniônico periférico (peripheral anionic site - PAS), o qual foi proposto com base na ligação de compostos bis quaternários (Bourne et al., 2005).

Existem duas importantes áreas a serem consideradas: a região de ligação e acomodação do substrato onde a ACh se liga à AChE por ligação iônica a um resíduo de aspartato ou glutamato e a ligação do hidrogênio ao resíduo de tirosina (Figura 4B). A outra região é responsável pelo mecanismo de hidrólise do substrato ACh, composta pelos aminoácidos serina e histidina (Figura 4B). O processo enzimático é extraordinariamente eficiente por causa da proximidade do nucleófilo serina e a catálise ácido/básica da histidina, sendo que uma molécula da ACh é hidrolisada em 100 microsegundos (Patrick, 2001).

A AChE tem numerosos inibidores: alguns como os organofosforados ligam-se exclusivamente no sítio esterásico, outros contendo grupo amônio quaternário similar a ACh ligam-se no sítio aniônico. Já os compostos bis ou tris-quaternários com mais de uma porção amônio, pode se ligar em ambos os

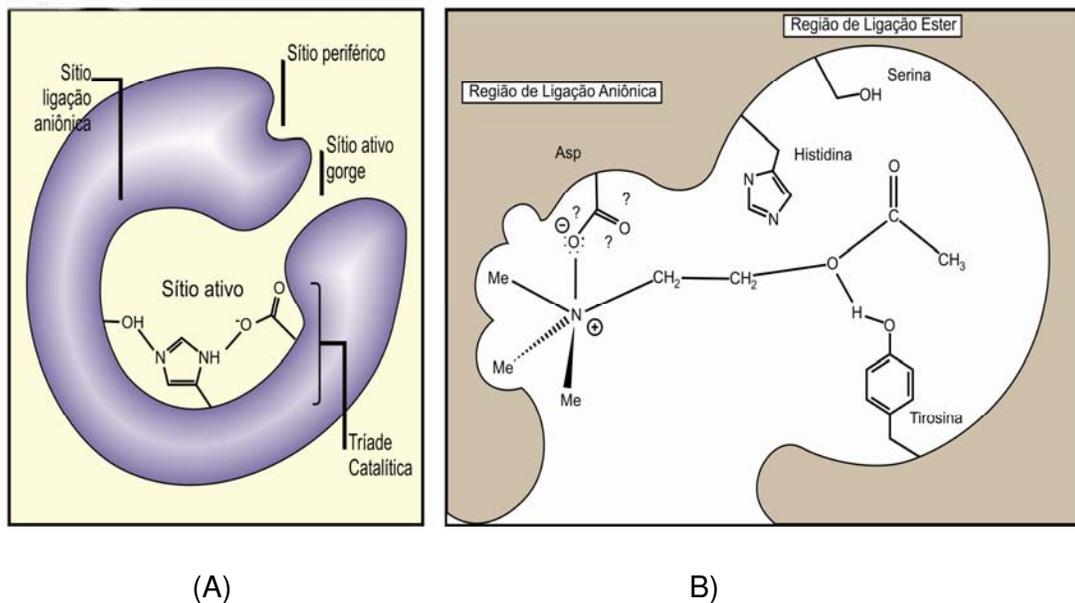


Figura 4: A) Ilustração do sítio esterásico contendo a tríade catalítica, externamente o sítio periférico aniônico (PAS). Adaptado de Soreq & Seidman (2001). B) Interação do substrato (ACh) com o sítio esterásico da AChE. Adaptado de Patrick (2001).

sítios. Outros ainda como o iodeto de propídio ou a fasciculina (peptídeo derivado de veneno de cobra), ligam exclusivamente no sítio aniônico periférico-PAS (Johnson & Moore, 1999).

1.3.1.3 AChE como alvo terapêutico

Por ser uma das mais eficientes e conhecidas catálises biológicas, a AChE tem sido investigada como um importante alvo terapêutico em várias doenças neurodegenerativas (Appleyard, 1994; Das et al., 2001). A classe terapêutica dos inibidores da AChE (IACHEs) melhora os sintomas cognitivos, comportamentais e funcionais relacionados às demências hipocolinérgicas como por exemplo a doença de Alzheimer (Gomes & Koszuoski, 2005). Desde a aprovação do donepezil, mais dois inibidores da AChE, a rivastigmina e a

galantamina, foram aprovados para o tratamento da doença de Alzheimer e também têm sido testados em outras desordens cognitivas como a doença de Parkinson (Werber & Rabey, 2001), a demência vascular (Kumar et al., 2000), a injúria traumática do encéfalo (Masanic et al., 2001) e inclusive na esclerose múltipla (Greene et al., 2000; Parry et al., 2003; Amato et al., 2006, Christodoulou et al., 2006; Porcel and Montalban, 2006).

Recentemente, foi relatado que IChEs possuem propriedades anti-inflamatórias, por inibir a proliferação de linfócito T e de citocinas pró-inflamatórias (Nizri et al., 2006). Além disso, recentes evidências também sugerem que uma porção dos receptores colinérgicos do encéfalo estão associados com células não excitáveis tais como os oligodendrócitos e os seus precursores. Deste modo, o tratamento com IChEs pode aumentar a estimulação colinérgica dos oligodendrócitos e promover assim o reparo da bainha de mielina (Bartzokis, 2006). Neste contexto AChEs podem representar um alvo de pesquisa importante e promissor em patologias que envolvam eventos desmielinizantes e inflamatórios associados à disfunções cognitivas como por exemplo a esclerose múltipla.

2. Sistema purinérgico

Os nucleotídeos extracelulares de adenina ATP e ADP, e o nucleosídeo adenosina 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, as situações de lise celular e a presença de enzimas como as ectonucleotidases (Rathbone et al., 1999).

2.1. Coexistência e coliberação do ATP e ACh

O ATP é um neurotransmissor e um neuromodulador do SNC e SNP (De Lorenzo et al., 2006). Este nucleotídeo é co-estocado e co-liberado juntamente com o neurotransmissor ACh (na taxa de aproximadamente 1:5) na sinapse neuromuscular, e uma vez na fenda sináptica ele é degradado até adenosina pela ação das ectonucleotidases (Ling et al., 2005). Assim, o ATP modula a liberação de ACh através de seu metabólito adenosina e também pela presença de seus receptores P2X e P2Y na sinapse neuromuscular (De Lorenzo et al., 2006). Apesar de existir pouca informação sobre a interação da sinalização purinérgica e colinérgica no SNC, foi demonstrado por Díaz-Hernández et al. (2002) que receptores de nucleotídeos ionotrópicos estão presentes nos terminais colinérgicos, sendo capazes de induzir a liberação de ACh sinaptossomal. Esses dados sugerem que receptores nucleotídicos podem co-existir com receptores nicotínicos no mesmo terminal, demonstrando a complexidade do funcionamento dos terminais colinérgicos e purinérgicos no SNC.

2.2. Nucleotídeos de adenina e plaquetas

As plaquetas também têm sido utilizadas como um excelente modelo para investigar doenças neurológicas, devido às características similares que estas células têm com os neurônios, como por exemplo, estocar e liberar neurotransmissores, além de apresentar receptores para os mesmos em sua

superfície (Chakrabarti et al., 1998). Estas similaridades entre plaquetas e neurônios reforçam a importância de se estudar parâmetros bioquímicos em plaquetas como marcadores clínicos de doenças neurológicas como a depressão, a esquizofrenia, a doença Alzheimer entre outras (Rainesalo et al., 2003; Cattabeni et al., 2004), facilitando assim uma melhor compreensão da etiopatogenia dessas doenças.

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

O ATP, ADP e a adenosina possuem importantes papéis nos mecanismos de tromboregulação regulando muitas funções plaquetárias. 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 (Anfossi 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; Remijn et al., 2002; Rozalski et al., 2005).

2.3. NTPDase (ATP difosfohidrolase, Apirase, Ecto/CD39, E.C. 3.6.1.5) e 5'-nucleotidase

E-NTPDases (Ecto – nucleosídeo trifosfato difosfohidrolases) 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; Biogonnese et al., 2004) (Figura 5). 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 NTPDase1 (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 (Figura 5) (Zimmermann, 2001). Esta enzima hidrolisa tanto o ATP como o ADP formando o AMP na presença de íons Ca^{2+} e Mg^{2+} (Ziganshin et al., 1994).

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 (Lunkes et al., 2003; Araújo et al., 2005). A função geral da apirase tem 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).

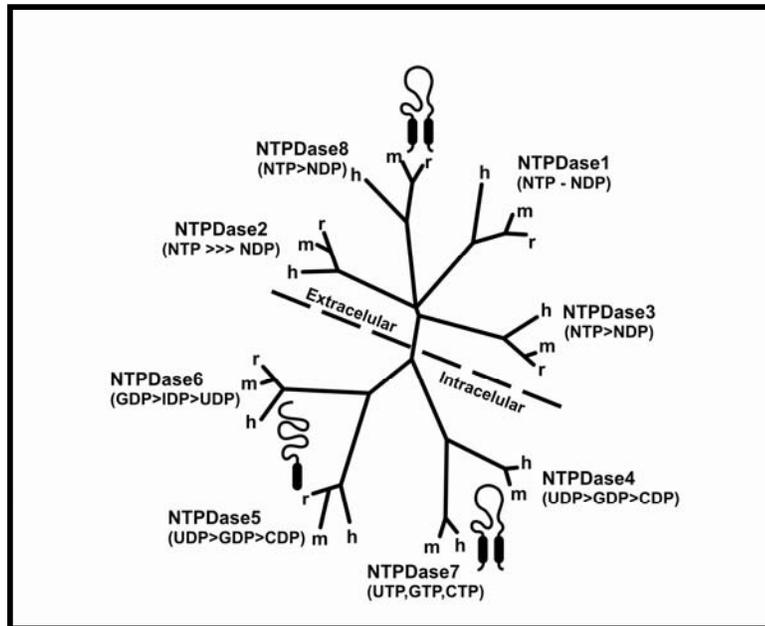


Figura 5 - Estrutura das enzimas da família NTPDase. Adaptado de Robson et al. (2006).

A ecto-5'-nucleotidase é uma enzima ancorada a membrana plasmática via uma molécula de GPI (glicosilfosfatidil inositol) com seu sítio catalítico voltado para o meio extracelular. 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; 1998; 2001).

3. Doenças desmielinizantes do sistema nervoso central

A doença desmielinizante é uma consequência da destruição das bainhas de mielina presentes no SNC e SNP. As alterações nestas bainhas podem ocorrer após uma variedade de eventos patológicos incluindo as reações auto-imunes, as intoxicações, as desordens metabólicas, as infecções virais e o

trauma mecânico que podem afetar tanto as células formadoras de mielina quanto os próprios neurônios mielinizados (Siegel, 1999).

Dentre as várias doenças que cursam com desmielinização no SNC destacam-se a cinomose dos cães e a esclerose múltipla em humanos (Bennet, 1997; Lazzarini, 2004), sendo que os mecanismos patogênicos de muitas destas condições são pouco compreendidos.

3.1 Esclerose múltipla

A esclerose múltipla é uma doença inflamatória crônica, a principal do grupo das doenças desmielinizantes, caracterizada por episódios repetidos de disfunção neurológica com remissão variável (Bjartmar et al., 2003; Costa et al., 2005). É uma doença de caráter imprevisível que afeta frequentemente a raça branca, o sexo feminino e é considerada a principal causa de incapacidade neurológica em adultos jovens (20 a 40 anos) (Puccioni-Sohler et al., 2001). Recentemente, também foi relatada em crianças, onde representa 10% dos casos de esclerose múltipla (Chabas et al., 2006).

Inicialmente, a maioria dos pacientes apresenta a forma surto-remissão (SR) da esclerose múltipla, caracterizada por episódios de desmielinização (surto) com conseqüentes deficiências neurológicas, seguido por episódios de remielinização (remissão) 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 esclerose múltipla (Reipert, 2004).

Não se conhece até o momento a causa da esclerose múltipla. Acredita-se que seja uma doença auto-imune, no qual um indivíduo com predisposição genética exposto a algum fator ambiental (p.ex. infecções virais), pode desencadear um processo auto-destrutivo mediado por linfócitos T contra a bainha de mielina e os oligodendrócitos do SNC (Kornek & Lassmann, 2003).

Os sintomas que ocorrem na esclerose múltipla podem variar dependendo do local onde ocorre à destruição da bainha de mielina, podendo incluir a perda da visão, a rigidez, a fraqueza, a falta de equilíbrio e a dormência (Kalb, 2000). Durante muitos anos considerou-se que essa doença raramente afetava as capacidades cognitivas. No entanto, com base em vários estudos ficou claro que o comprometimento cognitivo é muito comum nesta patologia, afetando 40 a 65% dos pacientes, os quais demonstram disfunções cognitivas envolvendo a memória, a atenção e o processamento de informações (Andrade et al., 1999; De Souza et al., 2002; Parry et al., 2003; Denney et al., 2005; Amato et al., 2006, Christodoulou et al., 2006; Porcel & Montalban, 2006). É importante ressaltar que esta disfunção cognitiva não é detectada somente na fase tardia da doença, mas também na fase inicial de surto-remissão, podendo progredir com o tempo (Denney et al., 2005; Amato et al., 2006; Chrstodoulou et al., 2006).

A procura de terapias que interfiram mais especificamente com diferentes componentes e fases da patogenia das doenças desmielinizantes ou induzam remielinização, esbarra na necessidade de uma melhor compreensão dos fenômenos celulares relacionados com a manutenção e reparo do tecido

nervoso (Levine & Reynolds, 1999; Franklin, 2002). Neste contexto, as células gliais, principalmente os astrócitos e os oligodendócitos têm um papel fundamental no restabelecimento das bainhas de mielina perdidas.

3.2 Células gliais

Os oligodendrócitos e células de Schwann são as células produtoras da bainha de mielina no SNC e SNP, respectivamente. Este processo de mielinização é considerado um marco significativo na evolução dos vertebrados por permitir a transmissão do impulso nervoso de forma rápida e eficiente, sendo capaz de integrar funções sensoriais, motoras e cognitivas (Lazzarini, 2004; Purves et al., 2005).

A desmielinização por sua vez se refere ao processo de remoção das bainhas de mielina previamente formadas (Franklin, 2002). Quando a perda se dá por instabilidade ou dano na célula mielinogênica (oligodendrócito e célula de Schwann) ou na própria bainha de mielina, com preservação dos axônios, a desmielinização é dita primária, devendo ser diferenciada daquela subsequente à degeneração axonal e denominada de secundária (Pereira et al., 1998). Já a reconstrução das bainhas de mielina perdidas no SNC refere-se ao processo de remielinização e pode ser efetuado pelas células precursoras de oligodendrócitos (OPC) as quais diferenciam - se em oligodendrócitos maduros ocupando a área desmielinizada, sendo este um evento importante para evitar um processo de degeneração (Stangel & Hartung, 2002; Zhao et al., 2005).

Os astrócitos compreendem 90% de toda a massa cerebral excedendo o número de neurônios de 10:1 (Benveniste, 1992). Essas células caracterizam-se pela presença de prolongamentos com filamentos intermediários (fibrilas

gliais), cujo componente principal é a proteína glial fibrilar ácida (GFAP) e a vimentina (VIM), que servem como meio de identificação desse tipo celular em estudos imuno-histoquímicos (Bondan et al., 2003). Independentemente da causa da lesão no SNC, o reparo do tecido é sempre realizado com a participação dos astrócitos, uma reação que inclui o aumento de seu número (astrocitose) e de suas dimensões (astrogliose), com conseqüente aumento da intensidade de marcação de GFAP. Estes fenômenos têm sido referidos como gliose astrocitária, cicatriz glial ou mesmo gliose (Montgomery, 1994).

Além de todas as funções referidas acima, as células gliais também desempenham um importante modo de sinalização no sistema nervoso (Fellin & Carmignoto, 2004), pela liberação de substâncias modulatórias (Newman, 2003), de substratos energéticos (Deitmer, 2001), além do importante papel na liberação de neurotransmissores como o glutamato, a acetilcolina e outros, os quais são essenciais para modular a transmissão sináptica (Näglner et al., 2001; Deng & Poretz, 2003; Mitterauer, 2004). Além disso, foi descoberta uma proteína de ligação da acetilcolina (AChBP) (Smit et al., 2001) sintetizada pela glia, onde a liberação pré-sináptica da ACh induz a secreção dessa proteína, e uma vez na fenda sináptica, ela age como uma “isca” molecular ligando-se à ACh, reduzindo assim sua viabilidade na sinapse, o que reforça a participação da glia juntamente com a AChE em modular a neurotransmissão colinérgica (Sixma & Smiti, 2003). Foi relatado por Sharma & Vijayaghavan (2002) que a AChPB também pode agir como uma proteína carreadora permitindo a difusão da ACh por longas distâncias e ao mesmo tempo protegendo-a da ação das colinesterases.

4. Modelos Experimentais de Desmielinização

Os modelos experimentais de desmielinização são importantes ferramentas para estudar estratégias terapêuticas, pois envolvem eventos de desmielinização e remielinização (Altmann & Boyton, 2004). Os principais modelos empregados para o estudo de eventos desmielinizantes do sistema nervoso incluem a indução de reações imunológicas contra componentes mielínicos (Reynolds et al., 1996; Gold et al., 2000; Buddeberg et al., 2004), a inoculação viral (Kelly et al., 1982), o uso de pressão mecânica sobre a mielina e a 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).

4.1. Modelo Experimental de Desmielinização pelo Brometo de Etídio (BE)

O BE (3,8-diamino-5-etil-6-fenil-fenatridina) é um composto púrpuro 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 (Figura 6) (Luedtke et al., 2003; Guazzo, 2005).

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 et al., 2003). Além disso, é considerada também uma droga gliotóxica indutora de desmielinização primária por destruir seletivamente células gliais - astrócitos e oligodendrócitos (Bondan, 1997; Graça et al., 2001).

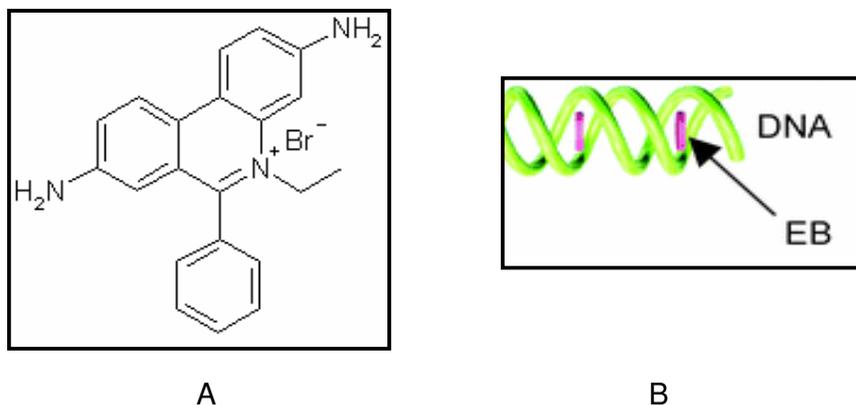


Figura 6 - Estrutura do brometo de etídio (3,8-diamino-5-etil-6-fenil-fenatridina) (A); intercalação entre os pares de bases do DNA (B). Adaptado de Luedtke et al. (2003).

O BE tem sido empregado para avaliar os eventos relacionados a desmielinização e remielinização na medula espinhal (Fushini & Shirabe, 2002), no pedúnculo cerebral (Penderis et al., 2003), no nervo óptico (Guazzo, 2005) e no 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 frequência cardíaca (Ekman, 2000; Purves et al., 2005). Injeções de BE na cisterna basal induzem lesões que se estendem desde o mesencéfalo até o corpo trapezóide, porém com maior gravidade na ponte, aonde chega a comprometer de 1/3 a 1/2 desta estrutura (Bondan, 1997). Um estudo realizado em pacientes com EM demonstrou que a ponte é o quarto local de maior incidência de lesões de desmielinização (28,6%) comparado com a substância branca periventricular, um local onde ocorre a maior prevalência de lesões (82,7%) (Skender-Gazibara et al., 2001; Minguetti, 2001).

Análises histológicas têm demonstrado que a injeção intracisternal de BE causa mudanças degenerativas nos oligodendrócitos e astrócitos após 72 horas da indução (Bondan et al., 2000). Os 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; Graça et al., 2001). Assim pode-se considerar que aos 3 dias pós-injeção (p.i) do brometo ocorre a morte e degeneração dos astrócitos e oligodendrócitos e início da desmielinização, sendo que aos 7 dias p.i ocorre o pico de desmielinização. Já aos 15 dias p.i ocorre o início da remielinização e aos 21 dias o pico da mesma, sendo que aos 30 dias ocorre uma remielinização completa. Neste contexto, a desmielinização tóxica pelo BE é um dos modelos mais utilizados para explorar a capacidade reparativa do SNC por apresentar boa reprodutibilidade de desmielinização e remielinização (Graça & Blakemore, 1986; Stangel & Hartung, 2002), o que torna este modelo uma importante ferramenta na compreensão de patologias desmielinizantes como a EM.

5. 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 ciclofosfamida, a azatiotropina e o metotrexato, e mais recentemente, a ciclosporina e a mitoxantrona (Faulds et al., 1993; Kappos, 1998; Neuhaus et al., 2003; Edan et al., 2004; Weiner, 2004). 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 o acetato de glatiramer e o interferon beta (IFN- β) (Neuhaus et al., 2003).

5.1. Ciclosporina (CsA)

A ciclosporina (CsA), é um peptídeo cíclico de 11 resíduos de aminoácidos (Figura 7) com potente atividade imunossupressora utilizada no tratamento de transplantes de órgãos e na terapia de doenças auto-imunes (Faulds et al., 1993). Foi relatado por Jolivalt et al. (2003) um efeito protetor da CsA na encefalomielite auto-imune experimental (EAE), um modelo utilizado para estudar a patogênese da EM. Já outros estudos randomizados em humanos com EM demonstraram que a CsA foi ineficaz no tratamento, além de apresentar importante toxicidade (Callegaro et al., 2002; Tullman et al., 2002). Além disso, numerosos efeitos colaterais desta droga têm sido relatados na literatura como hipertensão, nefrotoxicidade e neurotoxicidade (Rosendal et al., 2005). No modelo experimental de desmielinização pelo BE, a CsA aumentou a densidade dos oligodendrócitos nas margens da lesão de desmielinização, indicando que este agente imunossupressor foi capaz de afetar eventos

celulares envolvidos no processo de reparo do tecido desmielinizado (Bondan, 1997).

Por outro lado, têm sido demonstrado por Henrik et al. (2003) que a CsA inibe a atividade da AChE em várias estruturas cerebrais e que esta droga também exerce um efeito neuroprotetor e neurotrófico no sistema colinérgico por aumentar a imuno-reatividade da ChAT, enzima responsável pela síntese de ACh (Borlongan et al., 2000). Essas observações ajudam a confirmar que existe uma interação entre a CsA e o sistema colinérgico, o que pode fazer desta droga imunossupressora uma potente ferramenta terapêutica em desordens cerebrais associadas com as disfunções cognitivas.

5.2 Interferon beta (IFN- β)

Existem três formas de IFN- β que podem ser usadas na terapia clínica: o natural (nIFN- β) obtido de fibroblastos humanos, o interferon recombinante-1b (IFN- β -1b) obtido de bactérias e o interferon recombinante-1a (IFN- β -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 (Neuhaus et al., 2003; Revel, 2003).

Em portadores de EM, os dois tipos de IFNs recombinantes têm mostrado resultados satisfatórios por reduzir o número de surtos, por diminuir as lesões e por 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). Por outro lado, também foi relatado por Rao (2004), a eficácia do tratamento com IFN- β na disfunção cognitiva associada com o aprendizado, a memória e o processamento de informações em pacientes com EM, diminuindo assim a progressão desta patologia.

6. Vitamina E e Ebselen

A vitamina E (Vit E), um nutriente essencial em humanos é incorporado dentro das membranas celulares onde efetivamente desempenha um importante papel antioxidante (Steiner, 1993; Eidi et al., 2006). Além disso, a Vit E possui também uma variedade de funções não-antioxidantes como, por exemplo, a inibição da proteína quinase C (PKC) que regula a ativação de muitas proteínas envolvidas no processo inflamatório (Boscoboinik et al., 1991). Assim que este composto tem uma importante atividade anti-inflamatória como relatada por vários pesquisadores (Grammas et al., 2004; Saldeen & Saldeen, 2005; Walston et al., 2006; Reiter et al., 2007). Por outro lado, a Vit E inibe a agregação plaquetária in vitro (Quinn, 2004; Mabile et al., 1999; Freedman et al., 2000; Schoene, 2001), a reação de liberação granular (Steiner & Anastasi, 1976) e a adesão plaquetária (Szuwart et al., 2000). Neste contexto, a Vit E

pode promover efeitos benéficos em muitas doenças tais como a aterosclerose, nas doenças cardíacas e também nas doenças neurodegenerativas, como a esclerose múltipla (Johnson, 2000; Jialal et al., 2001).

Outros estudos também têm demonstrado o efeito neuroprotetor da Vit E tanto no dano neuronal induzido pelo glutamato (Schubert et al., 1992), como em proteger a barreira hematoencefálica durante uma crise epiléptica induzida experimentalmente (Kalayci et al., 2002). Além disto, a Vit E foi eficiente em restaurar parcialmente a hipofunção do sistema colinérgico no envelhecimento (Maneesub et al., 1993) e na retenção da memória (Eidi et al., 2006), focalizando também o envolvimento deste composto com a neurotransmissão colinérgica.

O ebselen (Ebs) [2-fenil-1,2-benzoisoselenazol-3-(2H)-one] é um composto seleno-orgânico solúvel em solventes orgânicos que reduz hidroperóxidos e lipoperóxidos por mimetizar a atividade da glutathione peroxidase, tendo um importante efeito antioxidante (Schewe, 1995). Além de possuir uma baixa toxicidade, o Ebs apresenta excelente propriedade anti-ateroesclerótica, citoprotetora (Dawson et al., 1995) e anti-inflamatória (Schewe, 1995). Por outro lado, foi demonstrado que o Ebs inibe in vitro a hidrólise dos nucleotídeos de adenina extracelular em plaquetas de ratos, indicando que este composto pode ter importantes efeitos nos mecanismos de tromboregulação (Furstenau et al., 2004). O papel neuroprotetor do Ebs também foi avaliado em modelos experimentais de isquemia cerebral e demonstraram uma importante recuperação neurológica, com redução das lesões nos neurônios, axônios e oligodendrócitos após o tratamento com este composto (Imai et al., 2001).

Por todas as funções relatadas acima a Vit E e o Ebs são ferramentas terapêuticas promissoras a serem testadas em doenças neurodegenerativas que envolvam eventos inflamatórios como, por exemplo, na esclerose múltipla.

Objetivos

Objetivo Geral

Investigar a atividade da enzima AChE em estruturas cerebrais de ratos experimentalmente desmielinizados pelo brometo de etídio (BE) e tratados com Interferon- β , Ciclosporina A, Vitamina E e Ebselen. A atividade da enzima NTPDase em plaquetas de ratos desmielinizados pelo BE e previamente tratados com Vitamina E e Ebselen também foi avaliada.

Objetivos Específicos

O presente estudo foi dividido em capítulos como se segue:

Capítulo 1: Investigar o efeito *in vitro* do BE na atividade da enzima AChE no estriado, hipocampo, córtex cerebral e cerebelo e também analisar os parâmetros cinéticos desta enzima na presença do BE nessas mesmas estruturas de ratos adultos.

Capítulo 2: Analisar *in vivo*, a atividade da enzima AChE em sinaptossoma de córtex cerebral e no sobrenadante do córtex cerebral, do estriado, do hipocampo, do cerebelo, do hipotálamo e da ponte de ratos normais e tratados com interferon beta após 7, 15 e 30 dias da injeção de BE.

Capítulo 3: Determinar *in vivo*, a atividade da enzima AChE no córtex cerebral, no estriado, no hipocampo, no hipotálamo, no cerebelo e na ponte de ratos normais e tratados com ciclosporina A após 21 dias da injeção de BE.

Capítulo 4: Avaliar *in vivo* a atividade da enzima AChE no córtex cerebral, no estriado, no hipocampo e em eritrócitos de ratos normais e tratados com vitamina E e ebselen após 3 e 21 dias da injeção de BE.

Capítulo 5: Determinar, *in vivo*, a atividade da NTPDase e 5'-nucleotidase em plaquetas de ratos normais e tratados com Vitamina E e ebselen após 3 e 21 dias da injeção de BE.

Material e Métodos referente ao modelo experimental de desmielinização pelo brometo de etídio utilizado nos capítulos 2, 3, 4 e 5.

Procedimento cirúrgico de craniotomia para acesso à cisterna basal

Para a realização da craniotomia e acesso à cisterna basal, os ratos foram submetidos à anestesia dissociativa composta por cloridrato de ketamina 10% e cloridrato de xilazina a 2%. Os fármacos foram misturados na mesma seringa na proporção de 5:1, respectivamente e dessa solução, foi administrada 0,1 mL para cada 100g de peso vivo, por via intraperitoneal. Uma vez em plano anestésico, foi realizada a tricotomia da região fronto-parieto-occipital, contenção em decúbito esternal e antisepsia com álcool-iodo-álcool.

Sobre os ossos frontal e parietal, foi feita uma incisão longitudinal de pele, subcutâneo e periósteo de aproximadamente 2,0 cm. Com o auxílio de uma perfuratriz elétrica de baixa rotação acoplada a uma broca sulcada de 2,0mm de diâmetro, foi realizada a craniotomia sobre o osso parietal situada a 0,85 cm póstero-diagonalmente e à direita do bregma. Em seguida e com auxílio de uma seringa de Hamilton de agulha removível de calibre 26s, introduzida no orifício previamente feito, numa posição perpendicular ao plano da superfície óssea da calota craniana e em direção à base do crânio foi perfurada a dura-máter e injetado 10 μ L de brometo de etídio na cisterna basal (grupo tratado) e 10 μ L de solução salina a 0,9% (grupo controle). Ao se atingir com a ponta da agulha a base craniana, a solução era injetada esperando-se cerca de 10 segundos para a retirada da agulha e com isso permitindo melhor difusão do líquido pelo sistema cisternal de forma a banhar a ponte. Foi realizada a sutura do periósteo com fio mononáilon 4-0 empregando sutura de

Wolff e a pele, utilizando o mesmo fio e padrão de sutura. Após o procedimento cirúrgico, os animais foram acondicionados em uma caixa plástica enriquecida com oxigênio a 100% para recuperação anestésica. Os pontos de pele foram removidos no décimo dia de pós-operatório.

Capítulo 1- Artigo publicado

Ethidium bromide inhibits rat brain acetylcholinesterase activity in vitro

Cinthia M. Mazzanti, Roselia Spanevello, Adriana Obregon, Luciane B. Pereira, Cristiane A. Streher, Mushtaq Ahmed, Alexandre Mazzanti, Dominguita L. Graça, Vera M. Morsch, Maria Rosa C. Schetinger

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Cinthia M. Mazzanti^a, Roselia M. Spanevello^a, Adriana Obregon^b, Luciane B. Pereira^b,
Cristiane A. Streher^b, Mushtaq Ahmed^b, Alexandre Mazzanti^c,
Dominguita L. Graça^d, Vera M. Morsch^a,
Maria Rosa C. Schetinger^{a,*}

^a Departamento de Bioquímica, Instituto de Ciências Básicas da Saúde, Universidade Federal do Rio Grande do Sul, Rua Ramiro Barcellos, 2600-Anexo 90035-003, Porto Alegre, RS, Brazil

^b Departamento de Química, Centro de Ciências Naturais e Exatas, Universidade Federal de Santa Maria, Campus Universitário, Camobi 97105-900, Santa Maria, RS, Brazil

^c Laboratório de Cirurgia Experimental, Centro de Ciências Rurais, Universidade Federal de Santa Maria, Campus Universitário, Camobi 97105-900, Santa Maria, RS, Brazil

^d Departamento de Patologia, Centro de Ciências da Saúde, Universidade Federal de Santa Maria, Campus Universitário, Camobi 97105-900, Santa Maria, RS, Brazil

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Abstract

Ethidium bromide (EtBr), a fluorescent dark red compound and stain for double-stranded DNA and RNA was used to study acetylcholinesterase (AChE) activity in vitro together with kinetic parameters of this enzyme in the striatum (ST), hippocampus (HP), cerebral cortex (CC) and cerebellum (CB) of adult rats. AChE activity in vitro in the ST, HP, CC and CB was significantly reduced ($p < 0.05$) in the presence of EtBr at concentrations of 0.00625, 0.0125, 0.025, 0.05 and 0.1 mM. For the analysis of the kinetic three concentrations of EtBr were tested (0.00625, 0.025 and 0.1 mM). An uncompetitive inhibition type was observed in the ST, HP and CC, whereas in the CB the inhibition type was mixed. These data indicate that EtBr should be considered a strong inhibitor of AChE activity demonstrating that there is an interaction between this compound and the cholinergic system.

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Keywords: Ethidium bromide; Acetylcholinesterase; Kinetic analysis; Rat; Cholinergic system

1. Introduction

Acetylcholinesterase (AChE, E.C. 3.1.1.7) is an important regulatory enzyme that rapidly hydrolyses the neurotransmitter acetylcholine (ACh) at brain cholinergic synapses as well as at the neuromuscular junction [1],

being one of the most efficient catalytic reactions known [2]. AChE shows some characteristics not found in any other enzyme, such as, its organization of the active site and its catalytic mechanism [3]. The active site of AChE was found to be located at the bottom of a narrow gorge and consists of two subsites, a negatively charged or anionic site and an esteratic site, containing the actual catalytic residues, also called the catalytic triad [4].

Apart from their classical role in cholinergic transmission, AChE has potent effects on cellular adhesion

* Corresponding author. Tel.: +55 55 3 2208665;
fax: +55 55 220 80 31.

E-mail address: mariorosa@smail.ufsm.br (M.R.C. Schetinger).

[4] and neurite extension [5], participates in the structural regulation of postsynaptic differentiation [6] and is present in lymphocytes, where it probably plays a role in the regulation of immune functions [7]. Therefore, an absence or inhibition of this enzyme may have devastating consequences on the brain and other organs [8].

Of toxicological and pharmacological significance, AChE is a sensitive target for both natural and synthetic cholinergic toxins, such as snake venom and insecticides [6]. Furthermore, it is also a target for therapeutically active compounds, including anti-Alzheimer's disease drugs [3]. AChE has numerous inhibitors, such as organophosphates, which bind exclusively to the esteratic site and others which have a quaternary ammonium group similar to acetylcholine, binding to either of the anionic sites. In addition, propidium iodide and the mamba snake venom peptide (fasciculin) bind exclusively to the peripheral site of AChE [4].

Ethidium bromide (3,8-diamino-5-ethyl-6-phenylphenanthridinium bromide; EtBr) is a fluorescent dark red compound originally used as trypanocide [9], but more recently has been used as stain for double-stranded DNA and RNA [10–12] and also as a gliotoxic agent in experimental studies on myelin lesions [13–15]. It was reported that EtBr, by presenting its quaternary ammonium structure, could interact with butyrylcholinesterase enzyme (BuChE) and reversibly inhibit its activity, demonstrating that there is an interaction between this phenanthridinium compound and the cholinergic system [16]. Moreover, EtBr has a structure very similar to that of propidium iodide, a well-known inhibitor of AChE [17,18] which leads to the hypothesis that this compound can also alter AChE activity.

Although there is a large literature about the toxic properties of EtBr, until now, no studies have demonstrated a relation of this compound with AChE activity, an enzyme that plays an indispensable role in the cerebral cortex, hippocampus and striatum, regions where the cholinergic inputs are extremely important. By the prerogatives outlined above, the purpose of the present investigation was to evaluate whether EtBr induces changes in AChE activity by studying kinetic parameters of AChE in the presence of this agent in brain tissues of adult rats.

2. Materials and methods

2.1. Chemicals

Acetylthiocholine iodide, ethidium bromide (EtBr), 5,5'-dithiobis 2-nitrobenzoic acid (DTNB), Tris

(hydroxymethyl)-aminomethane GR and Coomassie brilliant blue G were obtained from Sigma Chemical Co (St. Louis, MO, USA) and bovine serum albumin, K_2HPO_4 , from Reagen. All the other chemicals used in this experiment were of analytical grade.

2.2. Animals

Ten male Wistar 4–6-month-old rats with a body weight of 300–400 g were maintained on a 12 h light/12 h dark cycle, at a temperature of 22 °C, 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.

2.3. Brain tissue preparation

The animals were submitted to euthanasia, being previously anesthetized with ethyl ether and then decapitated and the brain structures were immediately removed and separated into striatum (ST), hippocampus (HP), cerebral cortex (CC) and cerebellum (CB) and placed in a solution of 10 mM Tris-HCl, pH 7.4, on ice. The brain structures were homogenized in a glass potter in Tris-HCl solution. Aliquots of resulting brain structure homogenates were stored at –8 °C until utilization. Protein varied for each structure: ST (0.4 mg/ml), HP (0.8 mg/ml), CC (0.7 mg/ml) and CB (0.6 mg/ml); these concentrations were determined by the Coomassie blue method according to Bradford [19] using bovine serum albumin as standard solution.

2.4. AChE enzymatic assay

The AChE enzymatic assay was determined by a modification of the spectrophotometric method of Ellman et al. [20] as previously described by Rocha et al. [21]. The reaction mixture (2 ml final volume) was composed of 50 mM K^+ -phosphate buffer, pH 7.5 and 1 mM 5,5'-dithiobisnitrobenzoic acid (DTNB). The method is based on the formation of the yellow anion, 4,4'-dithio-bis-acid-nitrobenzoic measured by absorbance at 412 nm during 2-min incubation at 25 °C. The enzyme (40–50 μ g of protein) was pre-incubated with different EtBr concentrations (0.00625, 0.0125, 0.025, 0.05 and 0.1 mM) for 2 min. The reaction was initiated by adding 0.8 mM acetylcholine iodide. All samples were run in duplicate or triplicate and enzyme activity was expressed in micromoles AcSCh/h/mg of protein.

2.5. Kinetic parameters

The kinetics of the interaction of EB with AChE were determined using the Lineweaver–Burk [22], double reciprocal plot, by plotting $1/V$ against $1/S$ analyzed over a range of acetylthiocholine concentrations (0.008–0.8 mM), either in the absence or presence of EB (0.00625, 0.025 and 0.1 mM).

K_m values were obtained by two different estimates, $1/V$ versus $1/S$ [22] and V versus V/S [23]. IC_{50} was calculated according to the Dixon and Webb [24] plot using inhibitor concentrations of 0.00625, 0.025 and 0.1 mM. K_i values (the dissociation constant of the enzyme–substrate–inhibitor (ESI) complex) were determined by the Dixon plot method [24], plotting the inverse of velocity ($1/V$) versus EB concentrations ($[I]$) and using five acetylthiocholine concentrations.

2.6. Statistical analysis

Data were analyzed using one-way analysis of variance (ANOVA) followed by the Tukey–Kramer's test. Differences between groups were considered significant when $p < 0.05$. All data were expressed as mean \pm S.E.M.

3. Results

3.1. In vitro effect of EtBr on AChE activity

AChE activity in the ST, HP, CC and CB was significantly reduced in the presence of EtBr. One-way analysis of variance (ANOVA) followed by the Tukey–Kramer's test revealed that AChE activity was significantly inhibited ($p < 0.05$) at all concentrations tested (0.00625, 0.0125, 0.025, 0.05 and 0.1 mM). These results are represented in Fig. 1A–D).

3.2. Kinetic determination of acetylcholinesterase

Analysis of kinetic data indicated that the inhibition caused by EtBr was uncompetitive in the ST, HP and CC (Fig. 2A–C). However, in the CB the inhibition was mixed (Fig. 2D). The values of K_m and V_{max} measured by $1/V$ versus $1/S$ (Table 1) and by V versus V/S confirmed the inhibition type. In the ST, HP and CC, where the inhibition was uncompetitive, a decrease in the K_m and V_{max} values was observed (Table 1 and Fig. 2A–C). In the mixed type inhibition, a decrease was seen in the V_{max} values and an elevation in the K_m values (Fig. 2D). The concentration of EtBr required to inhibit 50% (IC_{50})

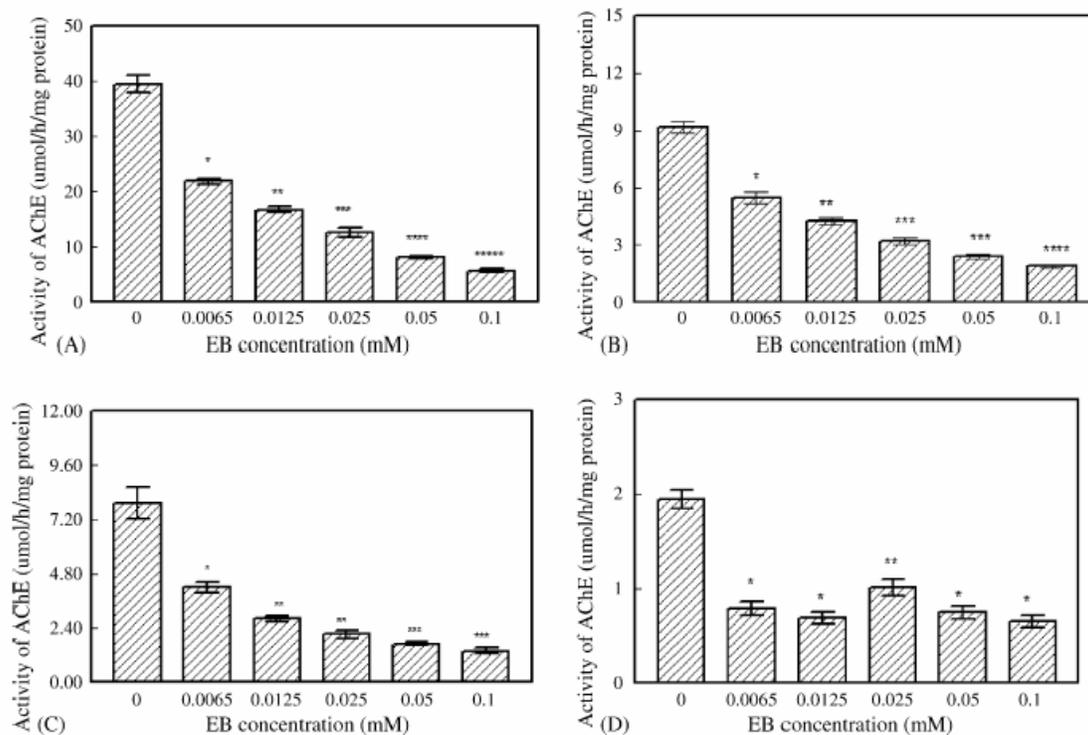


Fig. 1. Inhibition of AChE activity in vitro in the striatum (ST) (A), hippocampus (HP) (B), cerebral cortex (CC) (C) and cerebellum (CB) (D) in the presence of different concentrations of EtBr (0.00625, 0.0125, 0.025, 0.05 and 0.1 mM); ($n = 5$), $p < 0.05$.

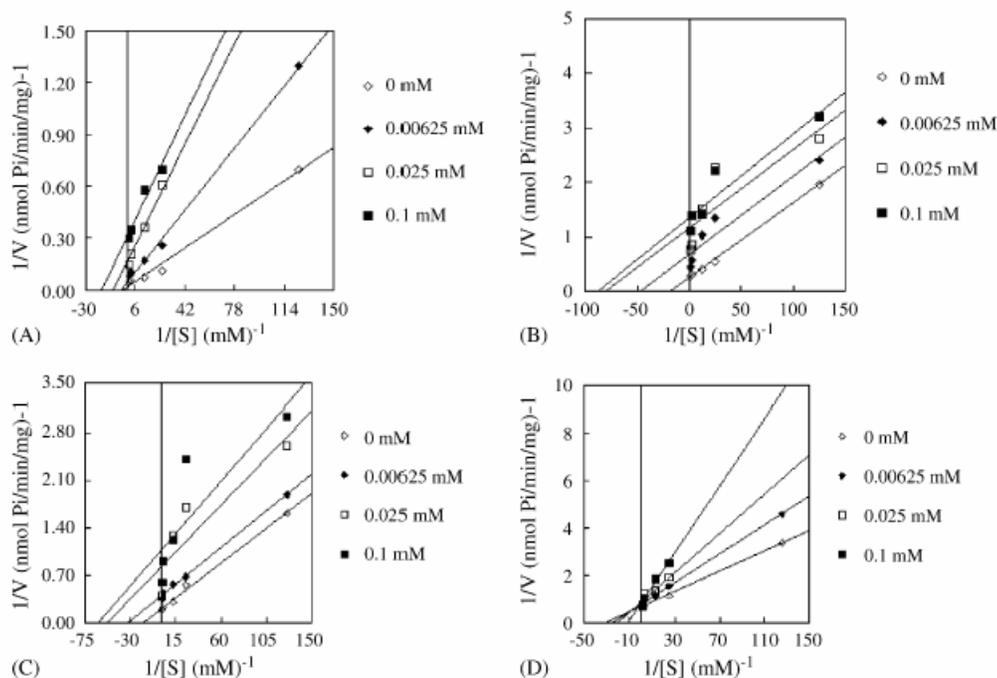


Fig. 2. Lineweaver–Burk representation of AChE inhibition by EtBr in the striatum (ST) (A), hippocampus (HP) (B), cerebral cortex (CC) (C) and cerebellum (CB) (D) with acetylthiocholine as substrate. Double reciprocal plot was constructed by plotting $1/V$ against $1/S$ analyzed over a range of substrate concentrations (0.008–0.8 mM) in the absence and in the presence of EtBr (0.00625, 0.025 and 0.1 mM). The plot represents the means of three experiments ($n=5$), S.D. < 10%, $p < 0.05$.

of AChE activity was calculated according to the Dixon and Webb method [24], using inhibitor concentrations of 0.00625, 0.025 and 0.1 mM and K_i values were measured by the Dixon and Webb method [24] plotting S/V versus $[I]$. The IC_{50} and K_i values for inhibiting AChE are shown in Table 2.

4. Discussion

There are several studies using EtBr as stain for double-stranded DNA [9–12,25] or in experimental studies on myelin lesions [13–15,26]. However, a study that correlates the effects of this compound on AChE activity in vitro with its kinetic analysis has not been found in the literature. Previous studies from our laboratory using EtBr in gliotoxic model have shown that the 0.025 mM concentration was able to induce demyelination in the central nervous system (CNS) [27]. Based on this study, we utilized concentrations of EtBr varying from 0.00625 to 0.1 mM to evaluate the effect of this compound in the AChE activity.

In this study, it was demonstrated that EtBr is a strong AChE inhibitor, causing an in vitro inhibition at the lowest concentration tested (0.0065 mM) in all cerebral structures. However, the analysis of the kinetic data indicated that the inhibition caused by EtBr was not homo-

geneous in all cerebral structures studied. For example, in the ST, HP and CC, the inhibition was uncompetitive, whereas in the CB, it was mixed. An important aspect to be discussed is that the HP and CC, which both receive cholinergic projections from the nucleus basalis of Meynert and the ST, which has an intrinsic cholinergic circuit, presented similar results. Taken together, we can postulate that in regions where the cholinergic inputs are extremely important, such as the ST, HP and CC, the action of EtBr was similar.

V_{max} and K_m decreased in the ST, HP and CC (Table 1 and Fig. 2A–C) characterizing an uncompetitive inhibition. On the other hand, in the mixed inhibition type, while V_{max} decreased, as observed in the CB, K_m increased (Table 1 and Fig. 2D). Thus, in the uncompetitive inhibition type, it is probable that the complex enzyme–substrate–inhibitor (ESI) will be formed and in the mixed inhibition type the complexes EI and ESI may be formed. Nonetheless, both an uncompetitive and a mixed inhibitor bind to another site, different from the substrate active site. In fact, the chemical structure of ACh and EtBr is not similar, thus the binding of EtBr to a site other than the active site is expected, as observed in the ST, HP, CC and CB. The IC_{50} and K_i values indicated that EtBr is a strong AChE inhibitor even at low mM concentrations. The IC_{50} values showed a similar

Table 1

K_m is expressed as mM concentration of AcSCh obtained by plotting $1/V$ vs. $1/S$

EtBr (mM)	K_m [S] ($\mu\text{moles AcSCh}$)	V_{max} (1/V) ($\mu\text{moles AcSCh/h/mg/protein}$)
	Striatum	
0	0.288	14.28
0.00625	0.189	11.11
0.025	0.097	6.66
0.1	0.054	3.70
	Hippocampus	
0	0.055	1.61
0.00625	0.020	1.51
0.025	0.012	1.16
0.1	0.011	0.96
	Cerebral cortex	
0	0.056	4.16
0.00625	0.030	3.33
0.025	0.018	1.44
0.1	0.016	1.40
	Cerebellum	
0	0.030	1.72
0.00625	0.039	1.42
0.025	0.047	0.89
0.1	0.089	0.83

Hydrolysis rates V were measured at various substrate (S) concentrations (0.008–0.8 mM) in 2 ml assay solution with 30 mM phosphate buffer, pH 7.0 and 0.454 mM DTNB (5,5'-dithiobis (2-nitrobenzoic acid) plus 30–50 μg of protein. Protein was preincubated for 2 min before substrate addition.

sensitivity to EtBr by ST, HP and CC, showing that these brain structures which the cholinergic inputs are very important presented related results, as observed above in the inhibitory mechanism (uncompetitive). Corroborating these results are observations by Patocka [16] that EtBr affects BuChE activity and reversibly inhibits its activity. BuChE's affinity for the substrate is reduced

Table 2

K_i and IC_{50} values for AChE

Cerebral structures	K_i (mM)	IC_{50} (mM)
Striatum	0.0032	0.009
Hippocampus	0.0068	0.008
Cerebral cortex	0.00236	0.0075
Cerebellum	0.00585	0.0049

IC_{50} was calculated according to the Dixon plot using inhibitor concentrations of 0.00625, 0.025 and 0.1 mM.

by EtBr and the breakdown of the ternary EIS complex is prevented, demonstrating that there is an interaction between this compound and the cholinergic system [16].

The results reported in this paper characterize EtBr as an AChE inhibitor similar to other inhibitors of this enzyme, as for example, propidium iodide [17,18]. Any important aspect of this study is that the EtBr structure is very similar to that of propidium (Fig. 3A and B), a well-known inhibitor of AChE activity [18]. Propidium specifically binds at the peripheral anionic site (PAS), a second binding site that is located on the surface of AChE next to the entry of the active site. This PAS exhibits a large negative surface potential and is thought to bind and orientate the substrate molecule prior to catalysis at the active site [17,18,28]. We can suggest that EtBr probably binds at this peripheral site of AChE, leading to an inhibition of this enzyme, as demonstrated in this study. EtBr, as well as propidium, presents a quaternary ammonium in its structure, which is demonstrated in the literature as an important condition for the effective binding of both the substrate and the inhibitors at the active site [29]. The anionic site of AChE presents a partially negative aspartate residue. An electrostatic interaction between the quaternary ammonium with EtBr can occur at the site, suggesting a probable binding place for this compound.

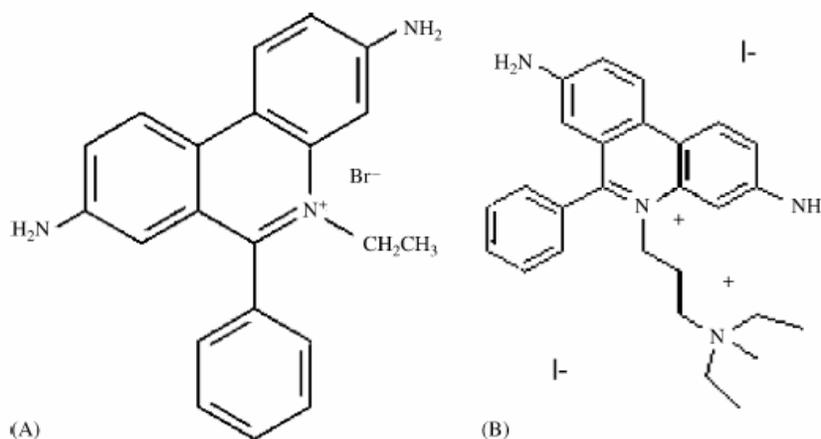


Fig. 3. Ethidium bromide (A) and Propidium iodide (B).

It is known that the inhibition of AChE is an important therapeutic target [6,7,30–34] for the treatment of neurodegenerative diseases [35,36]. A decrease in the activity of AChE indicates an increase of ACh levels in the synaptic cleft [1,2,37–39], enabling an improvement in cognitive functions, such as learning and memory [8,40,41]. On the other hand, an irreversible inhibition of this enzyme may have devastating consequences on the brain and other organs [3–5].

An important aspect to be discussed in this study is that at the low dose of EtBr tested (0.0065 mM) a significant inhibition of AChE activity occurred and this concentration is very far from the LD₅₀ in mice (300 µM; 100 mg/kg, subcutaneous) [12]. On the other hand, low doses of EtBr (10 µM) lead to an increase of total mitochondrial DNA and higher concentrations of EtBr (25 and 50 µM) induce the mtDNA deletion of the human neuronal cell line [25], indicating that EtBr has opposite biological effects.

In conclusion, EtBr is a strong inhibitor of AChE, as observed in this work. However, potential applications in human treatment have must be prevented due to its mutagenic effects.

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Capítulo 2 - Artigo publicado

Acetylcholinesterase activity in rats experimentally demyelinated with ethidium bromide and treated with interferon beta

C. M. Mazzanti, R. M. Spanevello, L. B. Pereira, J. F. Gonçalves, R. Kaizer, M. Côrrea, A. Mazzanti, R. Festugatto, D. L. Graça, V. M. Morsch, M. R. C. Schetinger

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Acetylcholinesterase Activity in Rats Experimentally Demyelinated with Ethidium Bromide and Treated with Interferon Beta

C. M. Mazzanti · R. M. Spanevello · L. B. Pereira · J. F. Gonçalves · R. Kaizer · M. Corrêa · M. Ahmed · A. Mazzanti · R. Festugatto · D. L. Graça · V. M. Morsch · M. R. C. Schetinger

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Abstract The ethidium bromide (EB) demyelinating model was associated with interferon beta (IFN- β) to evaluate acetylcholinesterase (AChE) activity in the striatum (ST), hippocampus (HP), cerebral cortex (CC), cerebellum (CB), hypothalamus (HY), pons (PN) and synaptosomes from the CC. Rats were divided into four groups: I control (saline), II (IFN- β), III (EB) and IV (EB and IFN- β). After 7, 15 and 30 days rats ($n = 6$) were sacrificed, and the brain structures were removed for enzymatic assay. AChE activity was found to vary in all the brain structures in accordance with the day studied (7–15–30 days) ($P < 0.05$). In the group III, there was an inhibition of the AChE activity in the ST, CB, HY, HP and also in synaptosomes of the CC ($P < 0.05$). It was observed that IFN- β per se was

capable to significantly inhibit ($P < 0.05$) AChE activity in the ST, HP, HY and synaptosomes of the CC. Our results suggest that one of the mechanisms of action of IFN- β is through the inhibition of AChE activity, and EB could be considered an inhibitor of AChE activity by interfering with cholinergic neurotransmission in the different brain regions.

Keywords Ethidium bromide · Acetylcholinesterase · Demyelination · Interferon beta · Synaptosomes · Rat

Introduction

The cholinergic system is one of the most important and, phylogenically, the oldest of the nervous system pathways [1]. Cholinergic neurons and their projections are widely distributed throughout the central nervous system (CNS) with an essential role in regulating many vital functions, such as learning, memory, cortical organization of movement and the control of cerebral blood flow [2, 3].

The enzyme acetylcholinesterase (AChE E.C. 3.1.1.7) is found in the cholinergic terminal where it performs the hydrolysis of the neurotransmitter acetylcholine (ACh) [4–8]. Besides its catalytic property [9], AChE has potent effects on cellular adhesion [10], neurite extension [11], as well as participating in the structural regulation of postsynaptic differentiation [12], and it is present in lymphocytes, where it probably plays a role in the regulation of immune functions [13]. Therefore, an absence or inhibition of this enzyme may have devastating consequences on the brain and other organs [2].

C. M. Mazzanti · R. M. Spanevello
 Departamento de Bioquímica, Instituto de Ciências Básicas da Saúde, Universidade Federal do Rio Grande do Sul, Rua Ramiro Barcellos, 2600-Anexo, 90035-003 Porto Alegre, RS, Brazil

L. B. Pereira · J. F. Gonçalves · R. Kaizer · M. Corrêa · M. Ahmed · V. M. Morsch · M. R. C. Schetinger (✉)
 Curso de Pós-Graduação em Bioquímica Toxicológica, Centro de Ciências Naturais e Exatas, Universidade Federal de Santa Maria, Campus Universitário, Camobi, 97105-900 Santa Maria, RS, Brazil
 e-mail: mariarosa@smail.ufsm.br

A. Mazzanti · R. Festugatto
 Laboratório de Cirurgia Experimental, Centro de Ciências Rurais, Universidade Federal de Santa Maria, Campus Universitário, Camobi, 97105-900 Santa Maria, RS, Brazil

D. L. Graça
 Departamento de Patologia, Centro de Ciências da Saúde, Universidade Federal de Santa Maria, Campus Universitário, Camobi, 97105-900 Santa Maria, RS, Brazil

The importance of AChE has been investigated in Alzheimer's disease [14–17] and other neurologic disorders including multiple sclerosis (MS). MS is a chronic demyelinating disease of the CNS, and cognitive impairment is a significant determinant of quality of life for patients with this pathology [18, 19]. It has been reported that MS patients show knowledge of disturbances regarding learning and memory [20] that may indicate the involvement of the cholinergic system in this pathology [21]. A study reported that inhibitors of AChE promoted memory improvement of MS patients, highlighting the importance of this enzyme as a therapeutic target [22].

Several studies on the biology of demyelination and remyelination in the CNS have been made on the gliotoxic agent ethidium bromide (EB) [23, 24]. EB selectively destroys glial cells—oligodendrocytes (the CNS myelin forming cells), and astrocytes that determine the processes of demyelination and remyelination [25].

Various immunosuppressive, anti-inflammatory and immunomodulating agents have been widely used in toxic and immune-mediated demyelinating models, as well as in some demyelinating diseases [26]. Interferon beta (IFN- β), an immunomodulating agent, has been shown to have beneficial effects on relapsing-remitting MS by reducing both the severity and frequency of attacks [27, 28]. It has also been demonstrated that IFN- β , besides regulating the lymphocyte T function, has the ability to protect astrocytes against apoptotic cell death [29] and it may additionally reduce astrocytosis and, thereby, promote endogenous repair [30]. Moreover, the efficacy of IFN- β was observed in the treatment of cognitive dysfunctions in patients with MS [31, 32], demonstrating the relevance of a study on AChE activity, a key enzyme of the cholinergic synapses in the CNS [33].

In this context, considering that MS represents the major disabling neurological illness of young adults and that the cholinergic system has a role in this pathology, this study was conducted in order to investigate the activity of the AChE enzyme in the gliotoxic demyelinating model in normal and IFN- β -treated rats.

Experimental procedure

Chemicals

Acetylthiocholine (ATC), Percoll, EB, 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, K_2HPO_4 , from

Reagen. IFN- β (Rebif[®] 44 mcg 12 MUI/ 0.5 ml) was purchased from SERONO BARI (Italy). All the others chemicals used in this experiment were of analytical grade.

Animals

Adult male Wistar rats (70–90 days; 220–300 g) were used in this experiment. The animals were maintained at a constant temperature ($23 \pm 1^\circ\text{C}$) 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 (saline $n = 18$); II-control (saline + IFN- β , $n = 18$); III-treated (EB, $n = 18$) and IV-treated (EB + IFN- β , $n = 18$). 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 0.85 cm to the right of the bregma was made until the duramater was exposed. With the use of a Hamilton syringe with a removable needle of caliber 26 s, the solutions were injected in the cisterna pontis (basal), an enlargement of the subarachnoid space on the ventral surface of the pons (PN). Ten microliters of EB (0.1%) 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 II and I. The duramater was left open and the skin, together with the remainder of subcutaneous tissue, was sutured with a nylon thread 4.0. To evaluate 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 hematoxylin and eosin (H&E).

Treatment

After the surgical procedure, the animals belonging to groups III and I were injected with saline solution and those in groups II and IV received IFN- β (500,000 U/

animal per day) subcutaneously until the end the experiment. Six animals of each group were sacrificed 7, 15 and 30 days after the surgical procedure, and their tissues were removed for the enzymatic assay.

Synaptosome preparation

The synaptosomes were isolated as described by Nagy and Delgado-Escueta [34] using a discontinuous Percoll gradient. The cerebral cortex (CC) was gently homogenized in 10 volumes of an ice-cold medium consisting of 32 mM sucrose, 0.1 mM EDTA and 5.0 mM Tris, pH 7.5, within a motor driven Teflon-glass homogenizer (15 strokes at 1500 rpm), and then centrifuged at 1000g for 10 min. An aliquot of 0.5 ml of crude mitochondrial pellet was mixed with 4.0 ml of an 8.5% Percoll solution and layered onto 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 15,000g for 20 min. to remove any contaminating Percoll. The pellet of 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 AChE activity assay.

Brain tissue preparation

Brain structures were immediately removed and separated into cerebral cortex (CC), striatum (ST), hippocampus (HP), cerebellum (CB), hypothalamus (HY) and pons (PN), and placed in a solution of 10 mM Tris-HCl, pH 7.4, on ice. The brain structures were homogenized in a glass potter in Tris-HCl solution and then centrifuged at 1500g for 10 min. Aliquots of resulting brain structure homogenates were stored at –8°C until utilization. Protein was determined previously and adjusted for each structure: ST (0.4 mg/ml), HP (0.8 mg/ml), CC and PN (0.7 mg/ml), CB (0.6 mg/ml), and HY (0.6 mg/ml) by the Coomassie blue method according to Bradford [35] using bovine serum albumin as standard solution.

AChE enzymatic assay

AChE enzymatic assay was determined by a modification of the spectrophotometric method of Ellman et al. [36] as previously described [37]. The assay medium (2 ml final volume) contained 100 mM K⁺-phosphate buffer, pH 7.5, and 1 mM 5,5'-dithiobisni-

trobenzoic acid. The enzyme (40–50 µg of protein) from the brain structures was pre-incubated for 2 min. The reaction was initiated by adding 0.8 mM ATC iodide as the substrate. After pre-incubation for 2 min, the reaction velocity was measured by increasing absorbance to 412 nm at 25°C. All samples were run in duplicate or triplicate. The enzyme activity was expressed in µmoles AcSCh/h/mg of protein.

Statistical analysis

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

Results

Regarding the model used in this study, the histological analysis of the ventral area of the PN revealed alterations caused by the EB injection. After 7 days loss of myelin sheaths was seen as status spongiosus of the tissue (Fig. 1A), while 15 days after EB injection was detected some vacuolated areas intermingled with macrophages (Fig. 1B). After 21 days many neuroglial cells processes were detected in the area where status spongiosus was formerly seen (Fig. 1C). Resolution of the lesions occurred through remyelination of the axons demyelinated by EB at 30 days (date not shown).

AChE activity was evaluated in all the cerebral structures, and in the CC the AChE activity was evaluated in the supernatant (S₁) and in the synaptosomes. In relation to the CC in the material S₁, post hoc comparisons by Duncan's multiple range test revealed that on days 7 and 30, there was not significant alteration in the activity of AChE. On day 15, there was a significant increase in the activity of AChE in group II ($P < 0.05$), when compared with group I (Fig. 2).

In the synaptosomes of the CC, post hoc comparisons by Duncan's test revealed that AChE activity on day 7 was significantly reduced in groups II, III and IV ($P < 0.05$), when compared with group I. On day 15, there was a significant increase ($P < 0.05$) in AChE activity in groups II, III and IV, compared with group I, while on day 30, there was a significant reduction in AChE activity in groups II, III and IV ($P < 0.05$) compared with group I (Fig. 3).

In the ST, post hoc comparisons by Duncan's test revealed that AChE activity was significantly decreased on days 15 and 30 in groups II, III and IV ($P < 0.05$), compared with group I, and no significant

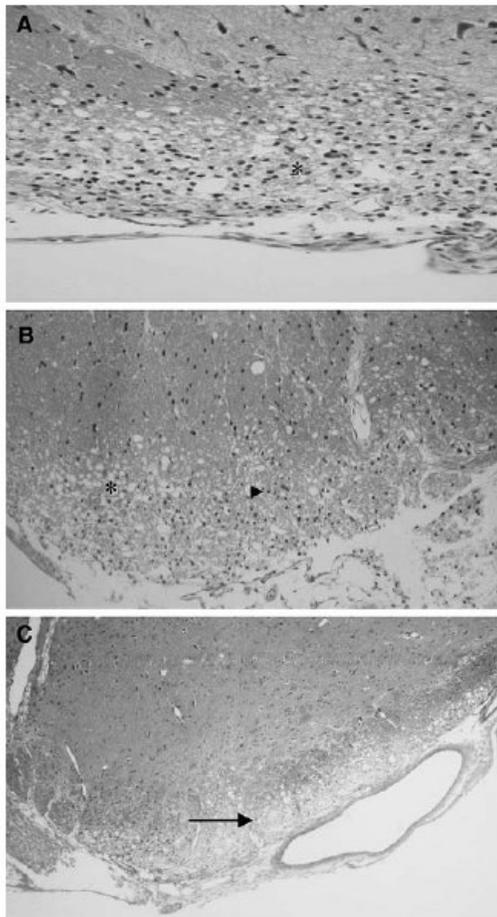


Fig. 1 Demyelinating lesion in the ventral area of the PN caused by the injection of EB in the brainstem of Wistar rats. After 7 days (A) spongiosis of the tissue (*) was observed (H&E, 160X). At 15 days (B) some vacuolated areas (*) intermingled with macrophages were detected (▴) (H&E, 100X), while after 21 days (C) extensive lesion (arrow) was seen where spongiosis subsided through remyelination (H&E, 100X)

alteration was found in AChE activity on day 7 (Fig. 4).

In the HP, post hoc comparisons by Duncan's test revealed that on days 7 and 15 there was a significant increase in AChE activity in groups III and IV ($P < 0.05$), compared with group I. On day 30, there was a significant decrease in the activity of AChE in groups II and III ($P < 0.05$) when compared with group I, and a significant increase in AChE activity in group IV ($P < 0.05$), when compared with group I (Fig. 5).

In the CB, post hoc comparisons by Duncan's test revealed a significant increase in AChE activity on day 7 in groups II and III ($P < 0.05$) compared with group

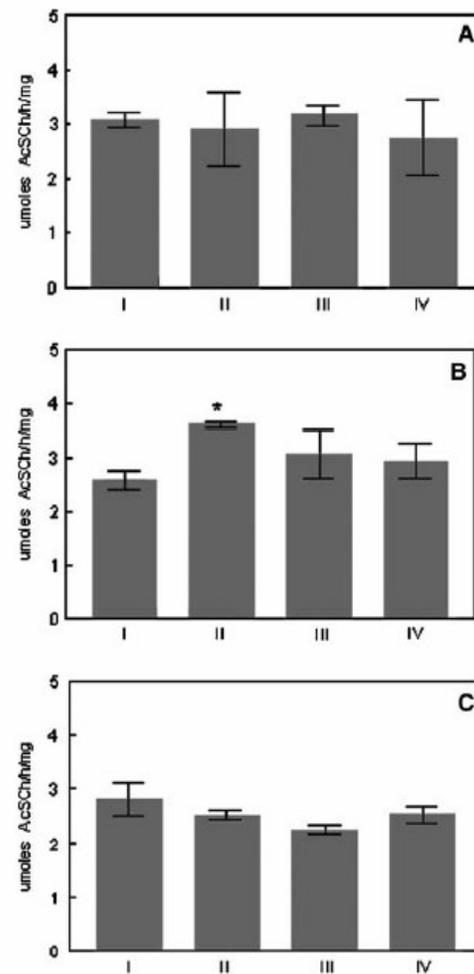


Fig. 2 AChE activity in the CC of rats at 7 (A), 15 (B) and 30 (C) days after the injection of EB in the brainstem. Groups I (control), II (IFN- β), III (EB) and IV (EB and IFN- β). (* $P < 0.05$; $n = 4-5$)

I. On day 15, groups II and IV presented a significant decrease ($P < 0.05$) in enzyme activity and in group III there was a significant increase of AChE ($P < 0.05$), compared with group I, while on day 30, there was a significant decrease of AChE activity in groups III and IV ($P < 0.05$), compared with group I (Table 1).

In the HY, post hoc comparisons by Duncan's test revealed that on day 7, there was a significant increase in AChE activity in groups II and IV ($P < 0.05$), compared with group I. On day 15, there was an increase in enzyme activity in group III ($P < 0.05$), and on day 30 there was a significant decrease in enzymatic activity in groups II and III ($P < 0.05$), compared with group I, and a significant increase in

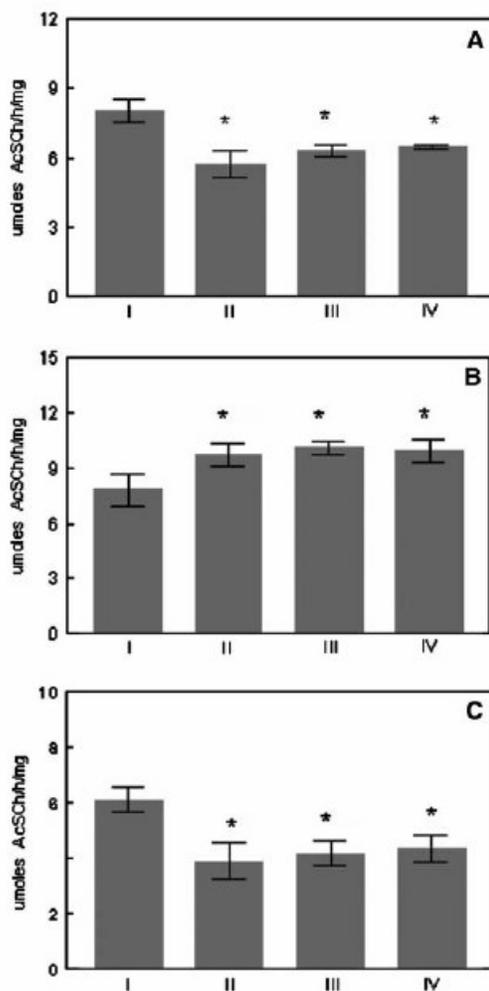


Fig. 3 AChE activity in synaptosomes of the CC of rats at 7 (A), 15 (B) and 30 (C) days after the injection of EB in the brainstem. Groups I (control), II (IFN- β), III (EB) and IV (EB and IFN- β). (* $P < 0.05$; $n = 4-5$)

AChE activity in group IV ($P < 0.05$), when compared with groups II and III (Table 1).

In the PN, post hoc comparisons by Duncan's test revealed no significant alterations on days 7 and 15, while on day 30, there was a significant increase in AChE activity in group II ($P < 0.05$) when compared with group I (Table 1).

Discussion

Toxic demyelination is one of the most commonly used models to explore the reparative capacity of the CNS, and toxins, such as EB, can be injected into the white matter of experimental animals leading to selective myelin loss [38]. There are several studies using EB as

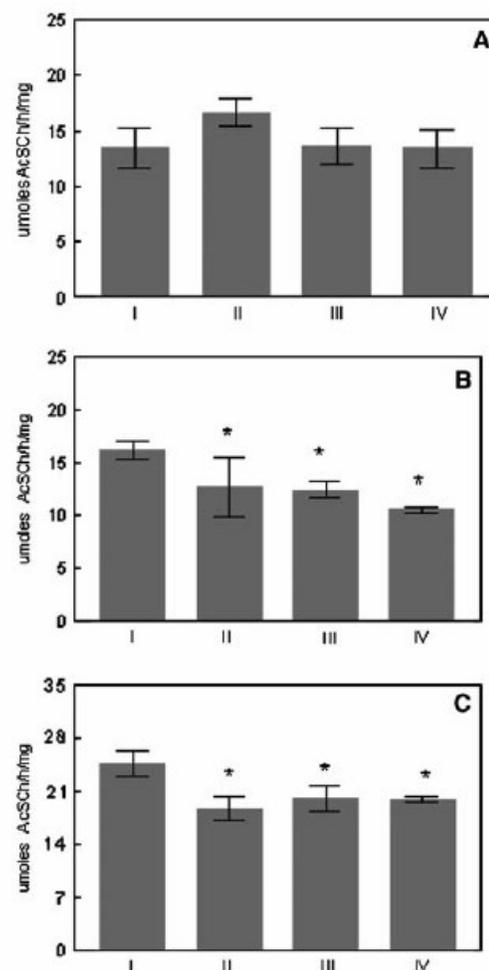


Fig. 4 AChE activity in the ST of rats at 7 (A), 15 (B) and 30 (C) days after the injection of EB in the brainstem. Groups I (control), II (IFN- β), III (EB) and IV (EB and IFN- β). (* $P < 0.05$; $n = 4-5$)

a demyelination experimental model [23–25, 39]. However, a study that correlates EB gliotoxic effects with the AChE activity in rats treated with IFN- β has not been found in the literature.

In the present study, AChE activity was found to vary in all the major areas—CC, ST, HP, CB, HY and PN, depending of the period (7–15–30 days) (Fig 3, 4, 5 and Table 1). In fact, this suggests that these alterations of AChE activity could be related with demyelination and remyelination events that occur during these periods. Histological analysis in this investigation showed those 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. Others morphologic

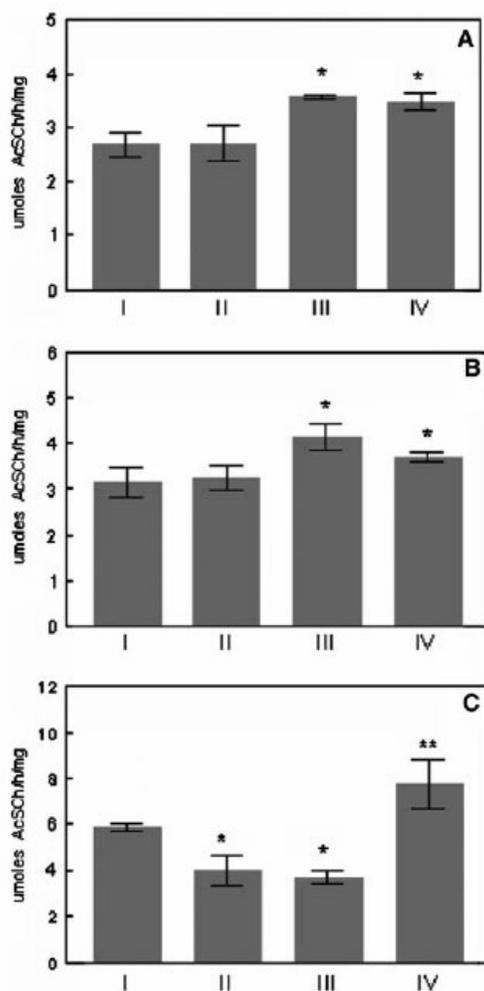


Fig. 5 AChE activity in the HP of rats at the 7 (A), 15 (B) and 30 (C) days after the injection of EB in the brainstem. Groups I (control), II (IFN- β), III (EB) and IV (EB and IFN- β). (* P < 0.05; ** P < 0.01; n = 4–5)

and immunohistochemical studies have proven that 7 days after the injection of EB, complete demyelination is attained, at 15 days the first signs of remyelination can be observed, and that remyelination is completed after around 25–30 days of the injection of EB [25, 26]. In this context, the white matter tracts arising from the nucleus basalis and projecting to distant forebrain regions could plausibly be interrupted by demyelinating plaques, creating a presynaptic cholinergic deficit responsive to treatment with cholinesterase inhibitors [40], which would make AChE an important therapeutic target [6, 8, 15–17, 41] for the treatment of neurodegenerative diseases [18–22].

In this study, it was observed a significant quantitative variation in the synaptosomal AChE activity when

Table 1 AChE activity in CB, HY, and PN of rats at 7, 15 and 30 days after injection of EB in the brainstem. Groups I (control), II (IFN- β), III (EB) and IV (EB and IFN- β). Values represent mean \pm SEM

Structures	Days		
	7	15	30
<i>Cerebellum</i>			
I	1.18 \pm 0.03	1.56 \pm 0.09	1.74 \pm 0.18
II	1.41 \pm 0.02*	1.15 \pm 0.05*	1.76 \pm 0.18
III	1.33 \pm 0.10*	1.99 \pm 0.24**	1.19 \pm 0.21*
IV	1.26 \pm 0.03	1.09 \pm 0.22*	1.14 \pm 0.05*
<i>Hypothalamus</i>			
I	5.07 \pm 0.44	5.39 \pm 0.54	6.90 \pm 0.45
II	6.86 \pm 0.15*	4.80 \pm 0.01	4.70 \pm 0.30*
III	5.25 \pm 0.05	7.30 \pm 1.12*	4.38 \pm 0.27*
IV	6.43 \pm 1.09*	5.69 \pm 0.39	5.68 \pm 1.07**
<i>Pons</i>			
I	4.38 \pm 0.28	2.83 \pm 0.14	5.58 \pm 1.08
II	4.30 \pm 0.05	3.07 \pm 0.39	6.59 \pm 0.9*
III	4.81 \pm 0.34	2.88 \pm 0.18	4.56 \pm 0.23
IV	4.73 \pm 0.39	3.31 \pm 0.36	5.23 \pm 0.20

* P < 0.05; ** P < 0.01; n = 4–5

compared with that of the supernatant (S_1) from the CC (Fig. 2 and 3). These results corroborate with previous studies from our laboratory showing that other enzymes, such as apyrase, also have their activities altered in synaptosomes after demyelination by EB [42].

Synaptosome is formed by nerve endings, useful particularly to pharmacological studies of ACh metabolism and release [43]. Thus, we can suggest that demyelinating events can lead to alterations in the synaptic membranes changing the conformational state of the AChE molecule, which would explain the altered activity observed in the present study. In addition, this difference between synaptosome and S_1 could be explained by the existence of both G4 (membrane bound) and G1 (cytosolic) AChE molecular forms in the different brain regions [5]. In the mammalian brain, the G4 form represents 60–90% of the total AChE, depending on the anatomical region, the remainder is composed by G1 and G2 forms [1].

Recent studies from our laboratory showed that novel benzodiazepines are inhibitors of the rat brain AChE [44] and antidepressants are inhibitors of cholinesterases from serum and erythrocytic membranes of humans [45], and enhanced AChE activity has also been observed in the CC and HP after the subchronic treatment with mercury chloride [3] as well as the enzyme is altered by diets [7] and organic solvents [8]. The lack of uniformity in the profile of AChE may be a reflection of the functional heterogeneity in the central cholinergic system observed from several works of various parameters [5, 33]. However, an absence or

inhibition of this enzyme may have devastating consequences on the brain and other organs, as AChE is involved in several key events demonstrated by various researchers [2, 10–13].

It was observed in this study that in the group treated with EB, there was an inhibition of AChE activity in the ST (15 and 30 days) (Fig. 4), HP, CB and HY (30 days) (Fig. 5, Table 1) and also in synaptosomes of the CC (7 and 30 days) (Fig. 3). AChE inhibition leads to an accumulation of ACh, causing overstimulation of the receptors [1, 9]. On the other hand, EB increased AChE activity in the HP and CB (7 and 15 days) (Fig. 5 and Table 1), HY (15 days) (Table 1) and synaptosomes of the CC (15 days) (Fig. 3). AChE activation leads to a fast ACh degradation and a subsequent downstimulation of ACh receptors [4]. These two mechanisms can promote undesirable effects. This may suggest that EB promotes a dysfunction in the synapses, interfering with cholinergic neurotransmission and modulation.

Recently, was demonstrated by our laboratory that EB is a strong inhibitor of AChE activity *in vitro*, and that this inhibition was not homogeneous in all cerebral structures evaluated [46]. In relation to the treatment with IFN- β , it was observed that this compound associate with EB did not alter AChE activity, suggesting that IFN- β was not able to prevent the inhibitory effects of EB.

One important data observed in this study is that IFN- β per se was capable to significantly inhibit AChE activity in ST, HP, HY and synaptosome of the CC (Fig. 3, 4, 5 and Table 1), structures rich in cholinergic pathways. The HP and CC receive cholinergic projections from the nucleus basalis of the Meynert and ST, which have an intrinsic cholinergic circuit, suggesting an interaction between IFN- β and the cholinergic system. Corroborating with these findings, it has been demonstrated by Rao [32], through cognitive tests, that IFN- β was effective in the treatment of patients with MS, improving memory, learning, attention and information processing, vital functions which are regulated by the cholinergic pathways. Moreover, the rate of cognitive dysfunction in MS is in the range of 45–65% and immunomodulating drugs such as IFN- β slow the disease progression [27, 28] as well as inhibit cognitive deterioration in this demyelinating pathology [31].

Although results from clinical trials have indicated that IFN- β improves cognition in patients with MS, little is known about its mechanisms of action [30]. Our results suggest that one of the mechanisms of action of IFN- β is through the inhibition of AChE activity, reinforcing that this agent could modulate cholinergic neurotransmission. In fact, we suggest that IFN- β can

contribute to the treatment of neurodegenerative disorders such as Alzheimer, MS and other dementia.

In conclusion, an experimental model of demyelination was used to evaluate AChE activity, and from the compiled results, we suggest that a cholinergic dysfunction exists, which is caused by the action of EB on the activity of this enzyme. We can also suggest that the beneficial effects of IFN- β on the cognition of patients with MS reported in many studies may be due, at least in part, to its ability to inhibit AChE activity, and this hypothesis could be investigated in future studies with the intention of finding a better therapy with cholinesterase inhibitors to benefit patients with cognitive disorders.

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Capítulo 3 - Artigo publicado

Cyclosporine A inhibits acetylcholinesterase activity in rats experimentally demyelinated with ethidium bromide

Cíntia M. Mazzanti, Roselia Spanevello, Mushtag Ahmed, Alexandre Mazzanti,
Fabiano Z. Salbego, Dominguita L. Graça, Eliza S. V. Sallis, Vera M. Morsch, Maria
Rosa C. Schetinger.

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Cyclosporine A inhibits acetylcholinesterase activity in rats experimentally demyelinated with ethidium bromide

Cinthia M. Mazzanti^a, Roselia Spanevello^a, Musthaq Ahmed^b, Roberta Schmatz^b,
Alexandre Mazzanti^c, Fabiano Z. Salbego^c, Dominguita L. Graça^d, Eliza S.V. Sallis^d,
Vera M. Morsch^b, Maria Rosa C. Schetinger^{b,*}

^aDepartamento de Bioquímica, Instituto de Ciências Básicas da Saúde, Universidade Federal do Rio Grande do Sul, Rua Ramiro Barcellos, 2600-Anexo, 90035-003 Porto Alegre, RS, Brazil

^bDepartamento de Química, Centro de Ciências Naturais e Exatas, Universidade Federal de Santa Maria, Campus Universitário, Camobi, 97105-900 Santa Maria, RS, Brazil

^cLaboratório de Cirurgia Experimental, Centro de Ciências Rurais, Universidade Federal de Santa Maria, Campus Universitário, Camobi, 97105-900 Santa Maria, RS, Brazil

^dDepartamento de Patologia, Centro de Ciências da Saúde, Universidade Federal de Santa Maria, Campus Universitário, Camobi, 97105-900 Santa Maria, RS, Brazil

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Abstract

Cyclosporine A is the major immunosuppressive agent used for organ transplantation and for the treatment of a variety of autoimmune disorders such as multiple sclerosis. In this work, we investigated the effect of the cyclosporine A on the acetylcholinesterase activity in the cerebral cortex, striatum, hippocampus, hypothalamus, cerebellum and pons of the rats experimentally demyelinated by ethidium bromide. Rats were divided into four groups: I control (injected with saline), II (treated with cyclosporine A), III (injected with 0.1% ethidium bromide) and IV (injected with 0.1% the ethidium bromide and treated with cyclosporine A). The results showed a significant inhibition ($p < 0.05$) of acetylcholinesterase activity in the groups II, III and IV in all brain structures analyzed. In the striatum, hippocampus, hypothalamus and pons the inhibition was greater ($p < 0.005$) when ethidium bromide was associated with cyclosporine A. In conclusion, the present investigation demonstrated that cyclosporine A is an inhibitor of acetylcholinesterase activity and this effect is increased after an event of toxic demyelination of the central nervous system. © 2007 ISDN. Published by Elsevier Ltd. All rights reserved.

Keywords: Ethidium bromide; Acetylcholinesterase; Cyclosporine A; Rat; Demyelination

1. Introduction

Toxic demyelination by ethidium bromide (EB) is one of the most commonly used models for exploring the reparative capacity of the central nervous system (CNS) (Yajima and Suzuki, 1979; Stangel and Hartung, 2002; Guazzo, 2005). EB induces focal demyelinating in the CNS (Bondan et al., 2000) by selectively damaging oligodendrocytes and astrocytes, and consequently interfering with the demyelination and remyelination processes (Graça et al., 2001). In fact, this model may be a useful tool to study experimental demyelinating mechanisms and, perhaps in the future, to help in understanding

multiple sclerosis (MS) (Levine and Reynolds, 1999; Smith and Franklin, 2001; Ohler et al., 2004).

Cognitive impairment is a significant determinant of the quality of life for patients with MS (Greene et al., 2000; DeSousa et al., 2002), the most common demyelinating disorder of the CNS. Cognitive dysfunction affects about half of the individuals with MS, and difficulties in learning and remembering new information represent the most common cognitive deficits (D'Intimo et al., 2005; Porcel and Montalban, 2006), with resultant losses of sensory and motor functions (Bjartmar et al., 2003; Ohler et al., 2004).

Many experimental and clinical studies have shown that the cholinergic system plays an important role in learning, memory and attention, highlighting the importance of acetylcholinesterase (AChE) as a therapeutic target by hydrolyzing the excitatory neurotransmitter, acetylcholine (ACh) (Grisaru et al.,

* Corresponding author. Fax: +55 55 220 80 31.

E-mail address: mariaschetinger@gmail.com (M.R.C. Schetinger).

1999). Acetylcholinesterase inhibitors (AChEI) may therefore represent a potential treatment option for impaired cognitive status in MS (Parry et al., 2003). Probably the cholinergic system is affected in the course of the disease as a consequence of demyelination and axonal transection (Gustavson and Cummings, 2003; Porcel and Montalban, 2006).

The treatment with AChEI has shown efficacy not only in Alzheimer's disease (Weinstock, 1995; Das et al., 2002; Rees et al., 2003; Kasa et al., 2004), but also in other cognitive disorders such as Parkinson's disease (Werber and Rabey, 2001), vascular dementia (Kumar et al., 2000), traumatic brain injury (Masanic et al., 2001) and even in MS (Greene et al., 2000; Parry et al., 2003; Christodoulou et al., 2006; Porcel and Montalban, 2006).

Numerous immunosuppressive and immunomodulating agents have been administered to patients with MS in an attempt to control the progression of the disease (Kappos, 1988). Recently, we demonstrated that the interferon β (IFN- β), an immunomodulating drug was capable of inhibiting AChE activity in cerebral structures of the normal and demyelinated rats, demonstrating that this agent could modulate cholinergic neurotransmission (Mazzanti et al., 2006a). In addition, cyclosporine A (CsA), an efficient immunosuppressive agent widely used after transplantation and in the therapy of immune disease such as MS (Faulds et al., 1993), also inhibited AChE activity in selected parts of the rat brain, demonstrating the interaction of this drug with the central cholinergic system (Herink et al., 2003). However, there is not evidence in the literature concerning the effects of CsA on the central cholinergic pathways associated with a demyelinating event.

In this context, due to the central role of the cholinergic system in a variety of neurodegenerative disease, it is relevant to study the effects of the CsA on the activity of AChE in a model of toxically induced CNS demyelination, in order to investigate the potentially therapeutic use of this drug in demyelinating/remyelinating events.

2. Experimental procedures

2.1. Chemicals

Acetylthiocholine iodide, ethidium bromide (EB), 5,5'-dithiobis 2-nitrobenzoic acid (DTNB), Tris(hydroxymethyl)-aminomethane GR and Coomassie brilliant blue G were obtained from Sigma Chemical Co. (St. Louis, MO, USA) and bovine serum albumin, K_2HPO_4 , from Reagen (São Paulo, Brazil). Cyclosporine A was purchased from SANDIMMUN[®] (Chile). All other reagents used in the experiments were of analytical grade of the highest purity.

2.2. Animals

Forty male Wistar 3–4-month-old rats with a body weight of 300–400 g were kept in separate animal cages, on a 12 h light/dark cycle, at a temperature of 22 °C, with free access to food and water. All animal procedures were approved by the Institutional Commission of the Federal University of Santa Maria.

2.3. Surgical procedure to access the basal cistern for injection of ethidium bromide (EB) or saline solution

The animals were divided into four groups: I-control (saline $n = 10$); II-treated (CsA: $n = 10$); III-treated (EB: $n = 10$); IV-treated (EB + CsA: $n = 10$).

The animals were anesthetized with ketamine, chloridate and xylazine (5:1:0.1 ml/100 g) and after shaving the fronto-parietal-occipital area antisepsis was performed with 2% iodine solution. With the aid of an orthodontic roof motor and a number 2 drill, a 0.85 cm hole was made to the right of the bregma until the duramater was exposed. With the use of a Hamilton syringe with a removable needle, 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 (0.1%) 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. After the surgical procedure, the animals from groups II and IV received 10 mg/kg of CsA intraperitoneally for seven consecutive days during the first week, followed by the application of the same amount every 48 h for 2 weeks. After the treatment period (3 weeks), the animals were submitted to euthanasia, being previously anesthetized with ethyl ether and the brain structures were separated for the subsequent enzymatic assay.

2.4. Histological studies

Three rats from each group were used for histological analysis of the lesion. These rats were perfused under deep anesthesia with 10% buffered formalin via the left ventricle at 21 days after injection (a.i). Brain stem coronal slices of the lesion were embedded in paraffin for routine processing and 5 μ m sections were produced and stained with hematoxylin and eosin (H&E).

2.5. Brain tissue preparation

Brain structures were immediately removed and separated into cerebral cortex (CC), striatum (ST) hippocampus (HP), hypothalamus (HY), cerebellum (CB) and pons (PN) and placed in a solution of 10 mM Tris-HCl, pH 7.4, on ice. The brain structures were homogenized in a glass potter in Tris-HCl solution. Aliquots of resulting brain structure homogenates were stored at -8 °C until utilization. Protein was determined previously and adjusted for each structure: ST (0.4 mg/ml), HP (0.8 mg/ml), CC and PN (0.7 mg/ml), CB (0.6 mg/ml) and HY (0.6 mg/ml) by the Coomassie blue method according to Bradford (1976) using bovine serum albumin as standard solution.

2.6. AChE enzymatic assay

The AChE enzymatic assay was determined by a modification of the spectrophotometric method of Ellmann et al. (1961) as previously described (Rocha et al., 1993). The reaction mixture (2 ml final volume) was composed of 100 mM K^+ -phosphate buffer, pH 7.5, 1 mM 5,5'-dithiobisnitrobenzoic acid (DTNB). The method is based on the formation of the yellow anion, 5,5'-dithiobis-acid-nitrobenzoic, measured by absorbance at 412 nm during 2-min incubation at 25 °C. The enzyme (40–50 μ g of protein) was pre-incubated for 2 min. The reaction was initiated by adding 0.8 mM acetylcholine iodide. All samples were run in duplicate or triplicate and the enzyme activity was expressed in micromoles AcSch/h/mg of protein.

2.7. Statistical analysis

The statistical analysis used was one-way ANOVA, followed by Duncan's multiple range tests. $p < 0.05$ was considered to represent a significant difference in both analyses used. All data were expressed as mean \pm S.E.M.

3. Results

3.1. Histological analysis of lesion

The lesions induced by EB consisted of areas of demyelination seen as status spongiosus of the tissue (Fig. 1A and B). More cellular activity and some new blood vessels were observed within the demyelination lesion after 21

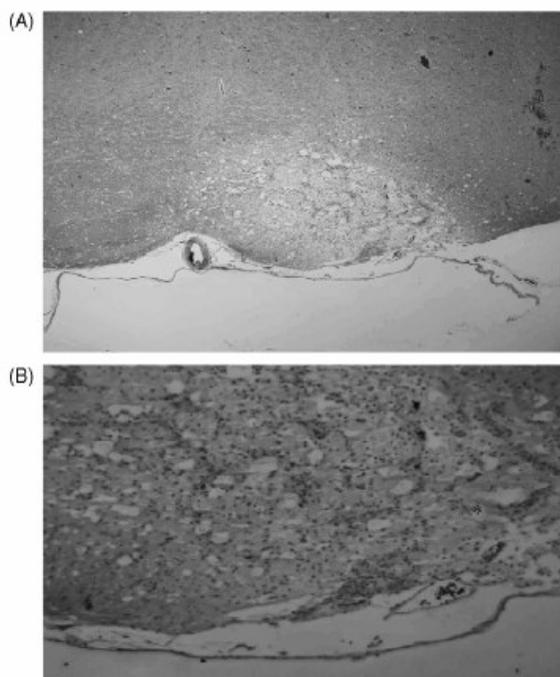


Fig. 1. Twenty-one days remyelinating lesion of group III in the pons on the right. Normal tissue on the left H&E 100 \times (A). Higher magnification of (A). Intense cell activity is observed intermingled with some residual vacuolated areas (*). H&E 260 \times (B).

days, an indication of remyelination of the lost myelin sheaths. The rats from the control group had a small traumatic lesion along the needle track (data not show).

3.2. AChE activity

The results showed that administration of EB (0.1%) or CsA affected AChE activity. When CsA was administered alone, post hoc comparison by Duncan's test revealed that AChE activity was inhibited in the CC (36.93%), ST (22.62%), HP (33.11%), HY (34.83%), CB (45.64%) and PN (33.21%), in relation to the control group (100%) ($p < 0.05$) (Figs. 2 and 3). In the group demyelinated with EB, the post hoc comparison by Duncan's test disclosed an inhibition of AChE activity in the CC (50.79%), ST (25.03%), HP (18.66%), HY (39.09%), CB (37.64%) and PN (64.91%) when compared with control group (100%) ($p < 0.05$) (Figs. 2 and 3). When the demyelinated rats were treated with CsA, the post hoc comparison by Duncan's test revealed an inhibition of AChE activity in all structures evaluated and this inhibition was more accentuated in the PN (73.56%), HY (52.33%), HP (49.88%), ST (38.32%) when compared with the control group (100%) ($p < 0.05$) (Figs. 2 and 3).

4. Discussion

There are several studies using EB as a demyelination experimental model (Yajima and Suzuki, 1979; Bondan et al.,

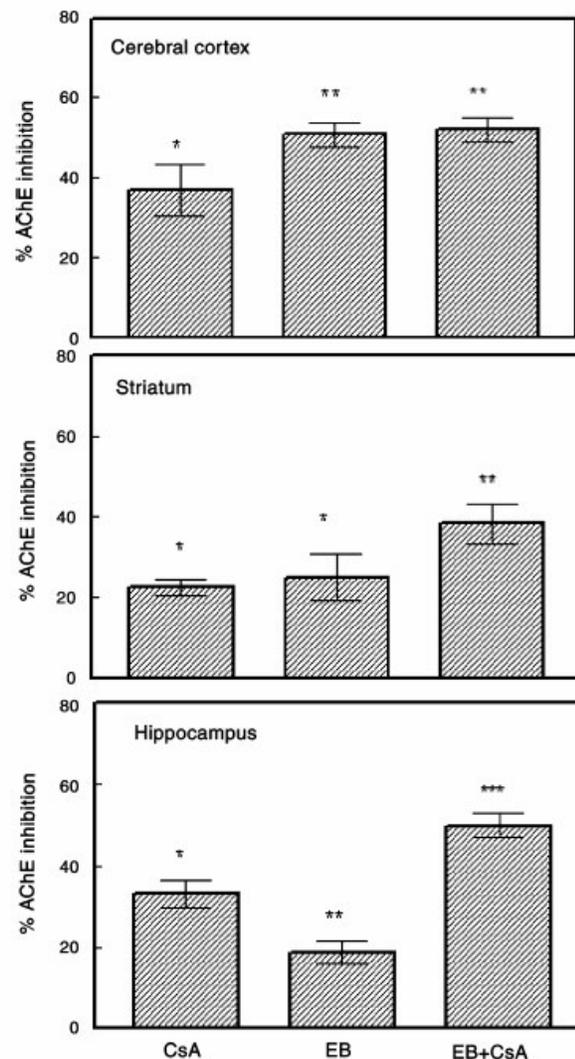


Fig. 2. Inhibition percentage of AChE in vivo in the cerebral cortex (CC), striatum (ST) and hippocampus (HP) in relation of the control group. Group CsA (treated with cyclosporine A); group EB (submitted to demyelination by ethidium bromide) and group EB + CsA (demyelinated by ethidium bromide and treated with cyclosporine A); $n = 7$ (* and ** $p < 0.05$; *** $p < 0.001$).

2000; Guazzo, 2005; Mazzanti et al., 2006a). The advantage of this method lies in the good reproducibility of demyelination and the predefined area where demyelination occurs (Stangel and Hartung, 2002). Concerning the histological changes induced by EB, this work demonstrated a lesion of the demyelization characterized by the spongiosus status of the tissue. In addition, the presence of new blood vessels and more cellular activity within the demyelination lesion after 21 days is an indication of remyelination of the lost myelin sheaths, indicating that this model can be used to study demyelination followed by the remyelination events.

In this study, an inhibition of AChE activity was observed in rats demyelinated by EB in all brain structures analyzed (Figs. 2 and 3). These results are in accordance with a previous study

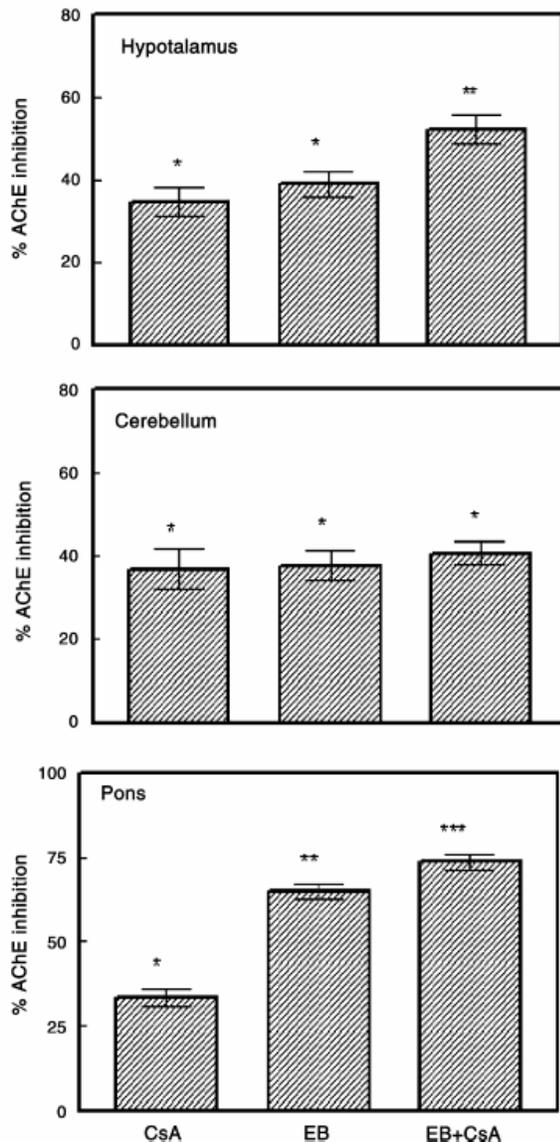


Fig. 3. Inhibition percentage of AChE in vivo in hypothalamus (HY), cerebellum (CB) and pons (PN) in relation to the control group. Group CsA (treated with cyclosporine A); group EB (submitted to demyelination by ethidium bromide) and group EB + CsA (demyelinated by ethidium bromide and treated with cyclosporine A); $n = 7$ (* and ** $p < 0.05$; *** $p < 0.001$).

from our laboratory demonstrating in vitro the inhibition of AChE by EB in the ST HP, CC and CB at various concentrations tested (Mazzanti et al., 2006b). We also carried out studies in vivo using EB as the demyelinating agent demonstrating that the AChE activity was inhibited on 7, 15 and 30 days after EB injection in the same brain structures as used in this study (Mazzanti et al., 2006a). Although MS is not known to be associated with a selective reduction in cholinergic neurons, it is likely that the demyelination and axonal damage that characterize this pathology can disrupt neuronal signaling by causing a conduction block and impairing axonal transport

(Christodoulou et al., 2006). In this context, demyelinating events could cause a presynaptic cholinergic deficit, which is responsive to treatment with cholinesterase inhibitors (Gustavson and Cummings, 2003). This would make AChE an important therapeutic target for the treatment of neurodegenerative diseases (Greene et al., 2000; Parry et al., 2003; Porcel and Montalban, 2006).

One important data observed in this study is that CsA per se inhibited the AChE activity in the CC, HY, ST and HP, regions where the cholinergic inputs are extremely important. Corroborating with these findings, it was demonstrated by Herink et al. (2002, 2003) that CsA inhibited AChE activity in the frontal cortex, hippocampus, medial septum and basal ganglia of rats. On the other hand, it was reported by Borlongan et al. (2000) that CsA exerted neuroprotective and neurotrophic effects on the cholinergic system by enhancing choline acetyltransferase (ChAT) immunoreactivity, which is for the synthesis of transmitter acetylcholine (ACh) (Prado et al., 2002). These observations help to confirm that there is an interaction between CsA and the cholinergic system, that may make this immunosuppressive drug a potent therapeutic and scientific tool in the brain disorders associated with cognitive impairment.

In this study when the demyelinating rats were treated with CsA there was an increase in the inhibitive effects on AChE activity in all structures evaluated. In addition, it was observed by our laboratory that interferon β (IFN- β), an immunomodulating drug also used in the treatment of MS patients, was capable of inhibiting AChE activity in the ST, HP, HY and CC in normal and demyelinated rats, demonstrating that this agent could modulate cholinergic neurotransmission (Mazzanti et al., 2006a). Taken together, these results help to confirm that drugs used in the treatment of MS patients such as CsA and IFN- β produce effects on the cholinergic system, highlighting the importance of investigating AChE activity, a key enzyme in the cholinergic synapses.

The treatment with of AChEI has shown a positive effect on the cognitive functions of patients with Alzheimer's disease (Potyk, 2005) and other conditions (Werber and Rabey, 2001), including MS (Greene et al., 2000; Freo et al., 2002; Parry et al., 2003; Porcel and Montalban, 2006; Amato et al., 2006; Christodoulou et al., 2006). A decrease in the activity of AChE indicates an increase of ACh levels in the synaptic cleft (Appleyard, 1992; Grisaru et al., 1999; Töugu and Kesvatera, 1996; Das et al., 2002) enabling an improvement in cognitive functions, such as learning and memory (Mesulam et al., 2002; Blum et al., 2002).

Although the results of this study have indicated that CsA inhibits AChE activity at a dose of 10 mg/kg, its clinical application is limited by relatively serious side effects, such as nephrotoxicity, hepatotoxicity and neurotoxicity (Rosecrantz et al., 2001; Rosendal et al., 2005). According to Durak et al. (2004), a depletion of hepatic glutathione and an accumulation of malondialdehyde, the final product of lipid peroxidation, have been shown to be some of main causative mechanisms of CsA-associated hepatotoxicity. In this study, an increase of lipid peroxidation was observed by using TBARS content determi-

nation in the serum, liver and kidney (data not show) of the rats demyelinated and treated with CsA, highlighting the involvement of this drug in reactive oxygen radical formation. In this context, we can suggest that CsA at the dose tested can cause collateral effects in other tissues.

Although the model of demyelination used in this study does not account for the inflammatory reaction in a disease like MS, it is well suited for the study of the principal mechanisms of demyelination and remyelination, as it mimics the initial relapsing-remitting (RR-MS) phase of the MS. Thus, we can suggest that future studies be carried out in order to determine an ideal dose and exposition time for the use of CsA, as its inhibitory effect on AChE activity could constitute a therapeutic window for cognitive impairment in MS.

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Capítulo 4 - Manuscrito

Effect of previous treatment with ebselen and vitamin E on acetylcholinesterase activity in rats exposed in an experimental model of toxic demyelination by ethidium bromide

Cinthia M. Mazzanti, Roselia Spanevello, Luciane B. Pereira, Jamile F. Gonçalves, Maisa Corrêa, Mushtaq Ahmed, Roberta Schmatz, Naiara Stefanello, Alexandre Mazzanti, Adriano T. Ramos, Tessie B. Martins, Dominguita L. Graça, Vera M. Morsch, Maria Rosa C. Schetinger

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ACETYLCHOLINESTERASE ACTIVITY IN RATS EXPOSED TO AN
EXPERIMENTAL MODEL OF TOXIC DEMYELINATION BY ETHIDIUM
BROMIDE**

**Cinthia M. Mazzanti¹, Roselia Spanevello¹, Luciane B. Pereira², Jamile F.
Gonçalves², Maisa Corrêa², Roberta Schamtz², Naiara Stefanello², Musthaq
Ahmed², Alexandre Mazzanti³, Adriano T. Ramos⁴, Tessie B. Martins⁴ Dominguita
L. Graça⁴, Vera M. Morsch², Maria Rosa C. Schetinger^{2*}**

¹Departamento de Bioquímica, Instituto de Ciências Básicas da Saúde, Universidade Federal do Rio Grande do Sul, Rua Ramiro Barcellos, 2600-Anexo, 90035-003. Porto Alegre, RS, Brazil.

²Departamento de Química, Centro de Ciências Naturais e Exatas, Universidade Federal de Santa Maria, Campus Universitário, Camobi, 97105-900 Santa Maria, RS, Brazil

³Laboratório de Cirurgia Experimental, Centro de Ciências Rurais, Universidade Federal de Santa Maria, Campus Universitário, Camobi, 97105-900 Santa Maria, RS, Brazil

⁴Departamento de Patologia, Centro de Ciências da Saúde, Universidade Federal de Santa Maria, Campus Universitário, Camobi, 97105-900 Santa Maria, RS, Brazil.

* Corresponding author: Fax: + 55-55-3220-8978

E-mail address: mariaschetinger@gmail.com or mariarosa@smail.ufsm.br (M. R. C. Schetinger)

ABSTRACT

An ethidium bromide (EB) demyelinating model was associated with vitamin E (Vit. E) and ebselen (Ebs) treatment to evaluate brain acetylcholinesterase (AChE) activity in the striatum (ST), hippocampus (HP) cerebral cortex (CC) and erythrocytes. Rats were divided into seven groups: I - Control (saline), II- (saline + canola); III- (saline + Ebs), IV - (saline + Vit E); V- (EB); VI - (EB + Ebs) and VII-(EB + Vit. E). After 3 days, AChE activity in the CC and HC was significantly reduced in groups III, IV, V, VI and VII ($p < 0.05$) and in the ST it was reduced in groups III and V ($p < 0.05$) when compared to the control group. After 21 days, AChE activity in the CC was significantly reduced in groups III, IV and V, while in groups VI and VII a significant increase was observed when compared to the control group. In the HC and ST, AChE activity was significantly reduced in groups V, VI and VII when compared to the control group ($p < 0.05$). In the erythrocytes, after 3 days AChE activity was significantly reduced in groups III, IV, V, VI and VII and at 21 days there was a significant reduction only in groups VI and VII ($p < 0.05$) when compared to the control group. In conclusion, this study demonstrated that Ebs and Vit E interfere with the cholinergic neurotransmission by altering the AChE activity in the different brain regions and in the erythrocytes.

Key Words: ethidium bromide, AChE, rat, demyelination, vitamin E, ebselen

1. INTRODUCTION

Acetylcholinesterase (AChE; EC 3.1.1.7) is one of the most efficient biological catalysts known and plays a key role in cholinergic neurotransmission by hydrolyzing the transmitter acetylcholine (ACh), thus terminating its action (Soreq and Seidman, 2001). However, the biological role of AChE is not limited to cholinergic transmission. AChE has been implicated in several non-cholinergic actions including cell proliferation (Appleyard, 1994), neurite outgrowth (Chacón et al., 2003) and other responses to various insults including stress and amyloid formation (Grisaru et al., 1999) making this enzyme an important therapeutic target. The first evidence of additional functions of the AChE protein was based on the high levels of this enzyme observed in non-neuronal tissues such as blood cells, notably erythrocytes and lymphocytes (Szelényi et al., 1982; Kawashima and Fujii, 2000), meninges and glia (Grisaru et al., 1999).

Glial cells also help in CNS signaling (Fellin and Carmignoto, 2004) by the release of modulator substances (Newman, 2003), energetic substrates (Deitmer, 2001) and neurotransmitters, such as glutamate, ACh and others that are essential for synaptic transmission (Nägler et al., 2001; Deng and Poretz, 2003; Mitterauer, 2004). A glia-derived acetylcholine-binding protein (AChBP) has been identified, a discovery that underlines glial participation in cholinergic neurotransmission (Smit et al., 2001). The presynaptic release of ACh induces the release of AChBP, which in turn reduces ACh viability within the synaptic cleft, reinforcing glial participation in cholinergic synapse (Sixma and Smit, 2003).

AChE inhibitors (AChEIs) were initially developed to treat cognitive dysfunction in Alzheimer's disease due to the loss of cholinergic neurons, which is a mark of this pathology (Weinstock, 1995; Das et al., 2002; Rees et al., 2003; Kasa et al., 2004), but also have been used in the treatment of various neurological disorders (Werber and Rabey, 2001, Masanic et al., 2001) including multiple sclerosis (MS) (Greene et al., 2000; Parry et al., 2003; Christodoulou et al., 2006; Porcel and Montalban, 2006).

MS is an inflammatory and demyelinating disease of the CNS that affects young adults (Bjartmar et al., 2003). The etiology and pathogenesis are still unclear, although there is evidence that MS is an autoimmune disease, in which CNS myelin is attacked by auto-aggressive T-cells (Matute and Pérez-Cerdá, 2005). Although MS is not known to be associated with a selective reduction in cholinergic neurons, it probably results from the disruption of cholinergic pathways occasioned by demyelination and axonal transection (Gustavson and Cummings et al., 2003; Porcel and Montalban, 2006). In fact, approximately 40 - 60% of people diagnosed with MS display cognitive impairment, most commonly in the area of new learning and memory, indicating the involvement of the cholinergic system in this pathology (Parry et al., 2003; Christodoulou et al., 2006).

AChEIs may therefore represent a potential therapeutic option for impaired cognitive status in MS (Porcel and Montalban, 2006). Moreover, it has recently been reported that AChEIs demonstrated anti-inflammatory properties by reducing lymphocyte proliferation and the secretion of pro-inflammatory cytokines (Nizri et al., 2006) and improving myelin integrity by cholinergic stimulation from oligodendrocytes (Bartzokis, 2006). Taken together, AChEI

may be a promising and important target of research for this demyelinating pathology that involves inflammatory events and cognitive dysfunction.

Toxic demyelination by ethidium bromide (EB) is one of the most commonly used models for exploring the reparative capacity of the CNS (Stangel and Hartung, 2002; Guazzo 2005). EB selectively destroys glial cells (oligodendrocytes and astrocytes), which control the processes of demyelination and remyelination. In fact, this model may be a useful tool to study experimental demyelinating mechanisms and, perhaps in the future, to help in understanding MS (Levine and Reynolds 1999; Smith and Franklin 2001; Ohler et al., 2004).

Vitamin E (Vit E) is an essential nutrient in humans and its antioxidant and anti-inflammatory role (Johnson, 2000; Rimbach et al., 2002; El-Demerdash, 2004) (Grammas et al., 2004; Reiter et al., 2007) is well established in the literature. Moreover, Vit E can partially restore the hypofunction of the cholinergic system in aging (Maneesub et al., 1993) and also has an effect on memory retention through the activation of this system (Eidi et al. 2006). In addition, ebselen (2-phenyl-1,2-benzisoselenazol-3-(2H)-one) is a nontoxic seleno-organic compound with anti-inflammatory and antioxidant properties (Schewe, 1995). Moreover, it has been demonstrated in experimental models that this compound exerts a neuroprotective role against brain ischemia (Imai et al., 2001) and against glutamate excitotoxicity (Porciúncula et al., 2001). However the effects of this drug on the cholinergic system have not been reported in the literature.

Therefore, considering the importance of AChE the aim of this study is to investigate the activity of this enzyme in a model of toxically-induced CNS

demyelination in normal and ebselen/vitamin E treated rats, in order to verify the participation of these compounds in the modulation of cholinergic neurotransmission. In addition, we intend to the participation of the astrocyte immunoreactivity to glial fibrillary acidic protein (GFAP) and vimentin (VIM) after EB injection.

2. Materials and Methods

2.1. Chemicals

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. Ebselen (2-phenyl-1,2-benzoisoselenazol-3-(2H)-one) was synthesized based on Engman and Hallberg (1989) and analysis of the 1H NMR and ^{13}C NMR spectra showed that this compound presents analytical and spectroscopic data in full agreement with its assigned structure (data not shown). Vitamin E (95% purity) was obtained from the Distribuidora Galena (São Paulo, Brazil). All other chemicals used in this experiment were of the highest purity.

2.2. Animals

Adult male Wistar rats (70-90 days; 220–300 g) were used. The animals were maintained at a constant temperature ($23\pm 1^\circ C$) on a 12h light/dark cycle with free access to food and water. The study was performed in accordance with the University Ethics Committee Guidelines for experiments with animals.

Treatment with Ebselen (Ebs) and Vitamin E (Vit .E)

The animals were randomly divided into seven groups: I - control (injected with saline; n=15), II- (injected with saline and treated with canola oil, n=15); III- (injected with saline and treated with Ebs, n=15), IV - (injected with saline and treated with Vit E, n=15); V- (injected with 0.1% EB; n=15); VI - (injected with 0.1% EB and treated with Ebs; n=15) and VII-(injected with 0.1% EB and treated with Vit E n=15). During four weeks before the demyelination procedure, the animals from groups III, IV, VI and VII were injected intraperitoneally (i.p.) for five consecutive days followed by two days of no treatment each week, completing a total of twenty applications of Ebs and Vit E diluted in canola oil (1 mL/Kg), using a dose of 2 mg/Kg.

2.3. Experimental demyelination with EB

For the surgical procedure, the animals from both treatments were anesthetized with ketamine chloridrate and xylazine (5:1; 0.1 ml/100g) and after shaving the fronto-parietal-occipital area, antiseptis 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 from groups III and IV, and the same volume of 0.9% saline solution were injected in the animals from groups I and II. The duramater was left open and the skin, together with the remainder of the subcutaneous tissue, was sutured with a nylon thread 4.0. In both

treatments studied, six animals from each group were sacrificed three and twenty one days after the surgical procedure being previously anesthetized with ethyl ether and the brain structures and blood were collected for the subsequent enzymatic assay.

2.4. Immunohistochemistry analysis

Three rats from every group were killed by perfusion with 0.09% saline with 0.01M EDTA under deep anesthesia at 3 and 21 days. Brain stem sections were collected, fixed in metacarn (60% methanol, 30% acetic acid, 10% chloroform), dehydrated and embedded in paraffin wax by standard methods. Sections (5 µm) were cut and stained with hematoxylin and eosin. Selected sections of these tissues were collected on glass slides pretreated with 4% sylvane, dewaxed and rehydrated in graded ethanol baths. The method used for the immunolabelling was the avidin-biotin complex. The sections were incubated during 2 hours at room temperature with the polyclonal primary antibody anti-GFAP (Rabbit anti-cow GFAP, code number Z0334, Dako) and with monoclonal antibody anti-VIM (Clone V9 VIM, code number MO725, Dako), standardized respectively in the dilutions 1:10000 and 1:100. The sections were incubated for 30 minutes with a biotinylated secondary antibody (anti-goat anti-mouse anti-rabbit/ Ig: DAKO LSAB+ Kit, peroxidase LV code number K0690) and for 30 minutes with the complex streptavidin-peroxidase (DAKO LSAB+ Kit, peroxidase LV code number K0690). The immunoreactivity was observed using diaminobenzidine (DAB-Sigma Biosciences, St Louis, USA: 0.072 g in 120 mL PBS + 1.2 mL H₂O₂ 10 vol) as the chromogen. The sections were counterstained with Harris

hematoxylin, dehydrated in graded ethanol baths, mounted under cover slips and observed by light microscopy.

2.5. Brain Tissue Preparation

Brain structures were immediately removed and separated into striatum (ST), hippocampus (HP) and cerebral cortex (CC) and placed in a solution of 10 mM Tris-HCl, pH 7.4, on ice. The brain structures were homogenized in a glass potter in Tris-HCl solution. Aliquots of resulting brain structure homogenates were stored at -8°C until utilization. Protein was determined previously in a strip that varied for each structure: ST (0.4 mg/ml), HP (0.8 mg/ml) and CC (0.7mg/ml) as determined by the Coomassie blue method according to Bradford (1976) using bovine serum albumin as standard solution.

2.6. Cerebral AChE Enzymatic Assay

The AChE enzymatic assay was determined by a modification of the spectrophotometric method of Ellman et al. (1961) as previously described (Rocha et al. 1993). The reaction mixture (2 ml final volume) was composed of 100 mM K⁺-phosphate buffer, pH 7.5 and 1 mM 5,5'-dithiobisnitrobenzoic 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 (40-50 µg of protein) was pre-incubated for 2 min. The reaction was initiated by adding 0.8 mM acetylthiocholine iodide. All samples were run in duplicate or triplicate and the enzyme activity was expressed in µmoles AcSCh/h/mg of protein.

2.7. Blood sample collection

The blood was collected in vacutainer tubes using EDTA as anticoagulant. The samples were hemolysed with phosphate buffer, pH 7.4 containing Triton X – 100 (0.03%) and stored at -20⁰C for one week.

2.8. Determination of AChE activity in erythrocytes

The AChE enzymatic assay was determined by the method of Ellman et al. (1961) modified by Worek et al. (1999). The specific activity of AChE in the erythrocytes was calculated from the quotient between AChE activity and hemoglobin content and the results are expressed as mU/ μ mol Hb.

2.9. Statistical Analysis

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

3. Results

3.1. Histological analysis of the lesion

At three days after the EB injection, a large lesion with a status of spongiosis was observed across the pons and many macrophages marked with vimentin (VIM) were observed in the lesion area when compared to the control group (Figure 1 A, B and C). The disappearance of astrocytes (GFAP + VIM) from the lesion centre was also observed (Figure 1 D, E and F). Regarding the treatment with vitamin E, a smaller demyelinated area was detected without

astrocytes at the center of the lesion whereas GFAP immunolabelled astrocytes were observed at the periphery of the lesion (Figure 1 G, H and I). The ebselen treated animals showed a smaller demyelinated lesion with vimentin and GFAP marked astrocytic processes at the periphery of the lesion (Figure 1 J, K and L).

At twenty-one days after the EB injection, a reduced area of demyelination with many neuroglial cell processes was detected in the area where the spongiosus status lesion was formerly seen, an indication of remyelination of the lost myelin sheaths. Vimentin marked macrophages and GFAP marked reactive astrocytes were observed mainly at the periphery of the lesions (Figure 2 A, B and C). In the rats treated with EB and Vitamin E, narrow lesions with cystic areas along the ventral aspects of the central pons were induced. VIM marked astrocytic processes within and around the periphery of the lesion and GFAP marked reactive astrocytic processes were also observed (Figure 2 D, E and F). In rats treated with ebselen, the cystic lesion was reduced and GFAP marked astrocytes were observed around the periphery of the lesion (Figure 2 G, H and I).

3.2. Activity of AChE in brain

AChE activity in brain regions was altered depending on the length of time from the demyelination by EB and treatment with ebselen or vitamin E. At three days, the results showed a significant alteration in AChE activity in all brain regions studied ($p < 0.05$) (Figure 3). In the cerebral cortex, AChE activity was significantly reduced in groups III, IV, V, VI and VII when compared to the control group (Figure 3A). Similarly, in the hippocampus an inhibition of AChE

also occurred in groups III, IV, V, VI and VII when compared to the control group ($p < 0.05$) (Figure 3B). However, in the striatum at three days AChE activity was significantly reduced only in group III and V ($p < 0.05$), while in group VI and VII the activity of this enzyme was increased when compared to the control group ($p < 0.05$) (Figure 3C).

At twenty one days, significant alterations in AChE activity also occurred ($p < 0.05$) (Figure 4). In the cerebral cortex, AChE activity was significantly reduced in groups III, IV and V while in the groups VI and VII a significant increase in AChE activity was observed when compared to the control group (Figure 4A). In the hippocampus and striatum the results were similar, the AChE activity in these two cerebral regions was significantly reduced in groups V, VI and VII when compared to the control group (Figure 4B and 4C).

It is important to note that controls were performed to correct for vehicle (canola) interference, and no differences between vehicle and control enzyme activity (saline) were observed (Figures 3, 4 A, B,C).

3.3. Activity of AChE in erythrocytes

In the erythrocytes, AChE activity was also altered depending on the length of time from the demyelination procedure. The results showed that at three days AChE activity was significantly reduced in groups III, IV, V, VI and VII when compared to the control group ($p < 0.05$) (Figure 5A). At twenty one days, an alteration in enzyme activity was also observed, and the results showed that AChE activity was significantly reduced only in groups VI and VII ($p < 0.05$) (Figure 5B).

In the erythrocytes there was also no difference between vehicle (canola) and control enzyme activity (saline) observed (Figure 5 A and B).

4. Discussion

Experimentally induced demyelination using EB provides a model for analyzing the cellular and enzymatic changes that occur during demyelinating pathologies, such as multiple sclerosis (MS) (Levine and Reynolds, 1999; Stangel and Hartung, 2002). This work was carried out in order to investigate possible changes in AChE activity in the rat brainstem at different lengths of time after the injection of EB associated with ebselen and vitamin E treatments.

Three days after the EB injection, a demyelinated lesion, which was characterized by macrophage infiltration and the absence of GFAP positive cells at the lesion center, was observed in the pons (Figure 1 D, E and F). On the other hand, 21 days after the injection, the lesion showed some cystic areas and reactive astrocytic processes (Figure 2 A, B, C). These results are in agreement with previous works regarding the EB gliotoxic model (Bondan et al., 2000; Bondan et al.; 2003; Graça et al., 2001; Sallis et al., 2006; Mazzanti, 2006ab, Spanevello et al. 2006, Mazzanti et al., 2007). In Vit E and ebselen treated rats, lesions induced by EB were smaller suggesting that these compounds somehow interfered in the development of the lesions. VIM+ macrophages and GFAP+ astrocytes had a similar distribution within the lesions after all treatments: the macrophage phagocytosed dead cells and decaying myelin sheaths; astrocytes absent from the center of the lesions; and recovery at 21 days where GFAP and VIM marked the reactive

processes. This cellular behavior was similar to that observed in the experiments cited above. Regardless of the cause of the aggression to the CNS, the repair of the tissue is performed with astrocyte participation. The astrocytic reaction entailed an increase in the number and dimension of the GFAP/VIM-rich processes immunolabelled as observed in the pictures.

In this study, an inhibition of AChE activity was observed in rats demyelinated by EB in all brain structures analyzed (Figure 3 and 4) at the periods of the 3 and 21 days. These results are in accordance with previous studies from our laboratory that demonstrated the *in vitro* inhibition of AChE activity by EB in the ST, HP, CC and CB at various concentrations tested (Mazzanti et al., 2006a). We also carried out studies *in vivo* using EB as the demyelinating agent demonstrating that AChE activity was also inhibited in the same brain structures used in this work (Mazzanti et al., 2006b, Mazzanti et al., 2007). Furthermore, other studies from our laboratory also showed that the demyelination caused by EB alters the activity of enzymes such as NTPDase and 5'-nucleotidase (Spanevello et al., 2006). Taken together, these findings indicate that demyelinating events interfere with the activity of enzymes that play crucial roles in the CNS.

Is important to emphasize that the demyelination and axonal damage that characterize MS can cause a neuronal conduction block and thus interrupt cholinergic neurotransmission (Christodoulou et al., 2006; Bartzokis, 2006). In this context, this presynaptic cholinergic deficit may be responsive to treatment with AChE inhibitors (Gustavson and Cumming, 2003), which makes this enzyme an important therapeutic target for the treatment of neurodegenerative diseases (Greene et al., 2000; Parry et al., 2003; Porcel and Montalban, 2006).

EB, beside inhibiting AChE activity, also interferes with the viability of glial cells, and, for this reason, it may damage the modulatory function of these cells in synaptic transmission, as for example in neurotransmitter release (Deitmer, 2001; Nagler et al., 2001; Araque et al., 2002; Deng and Poretz 2003; Newman, 2003; Fellin and Carmignoto, 2004; Mitterauer, 2004). Another perhaps even more interesting and speculative interpretation of these findings is that the cholinergic function may be altered in the synaptic cleft by the probable decrease of the basal level of AChBP (Smit et al., 2001; Sixma and Smit, 2003), due to the destruction of glial cells caused by EB as observed in this study. The inhibition of AChE observed in this study along with the hypothetical decrease of AChBP could bring about an increase in the concentration of the neurotransmitter ACh, causing an imbalance between glia and neurons, thus inducing cholinergic hyperactivity. In addition to a role in modulating synaptic ACh levels, AChE appears to possess non-cholinergic functions, some of which may involve cell proliferation and differentiation, responses to various insults including stress and amyloid formation, synaptogenesis and dendritic branching (Srivatisan and Peretz, 1997; Chacón et al., 2003). Therefore, an absence or inhibition of this enzyme may have devastating consequences on brain development (Mesulan et al. 2002).

In relation to the treatments used, it was observed that both ebselen and Vit E when associated with EB, were capable of increasing AChE activity in the ST (3 days), CC (21 days) and HP (21 days) (Figure 3C, 4A, 4B) suggesting that these compounds can prevent the inhibitor effects of EB in these brain regions. At 21 days, ebselen and vitamin E per se caused no alteration in AChE

activity in the HP and ST, probably, due to the fact that the blood level of these compounds may have been greatly reduced due to the length of time.

The antioxidant, anti-inflammatory and neuroprotective properties of ebselen are well established in the literature (Schewe, 1995; Imai et al., 2001; Porciúncula et al., 2001), but the interaction of this compound with cholinergic system has not been documented. One important data observed in this study is that ebselen *per se* was capable to significantly inhibit AChE activity in the ST, HP and CC, structures rich in cholinergic pathways, demonstrating its involvement with the cholinergic system. In addition has been reported by Nizri et al. (2005, 2006) that AChEIs possess anti-inflammatory properties promoting a cholinergic up-regulation by reducing lymphocyte proliferation and the secretion of pro-inflammatory cytokines. In this context, we can suggest that ebselen could be considered a bifunctional compound, because it presents not only anti-inflammatory properties, but also inhibits AChE activity as shown in our study, thus combining two different functions in one molecule.

In this study, Vit E *per se* also inhibited AChE activity in the CC and HP (Figure 3A and B). These cerebral regions receive cholinergic projections from the nucleus basalis of Meynert and play an important role in the learning and memory process. It has been demonstrated by Maneesub et al. (1993) that Vit E can partially restore the hypofunction of the cholinergic system in aging and also has an effect on memory retention through the activation of this system (Eidi et al., 2006). Although these studies have demonstrated that Vit E improves cognitive performance, little is known about its mechanism of action. Our results suggest that one of the mechanisms of action of Vit E is through the inhibition of AChE activity by its interaction with the cholinergic system.

The AChEIs have shown a positive effect on cognitive functions of patients both with Alzheimer's disease (Potyk, 2005) and other conditions (Werber and Rabey, 2001), including MS (Greene et al., 2000; Freo et al., 2002; Parry et al., 2003; Porcel and Montalban, 2006; Amato et al., 2006; Christodoulou et al., 2006). On the basis of these findings, we can suggest that Vit E is a promising candidate for the treatment of cognitive dysfunction in neurodegenerative diseases such as MS.

In relation to AChE activity in the erythrocytes, the results showed that at 3 days after the EB injection, an inhibition of this enzyme occurred in the groups treated (III, IV, VI, VII) and in the group demyelinated by EB (V) (Figure 5A). These results were similar with the results obtained in the cerebral regions. Corroborating with these findings, it was reported by Bernahardi et al. (2005) that the concentration of AChE in erythrocytes (RBC-AChE) is potentially a stable biomarker for the study of neurodegenerative disorders such as Alzheimer's disease. In addition, it was reported by Thiermann et al. (2005) that RBC-AChE could have similar functional properties as synaptic AChE and therefore may reflect the status at the synaptic site. In this context, we can infer that AChE activity in erythrocytes could be a good peripheral marker because it permits evaluation through the more accessible methods the action of this enzyme in CNS.

In conclusion, this is the first study to investigate the *in vivo* effect of ebselen and Vit E on AChE activity associated with a demyelinating model. Based on the results compiled, these drugs exert an effect on the cholinergic system by inhibiting AChE activity. Furthermore, treatment with Vit E and ebselen demonstrated a protective effect from the demyelination lesion caused

by EB. In this context, we can suggest that ebselen and Vit E should be considered potential therapeutics and scientific tools to be investigated in brain disorders associated with demyelinating events.

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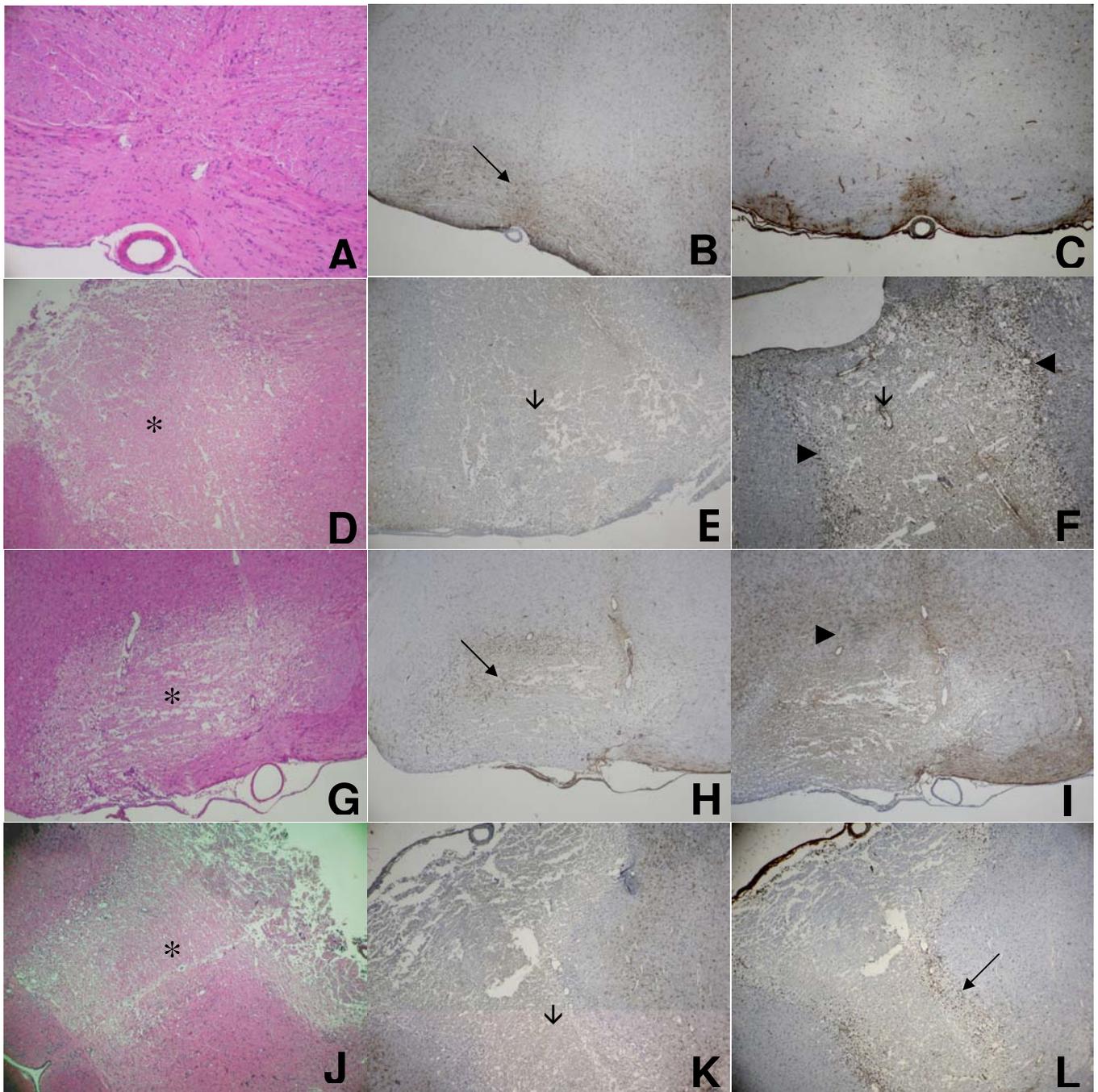


Figure 1

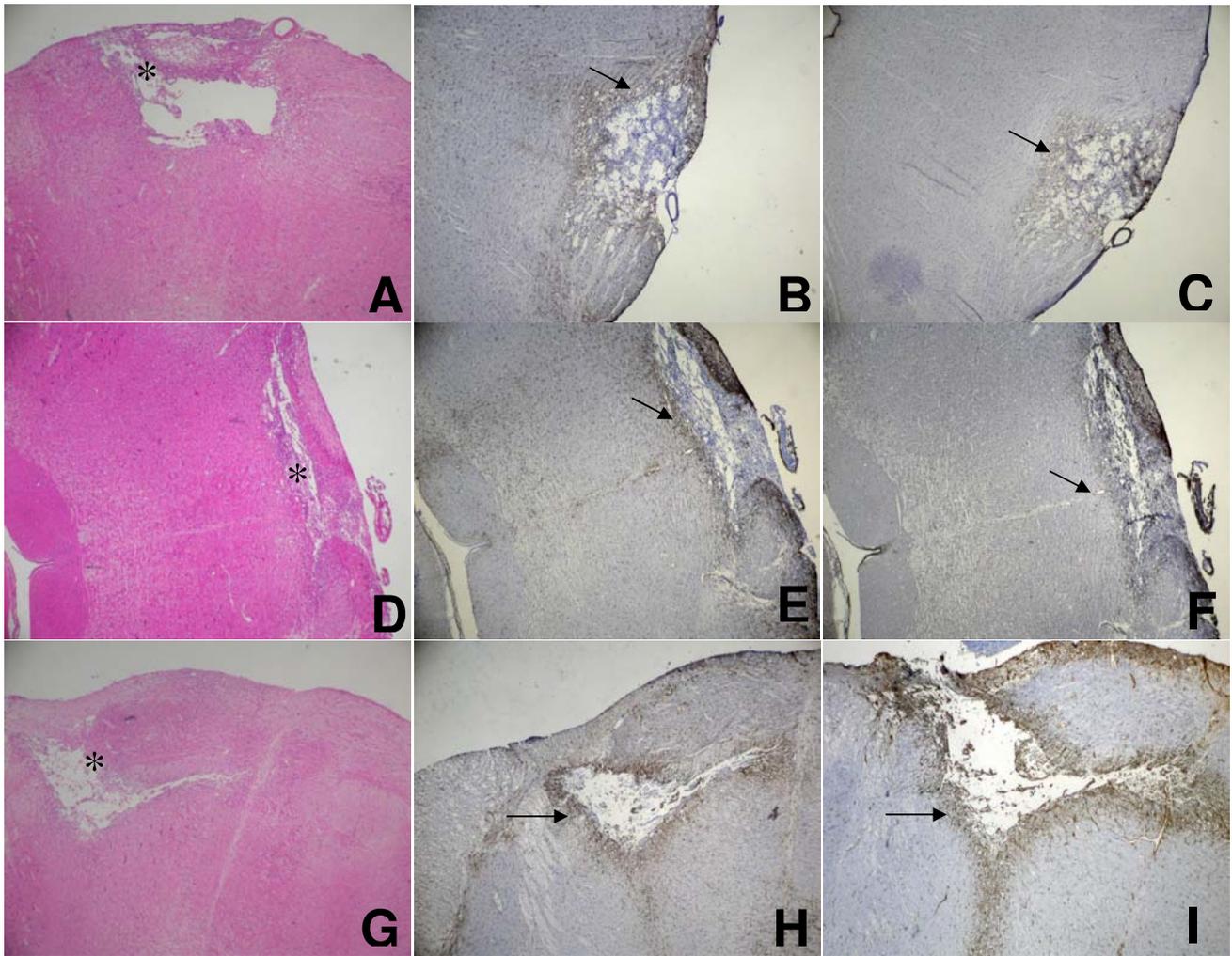


Figure 2

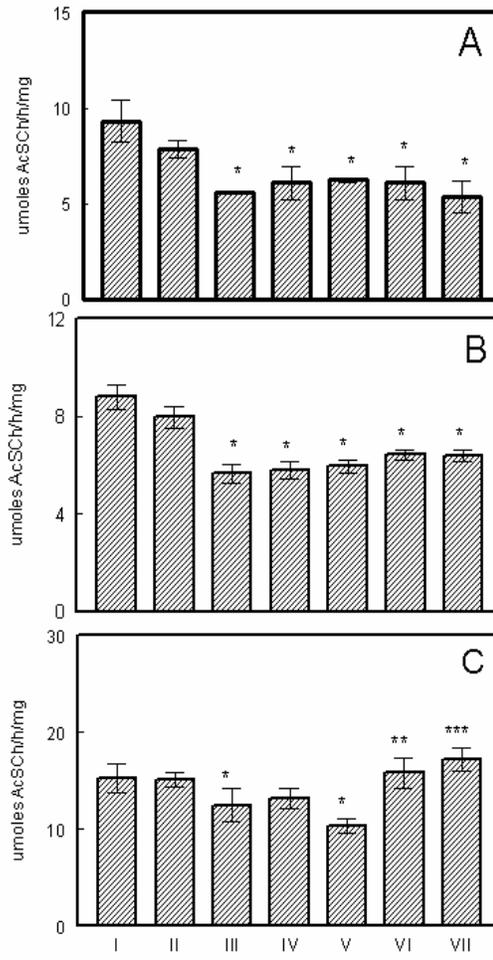


Figure 3

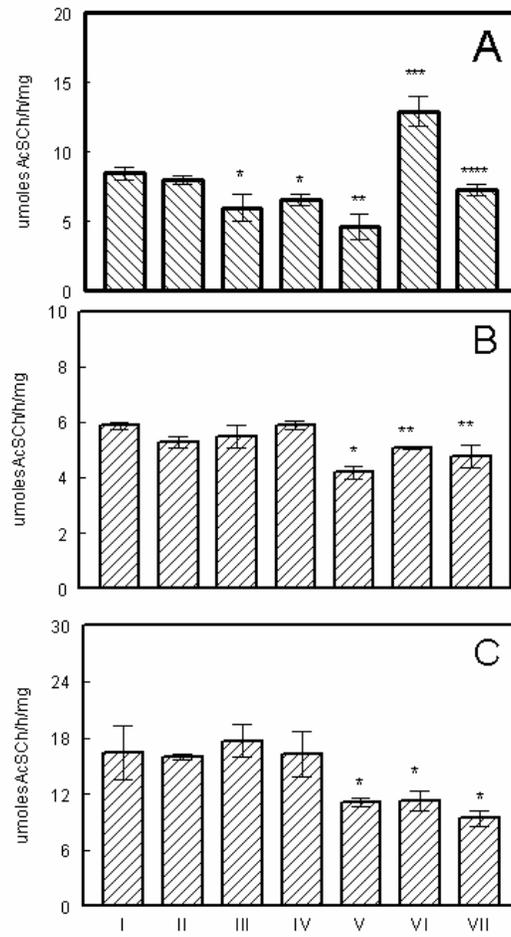


Figure 4

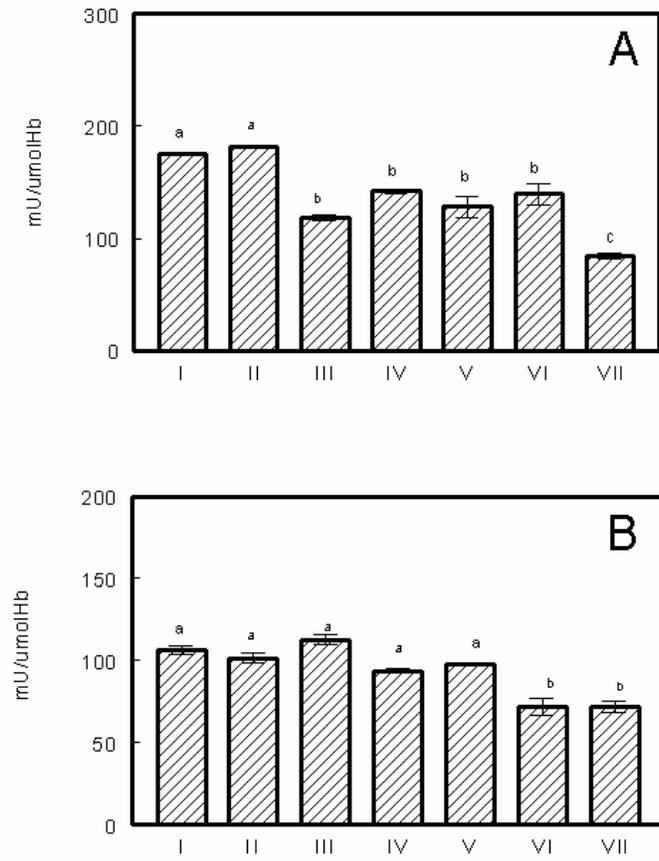


Figure 5

Legends

Figure. 1 Normal histological aspect of the pons H & E (A) and (B) astrocytic GFAP (↓) and (C) vimentin. Three days after EB injection there is a large demyelinating lesion across the pons (*) (D), absence of GFAP positive cells in the lesion center (↓) (E), blood vessels (↓) and macrophages (▶) are marked with vimentin (F). Ventral lateral aspect of the pons of the rats demyelinated and treated with Vit E (*) (G) H & E, in (H) Astrocytes are still absent from the center of the lesion although GFAP marked astrocytes are observed at the periphery of the lesion (▶) In (I) macrophage infiltration is marked with vimentin (↓). In (J) a long narrow lesion was induced along the pons following the injection of EB in ebselen treated rats (*) (H&E). In (K) absence of GFAP positive cells within the lesion and vimentin marked astrocytes are seen in long strips along the periphery of the lesion (↓) (L). Obj 10X.

Figure. 2 Twenty one days after EB injection there is a lesion in the ventral aspect of the pons with some cystic areas (*) (A). H&E. In (B) GFAP marks reactive astrocytes mostly at the periphery of the lesions and in (↓) (C) vimentin marked macrophages and reactive astrocytic processes (↓). Ventral aspects of the pons of the rats demyelinated and treated with Vit E (*) (D); GFAP marks reactive astrocytic processes (E) and vimentin marked astrocytic processes within and around the periphery of the lesion (↓) (F). Repair of cystic lesion induced by EB in ebselen treated rats (*) (G) H&E; GFAP

marked astrocytes around the periphery of the lesion (H) and (I) vimentin marked astrocytes along the periphery of the lesion (↓) Obj. 10 X.

Figure. 3 AChE activity in the CC (A), HP (B) and ST (C) of rats at three days after the injection of EB in the brainstem. Groups I (control), II (salina +canola); III (salina + Ebs), IV (saline + Vit E); V (EB); VI (EB + Ebs) and VII (EB + Vit. E). ($p^* < 0.05$; $p^{**} < 0.01$; $n=6$)

Figure. 4 AChE activity in the CC (A), HP (B) and ST (C) of rats at twenty one days after the injection of EB in the brainstem. Groups I (control), II (salina +canola); III (salina + Ebs), IV (saline + Vit E); V (EB); VI (EB + Ebs) and VII (EB + Vit. E). ($p^* < 0.05$; $p^{**} < 0.01$; $n=6$)

Figure. 5 AChE activity in erythrocytes of rats at three (A) and twenty one (B) days after the injection of EB in the brainstem. Groups I (control), II (salina +canola); III (salina + Ebs), IV (saline + Vit E); V (EB); VI (EB + Ebs) and VII (EB + Vit. E). ($p^a < 0.05$; $p^b < 0.01$; $n=6$)

Capítulo 5 - Artigo publicado

Previous treatment with ebselen and vitamin E alters adenine nucleotide hydrolysis in platelets from adult rats experimentally demyelinated with ethidium bromide

Cinthia M. Mazzanti¹, Roselia M. Spanevello¹, André Morsch², Rafael Zanin², Vanessa Battisti², Mushtaq Ahmed², Jamile F. Gonçalves²; Alexandre Mazzanti³, Dominguita L. Graça⁴, Vera M. Morsch² and Maria Rosa C. Schetinger^{2*}

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^a Departamento de Bioquímica, Instituto de Ciências Básicas da Saúde, Universidade Federal do Rio Grande do Sul, Rua Ramiro Barcellos, 2600-Anexo, 90035-003, Porto Alegre, RS, Brazil

^b Departamento de Química, Centro de Ciências Naturais e Exatas, Universidade Federal de Santa Maria, Campus Universitário, Camobi, 97105-900 Santa Maria, RS, Brazil

^c Laboratório de Cirurgia Experimental — DCPA, Centro de Ciências Rurais; Universidade Federal de Santa Maria, Campus Universitário, Camobi, 97105-900 Santa Maria, RS, Brazil

^d Departamento de Patologia, Centro de Ciências da Saúde, Universidade Federal de Santa Maria, Campus Universitário, Camobi, 97105-900 Santa Maria, RS, Brazil

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Abstract

Many aspects of the relationship between the demyelinating pathology and platelet function need to be elucidated. Thus, the activity of NTPDase and 5'-nucleotidase enzymes was analyzed in platelets from rats demyelinated with ethidium bromide (EB) and previously treated with ebselen (Ebs) and vitamin E (Vit. E). The animals were divided into four groups: for ebselen, the groups were: I — control (saline), II — (saline and Ebs), III — (EB) and IV — (EB and Ebs); and for vitamin E, the groups were: I — control (saline), II — (saline and Vit. E), III — (EB) and IV — (EB and Vit. E). After 3 and 21 days, the blood was collected and the platelets were separated for enzymatic assays. For the treatment with Ebs, the NTPDase activity for ATP substrate was significantly lower in groups II, III and IV ($p < 0.05$) after 3 days, while after 21 days, a reduction was observed in group III ($p < 0.05$). ADP hydrolysis was reduced in group II ($p < 0.05$) and increased in group IV ($p < 0.05$) after 3 days, while after 21 days there was an increase in group IV ($p < 0.05$). In the treatment with Vit. E, ATP hydrolysis was lower in groups II, III and IV ($p < 0.05$) after 3 and 21 days. ADP hydrolysis was increased in group II ($p < 0.05$) after 3 days, and in group IV ($p < 0.05$) after 21 days. However, 5'-nucleotidase activity was not altered by the treatments. These findings demonstrate that NTPDase activity in platelets is diminished in demyelinating events and the treatments with Ebs and Vit. E modulated adenine nucleotide hydrolysis.

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Keywords: Demyelination; Ebselen; NTPDase; 5'-nucleotidase; Platelets; Vitamin E

Introduction

Multiple sclerosis (MS) is a major demyelinating disease with an unknown etiology affecting the white matter of the central nervous system (CNS) (Smith and Franklin, 2001; Stangel and Hartung, 2002). Although the pathological

hallmark of this disease is primarily demyelination, other pathological events have been noted, such as changes in vascular permeability, which are considered crucial since they precede the development of MS lesions (Su et al., 2006).

Platelets have been recognized for decades as key pathological components of the processes associated with vascular inflammation and thrombosis (Atkinson et al., 2006). On this note, different authors have reported abnormalities in the platelet physiology and function in MS disease. For example, Neu et al. (1982) reported that platelets of MS patients exhibit a

* Corresponding author. Fax: +55 55 3220 8978.

E-mail addresses: mariaschetinger@gmail.com, mariorosa@smail.ufsm.br (M.R.C. Schetinger).

greater tendency to spontaneous aggregation and higher sensitivity to ADP-induced aggregation. Other studies have reported that platelets are involved in thrombi development in the demyelinating plaques, suggesting that these cells may play a role in the demyelination of white matter (Cananzi et al., 1987). In this context, a new methodology using biomarkers has been tested utilizing more accessible tissues such as platelets to determine peripheral indicators of the alteration localized in the central nervous tissue (Cattabeni et al., 2004).

Under such circumstance, the platelet model has been used in the investigation of a number of neurodegenerative disorders, such as Alzheimer's disease (Ferrarese et al., 2000). The similarities between platelet and neuron may even be clinically important, since a number of biochemical markers show parallel changes in CNS and platelets during the disease states (Odagaki and Koyama, 2002; Rainesalo et al., 2003). Thus, it is applicable to evaluate the effects of compounds using platelets as clinical markers for neurological disorders.

In more recent years, extracellular nucleotides such as ATP, ADP and adenosine have become clearly recognized for the important role that they play in modulating a variety of processes linked to vascular inflammation and thrombosis (Bohmer et al., 2003). ADP is the main promoter of platelet aggregation, whereas adenosine is one of its most potent inhibitors (Birk et al., 2002a). Moreover, studies have demonstrated that ATP has a complex role in the regulation of platelet aggregation (Soslau and Youngprapakorn, 1997; Birk et al., 2002b).

The most relevant ecto-enzymes involved in adenine nucleotide extracellular hydrolysis are NTPDase (E.C. 3.6.1.5, ectonucleoside triphosphate phosphohydrolase, E-NTPDase/CD39) and ecto-5'-nucleotidase (E.C. 3.1.3.5, CD73) (Schetinger et al., 1998; Lunkes et al., 2003; Araújo et al., 2005). NTPDase is a membrane-bound enzyme that hydrolyzes ATP and ADP to AMP, which is subsequently converted to adenosine by 5'-nucleotidase (Birk et al., 2002a). Both enzymes are present in the platelet membrane and play an important role in the control of adequate hemostasis and thrombogenesis by regulating ADP catabolism and adenosine production (Pilla et al., 1996; Buffon et al., 2004; Silva et al., 2005).

Ebselen (2-phenyl-1,2-benzisoxselenazol-3-(2H)-one) is a nontoxic seleno-organic compound with anti-inflammatory, anti-atherosclerotic and cytoprotective properties (Morin et al., 2003), which has also demonstrated antioxidant properties by mimicking glutathione peroxidase activity (Meotti et al., 2003; Nogueira et al., 2003; Borges et al., 2005). Of particular interest, it has been demonstrated that this drug has inhibited *in vitro* extracellular adenine nucleotide hydrolysis in platelets of rats indicating that this compound may have important effects on thromboregulation mechanisms (Furstenau et al., 2004). In addition, vitamin E (α -tocopherol) acts as an antioxidant (Steiner, 1993) and a stabilizer of membranes (Whitin et al., 1982; Quinn, 2004). The functions of vitamin E, however, are not restricted to membrane protection, but include a variety of other non-antioxidant functions, such as the inhibition of platelet aggregation *in vitro* (Quinn, 2004; Mabile et al., 1999; Freedman et al., 2000; Schoene, 2001; Kobzar et al., 2005), the inhibition of granular release reactions (Steiner and Anastasi, 1976) and the

inhibition of platelet adhesion (Szuwart et al., 2000). Thus, vitamin E may promote beneficial effects in many diseases, such as atherosclerosis, heart disease and also neurodegenerative diseases as MS (Johanson, 2000; Jialal et al., 2001).

Many aspects of the relation between the demyelinating pathology and the platelet function need to be elucidated since platelets are considered appropriate cells to study peripheral markers of neurodegenerative diseases. In order to address these questions, we have examined the potential role of the enzymes that participate in the hydrolysis of ATP, ADP and AMP in the platelets of rats experimentally demyelinated with ethidium bromide as well as the possible interference of treatments with ebselen and vitamin E in adenine nucleotide hydrolysis.

Materials and methods

Chemicals

Nucleotides, Trizma base, 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. Ebselen (2-phenyl-1,2-benzisoxselenazol-3-(2H)-one) was synthesized based on Engman and Hallberg (1989), and the analysis of the 1H NMR and ^{13}C NMR spectra showed that this compound presents analytical and spectroscopic data in full agreement with its assigned structure (data not shown). Vitamin E (95% purity) was obtained from the Distribuidora Galena (São Paulo, Brazil). All other chemicals used in this experiment were of the highest purity.

Animals

Adult male Wistar rats (70–90 days; 220–300 g) were used. The animals were maintained at a constant temperature ($23 \pm 1^\circ C$) on a 12 h light/dark cycle with free access to food and water. The study was performed in accordance with the University Ethics Committee Guidelines for experiments with animals.

Treatment with ebselen (Ebs)

The animals were randomly divided into four groups: I — control (injected with saline; $n=10$), II — (injected with saline and treated with Ebs; $n=10$), III — (injected with 0.1% EB; $n=10$) and IV — (injected with 0.1% EB and treated with Ebs; $n=10$). Four weeks before the demyelination procedure, the animals from groups II and IV were injected intraperitoneally (i.p.) for five consecutive days followed by two days of no treatment each week, completing a total of twenty applications of Ebs diluted in canola oil (1 mL/kg), using a dose of 2 mg/kg.

Treatment with vitamin E (Vit. E)

The animals were randomly divided into four groups: I — control (injected with saline; $n=10$), II — (injected with saline and treated with Vit. E; $n=10$), III — (injected with 0.1% EB, $n=10$) and IV — (injected with 0.1% EB and treated with Vit. E; $n=10$). Four weeks before the demyelination procedure,

the animals from groups II and IV were injected intraperitoneally (i.p.) for five consecutive days followed by two days of no treatment each week, completing a total of twenty applications of Vit. E diluted in canola oil (1 mL/kg), using a dose of 2 mg/Kg.

Experimental demyelination with EB

For the surgical procedure, the animals from both treatments were anesthetized with ketamine chloridate and xylazine (5:1; 0.1 ml/100 g), and after shaving the fronto-parietal-occipital area, antiseptis 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 0.85 cm to the right of the bregma until exposing the duramater. With the use of a Hamilton syringe with a removable needle of caliber 26 s, 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 was injected in the animals from groups III and IV, and the same volume of 0.9% saline solution was injected in the animals from groups I and II. The duramater was left open and the skin, together with the remainder of the subcutaneous tissue, was sutured with a nylon thread 4.0.

In both treatments studied, five animals from each group were sacrificed after three and twenty one days of the surgical procedure. After the sacrifices, the blood was collected for platelet preparation.

Histological studies

To confirm the demyelination used in the 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 21 days after injection (a.i). Brain stem coronal slices with the lesion were embedded in paraffin for routine processing and 5 μ m sections were produced and stained with hematoxylin and eosin (H & E).

Platelet preparation

The platelets were prepared by the method of Lunkes et al. (2004), with the following minor modifications. Total blood was collected from cardiac puncture into a flask with 0.120 M sodium citrate as anticoagulant. The total blood–citrate system was centrifuged at 160 \times g during 15 min to remove the residual blood cells. The platelet-rich plasma (PRP) was centrifuged at 1400 \times g for 30 min and washed twice by centrifugation at 1400 \times 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 by the Coomassie blue method according to Bradford (1976) using serum albumin as standard.

NTPDase and 5'-nucleotidase assays

The NTPDase enzymatic assay was carried out in a reaction medium containing 5 mM CaCl₂, 100 mM NaCl; 4 mM KCl,

5 mM glucose and 50 mM Tris–HCl buffer, pH 7.4, in a final volume of 200 μ l as described by Pilla et al. (1996). The 20 μ l of the enzyme preparation (8–12 μ g of protein) was added to the reaction mixture and the pre-incubation proceeded for 10 min at 37 °C. The reaction was initiated by the addition of ATP or ADP to a final concentration of 1.0 mM; the time of incubation was 60 minutes. For AMP hydrolysis, the 5'-nucleotidase activity was carried out as previously described, except that the 5 mM CaCl₂ was replaced by 10 mM MgCl₂, and the nucleotide added was 2 mM AMP (Furstenau et al., 2004).

Both enzyme assays were stopped by the addition of 200 μ l of 10% trichloroacetic acid (TCA) to provide a final concentration

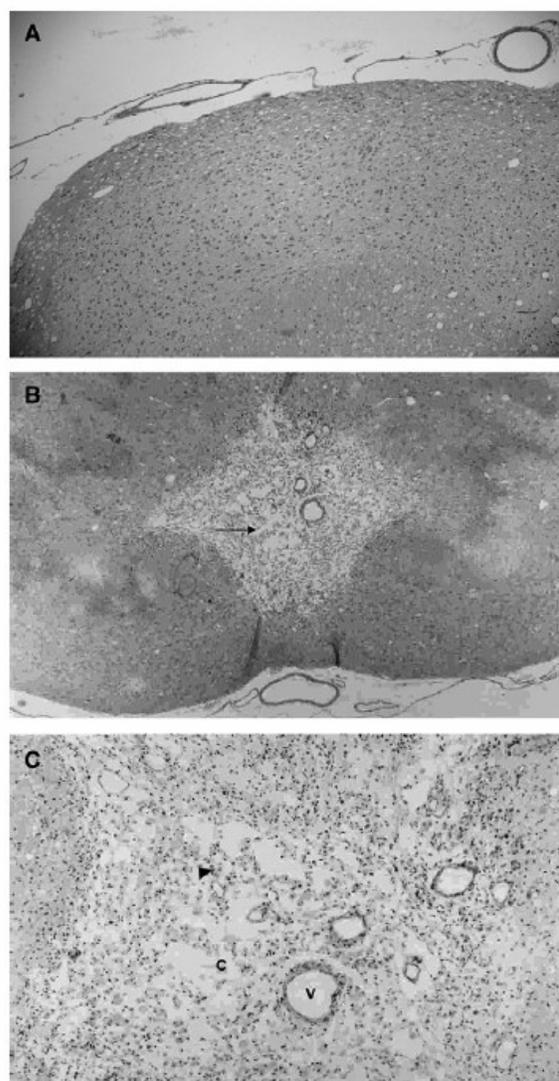


Fig. 1. Histological analysis of the control group demonstrating the normal aspect of the pons (A). In (B) area of demyelination induced by ethidium bromide (arrow) after twenty one-day-old a.i (HE \times 66). In C, close view of B showed numerous macrophages (black arrowhead), cystic cavities (c) and new blood vessels (v) (HE \times 10).

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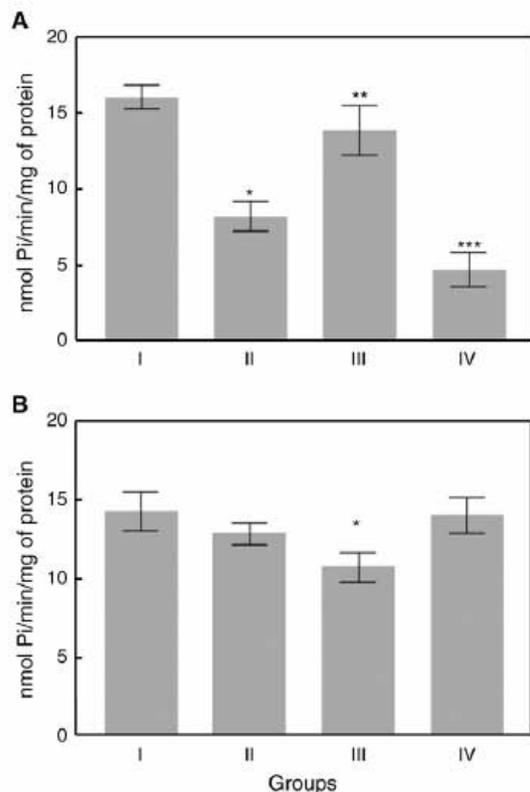


Fig. 2. NTPDase activity in platelets using ATP as substrate three (A) and twenty one (B) days after EB or saline injection. Group I: (saline); group II (Ebs); group III (EB) and group IV (EB+Ebs). Bars represent mean \pm SEM. (*) indicates a significant difference at $p < 0.05$, (**) indicates a significant difference at $p < 0.01$ and (***) indicates a significant difference at $p < 0.001$.

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_2PO_4 as standard. Controls were carried out to correct the non enzymatic hydrolysis 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, unless otherwise stated.

Statistical analysis

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

Results

Histological analysis of lesion

Concerning the model used in this study the histological analysis of the ventral area of the pons at 21 days showed

alterations caused by the EB injection. The lesions induced by EB consisted of areas of demyelination that were almost completely remyelinated at day 21 after injection (Fig. 1B and C).

Histological analysis of the ventral pons showed areas of spongiosis caused by EB Fig. 1B. The spongiosis was due to the loss of glial cells and myelin sheaths which started at 24 hours after injection (a.i.). At day 3 a.i., profuse invasion of macrophages could be seen, which were involved in the removal of tissue debris. From day 15 through day 21, remyelination of the lost myelin sheaths was observed. The new sheaths were thinner than the normal ones, and evidenced by special stains.

Treatment with ebselen

NTPDase activity in platelets was altered depending on the length of time from the demyelination procedure. The results showed a significant alteration in NTPDase activity after three days when ATP was used as substrate ($p < 0.05$), and post-hoc comparisons by Duncan's test revealed that ATP hydrolysis was significantly reduced in groups II, III and IV when compared to the control group I (Fig. 2A). After twenty one days, a significant alteration in enzyme activity ($p < 0.05$) was also

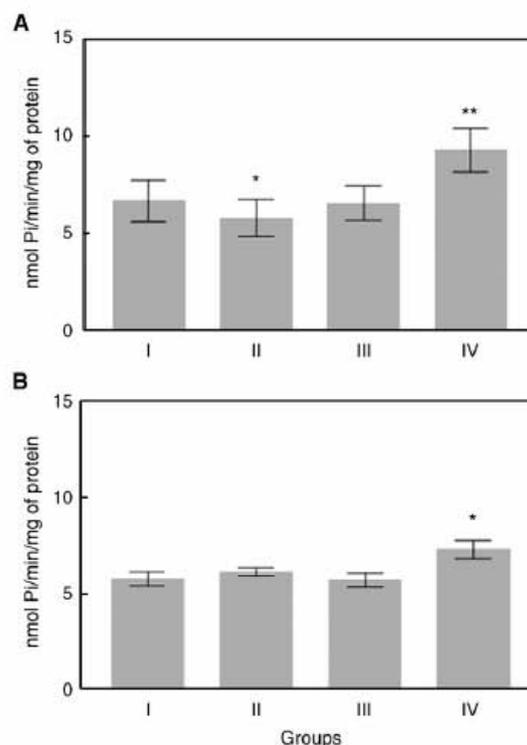


Fig. 3. NTPDase activity in platelets using ADP as substrate three (A) and twenty one (B) days after EB or saline injection. Group I: (saline); group II (Ebs); group III (EB) and group IV (EB+Ebs). Bars represent mean \pm SEM. (*) indicates a significant difference at $p < 0.05$ and (**) indicates a significant difference at $p < 0.01$.

observed, and post-hoc comparisons by Duncan's test revealed that ATP hydrolysis was significantly reduced in group III, while in groups II and IV there was no alteration in the hydrolysis of this nucleotide (Fig. 2B).

An inhibition of ADP hydrolysis was observed after three days ($p < 0.05$) and post-hoc comparisons by Duncan's test revealed that ADP hydrolysis was significantly reduced in group II when compared to group I, while in group IV the hydrolysis of this nucleotide was increased when compared to the control group I (Fig. 3A). After twenty one days a significant increase in enzyme activity ($p < 0.05$) was observed, and comparisons by Duncan's test revealed that ADP hydrolysis was significantly higher in group IV when compared to group I (Fig. 3B).

Ebselen did not alter AMP hydrolysis in rat platelets under the same conditions (data not shown).

Treatment with vitamin E

NTPDase activity in platelets was also altered depending on the length of time from the demyelination procedure. The results showed a significant alteration in NTPDase activity after three days when ATP was used as substrate ($p < 0.05$), and post-hoc comparisons by Duncan's test revealed that ATP hydrolysis was significantly reduced in groups II, III

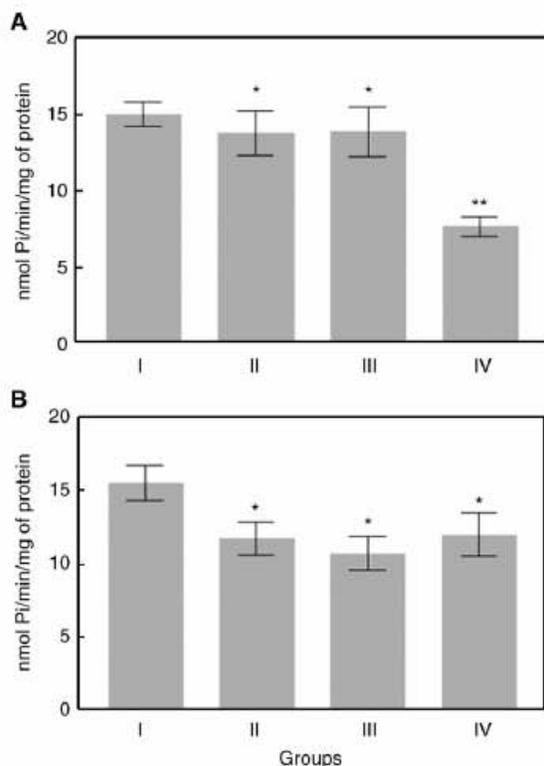


Fig. 4. NTPDase activity in platelets using ATP as substrate three (A) and twenty one (B) days after EB or saline injection. Group I: (saline); group II (Vit. E); group III (EB) and group IV (EB+Vit. E). Bars represent mean \pm SEM. (*) indicates a significant difference at $p < 0.01$.

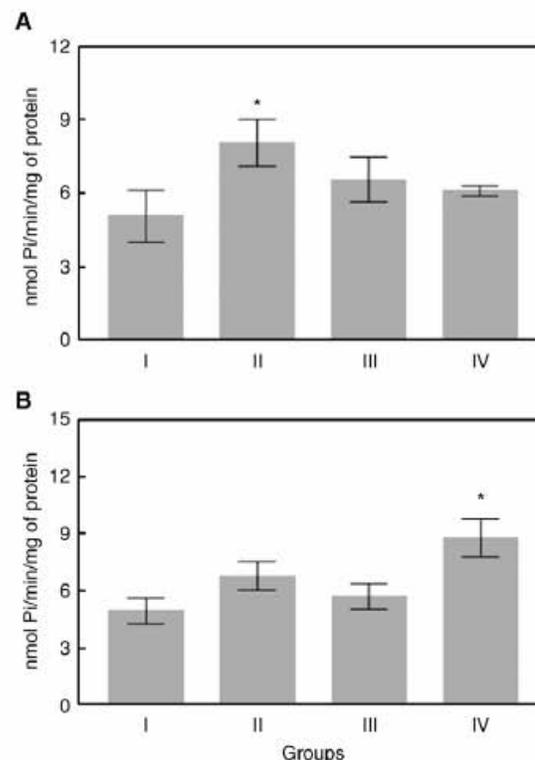


Fig. 5. NTPDase activity in platelets using ADP as substrate three (A) and twenty one (B) days after EB or saline injection. Group I: (saline); group II (Vit. E); group III (EB) and group IV (EB+Vit. E). Bars represent mean \pm SEM. (*) indicates a significant difference at $p < 0.05$.

and IV when compared to the control group I (Fig. 4A). After twenty one days a significant alteration in enzyme activity ($p < 0.05$) was also observed, and post-hoc comparisons by Duncan's test revealed that ATP hydrolysis was significantly reduced in groups II, III and IV when compared to control group I (Fig. 4B).

An increase in enzyme activity was observed after three days when ADP was used as substrate ($p < 0.05$), and post-hoc comparisons by Duncan's test revealed that ADP hydrolysis was significantly increased in group II when compared to group I (Fig. 5A). After twenty one days a significant increase in enzyme activity ($p < 0.05$) was observed, and comparisons by Duncan's test revealed that ADP hydrolysis was significantly higher in group IV when compared to group I (Fig. 5B).

Vitamin E did not alter AMP hydrolysis in rat platelets in the same conditions (data not shown).

Discussion

Experimentally induced demyelination using EB provides a model for analyzing the cellular and enzymatic changes that occur during demyelinating pathologies, such as multiple sclerosis (MS) (Levine and Reynolds, 1999; Stangel and Hartung, 2002). This work was carried out in order to investigate possible changes in NTPDase and 5'-nucleotidase

activities in platelets at different lengths of time after the injection of EB in the rat brainstem. The initial lesion was observed after three days of induction, with the death of oligodendrocytes and astrocytes, which was followed by the remyelination of the lost myelin sheaths between twenty one and thirty days (Reynolds et al., 1996). In fact, these periods (3 and 21 days) are very important for evaluating the role of the enzymes in demyelination and remyelination events in this experimental model. In this study, histological analysis in the 21 days of the injection showed a demyelinating lesion characterized by the loss of most myelin sheaths within the lesion area, however was observed the presence of new blood vessels, which indicates a remyelination of process.

In this study, it was observed that the platelets of rats injected with EB presented a reduction in ATP hydrolysis after three and twenty one days while no alteration occurred in ADP hydrolysis. Corroborating with these findings, some studies have suggested that alterations in the platelet function of patients with MS may be due to the breakdown of the neural tissue (Millar et al., 1966). This breakdown could give rise to the liberation of lipids and basic proteins in the blood, which could 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 matter, alterations in platelet membranes (Remijn et al., 2002; Wagner and Burger, 2003; Keating et al., 2004) could be decisive in the modification of the conformational state of the NTPDase molecule, which would explain the reduced activity observed in this present study after a period of three and twenty one days.

Previous studies from our laboratory have shown that NTPDase and 5'-nucleotidase activities were increased in synaptosomes from the cerebral cortex (Spanevello et al., 2006a) and diminished in platelets of rats demyelinated with EB (Spanevello et al., 2006b), indicating that there is an interaction between this enzyme and demyelinating events. On the other hand, an inhibition in NTPDase activity in platelets of the demyelinated rats and no alterations in 5'-nucleotidase activity were observed in the present study. Taken together, these results indicate that NTPDase and 5'-nucleotidase activities in platelets and synaptosomes play an important function in ATP, ADP and AMP hydrolysis in these different cellular compartments and may cause distinct cellular responses. On this basis, we may not infer that platelets could be a peripheral marker of the alteration observed in the cerebral cortex NTPDase and 5'-nucleotidase activities during the demyelinating events induced by EB.

An important aspect to be discussed is that ebselen per se reduced NTPDase activity after three days. This study supports the results found by Furstenau et al. (2004), which observed that ebselen inhibited *in vitro* ATP and ADP hydrolysis in platelets of rats, suggesting that an inhibition or modulation of this enzyme may prolong the effect of the nucleotides ATP and ADP and the activation of the receptors. On the other hand, after twenty one days, ebselen caused no alteration in NTPDase activity, probably, due to the fact that the blood level of ebselen may have been greatly reduced due to the length of time.

When ebselen was associated with EB, an increase was observed in ADP hydrolysis after 3 and 21 days. These results suggest that the modulation of NTPDase activity caused by

ebselen may be very important because the increase in ADP hydrolysis may have a beneficial effect by protecting platelets from excessive aggregation (Wadenvik et al., 1991; Gibbs et al., 1995; Tsiara et al., 2000; Panasiuk et al., 2004) contributing, this way, to the control of hemostasis, mainly in the demyelinating events of the disease.

The treatment with vitamin E also altered the nucleotide hydrolysis after the different time periods. Our results showed that a reduction in ATP hydrolysis by vitamin E after 3 and 21 days is important, since there is more free ATP in the circulation, protecting platelets from spontaneous aggregation. On the other hand, vitamin E per se increased ADP hydrolysis (Fig. 5A), the main promoter of platelet aggregation. These observations, taken together, show that vitamin E has a modulatory role on NTPDase activity in rat platelets. In addition, a number of studies have demonstrated that vitamin E has specific effects on cellular signaling at the enzymatic level by inhibiting platelet aggregation through a protein kinase C dependent mechanism (Boscoboinik et al., 1991; Freedman et al., 1996; Azzi et al., 2002; Rimbach et al., 2002; Zing and Azzi, 2004). These observations may represent a potential mechanism of platelet aggregation inhibition by vitamin E through enzymatic modulation, being that the exact molecular mechanisms of its action is not fully understood in the literature (Steiner, 1993; Mabile et al., 1999; Freedman et al., 2000; Schoene, 2001; Quinn, 2004; Kobzar et al., 2005).

An important aspect to be discussed in this study is that ATP and ADP hydrolysis by rat platelets is altered in different manners after treatment with vitamin E and ebselen. This different sensitivity of the NTPDase enzyme can be related with the physiological role of the ATP and ADP substrates. The ADP constituted the agonist main involved in the recruitment and aggregation of platelets in the local de vascular injury (Birk et al., 2002a) while the ATP competitively inhibits ADP-induced platelet aggregation (Soslau and Youngprapakorn, 1997; Birk et al., 2002b).

ATP, ADP and AMP hydrolysis in rat platelets occurs by the action of a surface-located enzyme cascade constituted by NTPDase and ecto-5'-nucleotidase (Zimmermann, 2001). The final product of the action of the two enzymes is adenosine, which is an important molecule for the modulation of vascular tone and a well known inhibitor of platelet aggregation (Afonso et al., 2002; Rozalski et al., 2005). Thus, our results demonstrated that ebselen and vitamin E are partial inhibitors of the enzyme cascade reported above because these drugs did not alter AMP hydrolysis activity when tested in the same conditions (data not shown).

In conclusion, our findings demonstrated alterations in nucleotide hydrolysis in platelets of rats demyelinated by EB, which might reinforce the abnormal hemostasis in demyelinating events. In addition, this is the first study to investigate the effect *in vivo* of ebselen and vitamin E on the activity of NTPDase and 5'-nucleotidase associated with the demyelinating model. These drugs modulated adenine nucleotide hydrolysis and may be important in the control of the platelet coagulant status in the demyelinating process. However, when comparing platelet and synaptosomal (cerebral cortex)

NTPDase and 5'-nucleotidase activities, in the EB demyelinating model, we may not infer that platelets could be good peripheral markers of such activities. Perhaps, the different physiological role of these enzymes in the SNC and in platelets is the main factor modulating these activities.

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DISCUSSÃO

Existem vários estudos utilizando o brometo de etídio (BE) como marcador de bandas de ácidos nucléicos em eletroforese (Vardevanyan et al., 2001), como agente tripanocida (Graça & Blakemore, 1986) e como droga gliotóxica para induzir desmielinização focal no SNC (Reynolds et al., 1996; Bondan et al., 2002; Stangel & Hartung, 2002). No entanto, não foi encontrado na literatura estudos que relacionassem o efeito do BE na atividade da AChE *in vivo* e *in vitro*.

Em relação ao estudo *in vitro* foi demonstrado que o BE é um potente inibidor da atividade da AChE já na menor concentração testada (0.0065 mM) em todas as estruturas cerebrais avaliadas (Figura 1, Artigo 1). A análise dos dados cinéticos indicou que a inibição causada pelo BE não foi homogênea em todas as estruturas estudadas. No estriado, hipocampo e córtex cerebral, a inibição foi incompetitiva, enquanto que no cerebelo foi do tipo mista. Um importante aspecto a ser discutido é que no hipocampo e córtex cerebral, os quais recebem projeções colinérgicas dos núcleos basais de Meynert e o estriado, o qual tem um circuito colinérgico intrínscio os resultados foram similares, sugerindo que em regiões onde as projeções colinérgicas são extremamente importantes, o efeito do BE foi similar (Figura 2, Artigo1).

A V_{max} e o K_m diminuíram no estriado, hipocampo e córtex cerebral (Tabela 1 e Figura 2, A-C) caracterizando uma inibição incompetitiva. Já na inibição mista observada no cerebelo, enquanto a V_{max} diminui o K_m aumenta (Tabela 1 e Figura 2 D, Artigo 1). Assim na inibição incompetitiva é provável que o complexo enzima-substrato-inibidor (ESI) foi formado e na inibição mista o tipo de complexo enzima-inibidor (EI) e ESI pode ser formado. Tanto na

inibição incompetitiva como na mista o inibidor se liga em outro local diferente do local de ligação do substrato. De fato a estrutura química da ACh e do BE não são similares, assim a ligação do BE em outro local que não seja o sítio ativo é esperado, como observado no estriado, hipocampo, córtex cerebral e cerebelo. Os valores de IC_{50} e K_i indicaram que o BE é um potente inibidor da AChE (Tabela 2, Artigo 1). Os valores de IC_{50} demonstraram uma sensibilidade similar do BE pelo estriado, hipocampo e córtex cerebral, estruturas ricas em vias colinérgicas como observado em relação ao mecanismo inibitório (incompetitivo). Foi relatado por Patocka et al. (1987) que o BE afeta a atividade da BuChE e inibe reversivelmente a sua atividade. A afinidade da BuChE para o substrato é reduzida pelo BE e a quebra do complexo ESI é prevenida, demonstrando que existe uma interação deste composto com o sistema colinérgico.

Os resultados demonstrados neste trabalho caracteriza o BE como um inibidor da AChE similar a outros inibidores desta enzima, como por exemplo o iodeto de propídio (Bourne et al., 2005). É importante ressaltar que a estrutura do BE é muito similar a do propídio, um bem conhecido inibidor da atividade da AChE (Teller et al., 2006) (Figura 3, Artigo 1). Ambos compostos apresentam um amônio quartenário na sua estrutura o qual é demonstrado na literatura como uma importante condição para efetuar a ligação de ambos os substratos e inibidores no sítio ativo (Patrick, 2001). O iodeto de propídio se liga especificamente ao sítio aniônico periférico (PAS), um segundo local de ligação que está localizado na superfície da AChE, próximo a entrada do sítio ativo desta enzima. Pode-se sugerir que o BE provavelmente se liga a este sítio

periférico da AChE, levando a inibição desta enzima como demonstrado neste estudo.

Em relação aos estudos *in vivo* a variação na atividade da AChE pode estar relacionada com eventos de desmielinização e remielinização que ocorrem nos períodos avaliados nestes estudos (3-7-15-21 e 30 dias) (Artigos 2, 3 e 5). As análises histológicas e imuno-histoquímica têm demonstrado que a injeção intracisternal de BE causa mudanças degenerativas nos oligodendrócitos e astrócitos caracterizadas por espongiose às 24 horas pós-injeção (p.i) e desaparecimento dos astrócitos (GFAP + VIM) no centro da lesão. No 3º dia p.i é observado uma maior celularidade representada por macrófagos, sendo que axônios desmielinizados aparecem somente a partir de 6 a 7 dias p.i. Os primeiros sinais de remielinização são evidentes a partir dos 12 dias, apresentando-se num estágio mais avançado aos 21 e 30 dias p.i (Reynolds et al., 1996; Bondan, 1997; Bondan et al., 2000; Graça et al., 2001; Bondan et al., 2003).

Em relação ao grupo desmielinizado com o BE, houve uma inibição na atividade da AChE no estriado (15 e 30 dias) (Figura 4 - Artigo 2), no hipocampo, no cerebelo, no hipotálamo (30 dias) (Figura 5, Tabela 1 - Artigo 2) e também em sinaptossomas de córtex cerebral (7 e 30 dias) (Figura 3, Artigo 2). Foi observado neste estudo que ocorreu uma variação quantitativa na atividade da AChE sinaptossomal em comparação com a do sobrenadante (S_1) no córtex cerebral. O sinaptossoma é formado por um terminal nervoso, utilizado frequentemente para estudos farmacológicos envolvendo o metabolismo e a liberação do neurotransmissor ACh (Moulian et al., 1994). Essa diferença entre o sinaptossoma e S_1 pode ser explicada pela existência

das diferentes formas moleculares da AChE, como a G4 (ligada à membrana) e a G1 (citosólica) nas diferentes regiões cerebrais (Das et al., 2001). No cérebro de mamíferos a forma G4 representa 60-90% da AChE total, dependendo da região anatômica e o restante é composta pelas formas G1 e G2 (Descarries et al., 1997).

Também foi observada uma inibição desta enzima aos 21 dias em ratos desmielinizados pelo BE no córtex cerebral, no estriado, no hipocampo, no hipotálamo, no cerebelo e na ponte (Figura 2 e 3, Artigo 3), enquanto que aos 3 e 21 dias a atividade da AChE também foi inibida no córtex cerebral, no estriado e no hipocampo, no mesmo grupo (Figura 3 e 4, Artigo 5). A inibição na atividade da AChE pode levar ao acúmulo da ACh e consequente superestimulação dos receptores (Tōugu & Kesvatera, 1996). Neste contexto, eventos desmielinizantes podem causar uma deficiência colinérgica pré-sináptica, a qual é responsiva ao tratamento com inibidores da AChE (Gustavson and Cummings, 2003), o que torna esta enzima um importante alvo terapêutico em desordens neurodegenerativas (Greene et al., 2000; Parry et al., 2003; Porcel & Montalban, 2006). Por outro lado, o BE aumentou a atividade da AChE no hipocampo e no cerebelo (7 e 15 dias) (Figura 5 e Tabela 1, Artigo 2), no hipotálamo (15 dias) (Tabela 1, Artigo 2) e em sinaptossoma de córtex cerebral (15 dias) (Figura 3, Artigo 2). A ativação da AChE leva a uma rápida degradação da ACh e uma baixa estimulação dos receptores (Grisaru et al., 1999). Esses dois mecanismos podem sugerir que o BE promove uma disfunção na sinapse, interferindo com a modulação da neurotransmissão colinérgica.

Desde a descoberta do envolvimento do sistema imune na patogênese da esclerose múltipla, muitos agentes imunossupressores e imunomoduladores têm sido utilizados no tratamento dessa 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 IFN- β é um agente imunomodulador atualmente empregado no tratamento da esclerose múltipla, porém os mecanismos celulares e moleculares envolvendo seus efeitos benéficos ainda permanecem pouco compreendidos (Revel, 2003).

Um importante dado observado neste estudo é que o IFN- β *per se* foi capaz de inibir significativamente a atividade da AChE no estriado, no hipocampo, no hipotálamo e sinaptossoma de córtex cerebral (Figura 3, 4, 5 e Tabela I- Artigo 2), regiões onde as projeções colinérgicas são extremamente importantes, sugerindo uma interação desta droga com o sistema colinérgico. Por outro lado, quando o IFN- β foi associado com o BE não houve alteração na atividade da AChE, sugerindo que esse composto não foi capaz de prevenir os efeitos inibitórios do BE sobre esta enzima. Foi demonstrado por Rao (2004) através de testes cognitivos que o IFN- β foi efetivo no tratamento de pacientes com esclerose múltipla, apresentando uma melhora significativa na memória, no aprendizado e no processamento de informações, funções vitais que são reguladas pelo sistema colinérgico. Além disso, a taxa de disfunção cognitiva na esclerose múltipla varia entre 45-65% e drogas imunomoduladoras como o IFN- β diminui a progressão da doença (Revel, 2003) e a disfunção cognitiva que envolve esta patologia desmielinizante (Barak & Achiron, 2002).

Embora resultados dos testes clínicos tenham indicado que o IFN- β melhora a cognição dos pacientes com esclerose múltipla, pouco é conhecido

sobre seu mecanismo de ação (Malik, 1998). Os resultados deste estudo sugerem que um dos mecanismos de ação do IFN- β é através da inibição na atividade da AChE, focalizando a importância deste composto em modular a neurotransmissão colinérgica.

Em relação ao tratamento com a CsA, foi observado que este composto *per se* também inibiu a atividade da AChE no córtex cerebral, no estriado, no hipocampo, no hipotálamo, no cerebelo e na ponte (Figura 2 e 3, Artigo 3). Corroborando com esses achados foi demonstrado por Herink et al. (2003) que a CsA inibiu a atividade da AChE no córtex frontal, no hipocampo, no septo medial e no gânglio basal de ratos. Por outro lado, Borlongan et al. (2000) demonstrou um efeito neuroprotetor e neurotrófico da CsA no sistema colinérgico por aumentar a imunoreatividade da colina-acetiltransferase (ChAT), uma enzima responsável pela síntese de ACh (Prado et al., 2002). Neste estudo quando ratos desmielinizados foram tratados com CsA, houve um aumento dos efeitos inibitórios na atividade da AChE em todas as estruturas avaliadas (Figura 2 e 3, Artigo 3).

Estes resultados ajudam a confirmar que drogas utilizadas no tratamento de pacientes com esclerose múltipla tais como o IFN- β e a CsA causam efeitos no sistema colinérgico, focalizando a importância de investigar a atividade da AChE, uma enzima chave na sinapse colinérgica. Além disso, pode-se sugerir que IFN- β e a CsA podem ser considerados potentes ferramentas terapêuticas e científicas para o tratamento de doenças neurodegenerativas como Alzheimer e outras demências.

Embora os resultados deste estudo tenham indicado que a CsA inibe a atividade da AChE na dose de 10 mg/Kg, sua aplicação clínica é limitada pelos

efeitos colaterais em órgãos vitais que podem levar à nefrotoxicidade, hepatotoxicidade e neurotoxicidade (Rosecrantz et al., 2001; Rosendal et al., 2005). A dose de CsA utilizada neste trabalho, foi baseada em estudos prévios com o modelo experimental de desmielinização pelo BE, onde a CsA aumentou a densidade dos oligodendrócitos nas margens da lesão de desmielinização, indicando que este agente imunossupressor foi capaz de afetar eventos celulares envolvidos no processo de reparo do tecido desmielinizado (Bondan, 1997).

Neste estudo, um aumento da peroxidação lipídica foi observado determinando o conteúdo de TBARS no soro, fígado e rim (dados não mostrados) de ratos desmielinizados e tratados com CsA, focalizando o envolvimento desta droga com a formação de espécies reativas de oxigênio. Corroborando com estes achados Durak et al. (2004) observou que a diminuição da glutathiona hepática e o acúmulo do malondialdeído, o produto final da peroxidação lipídica, são considerados os principais mecanismos de hepatotoxicidade associada ao tratamento com CsA. Nesse contexto, pode-se sugerir que estudos futuros possam ser conduzidos na ordem de investigar a dose ideal e o tempo de exposição para o uso da CsA, visto que o seu efeito inibitório na atividade da AChE pode constituir uma janela terapêutica para doenças auto-imunes associadas a uma disfunção cognitiva como a esclerose múltipla.

Outro estudo foi conduzido com o objetivo de testar drogas com funções neuroprotetoras como o ebselen (Ebs) e a Vitamina E (Vit E) no modelo experimental de desmielinização pelo BE. Neste estudo, foi observado após 3 dias uma lesão de desmielinização na ponte, caracterizada por infiltração de

macrófagos e ausência de células GFAP+ no centro da lesão, enquanto que aos 21 dias, a lesão demonstrou algumas áreas císticas e processos astrocíticos reativos. Em ratos tratados com Ebs e Vit E as lesões induzidas pelo BE foram menores, sugerindo que estes compostos interferem com o desenvolvimento das lesões. Os macrófagos (VIM+) e os astrócitos (GFAP+) tinham uma distribuição similar dentro das lesões após os tratamentos. Esse comportamento celular foi similar ao observado em vários estudos experimentais utilizando o BE como droga desmielinizante (Bondan et al., 2000; Bondan, et al.; 2003; Graça et al., 2001; Sallis et al., 2006). Independentemente da causa da agressão no SNC, o reparo do tecido acaba sempre contando com a participação astrocitária. A reação astrocitária pode incluir o aumento do seu número e/ou de suas dimensões, além de outras alterações estruturais e funcionais, como o espessamento dos feixes de filamentos gliais e conseqüente aumento da intensidade de marcação para GFAP/VIM como observado neste estudo (Bondan, et al.; 2003).

É bem estabelecido na literatura as propriedades anti-inflamatórias e antioxidantes do Ebs (Schewe, 1995), bem como da Vit E (Steiner, 1993). No entanto não tem sido documentada a interação desses compostos com a atividade da AChE. Neste trabalho o Ebs *per se* foi capaz de inibir significativamente a atividade da AChE no estriado, no hipocampo e no córtex cerebral (Figura 3 e 4, Artigo 5), estruturas ricas em projeções colinérgicas, demonstrando o envolvimento desta droga com este sistema. Foi relatado recentemente por Nizri et al. (2005, 2006) que IACHEs têm propriedades anti-inflamatórias por reduzir a proliferação de linfócitos e a secreção de citocinas pró-inflamatórias. Nesse contexto, sugere-se que o Ebs pode ser considerado

um composto bifuncional, pois além de apresentar propriedade anti-inflamatória, este estudo demonstrou que esse composto também possui um efeito inibidor na atividade da AChE, combinando duas diferentes funções em uma mesma molécula.

A Vit E *per se* também inibiu a atividade da AChE no córtex cerebral e no hipocampo (Figura 3 e 4, Artigo 5). Essas regiões recebem projeções colinérgicas dos núcleos basais de Meynert e representam um importante papel no processo de aprendizagem e memória. Tem sido demonstrado por Maneesub et al. (1993) que a Vit E pode restaurar parcialmente a hipofunção do sistema colinérgico no envelhecimento e também pode ativar este sistema na retenção da memória (Eidi et al., 2006). Embora esses estudos tenham demonstrado que a Vit E melhora a função cognitiva pouco é conhecido sobre seu mecanismo de ação. Pode-se sugerir que um dos mecanismos de ação deste composto seja através da inibição na atividade da AChE como observado neste estudo. A Vit E também possui uma variedade de funções não-antioxidantes como, por exemplo, a inibição da proteína quinase C (PKC), que regula a ativação de muitas proteínas envolvidas no processo inflamatório (Boscoboinik et al., 1991), demonstrando assim que este composto tem uma importante atividade anti-inflamatória como relatada por vários pesquisadores (Grammas et al., 2004; Saldeen & Saldeen, 2005; Walston et al., 2006; Reiter et al., 2007). Assim, pode-se inferir que a Vit E também pode ser considerado um composto bifuncional.

Uma outra e talvez mais interessante interpretação desses achados é que IACHEs podem aumentar a estimulação colinérgica dos oligodendrócitos e seus precursores via receptores e promover a mielinização (Bartzokis, 2006).

Isso demonstra a importância de se testar compostos com atividade anticolinesterásica em eventos de desmielinização e remielinização como ocorre no modelo gliotóxico do BE.

Em relação aos tratamentos utilizados foi observado que ambos, Ebs e Vit E quando associados com BE foram capazes de aumentar a atividade da AChE no estriado (3 dias), no córtex cerebral (21 dias) e no hipocampo (21 dias) (Figura 3C, 4A e 4B), sugerindo que essas drogas podem prevenir os efeitos inibitórios do BE nessas regiões cerebrais.

Outro importante dado observado neste estudo foi em relação à atividade da AChE no sangue. Esta enzima demonstrou uma inibição significativa após 3 dias da indução nos grupos tratados (III, IV, VI e VII) e no grupo desmielinizado pelo BE (V) (Figura 5A, Artigo 5). Esses resultados foram similares aos resultados obtidos nas estruturas cerebrais deste estudo (Artigo 5). Foi relatado por Bernhardt et al. (2005) que a concentração de AChE no sangue (RBC-AChE) pode funcionar como bom marcador periférico para estudar desordens neurodegenerativas, pois permite avaliar através de métodos mais acessíveis a ação desta enzima no SNC. Além disso, a RBC-AChE demonstrou ter propriedades funcionais similares com a AChE do SNC e assim pode refletir o seu estado com a AChE da fenda sináptica (Thiermann et al., 2005).

Foi também investigado neste estudo a atividade da NTPDase e 5' - nucleotidase em plaquetas de ratos desmielinizados com o BE e tratados com Vit E e Ebs. Foi observado que ratos injetados com BE apresentaram uma redução na hidrólise do ATP após 3 e 21 dias em ambos os tratamentos (Ebs e Vit E), enquanto que nenhuma alteração ocorreu na hidrólise do ADP (Figura 2, 3, 4 e 5, Artigo 4). Corroborando com esses achados, alguns estudos têm

sugerido que alterações na função da plaqueta de pacientes com esclerose múltipla pode ser devido à destruição e liberação de lipídeos e proteínas básicas no sangue, os quais podem alterar a superfície da membrana da plaqueta (Chiang et al., 1982; Neu et al., 1982; Khan et al., 1985; Cananzi et al., 1987). Assim uma alteração na membrana da plaqueta (Remijn et al., 2002; Wagner & Burger, 2003) pode ser um fator decisivo na modificação do estado conformacional da enzima NTPDase, o que pode explicar a reduzida atividade desta enzima neste estudo no período de 3 e 21 dias.

Estudos prévios têm demonstrado que a atividade da NTPDase e 5'-nucleotidase está aumentada em sinaptossoma de córtex cerebral (Spanevello et al., 2006) e diminuída em plaquetas de ratos desmielinizados pelo BE (Spanevello et al., 2007) indicando que existe uma interação desta enzima com eventos desmielinizantes. Por outro lado, no presente estudo foi observado uma inibição na atividade da NTPDase e nenhuma alteração na atividade da 5'-nucleotidase em plaquetas de ratos desmielinizados. Esses resultados indicam que a atividade da NTPDase e 5'-nucleotidase em plaquetas e sinaptossomas tem uma importante função na hidrólise do ATP, ADP e AMP nos diferentes compartimentos celulares e pode causar respostas celulares distintas. Nesse contexto, as plaquetas não podem ser consideradas um bom marcador periférico da alteração na atividade da NTPDase e 5'-nucleotidase durante eventos desmielinizantes induzidos pelo BE.

Um importante aspecto a ser discutido é que o Ebs *per se* reduziu a atividade da NTPDase após 3 dias (Figura 2-A, Artigo 4). Corroborando com esses resultados foi verificado por Furstenau et al. (2004) que o Ebs inibiu *in vitro* a hidrólise do ATP e ADP em plaquetas de ratos, sugerindo que uma

inibição ou modulação desta enzima pode prolongar os efeitos dos nucleotídeos ATP e ADP e a ativação de seus receptores. Por outro lado após 21 dias, Ebs não alterou a atividade da NTPDase (Figura 2-B, Artigo 4), provavelmente devido ao baixo nível sanguíneo dessa droga em relação ao tempo de exposição.

Quando o Ebs foi associado com BE, foi observado um aumento na hidrólise do ADP após 3 e 21 dias (Figura 3 A e B, Artigo 4). Estes resultados sugerem que a modulação na atividade da NTPDase causada pelo Ebs pode ser muito importante, visto que um aumento na hidrólise do ADP pode ter um efeito benéfico por proteger as plaquetas da agregação excessiva (Wadenvik et al., 1991; Gibbs et al., 1995; Tsiara et al., 2000; Panasiuk et al., 2004), contribuindo no controle da homeostasia, principalmente nos eventos desmielinizantes da doença.

O tratamento com Vit E também alterou a hidrólise dos nucleotídeos nos diferentes períodos de tempo avaliados. Os resultados demonstraram que a redução na hidrólise do ATP pela vitamina E aos 3 e 21 dias (Figura 4 A e B, Artigo 4) é importante, pois mais ATP livre na circulação vai estar presente, protegendo as plaquetas da agregação espontânea. Por outro lado, a Vit E *per se* aumentou a hidrólise do ADP (Figura 5 A, Artigo 4), o principal promotor da agregação plaquetária. Esses resultados demonstram que a Vit E tem um papel modulatório na atividade da NTPDase em plaquetas de ratos. Vários estudos têm demonstrado que a Vit E tem efeitos específicos na sinalização celular em nível enzimático por inibir a agregação plaquetária através da proteína quinase C (Boscoboinik et al., 1991; Freedman et al., 2000; Azzi et al., 2002; Rimbach et al., 2002; Zingg & Azzi, 2004). Essas observações, podem

representar um mecanismo potencial de inibição da agregação plaquetária pela Vit E através de modulação enzimática, sendo que o exato mecanismo de ação não está bem compreendido na literatura (Steiner, 1993; Quinn, 2004; Kobzar et al., 2005).

A hidrólise do ATP, ADP e AMP em plaquetas de ratos ocorre pela ação de uma cascata de enzimas localizadas na superfície constituídas pela NTPDase e ecto-5'-nucleotidase (Zimemermann, 2001). O produto final de ação destas duas enzimas é a adenosina, a qual é uma importante molécula para a modulação do tônus vascular e um bem conhecido inibidor da agregação plaquetária (Anfossi et al., 2002; Rozalski et al., 2005). Nesse contexto, foi demonstrado neste estudo que o Ebs e a Vit E são inibidores parciais da cascata enzimática relatada acima, visto que estas drogas não alteram a hidrólise do AMP quando testado nas mesmas condições (dados não mostrados).

Neste trabalho, um modelo experimental de desmielinização tóxica pelo BE foi utilizado para avaliar a atividade da AChE e pelos resultados compilados, pode-se sugerir que existe uma disfunção colinérgica, a qual é causada pela ação deste composto no SNC. Em relação aos tratamentos utilizados neste estudo, como o IFN- β e CsA, os resultados demonstraram uma interação destes compostos com o sistema colinérgico por inibirem a atividade da AChE. Também foram investigados os efeitos da Vit E e Ebs tanto no sistema colinérgico como no sistema purinérgico, demonstrando que estas drogas interferem com a atividade de enzimas como a AChE e NTPDase respectivamente, as quais são importantes para o funcionamento destes sistemas. Esses resultados podem ser investigados em estudos futuros com a

intenção de encontrar uma melhor terapia para beneficiar pacientes com patologias desmielinizantes.

CONCLUSÕES

Capítulo 1: O BE *in vitro* é um inibidor da atividade da AChE no estriado, no córtex cerebral, no hipocampo e no cerebelo. A análise dos dados cinéticos indicou que no estriado, no córtex cerebral e no hipocampo a inibição foi do tipo incompetitiva e no cerebelo foi do tipo mista, indicando que este composto pode interferir com a neurotransmissão colinérgica.

Capítulo 2: Alterações na atividade da AChE foram observadas no córtex cerebral, no estriado, no hipocampo, no cerebelo, no hipotálamo e na ponte após 7, 15 e 30 dias em ratos desmielinizados com BE e tratados com IFN- β , sugerindo que a ação do IFN- β na atividade desta enzima pode ser importante no tratamento de doenças desmielinizantes associadas com disfunção cognitiva.

Capítulo 3: A atividade da AChE foi inibida no córtex cerebral, no estriado, no hipocampo, no hipotálamo, no cerebelo e na ponte após 21 dias da injeção do BE. Quando associado ao tratamento com CsA esses efeitos inibitórios foram potencializados, indicando que esse agente interfere com a atividade da AChE podendo levar a alterações nos níveis extracelulares da acetilcolina.

Capítulo 4: Uma inibição na atividade da AChE no córtex cerebral, no estriado, no hipocampo e em eritrócitos foi observada após 3 e 21 dias da injeção do BE. O tratamento prévio com vitamina E e ebselen causou uma alteração na

atividade dessa enzima em ratos normais e desmielinizados pelo BE, sugerindo que esses dois compostos interagem com o sistema colinérgico.

Capítulo 5: A atividade da enzima NTPDase foi alterada em plaquetas de ratos desmielinizados pelo BE e tratados com Vitamina E e ebselen. O tratamento prévio com esses compostos foi capaz de modular a hidrólise dos nucleotídeos de adenina, o que pode contribuir para o controle dos mecanismos de trombo-regulação em eventos desmielinizantes.

Importância do trabalho e Perspectivas

Este foi o primeiro trabalho que avaliou a atividade da acetilcolinesterase (AChE) no modelo experimental de desmielinização tóxica pelo brometo de etídio, sendo observada uma alteração na atividade dessa enzima. O interferon beta e a ciclosporina que são empregados na terapia da esclerose múltipla demonstraram uma interação com a acetilcolinesterase, inibindo a atividade dessa enzima. Adicionalmente, os fármacos de ação neuroprotetora (Vitamina E e ebselen) também ocasionaram um efeito inibitório sobre a atividade da AChE. Esse resultado foi considerado um dos mais relevantes encontrado neste estudo, visto que, os inibidores da acetilcolinesterase podem representar uma importante opção terapêutica para melhorar a disfunção cognitiva de pacientes com esclerose múltipla, já que a neurotransmissão colinérgica está provavelmente afetada no curso dessa doença. Poderíamos aprofundar ainda mais estes estudos a partir da concretização dos seguintes objetivos:

- Identificar através de métodos histoquímicos a expressão da acetilcolinesterase no sistema nervoso central.
- Realizar testes comportamentais em ratos desmielinizados pelo brometo de etídio e tratados com os compostos analisados neste estudo.
- Avaliar parâmetros de estresse oxidativo no sistema nervoso central e em soro de ratos desmielinizados pelo brometo de etídio.
- Verificar as alterações na atividade das enzimas NTPDase e AChE em linfócitos de pacientes com esclerose múltipla.
- Utilizar a terapia regenerativa (células tronco retiradas da medula óssea) neste modelo de desmielinização pelo brometo de etídio.

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