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O envolvimento do cálcio e das junções gap na secreção de S100B em astrócitos de ratos

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Sumário

Parte I

Resumo

A S100B, uma proteína ligante de cálcio de 21 kDa, possui muitos alvos intracelulares envolvidos no ciclo celular e na regulação do citoesqueleto em astrócitos. Além disso, essa proteína é secretada e possui efeitos autócrinos e parácrinos na glia, plasticidade sináptica e microglia. A expressão de S100B, particularmente a S100B extracelular, é usada como um parâmetro de ativação e/ou morte glial em diversas situações de dano cerebral. Existem muitos imunoensaios para a dosagem de S100B, que diferem em relação à especificidade, sensibilidade, aplicação das amostras e custo. Nós padronizamos dois protocolos para a dosagem de S100B (variando em sensibilidade de 1,9 pg a 10 ng/mL) em amostras de humanos e de ratos, de tecido cerebral, tecido adiposo, soro, líquor, urina e amostras de cultura de células. Muitos secretagogos de S100B já foram identificados, mas o mecanismo de secreção dessa proteína ainda é desconhecido e envolve um mecanismo do tipo não-clássico de secreção. Nós investigamos o papel do Ca^{2+} na secreção de S100B em cultura primária de astrócitos. Nossos resultados mostraram que o DMSO é um potente secretagogo da S100B. A secreção de S100B induzida por DMSO foi dependente da mobilização de cálcio do retículo endoplasmático, mas não de reorganização do citoesqueleto. A propriedade do DMSO em induzir a secreção de S100B deve ser adicionada à lista de aplicações terapêuticas desse composto, especialmente considerando os efeitos neuroprotetores que têm sido observados da S100B em situações de dano cerebral agudo. Além disso, a utilidade do DMSO como uma ferramenta para investigar a mobilização de cálcio intracelular deve ser levada em consideração. Sabe-se que os astrócitos sentem, interagem e respondem a estímulos gerados pelos neurônios ou pelo dano neuronal e essa resposta envolve a comunicação por junção gap. A vulnerabilidade neuronal a danos é aumentada quando co-culturas de astrócitos e neurônios são expostas a inibidores de junção gap. Entretanto, a inibição das junções gap pode limitar a extensão de uma lesão. Nós investigamos uma possível relação entre a comunicação por junção gap e a secreção de S100B. Nossos dados indicam que o bloqueio das junções gap estimula a secreção de S100B em cultura de astrócitos, bem como em fatias agudas hipocampais. A secreção de S100B foi observada com o uso de diferentes tipos de bloqueadores de junção gap e o resultado foi dependente do tempo, da natureza do inibidor, de seu possível alvo intracelular e/ou do tipo de preparação celular utilizada. Fisiologicamente, um bloqueio local da comunicação por junção gap associado com a liberação de S100B em uma situação de dano favorece a idéia da existência de um mecanismo comum para limitar a extensão da lesão e, simultaneamente, aumentar as chances de sobrevivência celular.

Abstract

S100B, a calcium-binding protein of 21 kDa, has many putative intracellular targets involved in cell cycle and cytoskeleton regulation in astrocytes. In addition, this protein is also secreted and has autocrine and paracrine effects on glia, synaptic plasticity and microglia. S100B expression, particularly extracellular S100B, is used as a parameter of glial activation and/or death in several situations of brain injury. Several immunoassays for S100B measurement are available, which differ with regard to specificity, sensitivity, sample application, and, of course, economic costs. We standardized two protocols for S100B measurement (range between 1,9 pg and 10 ng/mL) in human and rat samples from brain and adipose tissues, blood serum, cerebrospinal fluid, urine and cell culture. Many S100B secretagogues have been identified, but the underlying mechanism of secretion remains unknown and involves a non-classic export. Herein, we investigate the role of Ca^{2+} in S100B secretion in primary cultured astrocytes. Results indicate that DMSO is a powerful S100B secretagogue. DMSO induced S100B secretion was dependent on increased intracellular Ca^{2+} mobilization from endoplasmatic reticulum and independent of cytoskeleton reorganization. Furthermore, the S100B-secreting property of DMSO should be added to the list of therapeutic applications of this compound, particularly considering the neuroprotective effects of S100B that have been observed in acute brain damage. In addition, the usefulness of DMSO *per se* as a tool to investigate intracellular calcium mobilization should be taken into consideration. Astrocytes sense, integrate, and respond to stimuli generated by neurons or neural injury; this response involves gap junction (GJ) communication. Neuronal vulnerability to injury increased when cocultures of astrocytes and neurons were exposed to GJ inhibitors. However, GJ uncoupling could limit the extension of a lesion. We investigated a possible link between GJ communication and S100B secretion. Our data indicate that GJ blocking stimulates S100B secretion in astrocyte cultures and acute hippocampal slices. S100B secretion was observed with different types of GJ inhibitors; the resulting event was dependent on time, the nature of the inhibitor, its putative molecular target of GJ blocking, and/or the cell preparation used. Physiologically, a local GJ closure associated with release of S100B in injury conditions favors the idea of a common mechanism available to limit the extension of lesion and increase the chances of cell survival.

Lista de Abreviaturas

ADP: adenosina difosfato

AMP: adenosine monofosfato

ATP: adenosine trifosfato

BAPTA-AM: ácido 1,2 bis (2-aminofenoxi)etano-N,N,N',N'-tetracético (éster acetoximetil)

cAMP: AMP cíclico

Cbx: carbenoxolona

COX-2: cicloxigenase 2

Cx: conexina

DMSO: dimetilsulfóxido

DTT: ditiotreitol

ECLIA: imunoensaio eletroquimioluminescente ("electrochemiluminescence immunoassay")

EDTA: ácido etilenodiaminatetracético

EGTA: ácido etileno glicol-bis(2-aminoetileter)- N,N,N',N'-tetracético

ELISA: imunoensaio enzimático ("enzyme-linked immunosorbent assay")

ET-1: endotelina-1

FFA: ácido flufenâmico

GFAP: proteína ácida fibrilar glial

Gza: ácido glicirrízico

Hal: haolotano

ICV: intracerebroventricular

IFMA: imunoensaio fluorimétrico (“immunofluorometric assay”)

IFN-γ: interferon gama

IL-1β: interleucina 1 beta

IL-8: interleucina 8

$IP_{3:}$ inositol trisfosfato

IRMA: imunoensaio imunorradiometrico (“immunoradiometric assay”)

LDH: lactate desidrogenase

LIA: imunoensaio quimioluminescente (“chemiluminescence immunoassay”)

LPA: ácido lisofosfatídico

LPS: lipopolissacarídeo

NF-kB: fator nuclear kapa B

Oct: octanol

PKA: proteína cinase A

PLC: fosfolipase C

PMSF: fluoreto de fenilmetanosulfonil

PVP-40: polivinilpirrolidona

RAGE: receptor para produtos terminais de glicação (“receptor for advanced glycation end products”)

SERCA: Ca^{2+} - ATPase do retículo sarco/endoplasmático

SFB: soro fetal bovino

SOC: canal de cálcio operado por estoque

TNF-α: fator de necrose tumoral alfa

VOC: canal de cálcio operado por voltagem

Introdução

1. Sistema nervoso central e proteínas S100

As proteínas S100 foram isoladas pela primeira vez em 1965 no tecido encefálico e foram assim denominadas por serem solúveis em solução 100% saturada de sulfato de amônio (Moore 1965). Até o presente momento já foram caracterizadas 25 proteínas pertencentes à família S100 (Donato *et al.* 2009). Essas proteínas são ligantes de cálcio do tipo EF-hand e são expressas de forma específica nos diferentes tipos celulares (Donato 2001, Goncalves *et al.* 2008). Por exemplo, no sistema nervoso central de mamíferos, a S100A1 é expressa em neurônios enquanto que a S100B é expressa em astrócitos.

As proteínas S100 apresentam uma grande diversidade de funções intra e extracelulares, como, por exemplo, a regulação do metabolismo energético, do ciclo celular, da inflamação, da coagulação e modulação de proteínas do citoesqueleto (Donato 2001).

1.1. Astrócitos e a proteína S100B

Os astrócitos são células gliais que, por muito tempo, foram considerados como sendo apenas um suporte estrutural para os neurônios. Há algumas décadas que essa visão está mudando, devido à descoberta de inúmeras outras funções desse tipo celular (Kimelberg & Norenberg 1989). Os astrócitos possuem um papel de suporte energético para os neurônios (Magistretti 2006, Magistretti & Pellerin 1999), sendo inclusive capazes de modular as sinapses (Perea & Araque 2006) através da liberação de glutamato e ATP, por exemplo. Atualmente, os astrócitos são considerados parte integrante das

sinapses (Perea *et al.* 2009). Dessa forma, neurônio e astrócitos formam uma unidade multifuncional no cérebro (Fellin & Carmignoto 2004, Kirchhoff *et al.* 2001). Além disso, os astrócitos fazem parte da barreira hematoencefálica, participam da manutenção da homeostase iônica cerebral e são responsáveis por captar neurotransmissores da fenda sináptica (Wang & Bordey 2008).

Os astrócitos são as células que expressam S100B em maior quantidade no sistema nervoso central. A S100B foi a primeira proteína da família S100 a ser isolada, juntamente com a S100A1, a partir de cérebro bovino. Essa proteína se apresenta na forma de homodímeros compostos por duas subunidades beta, cada uma contendo dois sítios de ligação para o cálcio (Donato 2001).

1.1.1. Células que expressam e secretam S100B

A proteína S100B foi inicialmente caracterizada como sendo uma proteína expressa especificamente no cérebro (Moore 1965) e por muito tempo foi considerada como sendo expressa exclusivamente em astrócitos. Entretanto, outros tipos celulares também expressam S100B, inclusive no sistema nervoso central (Goncalves et al. 2008).

Os astrócitos são as células que expressam S100B em maior quantidade no sistema nervoso central e também são capazes de secretar essa proteína de uma forma regulada (Goncalves et al. 2008). Por muito tempo a S100B foi considerada como uma proteína marcadora de astrócitos, mas esse conceito está sendo mudado, visto que outras células no sistema nervoso central também podem expressar S100B, como alguns neurônios colinérgicos (Yang *et al.* 1995), oligodendrócitos (Vives *et al.* 2003, Deloulme *et al.* 2004) e microglia (Adami *et al.* 2001). Além dos astrócitos, nenhum desses tipos

celulares é capaz de secretar S100B. Em um estudo com a linhagem OLN-93 de oligodendrócito, foi observada uma liberação de S100B para o meio extracelular (Steiner *et al.* 2008), bem como em estudos em células de melanoma (Donato 2001). No entanto, a liberação de S100B por outras células do sistema nervoso central, diferentes dos astrócitos, parece ocorrer vinculada à morte celular, o que não indica uma secreção regulada e sim uma liberação por rompimento de membrana celular (Goncalves et al. 2008).

A S100B originada do sistema nervoso central é capaz de atravessar a barreira hematoencefálica e contribuir para a concentração sérica dessa proteína. A concentração de S100B no soro é bastante baixa, na ordem de 0,05 ng/mL em humanos adultos saudáveis (Leite *et al.* 2008). Dessa forma, a S100B pode ser considerada como marcadora de lesão cerebral (Goncalves et al. 2008) e de dano na barreira hematoencefálica (Marchi *et al.* 2004).

A S100B é eliminada através da urina, mas em concentrações muito baixas, podendo ser detectada apenas em urina de crianças recém-nascidas (Leite et al. 2008, Goncalves et al. 2008), que possuem concentração de S100B no soro cerca de dez vezes maior que em adultos (Netto *et al.* 2005, Portela *et al.* 2002). O aumento de S100B na urina tem sido estudado como marcador de lesão com valor prognóstico em recém-nascidos (Gazzolo *et al.* 2009, Liu *et al.* 2010, Michetti & Gazzolo 2002).

Existem também fontes extracerebrais de S100B. Alguns tipos celulares como condrócitos, cardiomiócitos, células da medula óssea, linfócitos (Jonsson *et al.* 1999, Anderson *et al.* 2001) e células de melanoma (Harpio & Einarsson 2004) também expressam S100B. Os adipócitos não apenas expressam, mas também são capazes de

secretar S100B, contribuindo para a concentração sanguínea de S100B (Schulpis *et al.* 2007, Netto *et al.* 2006, Steiner *et al.* 2009a, Holtkamp *et al.* 2008), o que pode dificultar o uso dessa proteína como marcadora de lesão cerebral (Goncalves et al. 2008). Além disso, a S100B também pode ser encontrada no leite humano (Gazzolo *et al.* 2003).

1.1.2. Papéis intra e extracelulares da S100B

As proteínas da família S100, incluindo a S100B, possuem tanto funções intracelulares como também podem ser secretada e exercerem funções no meio extracelular (Donato 2003). Dentre as funções intracelulares da S100B podemos destacar a inibição da fosforilação protéica, regulação da atividade enzimática, modulação de citoesqueleto e regulação do crescimento e diferenciação celular. Por exemplo, a S100B é capaz de inibir a fosforilação das proteínas de citoesqueleto GFAP e vimentina (Ziegler *et al.* 1998, Frizzo *et al.* 2004), bem como de estimular a atividade da proteína fosfatase calcineurina (Leal *et al.* 2004).

As atividades extracelulares da S100B são bastante variadas. Estudos *in vitro* demonstraram efeitos tróficos da S100B quando em concentrações baixas, variando de altos valores na faixa de picomolar até baixos valores na faixa de nanomolar. Esses efeitos incluem aumento da sobrevivência neuronal, do crescimento de neuritos e da função sináptica. Por outro lado, concentrações mais altas de S100B, na faixa de altos valores de nanomolar e baixos valores na faixa de micromolar, podem causar disfunção e morte neuronal. Esses efeitos tóxicos são mediados pela ativação de astrócitos e microglia, que passam a produzir e secretar citocinas inflamatórias, como IL-1β e TNF-α, bem como NO (Van Eldik & Wainwright 2003). É importante ressaltar que esses estudos

foram realizados *in vitro* e que ainda não foi demonstrada uma correlação com valores de concentração de S100B observados *in vivo*.

A S100B secretada pode ter uma ação autócrina ou parácrina. Ela pode agir sobre astrócitos, estimulando a captação de glutamato (Tramontina *et al.* 2006), bem como agir sobre a microglia, potencializando o efeito do IFN-γ sobre a iNOS (Adami et al. 2001). O efeito da S100B extracelular pode ser diferente dependendo do tipo celular em que ela tem efeito estimulatório. Um estudo comparativo demonstrou que a S100B é capaz de potencializar a produção de NO estimulada por LPS em células BV-2 (uma linhagem de células de microglia); entretanto, esse efeito não é observado em astrócitos (Petrova *et al.* 2000). Além disso, a S100B é capaz de aumentar a expressão da COX-2, tendo efeito sinérgico com IL-1β e TNF-α, em microglia por um mecanismo envolvendo o fator de transcrição NF-kB (Bianchi *et al.* 2008).

Apesar de se conhecer muitos efeitos extracelulares da proteína S100B, pouco se sabe sobre os mecanismos de transdução de sinal envolvidos na sua ação. Até o presente momento não foi caracterizado nenhum receptor ou transportador específico para a S100B. O que se sabe é que ela é capaz de ligar no RAGE e que essa ligação é responsável por muitos de seus efeitos (Bianchi et al. 2008, Hofmann *et al.* 1999, Donato 2007). Já foi demonstrado que o receptor RAGE pode mediar tanto os efeitos tróficos quanto os efeitos tóxicos da S100B, ativando diferentes vias de sinalização intracelular, e que essa ativação depende da concentração de S100B extracelular (Donato et al. 2009).

1.1.3. Moduladores da secreção de S100B

Alterações na concentração de S100B no meio extracelular estão relacionadas tanto com o desenvolvimento normal (Tramontina *et al.* 2002), quanto com condições patológicas, como a esquizofrenia (Steiner *et al.* 2009b, Pedersen *et al.* 2008), a depressão maior (Zhang *et al.* 2009) e a doença bipolar (Andreazza *et al.* 2007), bem como doenças neurodegenerativas. Estudos mostram um aumento de S100B no líquido cefalorraquidiano de pacientes com a doença de Alzheimer (Peskind *et al.* 2001, Petzold *et al.* 2003) ou com a Síndrome de Down (Netto *et al.* 2005).

Estudos em culturas de células já identificaram muitos moduladores da secreção de S100B, como amônia (Leite *et al.* 2006), serotonina (Whitaker-Azmitia *et al.* 1990), fluoxetina (Tramontina *et al.* 2008), beta hidroxibutirato (Leite *et al.* 2004), altas concentrações de glicose (Nardin *et al.* 2007), glutamato (Ciccarelli *et al.* 1999, Goncalves *et al.* 2002), bloqueadores de junção gap (Leite *et al.* 2009), resveratrol (de Almeida *et al.* 2007), fármacos neurolépticos atípicos (Quincozes-Santos *et al.* 2008), IL1-β (de Souza *et al.* 2009), estresse metabólico (Gerlach *et al.* 2006), forskolina e LPA (Pinto *et al.* 2000).

Estudos *in vivo* também demonstraram alterações da concentração sérica de S100B ou no líquido cefalorraquidiano de S100B no jejum (Netto et al. 2006), em ratos tratados com dieta cetogênica (Ziegler *et al.* 2004), em modelos de demência (Rodrigues *et al.* 2009, Swarowsky *et al.* 2008, Vicente *et al.* 2009) e em filhotes de ratas intoxicadas com mercúrio (Vicente *et al.* 2004).

1.1.4. Mecanismo de secreção da S100B

A secreção protéica pode ser classificada em dois grandes grupos: secreção clássica e secreção não-clássica. No mecanismo de secreção clássico, a proteína é sintetizada e direcionada para o retículo endoplasmático através de uma seqüência peptídica sinalizadora específica em sua estrutura. A proteína é então encaminhada para o complexo de Golgi, de onde é exportada na forma de vesículas (Nickel 2003).

Já no mecanismo de secreção não-clássico, não há o envolvimento do sistema retículo endoplasmático/complexo de Golgi (Nickel 2003). Existem diferentes mecanismos já identificados para esse tipo de secreção, como o "flip-flop" de membrana, o "blebbing" de membrana, a secreção através de transportadores específicos e por vesículas endolisossômicas (Nickel 2003, Prudovsky *et al.* 2008). Atualmente já foram identificadas proteínas que, apesar de conterem a seqüência peptídica sinalizadora para a secreção clássica, não são secretadas via complexo de Golgi e, portanto, seu mecanismo de secreção também é considerado como sendo não-clássico (Nickel & Rabouille 2009). Apesar de muitos mecanismos já terem sido identificados, pouco se sabe sobre as vias de sinalização que modulam a secreção não-clássica (Prudovsky et al. 2008).

O mecanismo de secreção de S100B ainda não foi completamente elucidado; entretanto, sabe-se que sua secreção é via um mecanismo não clássico, ou seja, independente do complexo retículo endoplasmático/complexo de Golgi, visto que a S100B não possui a seqüência peptídica sinalizadora para a secreção clássica (Donato 2001). Além disso, um estudo em glioblastomas demonstrou que a S100B é secretada por um mecanismo vesicular e dependente da concentração de cálcio intracelular (Davey *et al.* 2001). É importante ressaltar que esse estudo foi realizado em linhagens celulares, que

por serem células transformadas podem apresentar mecanismos de secreção diferentes de células não tumorais. Até o presente momento nenhum estudo sobre o mecanismo de secreção de S100B foi realizado em astrócitos.

1.1.5. Dosagem de S100B

Atualmente existem muitos métodos diferentes para a dosagem de S100B, que variam desde técnicas caseiras (Green *et al.* 1997, Leite et al. 2008, Missler *et al.* 1995, Van Eldik & Griffin 1994) até kits comerciais. Todos os métodos disponíveis são imunoquímicos, com a forma de detecção empregada podendo ser enzimática (ELISA), fluorescente (IFMA), radiométrica (IRMA) ou quimioluminescente (ECLIA e LIA), dependendo da metodologia. Essas variações de metodologias ocasionam diferenças quanto à sensibilidade, anticorpos utilizados, reações cruzadas e interferentes de cada técnica, tornando muitas vezes difícil a comparação de resultados de diferentes grupos de pesquisa no que se refere à concentração de S100B em fluidos biológicos (Goncalves et al. 2008).

2. Comunicação entre astrócitos

2.1. Junções gap

A junção gap é um tipo de junção comunicante que une o citoplasma de duas células deixando passar de uma forma específica e regulada alguns compostos de uma célula para outra.

2.1.1. Estrutura e permeabilidade

As junções gap são formadas por proteínas transmembrana chamadas conexinas. Essas proteínas se agrupam em hexâmeros, que são chamados de conexons. Esses conexons formam um poro na membrana plasmática. Quando dois conexons se ligam não-covalentemente a junção gap está formada (Yeager & Harris 2007). A distância entre as duas células que participam da junção gap é apenas de 2-4 nm e o tamanho do poro formado é de cerca de 1 kDa (Mese *et al.* 2007). Sendo assim, compostos como glicose, glicose 6-fosfato, inositol trisfosfato, glutamato, glutamina, ATP, ADP, AMP e glutationa são capazes de passar de uma célula a outra através de junções gap (Tabernero *et al.* 2006).

Existem pelo menos 21 isoformas de conexinas no genoma humano. As junções gap podem ser formadas por apenas uma ou por várias isoformas, podendo ser classificadas como homoméricas ou heteroméricas (se as conexinas de um mesmo conexon são todas iguais ou se são diferentes, repectivamente) e homotípicas ou heterotípicas (se os dois conexons são idênticos ou se são diferentes entre si, respectivamente) (Mese et al. 2007). As isoformas de conexinas variam de acordo com o tipo celular, não sendo específicas de apenas um tipo celular. No sistema nervoso central, os neurônios expressam predominantemente Cx32 e Cx43 e os astrócitos expressam predominantemente Cx43, podendo também expressa Cx30, entre outras (Rouach *et al.* 2002, Rozental *et al.* 2000). A expressão de conexinas varia ontogeneticamente e também em algumas condições patológicas (Rouach et al. 2002).

2.1.2. Astrócitos e junções gap

A comunicação por junção gap no sistema nervoso central ocorre em grande quantidade nos astrócitos, formando uma verdadeira rede de comunicação entre esse tipo celular (Rouach et al. 2002, Rozental et al. 2000). Essas células podem formar junções gap do tipo astrócito-astrócito, astrócito-oligodendrócitos (Orthmann-Murphy *et al.* 2008) e até mesmo astrócito-neurônio (Froes *et al.* 1999). Os astrócitos podem até mesmo formar junções gap dentro da mesma célula, junções gap autocelulares, que unem diferentes prolongamentos celulares (Wolff *et al.* 1998). A função desse tipo de junção gap ainda não foi completamente elucidada, mas acredita-se que está envolvida com a facilitação da homeostase de íons e mensageiros intracelulares entre diferentes processos, independente de uma regulação do corpo celular (Rouach et al. 2002).

2.1.3. Hemicanais

Alguns conexons não formam junções gap; entretanto, permanecem funcionais na membrana celular, comunicando o meio intracelular com o meio extracelular. Esses canais são chamados hemicanais e estão presentes em astrócitos (Ye *et al.* 2003, Saez *et al.* 2003).

Pouco se sabe sobre a modulação dos hemicanais, apesar de já ter sido demonstrado que parecem ser regulados pela concentração de cálcio extracelular. Baixas concentrações de cálcio extracelular em culturas de astrócitos são capazes de abrir os hemicanais, liberando glutamato (Bennett *et al.* 2003, Ye et al. 2003) e também aminoácidos e glutationa (Rana & Dringen 2007, Stridh *et al.* 2008) para o meio extracelular.

2.1.4. Regulação metabólica e junção gap

Os astrócitos agem de forma conjunta com os neurônios, participando das sinapses e fornecendo suporte energético para que a atividade sináptica ocorra de maneira ideal. Os astrócitos liberam lactato para servir como fonte energética para os neurônios. Além disso, nas sinapses glutamatérgicas, os astrócitos captam o glutamato liberado na fenda sináptica, convertem em glutamina (pela ação da enzima glutamina sintetase), que é liberada para os neurônios, onde é novamente convertida em glutamato (pela ação da enzima glutaminase), fechando o chamado ciclo glutamato-glutamina (Magistretti 2006, Magistretti & Pellerin 1999, Pellerin 2008, Pellerin *et al.* 2007).

Essa cooperação dos astrócitos com os neurônios sempre foi, por muito tempo, atribuída a uma única célula glial interagindo com a sinapse, visto que um único astrócito pode emitir prolongamentos para mais de uma sinapse. Por outro lado, estudos mostram que a comunicação por junções gap está intimamente envolvida nesse processo, formando redes astrocíticas que comunicam os vasos, através dos pés terminais, com as sinapses, criando um fluxo de energia e uma melhor interação metabólica entre astrócitos e neurônios (Tabernero et al. 2006, Kirchhoff *et al.* 2001).

Além da comunicação por junção gap nos astróciots ser importante para a relação dessas células com os neurônios, essa rede formada pelos astrócitos também é de extrema importância para o metabolismo astrocítico. A inibição da comunicação por junção gap em astrócitos ocasiona um aumento na captação e no consumo de glicose, que é desviada para o ciclo das pentoses fosfato, fornecendo precursores para a síntese de DNA. Nessa situação ocorre um aumento na taxa de proliferação celular (Tabernero et al. 2006).

2.1.5. Regulação da junção gap

Sabe-se que a atividade neuronal regula a comunicação por junção gap (Kirchhoff et al. 2001, Rouach *et al.* 2004). Um estudo em cultura de astrócitos mostrou que ocorre uma facilitação na comunicação por junção gap e uma maior propagação das ondas de cálcio quando os astrócitos são cultivados juntos com neurônios (Rouach *et al.* 2000). Por muito tempo essa rede formada por astrócitos comunicados por junções gap foi chamada de "sinsício astrocítico", expressão que está sendo gradativamente substituída por rede astrocítica, visto que a comunicação por junção gap é bastante dinâmica e as células envolvidas nessa rede podem rapidamente ser alteradas devido a mudanças na atividade neuronal (Rouach *et al.* 2008), fornecendo assim um melhor suporte energético.

De fato, a inibição da comunicação por junção gap, tanto em co-culturas de astrócitos e neurônios quanto em culturas organotípicas, leva a um aumento da vulnerabilidade neuronal a um dano oxidativo (Blanc *et al.* 1998) e também à neurotoxicidade mediada por glutamato em um mecanismo que não altera a captação de glutamato (Ozog *et al.* 2002). Entretanto, em culturas puras de astrócitos, o bloqueio da comunicação por junção gap induz uma redução da passagem do sinal apoptótico induzido por depleção de ATP. É importante ressaltar que o sinal de início da apoptose nesse caso continua, sem ser transmitido para as células vizinhas, visto que a comunicação por junção gap está bloqueada, ocasionando uma menor morte celular (Nodin *et al.* 2005).

Muitos são os estímulos capazes de alterar a comunicação por junção gap em astrócitos, o que torna o estudo desse tipo de comunicação de extrema importância nos processos patológicos do sistema nervoso central. Aumentos de cálcio intracelular são

capazes de bloquear a comunicação por junção gap em astrócitos (Enkvist & McCarthy 1994), bem como aumentos de TNF-α e IL-1β (Haghikia *et al.* 2008, Meme *et al.* 2006). De fato, estudos mostram que processos inflamatórios podem modular a comunicação por junção gap (Kielian 2008, Kielian & Esen 2004). O glutamato aumenta essa comunicação (Enkvist & McCarthy 1994) e sabe-se que algumas proteínas ligantes de cálcio podem se ligar *in vitro* a polipeptídeos purificados de junções gap (Van Eldik *et al.* 1985). Apesar disso, ainda não foi demonstrado um efeito direto da S100B modulando junções gap como também um efeito da comunicação por junção gap sobre a secreção de S100B.

2.2. Sinalização por cálcio

O cálcio é um íon divalente que funciona como mensageiro intracelular, participando de uma diversidade de funções celulares, como proliferação, diferenciação e exocitose em diferentes tipos celulares (Berridge 2009) e que, em altas concentrações, pode sinalizar para a morte celular (Celsi *et al.* 2009). Dessa forma, em situação de repouso, o cálcio intracelular é mantido em concentrações em torno de 100 nM, enquanto que a concentração de cálcio extracelular é em torno de 1 mM. A célula possui uma diversidade de mecanismos e organelas envolvidas na manutenção desse gradiente, bem como nas variações de concentração de cálcio intracelulares quando em situações de estímulo (Rizzuto & Pozzan 2006).

2.2.1. Canais e trocadores de cálcio

A concentração de cálcio livre intracelular é mantida por transportadores presentes na membrana plasmática e também na membrana das organelas envolvidas no

estoque de cálcio. O gradiente de cálcio entre o meio intra e extracelular na ausência de estímulo é mantido essencialmente por proteínas da membrana plasmática, como a Ca^{2+}-ATPase, que bombeia Ca^{2+} para fora da célula com gasto de ATP, e o trocador Na^{+}/Ca^{2+}, que depende do funcionamento da Na^{+}/K^{+}-ATPase (Rizzuto & Pozzan 2006). Na membrana plasmática também estão os chamados canais operados por estoque (SOCs), que tem uma relação íntima com o retículo endoplasmático e são responsáveis por repor o cálcio do retículo logo após um estímulo (Berridge 2004). Outros canais de cálcio presentes na membrana plasmática são os canais de cálcio dependente de voltagem (VOCs) que, quando ativados, permitem a entrada de cálcio do meio extracelular para o meio intracelular (Rizzuto & Pozzan 2006). Em astrócitos, os canais do tipo L estão presentes em grande quantidade na membrana plasmática.

O retículo endoplasmático, por ser um importante reservatório de cálcio, possui transportadores específicos. Para a liberação dos estoques de cálcio dessa organela, existem os canais ativados por rianodina e os canais ativados por IP_3, mais comuns em astrócitos. Por outro lado, para a captação do cálcio intracelular pelo retículo, existe a bomba Ca^{2+} - ATPase do retículo sarco/endoplasmático (SERCA) (Rizzuto & Pozzan 2006). Essa bomba é inibida por tapsigargina.

2.2.2. Estoques intracelulares de cálcio

Existem organelas que estocam cálcio, como o retículo endoplasmático (Berridge 2002), a mitocôndria (Pizzo & Pozzan 2007), os endossomos, o complexo de Golgi e os lisossomos (Rizzuto & Pozzan 2006). O retículo endoplasmático é uma das organelas que não somente estoca cálcio, como também é responsável pela sinalização por cálcio. Em

astrócitos, o mecanismo de liberação de cálcio desse estoque ocorre principalmente por ativação da PLC, o que leva à produção de IP_3. O IP_3 se liga a canais de cálcio presentes na membrana do retículo endoplasmática e ativa a liberação de cálcio para o meio intracelular (Berridge 2002, Nowycky & Thomas 2002).

A mitocôndria também é um importante estoque de cálcio intracelular, sendo responsável por captar o cálcio quando esse se encontra em altas concentrações. Além disso, a função do retículo endoplasmático está intimamente conectada com a da mitocôndria, ocasionando um fluxo de cálcio entre essas organelas. A mitocôndria capta o excesso de cálcio livre e é capaz de repassar esse cálcio para o retículo em condições normais. O retículo pode, por sua vez, sinalizar através da liberação de cálcio para a abertura de poros na mitocôndria que levam à apoptose em situações de dano (Berridge 2002, Pizzo & Pozzan 2007).

2.2.3. Ondas de cálcio

Os astrócitos são células não excitáveis capazes de se comunicar por um fenômeno chamado ondas de cálcio. Quando um astrócito é estimulado, ocorre um aumento na sua concentração de cálcio intracelular e esse aumento é passado para células adjacentes, que não foram diretamente estimuladas, em um intervalo de milisegundos (Scemes & Giaume 2006). Essas ondas de cálcio fazem com que um maior número de células responda a um estímulo e que esse estímulo gere respostas em células mais distantes. As ondas de cálcio podem ser disparadas pela atividade neuronal e, como conseqüência, modulam a atividade sináptica, secretando glutamato, por exemplo

(Araque *et al.* 1998b, Araque *et al.* 1998a). Ou seja, as ondas de cálcio permitem uma comunicação bidirecional entre astrócitos e neurônios (Araque *et al.* 2001).

Existem dois mecanismos não mutuamente exclusivos que explicam como ocorrem as ondas de cálcio em astrócitos. O primeiro mecanismo ocorre por uma sinalização intracelular envolvendo a comunicação por junções gap. Nesse caso, o aumento de cálcio se propaga para as células adjacentes através da passagem do próprio cálcio, mas principalmente de IP_3 pelas junções gap. Dessa forma, o IP_3 sinalizará para que os estoques de cálcio do retículo endoplasmático sejam liberados na célula vizinha e assim sucessivamente por todas as células conectadas por junções gap (Scemes & Giaume 2006, Bennett et al. 2003).

O outro mecanismo ocorre através da sinalização extracelular, quando a célula estimulada secreta ATP, que através da ligação em receptores específicos sinaliza para a liberação dos estoques intracelulares de cálcio em células próximas. O principal mecanismo de secreção de ATP parece ser vesicular (Bowser & Khakh 2007), embora ele também possa ser liberado através de hemicanais (Bennett et al. 2003).

Ainda existe muita controvérsia sobre qual mecanismo é mais importante para a formação das ondas de cálcio, mas o que parece é que eles atuam em conjunto (Araque et al. 2001, Bennett et al. 2003) ou ainda que o mecanismo predominante possa variar de acordo com o estímulo (Suadicani *et al.* 2004). Em linhagens de glioblastoma, há evidências de que a mobilização de Ca^{2+} está envolvida com a secreção de S100B (Davey et al. 2001), porém nenhum estudo em astrócitos foi realizado até o presente momento.

Objetivos

Objetivo Geral

Estudar o efeito do bloqueio da comunicação por junção gap sobre a secreção de S100B em astrócitos e fatias agudas hipocampais, bem como o envolvimento da mobilização de cálcio intracelular no processo de secreção dessa proteína.

Objetivos Específicos

- Padronizar uma técnica para dosar S100B, que seja simples, sensível, amplamente aplicável e de baixo custo, possibilitando o estudo da secreção dessa proteína em pequenos intervalos de incubação.
- Estudar o efeito do bloqueio da comunicação por junção gap em astrócitos primários e fatias agudas hipocampais sobre a secreção de S100B.
- Estudar o efeito da mobilização de cálcio intracelular, especialmente do retículo endoplasmático, sobre a secreção de S100B estimulada por secretagogos clássicos dessa proteína, como a forscolina.

Parte II

Capítulo I

A simple, sensitive and widely applicable ELISA for S100B: Methodological features of the measurement of this glial protein.

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A simple, sensitive and widely applicable ELISA for S100B: Methodological features of the measurement of this glial protein

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Abstract

S100B expression, particularly extracellular S100B, is used as a parameter of glial activation and/or death in several situations of brain injury. Several immunoassays for S100B measurement are available, which differ with regard to specificity, sensitivity, sample application, and, of course, economic costs. We standardized two protocols for S100B measurement (range between 1.9 pg and 10 ng/mL) in human and rat samples from brain and adipose tissues, blood serum, cerebrospinal fluid, urine and cell culture. Abundance and secretion of this protein in adipose tissue reinforces the caution about its origin in blood serum. Interestingly, S100B recognition was affected by the redox status of the protein. This aspect should be considered in S100B measurement, assuming that oxidized and reduced forms possibly coexist *in vivo* and the equilibrium can be modified by oxidative stress of physiological or pathological conditions or even by obtaining sample conditions.

Keywords: Adipocyte; Astrocyte; Brain injury; ELISA; Oxidative stress; S100B protein

1. Introduction

The search for general and specific peripheral markers of brain damage has increased over the last 10 years. One putative marker is S100B, which is a calcium-binding protein, predominantly expressed by astrocytes in vertebrate brain (Donato, 2001; Marenholz et al., 2004). This protein is secreted (Van Eldik and Zimmer, 1987) and the extracellular effect of S100B, observed in neural cultures, depends on its concentration, since it is neurotrophic at nanomolar levels and apoptotic at micromolar levels (Donato, 2003; Van Eldik and Wainwright, 2003).

S100B expression, particularly extracellular S100B, is used as a parameter of glial activation and/or death in several situations of brain injury (Rothermundt et al., 2003; Kleindienst and Ross Bullock, 2006). Brain disorders associated with peripheral increments of S100B include traumatic brain damage (McKeating et al., 1998; Ingebrigtsen et al., 1999), brain ischemia (Kim et al., 1996; Missler et al., 1997; Wunderlich et al., 1999), neurodegenerative diseases (Green et al., 1997; Otto et al., 1997; Peskind et al., 2001; Netto et al., 2005, 2006) and psychiatric disorders (Rothermundt et al., 2001; Lara et al., 2001; Andreazza et al., 2007). The significance of these increases in S100B, however, is often unknown and debatable due to the possible extra brain sources of S100B or methodological problems surrounding the measurement of S100B.

Several immunoassays for S100B measurement are available, these are include commercial kits for immunoradiometric assay (IRMA) and immunoluminometric assay (LIA) from Sangtec, an enzyme-linked immunosorbent assay (ELISA) from CanAg and home made ELISAs that employ commercial antibodies (Missler et al., 1997; Green et al., 1997; Tramontina et al., 2000). All these available methods differ with regard to specificity, sensitivity, sample application, and, of course, economic costs.

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Our group has studied S100B protein in a number of physiological and pathological conditions, in cell cultures, brain and adipose tissues of rats and blood and cerebrospinal fluid (CSF) samples from humans. For these studies, we originally utilized an ELISA for S100B (Tramontina et al., 2000), based on Green et al. (1997). Reviewing the literature, it may be noted that a number of controversial aspects exist with regard to the use of S100B as a marker of brain damage, since several biochemical features of this protein and its physiological and pathological variations are not well characterized; furthermore, possible methodological pitfalls in the immunoassays could contribute to those discrepancies.

This study present and discuss two specific and sensitive protocols for S100B measurement, applicable to many biological samples. We found that the oxidation state affects the immunorecognition and that this aspect is relevant, assuming that oxidized and reduced forms possibly coexist *in vivo* and that the equilibrium can be modified in oxidative stress conditions.

2. Materials and methods

2.1. Material

Anti-S100B antibody (SH-B1, S2532, lot 115K4846), S100B protein (S6677), phenylmethyl-sulfonylfluoride (PMSF), ethylene glycol-bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetracetic acid (EGTA), 4-(2-hydroxyethyl)piperazine-1-ethanesulfonic acid (HEPES), carbonate–bicarbonate buffer, bovine serum albumin (BSA), albumin from chicken egg white grade II, polyvinylpyrrolidone-40 (PVP-40), Tween-20, Triton X-100, Sigma fast *O*-phenylenediamine dihydrochloride tablets sets (OPD), 1,4-dithio-DL-threitol (DTT) and other material for cell cultures were purchased from Sigma. Polyclonal anti-S100 antibody (Z0311, lot 00020766) and anti-rabbit peroxidase-conjugated anti-IgG (NA934V, lot 349195) were purchased from DAKO and Amersham, respectively. Fetal calf serum (FCS) was purchased from Cultilab (São Paulo, Brazil). Dulbecco's modified Eagle's medium (DMEM) was from Gibco and H_2O_2 was from MERCK. High-binding flat-bottomed plates from Greiner Bio-One were used in ELISA.

2.2. ELISA for S100B

Protocol 1: Plates were previously coated overnight at 4 °C with 100 μL of a fresh 1/1000 dilution of monoclonal anti-S100B in 50 mM carbonate–bicarbonate buffer (pH 9.5). Plates were washed three times with 200 μL of washing buffer (0.1% BSA in PBS containing 0.05% Tween-20). Blocking solution (2% BSA, 150 μL) were incubated for 1 h at room temperature. Plates were washed once with 200 μL of washing buffer. Then, 50 μL of 60 mM barbital or 50 mM Tris buffer (pH 8.6) containing, or not, 0.2 mM $CaCl_2$ plus 50 μl of samples (diluted with PBS containing 0.2% BSA) or standard curve (range from 0.0019 to 1 ng/mL) were incubated for 2 h at 37 °C on a warming plate. Plates were then washed three times with 200 μL of washing buffer. Polyclonal anti-S100B antibody, diluted 1/5000 in 0.5% BSA (100 μL), containing or not 0.2 mM $CaCl_2$ was then incubated for 30 min at 37 °C. Plates were washed three times with 200 μL of washing buffer. Then, 100 μL of anti-rabbit peroxidase-conjugated diluted 1/5000 in 0.5% BSA were incubated for 30 min at 37 °C. Plates were washed three times with 200 μL of washing buffer and once with 200 μL PBS. Finally, 200 μL of a fresh solution of Sigma Fast OPD were incubated in the dark, for 30 min at room temperature. Microplates were immediately read at 450 nm or 50 μL of 3 M HCl were added and plates were read at 492 nm. Values indicated in the figures refer to 492 nm reading. *Protocol 2*: All buffers and antibodies used in protocol 1 were also used in protocol 2. The main difference was that both polyclonal anti-S100B and anti-rabbit peroxidase-conjugated antibodies were incubated together, for 1 h at 37 °C, on a warming plate. Both antibodies were diluted 1/5000 in PBS containing 0.5% BSA and in the presence or absence of 0.2 mM $CaCl_2$. Using this protocol, the standard curve was linear and ranged from 0.019 to 10 ng/mL.

2.3. Human samples

Five milliliters of blood from 32 adult (older than 18 years; 21 males and 11 females) healthy volunteers were collected by venipuncture without anticoagulants. Serum was obtained by centrifugation at 3000 × *g* for 5 min and kept frozen at −70 °C (for up to 6 months) until the biochemical assay. These individuals did not have any history of major psychiatric disorders, dementia or cancer and were not on medication. Urine samples were collected from 13 children at the ages indicated in Fig. 6. Children did not have any history of major neurologic disorder, cancer or renal impairment and were not on medication. Urine samples were immediately frozen at −70 °C (for up to 1 month) until the ELISA for S100B. Samples of human peritumoral tissues were obtained by surgical resection from previously untreated patients at the Neurosurgical Unit of the Hospital de Clínicas de Porto Alegre. The local ethical committee approved this study, and all subjects (or parents) signed their informed consent before entering the study.

2.4. Rat samples

Male 10- or 60-day-old Wistar rats, from the local breeding colony, were anaesthetized by intraperitonial injection of a mixture of ketamine and xylazine (75 and 10 mg/kg, respectively). Animals were then positioned in a stereotactic holder and cerebrospinal fluid was obtained by cisterna magna puncture using an insulin syringe (27 gauge × 1/2 in. length). A maximum volume of 30 μL was collected in a 3 min period to minimize the risk of brainstem damage. Using a similar syringe, intracardiac puncture was carefully performed to obtain 1 mL of blood. Blood was transferred to an Eppendorf tube, which was rested for 5 min and then centrifuged (3000 × *g*) for another 5 min to obtain the serum fraction. CSF and serum samples were frozen (−20 °C) until further analysis. After decapitation, the hippocampi were dissected and cut into 0.4 mm slices with a McIlwain chopper. Epididymal fat pads were dissected out. Hippocampal and epididymal tissues were homogenized in phosphate-buffered saline

(pH 7.4), containing 1 mM EGTA and 1 mM PMSF and stored at −20 °C. Crude epididymal tissue homogenate was delipidated by mixing with Triton X-100 and sucrose at final concentrations of 0.5% and 30%, respectively, and centrifuged at 10,000 × *g* for 10 min (4 °C). All procedures with animals were in accordance with the NIH guidelines (USA) for the care and use of laboratory animals and were approved by the local authorities.

2.5. Cell cultures

Astrocyte culture: Primary astrocyte cultures from Wistar rats were prepared as previously described (Gottfried et al., 1999). Briefly, the cerebral cortex of newborn Wistar rats (1–2 days old) were removed and mechanically dissociated in Ca^{2+}- and Mg^{2+}-free balanced salt solution (BSS), pH 7.4, containing (in mM): 137 NaCl; 5.36 KCl; 7.9 $NaHCO_3$; 0.27 Na_2HPO_4; 1.1 KH_2PO_4 and 6.1 glucose. The cortex were cleaned of meninges and mechanically dissociated by sequential passage through a Pasteur pipette. After centrifugation at 1000 RPM for 5 min, the pellet was resuspended in DMEM (pH 7.6) supplemented with 8.39 mM HEPES, 23.8 mM $NaHCO_3$, 0.1% fungizone, 0.032% garamycin and 10% FCS. Cultures were maintained in DMEM containing 10% FCS in 5% CO_2/95% air at 37 °C and allowed to grow to confluence and used at 15 days *in vitro*. The medium was replaced by DMEM without serum for S100B measurement. *C6 glioma cell culture*: A rat glioblastoma cell line was obtained from the American Type Culture Collection (Rockville, Maryland, USA). Cells were seeded on 24-well plates at densities of 10^4 cells/well and cultured in DMEM, containing 5% FCS in 5% CO_2/95% air at 37 °C for 5 days. *Adipocyte culture*: Epididymal fat pads from 60-day-old rats were dissected out, incubated in BSS containing collagenase, and mechanically dissociated. The cell suspension was diluted in DMEM (pH 7.6), supplemented with 8.4 mM HEPES/24 mM $NaHCO_3$, seeded on 24-well plates at a density of 10^4 cells/well and maintained in a CO_2 incubator at 37 °C for 6 h (Marshall et al., 1983).

2.6. S100B oxidation procedures

S100B solution at 1 μg/mL was incubated, or not, with 0.1 mM H_2O_2, in the dark, for 30 min. Standard curves of S100B (from 0.015 to 0.25 ng/mL) were prepared and measured by ELISA (protocol 1). This first procedure is commonly used to induce oxidative stress in cell cultures or tissue slices. Another stronger procedure, utilizing cystein oxidation or cystein alkylation, was performed on purified S100B to investigate immunorecognition. A fresh solution of S100B (assumed to be non-oxidized S100B) was incubated with 5 mM of sodium tetrathionate in the presence of 1 mM $CaCl_2$ for 1h or alkylated with iodoacetamide (Scotto et al., 1998; Gonçalves et al., 2000). Protein was dialyzed against 20 mM Tris–HCl, pH 7.4 and analyzed by 11% SDS-PAGE, as described by Schägger and von Jagow (1987), for separation of S100B 10 kDa monomers (non-oxidized S100B) from 21 kDa dimeric S100B (oxidized S100B). Molecular weight standards of 2512, 6214, 8159, 14,404 and 16,949 Da were purchased from LKB.

2.7. Protein content

The total protein content was determined by the modified method of Lowry (Peterson, 1977), using BSA as standard.

3. Results

3.1. Performance characteristics of the protocols

As shown in Fig. 1, the S100B assay using protocols 1 and 2 covered concentration ranges of between 1.9 pg and 1 ng/mL and 19 pg and 10 ng/mL, respectively. The immunoassays were extremely linear, presenting equations of $y = 1.620x + 0.142$ and $R = 0.9875$ for protocol 1, and $y = 0.203x + 0.206$ and $R = 0.9796$, for protocol 2. The intra-assay and inter-assay coefficients of variance (CV) are indicated in Table 1.

3.2. Linearity for different samples

Linearity of the assays was examined by serial dilutions of different samples: brain and adipose tissues from rats for protocol 1; serum and CSF from humans for protocol 2. The *R* values were 0.996 and 0.998 for protocols 1 and 2, respectively. Val-

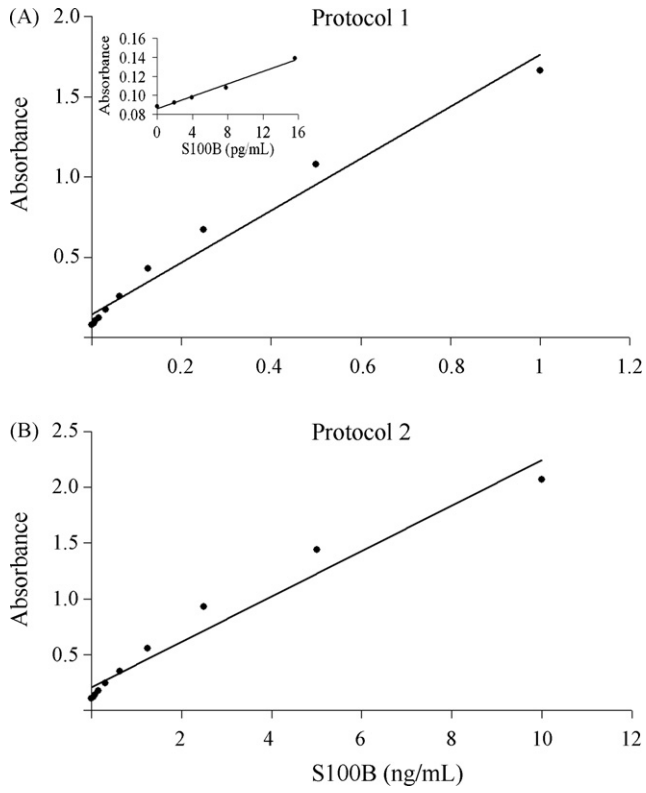

Fig. 1. Standard curves of S100B measured by ELISA. (A) Diluted samples of S100B (between 1.9 pg and 1 ng/mL) were measured using a three-step sandwich ELISA, according to the protocol 1 described in Section 2. Lower values are shown in the insert. (B) Diluted samples of S100B (between 19 pg and 10 ng/mL) were measured using a two-step sandwich ELISA, according to the protocol 2 described in Section 2. Absorbance values correspond to the means ± standard errors from 10 to 12 independent experiments. The intra-assay and inter-assay coefficients of variance (CV) are indicated in Table 1.

Table 1
Coefficients of variance (CV) in ELISA for S100B

ng/mL	Intra-CV (%) ($N=4$)	Inter-CV (%) ($N=10$)
Protocol 1		
0.015	2.5	4.9
0.03	3.6	7.3
Protocol 2		
0.15	3.2	6.4
0.3	2.8	5.5

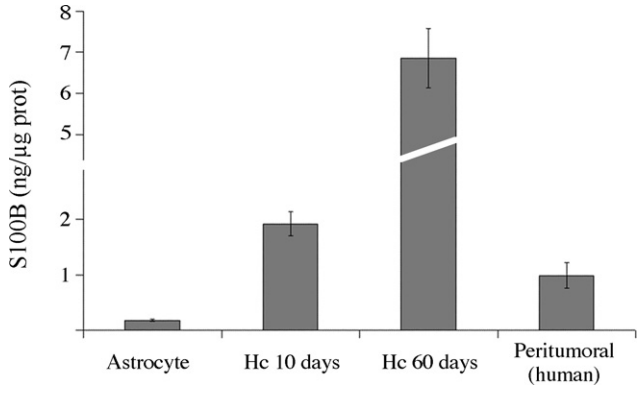

Fig. 2. S100B content in rat astrocyte cultures, rat hippocampus and human peritumoral tissue. S100B content was measured by ELISA (protocol 2) in primary astrocyte cultures prepared from the cerebral cortex of neonate rats (astrocyte, $N=6$); in hippocampal tissue of 10-day-old rats (Hc 10 days, $N=5$) and 60-day-old rats (Hc 60 days, $N=5$); and in peritumoral tissues from human brain obtained by surgical resection (peritumoral, $N=7$). Values (expressed in nanograms of S100B per microgram of protein) correspond to the means ± standard errors.

ues obtained in hippocampal tissue and astrocytes from rats, as well as peritumoral hippocampal tissue from humans are shown in Fig. 2. Values of S100B secretion (under serum-deprived conditions, for 6 h) in different cell types are also presented in Fig. 3.

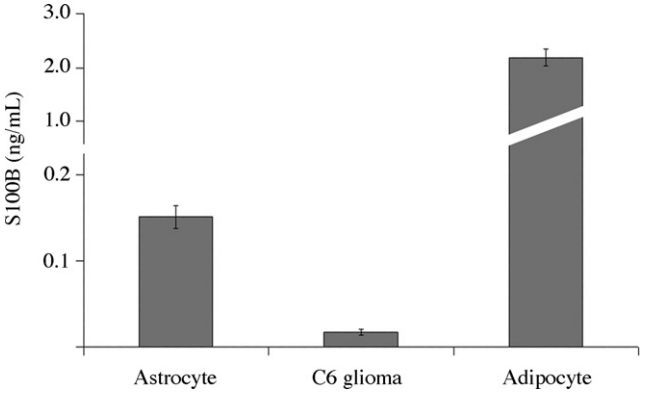

Fig. 3. S100B secretion in cell cultures. Extracellular S100B was measured by ELISA in culture medium of primary astrocytes from the cerebral cortex of neonate rats (astrocyte); C6 glioma cells obtained from the American Type Culture Collection (C6 glioma); and adipocytes prepared from epididimal adipose tissue from 60-day-old rats (adipocyte). Media samples were collected 6 h after serum deprivation. Values (expressed in nanograms of S100B per milliter of medium) correspond to the means ± standard errors, from three independent experiments.

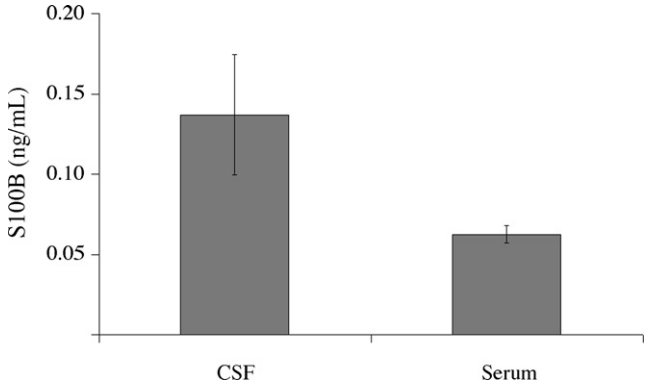

Fig. 4. Serum and cerebrospinal fluid S100B content in rats. Rats were anaesthetized and CSF was collected by cisterna magna puncture. Blood was colleted by intracardiac puncture and serum obtained by centrifugation. Serum and CSF S100B contents (from 11 to 12 rats) were measured by ELISA. Values (expressed in nanograms of S100B/mL) correspond to the means ± standard errors.

It should be noted that the S100B content in serum and CSF from rat samples was always detected (>1.9 pg/mL) (Fig. 4). Notice that the CSF/serum ratio for S100B (in rats) is approximately 3. This ratio dropped, however, to less than 1 when shed blood was collected after decapitation (data not shown). Similarly, S100B content in human serum was also always detected (Fig. 5A), but in the case of urine samples from children, S100B was not detected in 15% of samples ($N=13$, Fig. 5B).

3.3. Sample stability tests

Since serum and brain tissue S100B samples are commonly stored before measurement, the effect of freezing and thawing was examined. Serum, CSF and urine were immediately frozen at −20 °C and analyzed 24 h after. Brain tissue slices (diameter around 1 mm) were stored at −70 °C without the addition of protease inhibitors for 24 h, they were then thawed and homogenized in the presence of 1 mM PMSF, 1 mM EDTA and 1 mM EGTA. Values of homogenates from frozen or fresh tissue samples did not present significant differences (inter-assay CV of less than 8%). Results obtained for frozen and fresh samples of serum and CSF were also similar (data not shown).

3.4. Peroxide-induced oxidation and calcium-binding status of S100B

In a set of experiments, the S100B curve was prepared from a protein sample exposed to 0.1 mM H_2O_2 for 30 min (Fig. 6A). The immunoassay for oxidized S100B was also extremely linear between the range of 0.015 and 1 ng/mL S100B ($R=0.9969$). However, the angular coefficient changes after oxidation decreased by about 37%. DTT was not able to able to reverse this change (data not shown). Both antibodies for capture (SH-B1 clone from Sigma) and detection (polyclonal anti-S100 from DAKO) were not affected by the presence of calcium (data not shown).

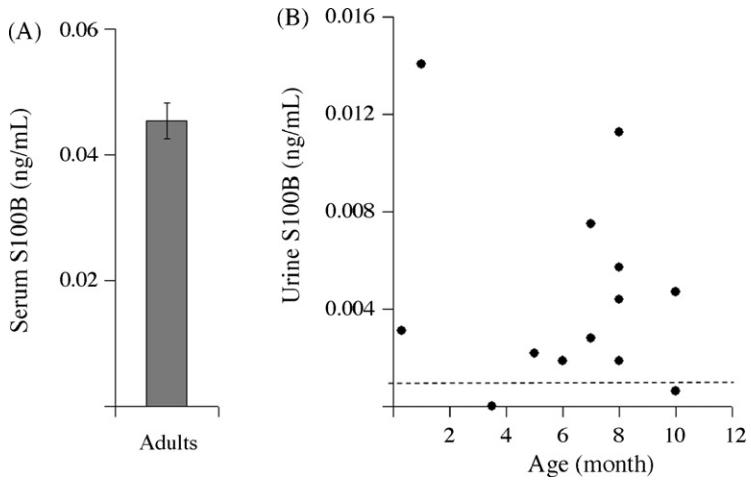

Fig. 5. Serum and urine S100B contents in humans. (A) Serum S100B in samples of humans (older than 18 years old, $N = 32$) were measured by ELISA (protocol 1). (B) Urine S100B in samples from children (at indicated age, $N = 13$) were measured by ELISA (protocol 1). Dashed line indicates the detection limit (1.9 pg/mL). Values (expressed in nanograms of S100B/mL) correspond to the means ± standard error.

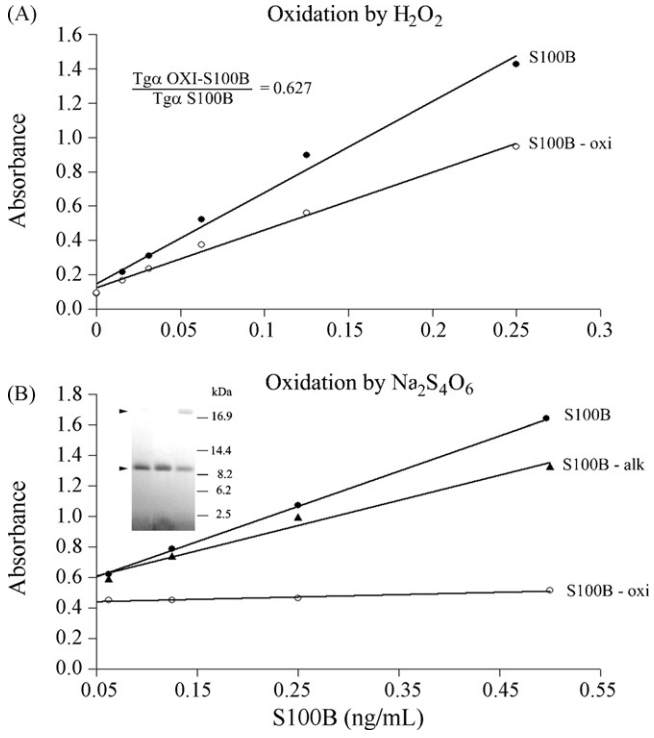

Fig. 6. Measurement of S100B in samples exposed to peroxide-induced oxidation. (A) Samples of S100B were incubated or not with 0.1 mM H_2O_2 for 30 min. After this procedure, S100B was measured by ELISA. Each value corresponds to the mean ± standard error from four independent experiments. Notice that linearity was maintained after oxidation, but the angular coefficient changed from basal (closed circles) to the oxidized (open circles) condition. (B) Non-oxidized (closed circles), alkylated (with iodoacetamide; triangles) and oxidized S100B (with sodium tetrathionate; open circles) were quantified by ELISA. SDS-PAGE of S100B samples is shown in the inset. Lanes 1, 2 and 3 correspond to non-oxidized, alkylated and oxidized S100B, stained with Comassie-blue, respectively. In the right margin are the positions of molecular weight standards. Arrowheads indicate dimeric (21 kDa) and monomeric (10 kDa) S100B. Notice that sensitivity to oxidized S100B strongly decreased. Each value corresponds to the mean ± standard error from three independent experiments.

3.5. Sodium tetrathionate-oxidation and iodoacetamide-alkylation of S100B

In order to confirm the altered antibody recognition, dependent on the oxidation state of S100B, we induced cystein oxidation with sodium tetrathionate and cystein alkylation with iodoacetamide. Comparative curves were prepared based on the content of oxidized and non-oxidized proteins, as evaluated by Comassie-blue staining. After oxidation assay about 50–60% of S100B consisted of disulfide-bonded dimers, in contrast to the non-oxidized or alkylated form, where about 90% were found as monomers. The absorbance curve for the immunoassay of S100B shows a clear difference in the angular coefficient between oxidized and non-oxidized S100B (Fig. 6B).

3.6. Delipidation of adipose tissue samples

Considering that S100B is abundant in adipose tissue and that lipid content can affect immunoassay we standardized a simple procedure for the delipidation of adipose tissue samples. Sample delipidation improved the accuracy of S100B measurement in these samples (Fig. 7).

3.7. Buffers and blockers

Additional buffers and blockers were investigated in order to reduce the economical costs of these assays. Tris–HCl buffer suitably replaced barbital buffer in both protocols; and PVP-40 and ovoalbumin at 2% efficiently replaced the blocker activity of 2% BSA. Non-fat powered milk cannot be used as a blocker in either of the ELISA protocols for S100B.

4. Discussion

There are many possible methods available for S100B measurement and, sometimes, contrasting results are enthusiastically discussed, ignoring or underestimating methodological

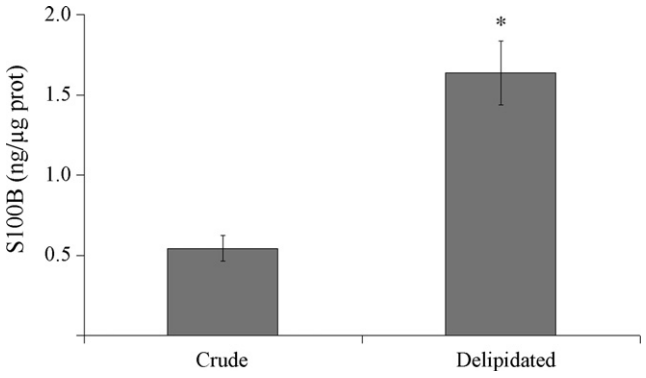

Fig. 7. Measurement of S100B in the adipose tissue of rats. Epididymal fat pads from 60-day-old rats were homogenized in phosphate-buffered saline solution (crude sample) and then delipidated with 0.5% Triton X-100 and 30% sucrose (delipidated sample), as described in Section 2. S100B content was measured in crude and delipidated samples by ELISA. Values (expressed in nanograms of S100B/μg of protein) correspond to the means ± standard errors. *, a significant increase was observed after delipidation (Student's *t* test, $p < 0.01$).

differences. All available methods are based on the antibody recognition of S100B protein, particularly with commercial antibodies. In theory, the use of these antibodies should facilitate the comparison of results from independent groups; however, a number of details in the methodological procedures could affect the specificity and affinity of S100B detection in the different biological samples, suggesting caution in the comparison of results.

Although the two ELISA protocols, described herein, have different sensitivities they are quite similar in the procedure steps and together allow a wide application for S100B containing samples. Protocol 1 was 10 times more sensitive than protocol 2, with similar reproducibility. Protocol 2 had a sensitivity and reproducibility that was comparable to the commercial kits available for S100B, particularly from Sangtec (Tort et al., 2003).

The S100B recognition in diverse samples, based on this assay, was not affected by freezing and thawing. Addition of common protease inhibitors (PMSF, EDTA and EGTA) to the homogenate tissue was enough to preserve protein stability. No protease inhibitors were added to serum, CSF or urine samples. Another interesting aspect of the assay was the use of blocking agent during. Both 2% ovoalbumin and PVP-40, instead of bovine serum albumin, efficiently blocked unspecific interactions in the immunoassay without altering the blank reading. However, confirming the presence of S100B in milk (Gazzolo et al., 2003), it was not possible to use non-fat milk as a blocking agent.

Post-translational modifications and/or non-covalent ligants are elements that interfere in the antibody–antigen interaction, however these modifications are often not fully considered during the elaboration and interpretation of immunoassays (Tramontina et al., 2007). S100B is a calcium-binding protein and its calcium-binding status greatly affects its antibody recognition (Gonçalves et al., 1997). Previously, we showed that the monoclonal antibody (SH-B1, from Sigma) used for capture in this assay is not affected by the presence of calcium (Gonçalves et al., 1997), but the secondary peroxidase-conjugated anti-S100 antibody (from DAKO), used for detection, had its recognition activity improved by Ca^{2+} addition (Green et al., 1997; Tramontina et al., 2000). Conversely, calcium-chelators improve the antigen recognition in the immunoassay with Sangtec antibodies (Tort et al., 2003). In this ELISA for S100B, neither of the antibodies (for capture and detection) was affected by the presence of calcium.

S100B interferes in the phosphorylation of many protein substrates (Donato, 2001), but it does not appear to be the target of protein kinases *in vivo*. Therefore, this aspect is not relevant for immunoassays. However, other feature is its redox state is a condition that could affect antibody recognition, as it interferes with the interaction to specific protein targets (Scotto et al., 1998; Gonçalves et al., 2000). In fact, we observed that incubation of S100B with 0.1 mM H_2O_2 for 30 min diminished its recognition. A vigorous oxidation of S100B, induced by sodium tetrathionate, significantly decreased recognition by the antibody.

All amino acids can be oxidized, but some of them are more susceptible to oxidation in physiological or pathological conditions, particularly cystein, methionine and tyrosine (Spickett et al., 2006). DTT was not able to recover the basal recognition of S100B, suggesting that the peroxide-induced oxidation was not limited to the disulfide bond formation. Moreover, the oxidized form of S100B, as occurs in some other proteins, is highly resistant to reducing agents, suggesting that *in vivo* reduced and oxidized forms are not in spontaneous equilibrium (Mely and Gérard, 1988). Therefore, this aspect needs to be considered in the immunoassay and in the interpretation of results, particularly when samples were exposed to oxidative stress, including the extraction process. It should be noted that EDTA, used in the preparation of the tissue sample, possibly contributes to diminish the metal-mediated oxidation of the protein. Moreover, S100B is a very soluble and interacting protein (Donato, 2001) and its recognition by the antibody could be affected by other neighbor proteins, independently of possible cross-immune reactions. We used a monoclonal anti-S100B that does not react with S100A1, a protein that is abundant in neural tissue. Anti-S100B antibodies from Sangtec have been reported to weakly react with S100A1 (Heizmann, 2004); moreover, their S100B recognition may be affected by neighboring proteins (Fazio et al., 2004), demanding caution in the measurement of this protein in tissue homogenates (Mazzini et al., 2005).

In our ELISA for S100B, we found a linear correlation in the dilutions of the analyzed samples, but delipidation was essential to ensure this correlation for adipose tissue samples. This may be due to the general interference of lipids in the hydrophobic interaction between antigen and antibody.

In summary, we have described to two specific and sensitive protocols for S100B measurement, applicable to many biological samples, including brain and adipose tissues, cell culture, cerebrospinal fluid, blood serum and urine. We observe that the oxidation state of S100B affects antibody recognition. This aspect is quite important and should be considered, assuming that oxidized and reduced forms may coexist *in vivo* and that the equilibrium can be modified by oxidative stress of physiological or pathological conditions or even by obtaining sample conditions.

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References

Andreazza AC, Cassini C, Rosa AR, Leite MC, de Almeida LM, Nardin P, et al. Serum S100B and antioxidant enzymes in bipolar patients. J Psychiatr Res 2007;41:523–9.

Donato R. S100: a multigenic family of calcium-modulated proteins of the EF-hand type with intracellular and extracellular functional roles. Int J Biochem Cell Biol 2001;33:637–68.

Donato R. Intracellular and extracellular roles of S100 proteins. Microsc Res Tech 2003;60:540–51.

Fazio V, Bhudia SK, Marchi N, Aumayr B, Janigro D. Peripheral detection of S100beta during cardiothoracic surgery: what are we really measuring? Ann Thorac Surg 2004;78:46–52.

Gazzolo D, Monego G, Corvino V, Bruschettini M, Bruschettini P, Zelano G, et al. Human milk contains S100B protein. Biochim Biophys Acta 2003;1619:209–12.

Gonçalves CA, Gottfried C, Kommers T, Rodnight R. Calcium-modulated proteins change their immunoreactivity in the presence of Ca^{2+}: a study of antibody recognition in a dot immunoassay for calmodulin, calcineurin (beta-subunit), and S100B. Anal Biochem 1997;253:127–30.

Gonçalves DS, Lenz G, Karl J, Gonçalves CA, Rodnight R. Extracellular S100B protein modulates ERK in astrocyte cultures. Neuroreport 2000;11:807–9.

Gottfried C, Valentim L, Salbego C, Karl J, Wofchuk ST, Rodnight R. Regulation of protein phosphorylation in astrocyte cultures by external calcium ions: specific effects on the phosphorylation of glial fibrillary acidic protein (GFAP), vimentin and heat shock protein 27 (HSP27). Brain Res 1999;833:142–9.

Green AJ, Keir G, Thompson EJ. A specific and sensitive ELISA for measuring S-100b in cerebrospinal fluid. J Immunol Methods 1997;205:35–41.

Heizmann CW. S100B protein in clinical diagnostics: assay specificity. Clin Chem 2004;50:249–51.

Ingebrigtsen T, Waterloo K, Jacobsen EA, Langbakk B, Romner B. Traumatic brain damage in minor head injury: relation of serum S-100 protein measurements to magnetic resonance imaging and neurobehavioral outcome. Neurosurgery 1999;45:468–75.

Kim JS, Yoon SS, Kim YH, Ryu JS. Serial measurement of interleukin-6, transforming growth factor-beta, and S-100 protein in patients with acute stroke. Stroke 1996;27:1553–7.

Kleindienst A, Ross Bullock M. A critical analysis of the role of the neurotrophic protein S100B in acute brain injury. J Neurotrauma 2006;2:1185–200.

Lara DR, Gama CS, Belmonte-de-Abreu P, Portela LV, Gonçalves CA, Fonseca M, et al. Increased serum S100B protein in schizophrenia: a study in medication-free patients. J Psychiatr Res 2001;35:11–4.

Marenholz I, Heizmann CW, Fritz G. S100 proteins in mouse and man: from evolution to function and pathology. Biochem Biophys Res Commun 2004;322:1111–22.

Marshall S, Garvey WT, Geller M. Primary culture of isolated adipocytes. J Biol Chem 1983;259:6376–84.

Mazzini GS, Schaf DV, Oliveira AR, Gonçalves CA, Belló-Klein A, Bordignon S, et al. The ischemic rat heart releases S100B. Life Sci 2005;77:882–9.

McKeating EG, Andrews PJ, Mascia L. Relationship of neuron specific enolase and protein S-100 concentrations in systemic and jugular venous serum to injury severity and outcome after traumatic brain injury. Acta Neurochir Suppl 1998;71:117–9.

Mely Y, Gérard D. Large-scale, one-step purification of oxidized and reduced forms of bovine brain S100b protein by HPLC. J Neurochem 1988;50:739–44.

Missler U, Wiesmann M, Friedrich C, Kaps M. S-100 protein and neuron-specific enolase concentrations in blood as indicators of infarction volume and prognosis in acute ischemic stroke. Stroke 1997;28:1956–60.

Netto CB, Portela LV, Ferreira CT, Kieling C, Matte U, Felix T, et al. Ontogenetic changes in serum S100B in Down syndrome patients. Clin Biochem 2005;38:433–5.

Netto CB, Portela LV, Felix TM, Souza DO, Goncçalves CA, Giugliani R. Serum S100B levels in patients with neural tube defects. Clin Chim Acta 2006;364:275–8.

Otto M, Stein H, Szudra A, Zerr I, Bodemer M, Gefeller O, et al. S-100 protein concentration in the cerebrospinal fluid of patients with Creutzfeldt-Jakob disease. J Neurol 1997;244:566–70.

Peskind ER, Griffin WS, Akama KT, Raskind MA, Van Eldik LJ. Cerebrospinal fluid S100B is elevated in the earlier stages of Alzheimer's disease. Neurochem Int 2001;39:409–13.

Peterson GL. A simplification of the protein assay method of Lowry et al. which is more generally applicable. Anal Biochem 1977;83:346–56.

Rothermundt M, Arolt V, Wiesmann M, Missler U, Peters M, Rudolf S, et al. S-100B is increased in melancholic but not in non-melancholic major depression. J Affect Disord 2001;66:89–93.

Rothermundt M, Peters M, Prehn JH, Arolt V. S100B in brain damage and neurodegeneration. Microsc Res Tech 2003;60:614–32.

Scotto C, Mely Y, Ohshima H, Garin J, Cochet C, Chambaz E, et al. Cysteine oxidation in the mitogenic S100B protein leads to changes in phosphorylation by catalytic CKII-alpha subunit. J Biol Chem 1998;273:3901–8.

Schägger H, von Jagow G. Tricine-sodium dodecyl sulfate-polyacrylamide gel electrophoresis for the separation of proteins in the range from 1 to 100 kDa. Anal Biochem 1987;166:368–79.

Spickett CM, Pitt AR, Morrice N, Kolch W. Proteomic analysis of phosphorylation, oxidation and nitrosylation in signal transduction. Biochim Biophys Acta 2006;1764:1823–41.

Tort AB, Dietrich MO, Goncalves CA, Souza DO, Portela LV. Influence of anticoagulants on the measurement of S100B protein in blood. Clin Biochem 2003;36:519–22.

Tramontina F, Karl J, Gottfried C, Mendez A, Gonçalves D, Portela LV, et al. Digitonin-permeabilization of astrocytes in culture monitored by trypan blue exclusion and loss of S100B by ELISA. Brain Res Protoc 2000;6:86–90.

Tramontina F, Leite MC, Cereser K, de Souza DF, Tramontina AC, Nardin P, et al. Immunoassay for glial fibrillary acidic protein: antigen recognition is affected by its phosphorylation state. J Neurosci Methods 2007;162:282–6.

Van Eldik LJ, Wainwright MS. The Janus face of glial-derived S100B: beneficial and detrimental functions in the brain. Restor Neurol Neurosci 2003;21:97–108.

Van Eldik LJ, Zimmer DB. Secretion of S-100 from rat C6 glioma cells. Brain Res 1987;436:367–70.

Wunderlich MT, Ebert AD, Kratz T, Goertler M, Jost S, Herrmann M. Early neurobehavioral outcome after stroke is related to release of neurobiochemica markers of brain damage. Stroke 1999;30:1190–5.

Capítulo II

Gap junction inhibitors modulate S100B secretion in astrocyte cultures and acute hippocampal slices.

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Gap Junction Inhibitors Modulate S100B Secretion in Astrocyte Cultures and Acute Hippocampal Slices

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Astrocytes sense, integrate, and respond to stimuli generated by neurons or neural injury; this response involves gap junction (GJ) communication. Neuronal vulnerability to injury increased when cocultures of astrocytes and neurons were exposed to GJ inhibitors. However, GJ uncoupling could limit the extension of a lesion. We investigated a possible link between GJ communication and S100B secretion. S100B is a calcium-binding protein of 21 kDa that is predominantly expressed and secreted by astrocytes, which has trophic paracrine activity on neurite growth, glial proliferation, and neuronal survival. GJ inhibitors were analyzed in isolated astrocytes in primary cultures from hippocampus, acute hippocampal slices, and C6 glioma cells, which were used as a negative control. Our data indicate that GJ blocking stimulates S100B secretion in astrocyte cultures and acute hippocampal slices. Different assays were used to confirm cell integrity during exposure to GJ inhibitors. S100B secretion was observed with different types of GJ inhibitors; the resulting event was dependent on time, the nature of the inhibitor, its putative molecular target of GJ blocking, and/or the cell preparation used. Only carbenoxolone induced a fast and persistent increase in S100B secretion in both preparations. Endothelin-1 increased S100B secretion in astrocyte cultures at 1 hr, but a decrease was observed at 6 hr or in acute hippocampal slices. Physiologically, a local GJ closure associated with release of S100B in injury conditions favors the idea of a common mechanism available to limit the extension of lesion and increase the chances of cell survival.

Key words: carbenoxolone; endothelin-1; flufenamic acid; gap junction; S100B

S100B is a calcium-binding protein of 21 kDa that is predominantly expressed and secreted by astrocytes in vertebrate brain (Marenholz et al., 2004). Intracellularly, many putative protein targets of S100B have been characterized, particularly modulating cytoskeleton plasticity and cell proliferation (Donato, 2001). At subnanomolar concentrations, extracellular S100B stimulates in vitro neurite growth, results in glial proliferation, and promotes neuronal survival (Van Eldik and Wainwright, 2003).

We have observed an accumulation of S100B in brain tissue, accompanied by a negative correlation of this protein in cerebrospinal fluid, during the development of rats (Feoli et al., 2008; Tramontina et al., 2002), as well as a negative correlation between intracellular content and secretion of S100B observed during the increment of cell density and confluence in culture preparations (Tramontina et al., 2002). Because evidence suggests an increase in gap junction (GJ) communication in astroglial cultures at confluence (Tabernero et al., 2006), we hypothesized that the decrease in basal S100B secretion, at confluence, could be associated with changes in this type of cell communication.

GJ are closely packed hemichannels formed by a hexameric pore of connexins that align themselves between neighboring cells head to head to form functional and regulated intercellular channels (see Kielian, 2008, for a review). Moreover, isolated hemichannels are able to release glutathione and amino acids (Stridh et al., 2008). GJ are developmental regulated, and the expression of connexins, particularly Cx43, has been associated with glial proliferation (Tabernero et al., 2006). The internal gating control of these channels is regulated by intracellular Ca^{2+}, pH, and protein kinases (Salameh and Dhein, 2005). However, much of the information regarding the protein kinases, particularly PKC and PKA, is still conflicting. A number of S100B secretagogues have been identified, but the underlying secretion mechanism remains unknown and involves a nonclassic vesicular export (Davey et al., 2001; Gon-

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calves et al., 2008). Some of these S100B secretagogues also act on GJ, e.g., forskolin and lysophosphatidic acid (Pinto et al., 2000). However, so far, a possible link between GJ and S100B secretion has not been addressed.

GJ have been extensively investigated in neural (and nonneural) cells by uncoupling chemicals, but generally, their effects on other ionic channels and molecular binding sites are not well characterized (Salameh and Dhein, 2005). Four classes of uncoupling GJ agents were used in this study: 1) small lipophilic agents (halothane and octanol) that apparently incorporate into the lipid bilayer and interfere in ionic channels, including GJ; 2) carbenoxolone (CBX), which affects connexins and is a synthetic drug derived from glycyrrhizic acid (GZA) that has no effect on GJ; 3) flufenamic acid (FFA), a nonsteroid anti-inflammatory molecules that independently inhibits cyclooxygenase-2 and GJ; and 4) endothelin-1, which in contrast to the other GJ blockers acts via specific signaling pathways on astrocytic GJ rather than at the connexin proteins directly. The action of these compounds has been characterized on Cx43, the most abundant astrocytic connexin in cell culture and brain tissue (Rozental et al., 2000; Saez et al., 2003; Tabernero et al., 2006).

To investigate a putative role of GJ in S100B secretion, we analyzed the effect of different classes of chemical inhibitors in three preparations from rat brain: primary cultures of astrocytes, acute hippocampal slices, and C6 glioma cells, which contain low levels of GJ channels when compared with astrocytes and which were used as a negative control.

MATERIALS AND METHODS

Materials

Poly-l-lysine anti-S100B antibody (SH-B1), octanol, GZA, FFA, endothelin-1, methylthiazolyldiphenyl-tetrazolium bromide, neutral red, and propidium iodide were purchased from Sigma. Fetal calf serum (FCS), Dulbecco's modified Eagle medium (DMEM), and other materials for cell culture were purchased from Gibco. Halothane was purchased from Cristalia. Polyclonal anti-S100B and anti-rabbit peroxidase linked were purchased from Dako and GE, respectively.

Astrocyte Culture

Primary astrocyte cultures from Wistar rats were prepared as previously described (Gottfried et al., 2003). Procedures were in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals and were approved by the local authorities. Briefly, the cerebral cortex of newborn Wistar rats (1–2 days old) were removed and mechanically dissociated in Ca^{2+}- and Mg^{2+}-free Hanks' balanced salt solution (HBSS), pH 7.4, containing (in mM): 137 NaCl, 5.36 KCl, 0.27 Na_2HPO_4, 1.1 KH_2PO_4, and 6.1 glucose. The cortex was cleaned of meninges and mechanically dissociated by sequential passage through a Pasteur pipette. After centrifugation at 1,000 rpm for 5 min, the pellet was resuspended in DMEM (pH 7.6) supplemented with 8.39 mM HEPES, 23.8 mM $NaHCO_3$, 0.1% amphotericin B, 0.032% Garamycin, and 10% FCS. Approximately 300,000 cells were seeded in each well in 24-well plates and maintained in DMEM containing 10% FCS in 5% CO_2/95% air at 37°C, then allowed to grow to confluence and used at 15 days in vitro. The medium was replaced by DMEM without serum in the absence or presence of GJ blockers or GZA. Preconfluent astrocyte cultures, obtained by seeding 50,000 cells per well, were used as negative controls for GJ inhibitors.

C6 Glioma Cell Culture

The C6 glioma cell line was obtained from the American Type Culture Collection (Rockville, MD). Late passage cells (i.e., after at least 100 passages) were seeded in 24-well plates at densities of 10^4 cells/well, then cultured in DMEM (pH 7.4) supplemented with 5% fetal bovine serum, 2.5 mg/mL amphotericin B, and 100 U/L gentamicin in 5% CO_2/95% air at 37°C (de Souza et al., 2009). After cells reached confluence, the culture medium was replaced by DMEM without serum in the absence or presence of GJ blockers or GZA. The preparations, which contain low levels of GJ, were also used as negative controls for GJ inhibitors (Tabernero et al., 2006).

Preparation and Incubation of Hippocampal Slices

Rats were decapitated and their hippocampi quickly dissected out. Transverse sections (300 μm) were rapidly obtained with a McIlwain tissue chopper. One slice was placed into each well of a 24-well culture plate. Slices were incubated in oxygenated physiological medium containing 120 mM NaCl, 2 mM KCl, 1 mM $CaCl_2$, 1 mM $MgSO_4$, 25 mM HEPES, 1 mM KH_2PO_4, and 10 mM glucose, pH 7.4, at room temperature and the medium was changed every 15 min with fresh medium. After a 120-min equilibration period, slices were incubated in medium in the absence or presence of GJ blockers or glycyrrhizic acid for 1 hr at 30°C.

Drug Treatment

Cells and slices were incubated in DMEM without serum or physiological medium, respectively, in the absence or presence of GJ blockers or GZA. For GJ communication assay with Lucifer yellow, cells were incubated in HBSS. CBX (100 μM), halothane (0.02%), and endothelin-1 (1 μM) were diluted in water. GZA (100 μM), FFA (100 μM), and octanol (1 mM) were diluted in 0.1% dimethyl sulfoxide (DMSO), which did not affect basal S100B secretion. Results were expressed as the percentage of basal in each experiment.

S100B Measurement

S100B was measured by enzyme-linked immunosorbent assay as previously described (Leite et al., 2008). Briefly, 50 μl of sample plus 50 μl of Tris buffer were incubated for 2 hr on a microtiter plate previously coated with monoclonal anti-S100B (SH-B1). Polyclonal anti-S100B was incubated for 30 min, and then peroxidase-conjugated anti-rabbit antibody was added for a further 30 min. A colorimetric reaction with *o*-phenylenediamine was measured at 492 nm.

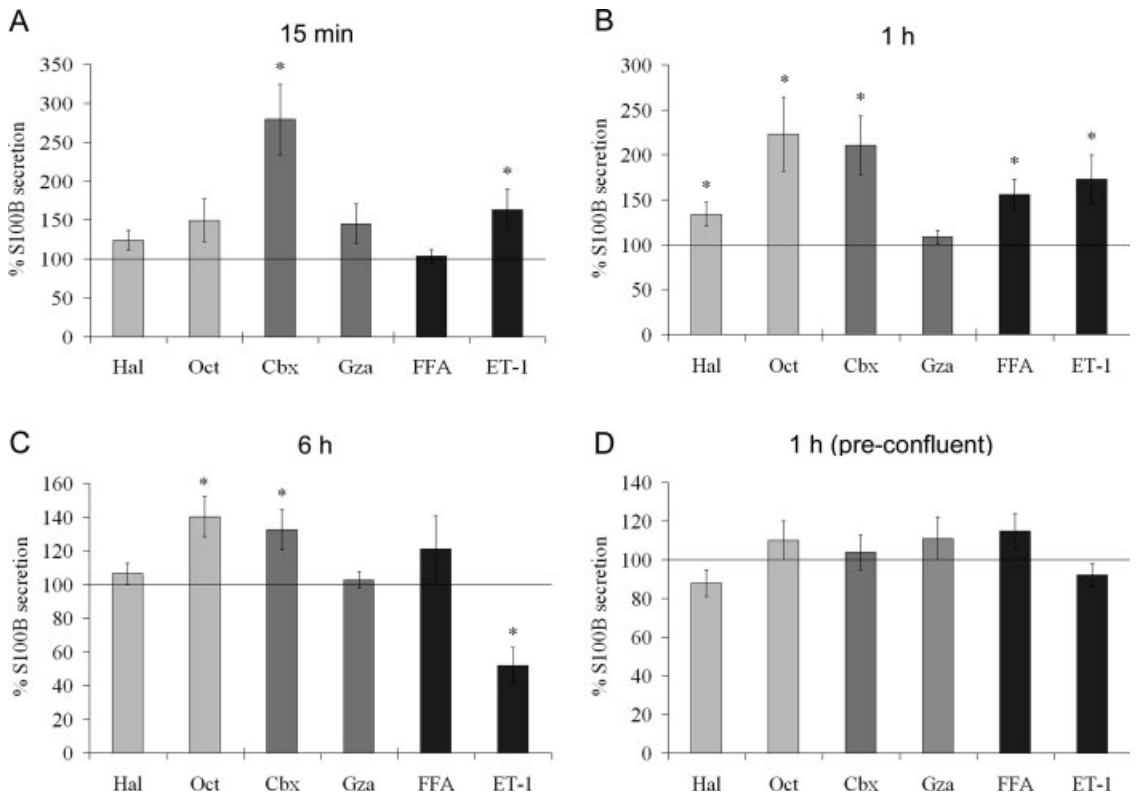

Fig. 1. GJ inhibitors stimulate S100B secretion in astrocyte cultures. Rat primary astrocyte cultures and C6 glioma cells in serum-free medium were exposed to different GJ inhibitors: halothane (Hal, at 0.02%), octanol (Oct, at 1 mM), carbenoxolone (Cbx, at 100 μM), glycyrrhizic acid, inactive analogue of Cbx (Gza, at 100 μM), flufenamic acid (FFA, at 100 μM), and endothelin-1 (ET-1, at 1 μM), and astrocyte samples from media were collected at 15 min (**A**), 1 hr (**B**), and 6 hr (**C**) for extracellular S100B measurement. Basal values were assumed as being 100% in nine independent experiments performed in triplicate. **D:** Negative control using preconfluent astrocyte cultures exposed to the GJ inhibitors (media collected at 1 hr) in five independent experiments. *Significant statistical difference from basal secretion (Student *t*-test, assuming $P < 0.05$).

GJ Communication

Scrape loading/dye transfer was carried out as previously described (Nodin et al., 2005). After treatment, the conditioned incubation buffer was removed and saved. The cultures were quickly and carefully rinsed in Ca^{2+}-free HBSS to prevent uncoupling of the cells as a result of high Ca^{2+} levels. Two parallel scrapes were performed with a scalpel blade in the Ca^{2+} free HBSS with 0.1% (w/v) Lucifer yellow at room temperature. Disruption of the cell membrane allows the dye to permeate the cell and to diffuse to surrounding cells through GJ channels. The dye was rinsed away with HBSS after 1 min. The conditioned incubation buffer was reintroduced and cultures were left for 7 min. After that, cells were viewed with a Nikon inverted microscope and images transferred to a computer with a digital camera. All images are representative fields from at least three experiments carried out in triplicate.

MTT Reduction Assay

Cells were treated with methylthiazolyldiphenyl–tetrazolium bromide (MTT) 50 μg/mL for 30 min in 5% CO_2/95% air at 37°C. After, the media was removed and MTT crystals were dissolved in DMSO. Absorbance values were measured at 560 and 650 nm. The reduction of MTT was calculated by the following formula: [(abs 560 nm) − (abs 650 nm)]. Results were expressed as percentage of control.

Neutral Red Incorporation Assay

Cells were treated with 50 μg/mL neutral red (NR) for 30 min in 5% CO_2/95% air at 37°C. Afterward, cells were rinsed twice with phosphate-buffered saline for 5 min each, and the NR dye taken up by viable cells was extracted with 500 μl of acetic acid/ethanol/water (1/50/49, v/v). Absorbance values were measured at 560 nm. Results were expressed as a percentage of control.

Lactate Dehydrogenase Assay

The lactate dehydrogenase (LDH) assay was carried out in 50 μl of extracellular medium with a commercial colorimetric assay from Doles (Goiania, Brazil).

Propidium Iodide Uptake Assay

Cells were treated with GJ blockers concomitantly with 7.5 μM propidium iodide and incubated for 1 hr. Cells were

viewed with a Nikon inverted microscope with a TE-FM Epi-Fluorescence accessory; images were transferred to a computer with a digital camera. All images are representative fields from at least three experiments carried out in triplicate. Optical density was determined with the Optiquant version 02.00 software (Packard Instrument Company). Density values obtained were expressed as density light units.

Statistical Analysis

Parametric data are reported as mean ± standard error and were analyzed by Student's *t*-test. Significance was considered when $P < 0.05$. All analyses were carried out in a PC-compatible computer by SPSS software.

RESULTS

Basal S100B secretion in primary astrocytes was modified in the presence of the GJ inhibitors (Fig. 1). A rapid increment (at 15 min) was observed when cells were exposed to CBX and endothelin-1 (Fig. 1A). Later (at 1 hr), all GJ inhibitors induced an increase in S100B secretion. GZA, a precursor of CBX but inactive on GJ, did not alter S100B secretion. However, at 6 hr, only octanol and CBX remained, stimulating S100B secretion (Fig. 1C). Interestingly, endothelin-1 caused a decrease in S100B secretion at this time. Preconfluent astrocyte cultures were used as negative controls (Fig. 1D). No significant changes in S100B secretion were observed in the presence of GJ inhibitors in these preparations.

In order to investigate the effect of neuronal neighbors on S100B secretion, we used acute hippocampal slice preparations and measured the protein secretion 1 hr after GJ inhibitor exposure (Fig. 2A). Again, as occurred with astrocytes at 6 hr, CBX caused an increase in S100B secretion, while endothelin-1 decreased S100B secretion. Octanol, halothane, and FFA did not affect S100B secretion in hippocampal slice preparations. However, unexpectedly, GZA also caused an increase in S100B secretion equivalent to CBX. C6 glioma cells were used as negative controls (Fig. 2B). Again, no significant changes in S100B secretion were observed in the presence of GJ inhibitors in these preparations.

To confirm whether GJ inhibitors were, in fact, blocking the connexin channels rather than affecting cell integrity, a scrap loading assay was carried out with Lucifer yellow and the propidium iodide incorporation assay, respectively (Fig. 3). CBX and endothelin-1 did not affect cell integrity at 1 hr (Fig. 3D–F) or at 6 hr (data not shown). Moreover, these GJ blockers were able to inhibit GJ communication (Fig. 3B,C), compared with control astrocytes also submitted to scrap loading (Fig. 3A).

It is important to emphasize that we are assuming S100B "secretion" (instead of "release") also on the basis of cell integrity, as evaluated by measurement of extracellular LDH activity carried out in parallel with S100B measurement in extracellular medium. The increases observed in Figures 1 and 2 were not corrected by extracellular LDH activity, which could overestimate

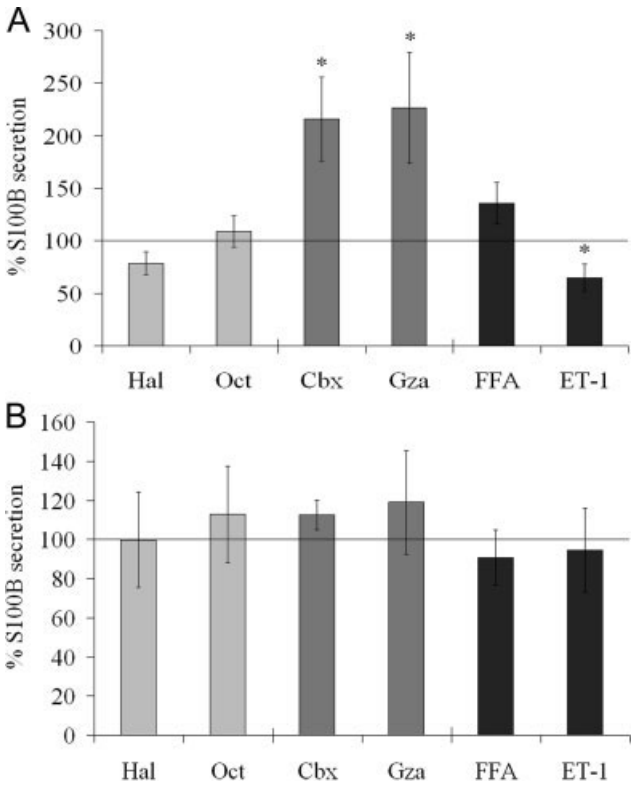

Fig. 2. Carbenoxolone and glycyrrhizic acid (GZA) stimulate S100B secretion in hippocampal slice, but not in C6 glioma cells. **A:** Acute hippocampal slices, after an equilibration phase, were incubated in HBSS with different GJ inhibitors: halothane (Hal, at 0.02%), octanol (Oct, at 1 mM), carbenoxolone (Cbx, at 100 μM), GZA, an inactive analogue of Cbx (Gza, at 100 μM), flufenamic acid (FFA, at 100 μM), and endothelin-1 (ET-1, at 1 μM). A sample was then collected from the medium at 1 hr for extracellular S100B measurement. Basal values were assumed as being 100% in nine independent experiments performed in triplicate. **B:** Negative control using C6 glioma cell preparations exposed to the GJ inhibitors (media collected at 6 hr) in five independent experiments. *Significant statistical difference from basal secretion (Student *t*-test, assuming $P < 0.05$).

these values. In fact, three GJ inhibitors (FFA, CBX, and endothelin-1) per se caused a decrease in LDH activity at 6 hr in astrocyte cultures (Table I). In agreement, FFA and endothelin-1 caused a decrease in LDH activity in the incubation medium of slice preparations at 1 hr. C6 glioma cells, also at 6 hr, exhibited a decrease in extracellular LDH activity when exposed to FFA and CBX. The enzymatic assay was not able to detect extracellular alterations in LDH activity at 1 hr in astrocyte and C6 cultures. No direct effect of these GJ inhibitors was observed on rat serum LDH activity (data not shown).

Two other assays (NR incorporation assay and MTT reduction assay) were performed to evaluate cell viability in astrocyte cultures and hippocampal slice preparations when exposed to GJ inhibitors and GZA. Only FFA altered MTT reduction in astrocyte cultures,

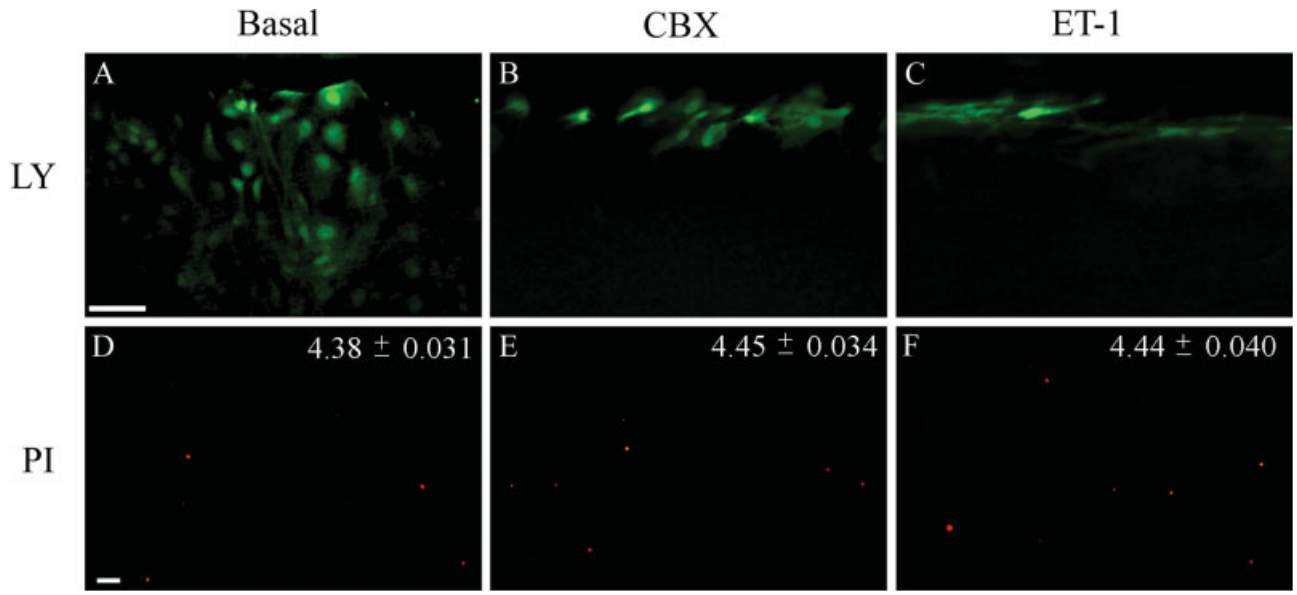

Fig. 3. Influence of GJ inhibitors on membrane integrity and GJ communication, evaluated by fluorescence staining. **A–C:** Astrocytes were exposed to GJ inhibitors (carbenoxolone or endothelin-1) for 1 hr and then submitted to scrape loading with Lucifer yellow for 10 min, as detailed in Materials and Methods. Shown are basal cell fluorescent staining (A), carbenoxolone (B), and endothelin-1 (C). **D–F:** Astrocyte cultures were exposed to GJ inhibitors (carbenoxolone at 100 μM or endothelin-1 at 1 μM) for 1 hr in the presence of propidium iodide (PI). Shown are basal nuclear fluorescent staining (D), carbenoxolone (E), and endothlin-1 (F). Values of gross density luminescence unit (mean ± SE) from three independent experiments performed in triplicate are indicated. Scale bar = 100 μm. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

inducing an increase of 25% (Fig. 4A). In contrast, hippocampal slices incubated with GJ inhibitors exhibited a decrease (approximately 20%) in MTT reduction (Fig. 4B). GZA did not alter MTT reduction in slice preparations.

Confirming the MTT assay, NR incorporation assay indicated that astrocyte cultures were viable when exposed to GJ inhibitors (Fig. 5A). Again, FFA was an exception. This compound caused a decrease in NR incorporation. In hippocampal slices, this assay indicated that no change in cell viability was observed in hippocampal slices exposed to GJ inhibitors (Fig. 5B). However, CBX caused a small decrease (<8%) in NR incorporation.

TABLE I. Extracellular LDH Activity in the Presence of GJ Inhibitors*

	1 hr		6 hr	
Inhibitor	Astrocytes	Hippocampal slices	Astrocytes	C6 glioma
Basal (%)	ND	100	100	100
FFA	ND	60.1 ± 7.9	43.8 ± 6.4	32.3 ± 8.2
ET-1	ND	58.0 ± 9.6	63.4 ± 7.2	98.6 ± 3.1
CBX	ND	106.7 ± 9.0	33.6 ± 12.3	12.0 ± 4.9

*Values of extracellular LDH activity (mean ± SE) in different cell preparations (primary astrocyte cultures, acute hippocampal slices, and C6 glioma cells) exposed to gap junction (GJ) inhibitors (FFA, 100 μM flufenamic acid; ET-1, 1 μM endothelin-1; CBX, 100 μM carbenoxolone) for 1 and 6 hr. Basal values were assuming as being 100% in nine independent experiments. ND, not detected.

DISCUSSION

Our results show that S100B secretion is increased by the blocking of GJ in both astrocyte cultures and hippocampal slices. Specific variations are likely due to the nature of the GJ inhibitor, its molecular target of GJ blocking, and/or the cell preparation used. This investigation involved four classes of uncoupling GJ agents; however, their mechanisms of action remain unknown. Two small lipophilic agents, halothane and octanol, were able to induce S100B secretion in astrocyte cultures at 1 hr (but only octanol at 6 hr). These agents were not S100B secretagogues in hippocampal slice preparations. These molecules, which putatively act in the lipid bilayer, apparently had different molecular targets or metabolization in cell and slice preparations. Similarly to halothane, FFA was also not as effective as the S100B secretagogue in astrocytes at 6 hr or in hippocampal slices. The mechanism of FFA on GJ is unknown. The only common aspect between halothane and FFA is the trifluorocarbon in their structures; however, this feature does not allow us to draw conclusions about a common molecular target or metabolic inactivation.

In contrast, CBX, a synthetic molecule derived from GZA, induced a rapid and persistent effect on S100B secretion, in both astrocyte and slice preparations. This uncoupling GJ agent is proposed to act directly on connexin (Salameh and Dhein, 2005). However, its specificity to GJ has been argued and an effect on astro-

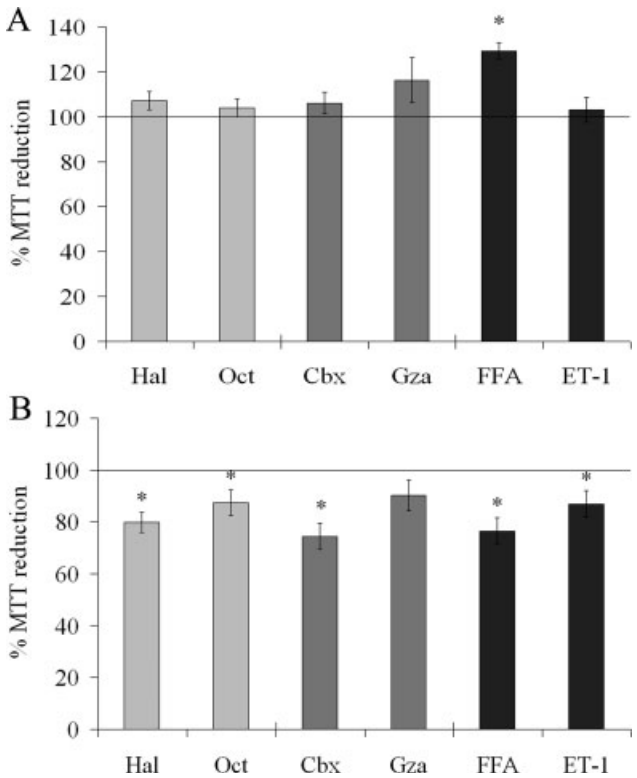

Fig. 4. Effect of GJ inhibitors on MTT reduction capacity in astrocyte cultures and hippocampal slices. Rat primary astrocyte cultures (**A**) and acute hippocampal slices (**B**) were exposed to different GJ inhibitors: halothane (Hal, at 0.02%), octanol (Oct, at 1 mM), carbenoxolone (Cbx, at 100 μM), glycyrrhizic acid, inactive analogue of Cbx (Gza, at 100 μM), flufenamic acid (FFA, at 100 μM), and endothelin-1 (ET-1, at 1 μM) for 1 hr. MTT reduction assay was performed as described in Materials and Methods. Basal absorbance values for formazan were assumed as being 100% in six independent experiments performed in triplicate. *Significant statistical difference from basal secretion (Student *t*-test, assuming $P < 0.05$).

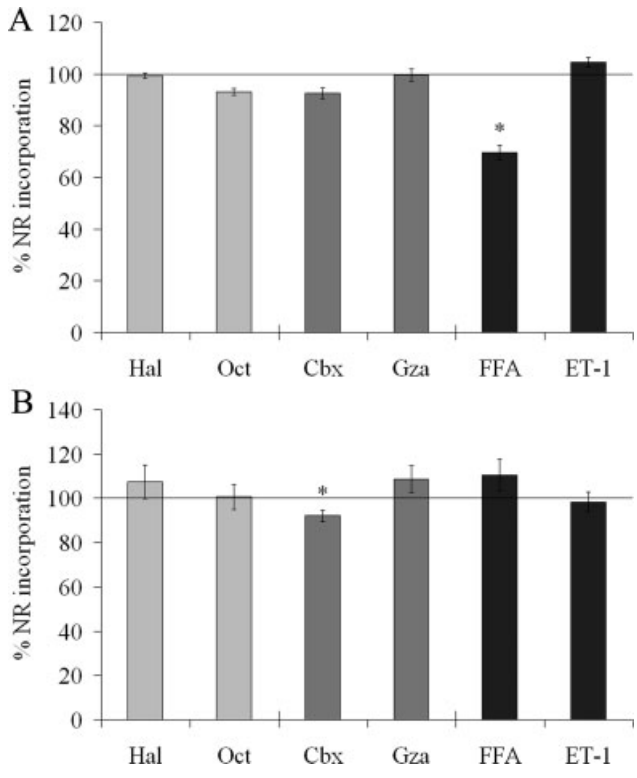

Fig. 5. Effect of GJ inhibitors on neutral red (NR) uptake in astrocyte cultures and hippocampal slices. Rat primary astrocyte cultures (**A**) and acute hippocampal slices (**B**) were exposed to different GJ inhibitors: halothane (Hal, at 0.02%), octanol (Oct, at 1 mM), carbenoxolone (Cbx, at 100 μM), glycyrrhizic acid, inactive analogue of Cbx (Gza, at 100 μM), flufenamic acid (FFA, at 100 μM), and endothelin-1 (ET-1, at 1 μM) for 1 hr. NR uptake assay reduction was performed as described in Materials and Methods. Basal absorbance values for incorporated NR were assumed as being 100% in six independent experiments performed in triplicate. *Statistical difference from basal secretion (Student *t*-test, assuming $P < 0.05$).

cytes secondary to its neuronal activity is possible as well (Rouach et al., 2002).

Endothelin-1 activity on GJ communication in astrocytes involves specific intracellular signaling (Salameh and Dhein, 2005). In agreement with our hypothesis, endothelin-1 was able to induce S100B secretion in astrocytes at 1 hr. However, possibly as a result of long-term changes induced in astrocytes and/or neuronal neighbors, endothelin-1 caused a decrease in S100B secretion in astrocyte cultures at 6 hr and in acute hippocampal slices. Dye-coupling studies have demonstrated that the inhibition of GJ communication by endothelin-1 and -3 occurs rapidly and returns to control levels after 90 min of exposure (Blomstrand and Giaume, 2006). Moreover, these authors showed that long-term exposure of cells to endothelins could desensitize receptors for endothelin-1, whereas CBX did not show any time-dependent desensitization (Blomstrand and Giaume, 2006). The long-term effect of endothelin-1 in cell culture also includes a decrease in the expression of glutamate receptors and glutamine synthetase (Lehmann et al., 2008). However, there is no current information regarding the desensitization of endothelin-1 receptors in hippocampal slice preparations, although this possibility cannot be ruled out. An alternative explanation to the endothelin-1-induced decrease S100B secretion in hippocampal slices could be due to the increase in neuronal activity mediated by glutamate (Goncalves et al., 2002; Shihara et al., 1998).

Another interesting aspect regarding the differences between astrocyte and slice preparations is the effect of GZA. No effect of GZA on GJ has been described, and in fact, no effect was observed on S100B secretion in astrocyte cultures. However, a possible effect of GZA on other cell activities could trigger S100B secretion observed in acute hippocampal slices. In agreement, an effect of GZA on neuronal excitability was recently described, particularly on K^+ permeability (Elsen et al., 2008). Moreover, through a mineralocorticoid-like

activity, GZA could affect the neuronal membrane K^+/Na^+ ATPase (Zhou et al., 1996).

The scrape loading assay with Lucifer yellow confirms that the GJ inhibitors used worked in our preparations. Moreover, cell integrity and/or viability assays (propidium iodide, LDH release, MTT reduction, and NR uptake) helped us to characterize the effect of these inhibitors on S100B secretion, as well as to identify some apparent side effects of GJ inhibitors in the different cell preparations.

MTT reduction assays in astrocyte cultures (Fig. 4A) indicate that the reductive capacity was not affected by GJ inhibitors and that FFA in particular increased this capacity. However, when this capacity was evaluated in acute hippocampal slices, all uncoupling GJ agents (but not GZA) caused a decrease in reductive capacity. This suggests that these effects in brain slices, which are more vulnerable to oxidative stress than cultured cells, do not necessarily involve GJ targets. However, it is important to mention that the MTT reduction assay does not exclusively measure the reductive mitochondrial capacity, but also the membrane activities of endocytosis (to uptake MTT) and exocytosis (to export the MTT reduced product) (Liu et al., 1997). NR uptake assay indicated that in general, GJ inhibitors did not alter cell viability in both cultured astrocytes and acute hippocampal slices. The NR uptake assay indicated that FFA seems to impair cell viability; however, the MTT, LDH, and propidium iodide assays did not show a similar effect. Although the reason for this is unclear at moment, this emphasizes the importance of the use of different assays for the evaluation of cell viability.

All embryonic neural cells express high levels of Cx43, but with development, this connexin remains expressed predominantly in astrocytes, which are found in GJ in vivo and under culture conditions (Rozental et al., 2000). A decrease in basal S100B secretion has been observed after astroglial confluence in vitro (Tramontina et al., 2002; Van Eldik and Zimmer, 1987), which could be negatively modulated by changes in GJ communication in these preparations. In agreement with this, our present data reinforce the existence of an inverse relationship between GJ communication and S100B secretion.

Today we know that astrocytes sense, integrate, and respond to stimuli generated by neurons or neural injury and that this response involves Cx43 channels and hemichannels. In support of this hypothesis, GJ blockers increased neuronal vulnerability when cocultures of astrocytes and neurons were exposed to oxidative stress (Blanc et al., 1998) or to high glutamate (Ozog et al., 2002). On the other hand, considering the astrocytic syncytium in the brain, GJ uncoupling could limit the extension of a lesion (Nodin et al., 2005). Therefore, on the basis of the neurotrophic activity of S100B observed in many conditions of injury (Van Eldik and Wainwright, 2003), it may be proposed that secreted S100B, as a result of GJ uncoupling, may be of benefit to cell survival in the affected area. As such, the altered signaling during an injury (for example, mediated by Ca^{2+} or adenosine triphosphate) could locally and transiently close the functional astrocyte syncytium and this could trigger other signals for cell survival, including S100B secretion. In support of the inverse correlation between GJ communication S100B secretion, high glutamate or K^+ (as observed in excitotoxic conditions) increases GJ communication (Enkvist and McCarthy, 1994) and decreases S100B secretion in astrocyte cultures (Goncalves et al., 2002) and acute hippocampal slices (Nardin et al., 2009). However, there are many putative S100B secretagogues, and it would be reasonable to consider that all they trigger S100B secretion mediated by GJ communication.

In summary, our data indicate that GJ blocking stimulates S100B secretion in astrocyte cultures and in acute hippocampal slices. Different assays were used to confirm cell integrity during exposure to GJ inhibitors. S100B secretion was observed with different types of GJ inhibitors, and the resulting event was dependent on time, the nature of the inhibitor, its putative molecular target of GJ blocking, and/or the cell preparation used. Only CBX induced a fast and persistent increase of S100B secretion in both preparations. Physiologically, a local GJ closure, associated with release of S100B in injury conditions, favors the idea of a common mechanism available to limit the extension of lesion and increase the chances of cell survival.

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REFERENCES

Blanc EM, Bruce-Keller AJ, Mattson MP. 1998. Astrocytic gap junctional communication decreases neuronal vulnerability to oxidative stress–induced disruption of Ca^{2+} homeostasis and cell death. J Neurochem 70:958–970.

Blomstrand F, Giaume C. 2006. Kinetics of endothelin-induced inhibition and glucose permeability of astrocyte gap junctions. J Neurosci Res 83:996–1003.

Davey GE, Murmann P, Heizmann CW. 2001. Intracellular Ca^{2+} and Zn^{2+} levels regulate the alternative cell density–dependent secretion of S100B in human glioblastoma cells. J Biol Chem 276:30819–30826.

de Souza DF, Leite MC, Quincozes-Santos A, Nardin P, Tortorelli LS, Rigo MM, Gottfried C, Leal RB, Goncalves CA. 2009. S100B secretion is stimulated by IL-1beta in glial cultures and hippocampal slices of rats: likely involvement of MAPK pathway. J Neuroimmunol 206:52–57.

Donato R. 2001. S100: a multigenic family of calcium-modulated proteins of the EF-hand type with intracellular and extracellular functional roles. Int J Biochem Cell Biol 33:637–668.

Elsen FP, Shields EJ, Roe MT, Vandam RJ, Kelty JD. 2008. Carbenoxolone induced depression of rhythmogenesis in the pre-Botzinger complex. BMC Neurosci 9:46.

Enkvist MO, McCarthy KD. 1994. Astroglial gap junction communication is increased by treatment with either glutamate or high K^+ concentration. J Neurochem 62:489–495.

Feoli AM, Leite MC, Tramontina AC, Tramontina F, Posser T, Rodrigues L, Swarowsky A, Quincozes-Santos A, Leal RB, Gottfried C, Perry ML, Goncalves CA. 2008. Developmental changes in content of glial marker proteins in rats exposed to protein malnutrition. Brain Res 1187:33–41.

Goncalves D, Karl J, Leite M, Rotta L, Salbego C, Rocha E, Wofchuk S, Goncalves CA. 2002. High glutamate decreases S100B secretion stimulated by serum deprivation in astrocytes. Neuroreport 13:1533–1535.

Goncalves CA, Concli Leite M, Nardin P. 2008. Biological and methodological features of the measurement of S100B, a putative marker of brain injury. Clin Biochem 41:755–763.

Gottfried C, Cechin SR, Gonzalez MA, Vaccaro TS, Rodnight R. 2003. The influence of the extracellular matrix on the morphology and intracellular pH of cultured astrocytes exposed to media lacking bicarbonate. Neuroscience 121:553–562.

Kielian T. 2008. Glial connexins and gap junctions in CNS inflammation and disease. J Neurochem 106:1000–1016.

Lehmann C, Eisner F, Engele J. 2008. Role of endothelins as mediators of injury-induced alterations of glial glutamate turnover. J Neurosci Res 86:660–667.

Leite MC, Galland F, Brolese G, Guerra MC, Bortolotto JW, Freitas R, Almeida LM, Gottfried C, Goncalves CA. 2008. A simple, sensitive and widely applicable ELISA for S100B: methodological features of the measurement of this glial protein. J Neurosci Methods 169:93–99.

Liu Y, Peterson DA, Kimura H, Schubert D. 1997. Mechanism of cellular 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) reduction. J Neurochem 69:581–593.

Marenholz I, Heizmann CW, Fritz G. 2004. S100 proteins in mouse and man: from evolution to function and pathology (including an update of the nomenclature). Biochem Biophys Res Commun 322:1111–1122.

Nardin P, Tortorelli L, Quincozes-Santos A, de Almeida LM, Leite MC, Thomazi AP, Gottfried C, Wofchuk ST, Donato R, Goncalves CA. 2009. S100B Secretion in Acute Brain Slices: Modulation by Extracellular Levels of Ca^{2+} and K^+. Neurochem Res [Epub ahead of print] doi 10.1007/s11064-009-9949-0.

Nodin C, Nilsson M, Blomstrand F. 2005. Gap junction blockage limits intercellular spreading of astrocytic apoptosis induced by metabolic depression. J Neurochem 94:1111–1123.

Ozog MA, Siushansian R, Naus CC. 2002. Blocked gap junctional coupling increases glutamate-induced neurotoxicity in neuron–astrocyte co-cultures. J Neuropathol Exp Neurol 61:132–141.

Pinto SS, Gottfried C, Mendez A, Goncalves D, Karl J, Goncalves CA, Wofchuk S, Rodnight R. 2000. Immunocontent and secretion of S100B in astrocyte cultures from different brain regions in relation to morphology. FEBS Lett 486:203–207.

Rouach N, Avignone E, Meme W, Koulakoff A, Venance L, Blomstrand F, Giaume C. 2002. Gap junctions and connexin expression in the normal and pathological central nervous system. Biol Cell 94:457–475.

Rozental R, Giaume C, Spray DC. 2000. Gap junctions in the nervous system. Brain Res Brain Res Rev 32:11–15.

Saez JC, Contreras JE, Bukauskas FF, Retamal MA, Bennett MV. 2003. Gap junction hemichannels in astrocytes of the CNS. Acta Physiol Scand 179:9–22.

Salameh A, Dhein S. 2005. Pharmacology of gap junctions. New pharmacological targets for treatment of arrhythmia, seizure and cancer? Biochim Biophys Acta 1719:36–58.

Shihara M, Hirooka Y, Hori N, Matsuo I, Tagawa T, Suzuki S, Akaike N, Takeshita A. 1998. Endothelin-1 increases the neuronal activity and augments the responses to glutamate in the NTS. Am J Physiol 275(2 Pt 2):R658–R665.

Stridh MH, Tranberg M, Weber SG, Blomstrand F, Sandberg M. 2008. Stimulated efflux of amino acids and glutathione from cultured hippocampal slices by omission of extracellular calcium: likely involvement of connexin hemichannels. J Biol Chem 283:10347–10356.

Tabernero A, Medina JM, Giaume C. 2006. Glucose metabolism and proliferation in glia: role of astrocytic gap junctions. J Neurochem 99: 1049–1061.

Tramontina F, Conte S, Goncalves D, Gottfried C, Portela LV, Vinade L, Salbego C, Goncalves CA. 2002. Developmental changes in S100B content in brain tissue, cerebrospinal fluid, and astrocyte cultures of rats. Cell Mol Neurobiol 22:373–378.

Van Eldik LJ, Wainwright MS. 2003. The Janus face of glial-derived S100B: beneficial and detrimental functions in the brain. Restor Neurol Neurosci 21:97–108.

Van Eldik LJ, Zimmer DB. 1987. Secretion of S-100 from rat C6 glioma cells. Brain Res 436:367–370.

Zhou QL, Zhang ZQ, Nagasawa T, Hiai S. 1996. [The structure activity relationship of saikosaponins and glycyrrhizin derivatives for Na^+, K^+-ATPase inhibiting action]. Yao Xue Xue Bao 31:496–501.

Capítulo III

S100B secretion is mediated by Ca^{2+} from endoplasmic reticulum: a study using DMSO as a tool for intracellular Ca^{2+} mobilization.

Artigo submetido no periódico Journal of Neurochemistry

S100B SECRETION IS MEDIATED BY CA^{2+} FROM ENDOPLASMIC RETICULUM: A STUDY USING DMSO AS A TOOL FOR INTRACELLULAR CA^{2+} MOBILIZATION

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Abbreviations: BAPTA-AM, 1,2-bis-(o-Aminophenoxy)-ethane-N,N,N',N'-tetraacetic acid - tetraacetoxymethyl ester; cAMP, cyclic AMP; DMSO, dimethyl sulfoxide; ICV, intracerebroventricular; LDH, lactate dehydrogenase; LPS, lipopolysaccharide; PBS, phosphate-buffered saline; PKA, Protein Kinase dependent on cAMP; PLC, Phopholipase C; MTT, methylthiazolyl-diphenyl-tetrazolium bromide; RAGE, Receptor for Advanced Glycation End products; T1R/T2R, calcium response type 1/calcium response type 2

Abstract

S100B, a calcium-binding protein of 21 kDa, has many putative intracellular targets involved in cell cycle and cytoskeleton regulation in astrocytes. In addition, this protein is also secreted and has autocrine and paracrine effects on glia, synaptic plasticity and microglia. Many S100B secretagogues have been identified, but the underlying mechanism of secretion remains unknown and involves a non-classic export. Herein, we investigate the role of Ca^{2+} in S100B secretion in primary cultured astrocytes. Results indicate that dimethyl sulfoxide (DMSO, at concentrations of higher than 0.1%) is a powerful S100B secretagogue. DMSO-induced S100B secretion was dependent on increased intracellular Ca^{2+} mobilization from endoplasmatic reticulum and independent of cytoskeleton reorganization. This issue highlights the fact that secreted S100B is a possible mediator of the effects (and side-effects) of DMSO and that caution is required in the use of this vehicle, since some secretable proteins, including S100B, are involved in intercellular communication. Furthermore, the S100B-secreting property of DMSO should be added to the list of therapeutic applications of this compound, particularly considering the neuroprotective effects of S100B that have been observed in acute brain damage. In addition, the usefulness of DMSO *per se* as a tool to investigate intracellular calcium mobilization should be taken into consideration. DMSO-induced secretion of S100B has also been observed in hippocampal slices and *in vivo* in the cerebrospinal fluid of rats.

Key words: Astrocyte, calcium, protein secretion, DMSO, S100B

Running title: Astroglial S100B secretion induced by DMSO

Introduction

S100B is a homodimeric calcium-binding protein of 21 kDa, belonging to the S100 family of proteins (Marenholz *et al.* 2004) that are characterized by their high solubility and, currently, comprises 21 members that are expressed in a cell-specific manner [see (Donato *et al.* 2009) for a review]. Astrocytes appear to be the major S100B-containing compartment in the gray matter of the central nervous system (Steiner *et al.* 2007, Goncalves *et al.* 2008). This protein has many putative intracellular targets involved in cell cycle and cytoskeleton regulation, but it is also secreted (less than 1%) and has autocrine and paracrine effects on glia, neurons and microglia. The effect of extracellular S100B on each cell target, whether it be trophic or toxic, will depend upon its concentration, redox environment and, evidently, active signaling pathways (Donato *et al.* 2008, Pichiule *et al.* 2007).

Many S100B secretagogues have been identified, including forskolin, lyso-phosphatidic acid (Pinto *et al.* 2000), fluoxetin (Tramontina *et al.* 2008), kainate (Sakatani *et al.* 2008) and carbenoxolone (Leite *et al.* 2009). Moreover, it is known that metabolic stress conditions affect S100B secretion, such as elevated concentrations of glutamate (Buyukuysal 2005), glucose (Nardin *et al.* 2007), beta-hydroxy-butyrate (Leite *et al.* 2004) and ammonium (Leite *et al.* 2006). However, the underlying mechanism of secretion remains unknown and involves a non-classic vesicular export (Davey *et al.* 2001, Goncalves et al. 2008, Donato et al. 2008). Commonly, S100B secretagogues in astrocyte cultures are characterized in the absence of serum [e.g. (Eriksen *et al.* 2002)]. Furthermore, serum deprivation, *per se*, is able to stimulate S100B secretion (Goncalves *et al.* 2002, Pinto et al. 2000). Therefore, some putative secretagogues may act via a mechanism triggered by serum-deprivation, a complex event where Ca^{2+} is a key mediator. As an example, serum-deprivation in astrocyte cultures is able to induce Ca^{2+} release from the endoplasmic reticulum and this, in turn, underlies the release of fibroblast growth factor

-1 (Matsunaga & Ueda 2006). In addition, we have observed that serum deprivation in astrocytes induces an early transitory increment in cyclic AMP (cAMP) (Goncalves et al. 2002), which could be associated with intracellular Ca^{2+} changes following this event. Therefore, S100B release is directly or indirectly modulated by Ca^{2+}. However, a clear connection between intracellular Ca^{2+} in astrocytes and S100B release is lacking.

We recently found that Ca^{2+}-free medium or exposure to 1 mM EGTA in acute hippocampal slices causes a significant increase in S100B secretion (Nardin *et al.* 2009), in agreement with previous observations in brain slices (Buyukuysal 2005), possibly due to the mobilization of internal stores of Ca^{2+}. In fact, Heizmann's group, using the U87 glioblastoma cell line, exposed to EGTA (10 mM for 6h) in a serum-free medium, observed an increase in S100B secretion (Davey et al. 2001). In addition, calcium channel blockers (verapamil and $CoCl_2$) reduced S100B secretion in acute hippocampal slices (Nardin et al. 2009), suggesting an involvement of extracellular Ca^{2+}. Hence, we herein investigate the role of extra and intracellular Ca^{2+} in S100B secretion in primary cultured astrocytes.

During this investigation, serendipitously, we discovered that dimethyl sulfoxide (DMSO) is a powerful S100B secretagogue. DMSO is an amphipathic molecule with a highly polar sulfoxide [-S-O] and two apolar dimethyl groups [$-(CH_3)_2$], making it a very good vehicle in both aqueous and organic media. This compound is also used as a hydroxyl radical scavenger, hydrogen-bound disrupter and cryoprotectant [see (Santos *et al.* 2003) for a review]. Moreover, DMSO itself appears to affect the secretion of some proteins such as interleukin-1 (Xing & Remick 2005), interleukin-8 (Zeng *et al.* 2003) and insulin (Kemp & Habener 2002). However, the molecular basis of the effect of DMSO remains uncertain. The present study investigates the involvement of Ca^{2+} in S100B secretion in astrocyte cultures, as well as providing a possible explanation for the S100B secretagogue effect of DMSO. This effect was also investigated *in*

vivo by intracerebroventricular (ICV) administration of DMSO and in acute hippocampal slice preparations.

Experimental Procedures

Material- Poly-L-lysine, antibody anti-S100B (SH-B1), propidium iodide, methylthiazolyldiphenyl-tetrazolium bromide (MTT), ethylene glycol-bis (2-aminoethylether)-*N,N,N′,N′*-tetraacetic acid (EGTA), ionomycin, A23187, 1,2-bis-(o-Aminophenoxy)-ethane-N,N,N',N'-tetraacetic acid - tetraacetoxymethyl ester (BAPTA-AM), thapsigargin, forskolin, verapamil, cobalt chloride, rhodamine-phalloidin, fura-2-AM and pluronic F-127 were purchased from Sigma. Fetal calf serum (FCS), Dulbecco's modified Eagle's medium (DMEM) and other materials for cell culture were purchased from Gibco. Polyclonal anti-S100B and anti-rabbit peroxidase-conjugated antibody were purchased from DAKO and GE, respectively. DMSO was purchased from Merck.

Cell culture- Primary astrocyte cultures from Wistar rats were prepared as previously described (Gottfried *et al.* 2003). Procedures were in accordance with the NIH Guide for the Care and Use of Laboratory Animals and were approved by the local authorities. Briefly, cerebral cortex of newborn Wistar rats (1–2 days old) were removed and mechanically dissociated in Ca^{2+}- and Mg^{2+}-free balanced salt solution, pH 7.4, containing (in mM): 137 NaCl; 5.36 KCl; 0.27 Na_2HPO_4; 1.1 KH_2PO_4 and 6.1 glucose. The cortex were cleaned of meninges and mechanically dissociated by sequential passage through a Pasteur pipette. After centrifugation at 200 x *g* for 5 min the pellet was resuspended in DMEM (pH 7.6) supplemented with 8.39 mM HEPES, 23.8 mM NaHCO3, 0.1% amphotericin, 0.032% gentamicin and 10% fetal calf serum (FCS). Cultures were maintained *in vitro* in DMEM containing 10% FCS in 5% CO_2/95% air at 37°C, allowed to grow to confluence and used at 15 days.

Culture of Cells on Coverslips for Calcium Measurements- When primary cortical astrocyte cultures reached confluence, they were trypsinized (0.25% trypsin/EDTA), re-plated onto 25 mm poly-L-lysine-coated coverslips, and employed in experiments within two days (Carvalho *et al.* 2004).

Calcium Measurements by Real Time Fluorescence Microscopy- For intracellular calcium measurements, cells were incubated with appropriate indicator in microscopy buffer containing (in mM): 1.5 $CaCl_2$, 130 NaCl, 5.6 KCl, 0.8 $MgSO_4$, 1 Na_2HPO_4, 25 glucose, 2 HEPES and 2.5 $NaHCO_3$, pH 7.3. For cytoplasmic calcium measurements, cells were loaded with Fura 2-AM (5 μM) in microscopy buffer containing 5 μl nonionic surfactant Pluronic F-127 (0.02%) for 30 min at room temperature. After incubation, cells were rinsed and placed in a Leiden coverslip chamber on the microscope stage. The temperature of the specimen was maintained at 37°C during the experiments. Calcium levels were investigated in isolated cells using an inverted microscope (TE300 Nikon, Osaka, Japan) coupled to a high-resolution cooled CCD camera (Roper Sci or CoolSnap, both from Princeton Instruments, USA) and controlled by imaging software (BioIP, Wilmington, DA). Cells were stimulated with 0.5% DMSO, 2 mM EGTA or 10 μM forskolin. Fura-2 fluorescence was acquired using 340 and 380 nm excitation and 510 nm emission filter sets. Calibrations were performed using digitonin (100 μM) to obtain maximum fluorescence (R_{max}), and $MnCl_2$ (2 mM), for minimum fluorescence (R_{min}) of the system.

Changes in cytosolic [Ca^{2+}] were determined by temporal analyses of single cells and expressed as fluorescence intensity F/F_0 ratios. Fluorescence was extracted from Regions of Interested (ROI), determined as previously described (Smaili *et al.* 2008). The resting fluorescence value, F_0, was determined at the beginning of each experiment. Results were normalized in relation to the basal fluorescence and data were expressed as percentages in relation to the total Ca^{2+} (obtained with digitonin) in the system.

Surgical Procedure for ICV DMSO Infusion- Procedures were in accordance with the NIH Guide for the Care and Use of Laboratory Animals and were approved by the local authorities. Adult Wistar rats (60 days old) were used. For ventricle access, the animals were anesthetized with ketamine/xylazine (75 and 10 mg/Kg, respectively, i.p.) and placed in a stereotaxic apparatus. A midline saggital incision was made in the scalp and one burr hole was drilled in the skull over the right lateral ventricle. The following coordinates were used: 0.9 mm posterior to bregma; 1.5 mm lateral to saggital suture; 3.6 mm beneath the surface of brain (Rodrigues *et al.* 2009). Rats received 2 µL ICV of pure DMSO or phosphate-buffered saline (control). Assuming a CSF volume of 200 µL (excluding subarachnoid space), final DMSO concentration would be approximately 1%. After the surgical procedure, rats were kept in a stereotactic holder for 1 h and the CSF was obtained by puncture of the cisterna magna using an insulin syringe. A maximum volume of 30 µL was collected over a 3 min period to minimize risk of brain stem damage. Cerebrospinal fluid samples were frozen (–20°C) until S100B analysis. The blood samples were obtained with intracardiac puncture, and the animals were killed by decapitation. The blood samples were incubated at room temperature (25°C) for 5 min and centrifuged at 3200 rpm for 5 min. Serum was stored at -20 °C until the day of analysis.

Hippocampal Slices- Hippocampal slices were prepared as previously described (Nardin et al. 2009). Sixty-day-old Wistar rats were killed by decapitation and their brains were removed and placed in cold HEPES-buffered saline medium with the following composition (in mM): 120 NaCl; 2 KCl; 1 $CaCl_2$; 1 $MgSO_4$; 25 HEPES; 1 KH_2PO_4, and 10 glucose, adjusted to pH 7.4, and previously aerated with O_2. The hippocampi were dissected and transverse slices of 0.3 mm were obtained using a McIlwain Tissue Chopper. Slices were then transferred immediately to 24-well culture plates, each well containing 0.3 ml of physiological medium and only one slice. The medium was exchanged for fresh saline medium every 15 min at room temperature (maintained

at 25°C). Following a 120-min equilibration period, the medium was removed and replaced with HEPES-buffered medium (containing, or not, DMSO) for 60 min at 30°C in a warm plate. Subsequently, 10 μL of media were collected and stored at -20°C until S100B measurement.

S100B Measurement- S100B was measured by ELISA, as previously described (Leite *et al.* 2008). Briefly, 50 μl of sample plus 50 μl of Tris buffer were incubated for 2 h on a microtiter plate previously coated with monoclonal anti-S100B. Polyclonal anti-S100 was incubated for 30 min and then peroxidase-conjugated anti-rabbit antibody was added for a further 30 min. The color reaction with OPD was measured at 492 nm. The standard S100B curve ranged from 0.002 to 1 ng/ml.

Astrocyte Morphology- After 1 h of drug treatment, cells were fixed for 20 min with 4% paraformaldehyde in phosphate-buffered saline (PBS), rinsed with PBS and permeabilized for 10 min in PBS containing 0.2% Triton X-100. Fixed cells were then blocked for 60 min with PBS containing 5% bovine serum albumin and incubated 20 min with rhodamine-phalloidin at 2.5 U/mL. Cells were viewed with a Nikon inverted microscope and images transferred to computer with a digital camera (Sound Vision Inc., Wayland, MA, USA). All images are representative fields from four experiments carried out in triplicate.

MTT Reduction Assay- Cells were treated with 50 μg/mL Methylthiazolyldiphenyl-tetrazolium bromide (MTT) for 30 min in 5% CO_2/95% air at 37°C. The medium was then removed and MTT crystals were dissolved in DMSO. Absorbance values were measured at 560 and 650nm. The reduction of MTT was calculated as (abs 560 nm) – (abs 650 nm).

Propidium Iodide Uptake Assay- Cells were treated with DMSO, concomitant with 7.5 μM propidium iodide, and incubated for 6 h. Cells were viewed with a Nikon inverted microscope with a TE-FM Epi-Fluorescence accessory and images were transferred to a computer with a

digital camera. All images are representative fields from at least three experiments carried out in triplicate. Optical density was determined with the Optiquant version 02.00 software (Packard Instrument Company). Density values obtained were expressed as density light units.

Statistical Analysis- Parametric data are reported as mean ± standard error and were analyzed by Student's *t* test (when two groups were considered) or by one-way analysis of variance (ANOVA), in the SPSS-16.0. Data from S100B measurements were log transformed to satisfy the assumption of statistical tests when necessary. Tests are specified in the legends, assuming $p< 0.05$.

Results

S100B secretion is increased by removal of external calcium. Generally, S100B secretion has been studied under serum-free conditions, in which Ca^{2+} and cAMP are putatively triggered messengers. A time-dependent increase in S100B secretion from astrocytes was observed after serum deprivation for 15 min, 1 h and 6 h (Fig 1A). Fig 1B demonstrates that EGTA addition (from 1mM to 3 mM) significantly increased S100B secretion after 1 h of serum deprivation. This secretion was apparently negatively correlated with free external Ca^{2+}. In addition, $CoCl_2$ (at 1mM) caused a decrease in basal secretion of S100B (Fig 1C). Ionophores (1 μM ionomycin and 1 μM A23187) were also able to increase S100B secretion within 1 h; this increase was even greater than that induced by forskolin, a classical secretagogue for S100B. Similar results were observed in 15 min for all these compounds. However, the secretion induced by ionophores, within 6 h, affected cell integrity (as evaluated by propidium iodide - data not shown).

DMSO induces S100B secretion and this effect is prevented by thapsigargin or BAPTA-AM. DMSO is the most common solvent for compounds used to mobilize or measure

intracellular calcium. Therefore, we first investigated the effect of this compound on S100B secretion. Interestingly, we found a positive correlation between DMSO concentration (over 0.1%) and S100B secretion (Fig 2A) at all times examined (15 min, 1h and 6 h). This dramatic DMSO-induced S100B secretion was also observed in the presence of serum (Fig 2B). DMSO-induced S100B secretion was significantly reduced by 1μM thapsigargin or 10 μM BAPTA-AM, in the presence or absence of serum (Fig 2C and D, respectively). Moreover, U73122 (at 1 μM), a Phopholipase C (PLC) inhibitor, and Ca^{2+}-channel blockers (1mM $CoCl_2$ and 50 μM verapamil) also reduced DMSO-stimulated S100B secretion.

DMSO induces an increase in intracellular Ca^{2+}. Representative images of astrocytes loaded with Fura-2 are shown in Fig 3 A. The effect of DMSO was investigated and two types of Ca^{2+}response were induced, as shown by representative graphs in Fig 3B, where a fast, short and transient increment (type 1 response, T1R) and a slower and more sustained increase (type 2 response, T2R) can be observed. Quantitative analyses of assays with Fura-2 indicated the percentage and type of cells responsive to 0.5% DMSO or 2 mM EGTA (calcium-free medium) or 10 μM forskolin (Fig 3C) and the intensity of these responses, compared to the maximum fluorescence obtained with digitonin (Fig 3D). Fig 3C demonstrates the percentage of cells that responded with only T1R; only T2R; both T1R and T2R and cells with no response (none) after DMSO, EGTA or forskolin additions. Note that DMSO and forskolin induced only TR1 in 25 and 30 % of cells, respectively, but only TR1 was not observed during incubation with EGTA. Moreover, less than 20% of cells exhibited only TR2 induced by DMSO or forkolin, but more than 40% exhibited only TR2 during incubation with EGTA. Under all conditions, the percentage of cells that exhibited TR1 and TR2 was higher than 50%, and less than 5% of cells were non-responsive, i.e., neither TR1 nor TR2.

When the profile of the DMSO-induced Ca^{2+} increment was analyzed, it was seen to be more similar to that evoked by forskolin than that caused by external addition of EGTA. In addition, the intensities of TR1 and TR2 (Fig 3D) during incubation with EGTA were not different, but they differed (TR2 > TR1) when they were induced by DMSO or forskolin.

DMSO-induced calcium increment involves mobilization from endoplasmatic reticulum. In another set of experiments, we investigated the involvement of calcium from the external medium (by removing it) and/or from endoplasmatic reticulum (by adding thapsigargin), in the types of responses: TR1; TR2; TR1 and TR2; neither T1R nor T2R (none) (Fig 4A). About 25% of cells exhibited T1R in the presence of DMSO and this number fell to 10% in the absence of external calcium; however, this response disappeared when cells were incubated with thapsigargin in a calcium-free medium. On the other hand, the percentage of cells that exhibited TR2 in the presence of DMSO was not altered by external calcium removal, but dramatically increased when cells were also incubated in the presence of thapsigargin. The percentage of cells that exhibited TR1 and TR2 under the influence of DMSO (about 60%) was not altered by removal of external calcium as well, but disappeared when thapsigargin was added. It was also possible to observe an increase in the percentage of non-responsive cells, by removal of external calcium and even more when thapsigargin was added.

In addition, the intensity of the type 1 response (Fig 4B) induced by DMSO was higher in Ca^{2+}-free medium and, interestingly, this was fully abolished when thapsigargin was added. The intensity of the type 2 response was not modified by removal of calcium, but significantly decreased when thapsigargin was added to the Ca^{2+}-free medium.

DMSO does not affect MTT reduction capacity or membrane integrity. Extracellular lactate dehydrogenase (LDH) activity was measured to evaluate membrane integrity (in parallel with S100B measurement). No changes were induced by DMSO at 1or 6 h, independently of the

presence of serum (data not shown). Two other assays were performed to characterize cell viability; the MTT reduction assay (Fig 5A,B) and PI exclusion (Fig 5C). DMSO (0.1-1%) did not affect the cell capacity of MTT reduction at 1 or 6 h (Fig 5A). Notice that this also occurred in the presence of serum (Fig 5B). It should be noted that the MTT reduction was significantly higher (~60%) in serum-deprived astrocytes (Student t test, $p<0.05$, in basal conditions or in the presence of DMSO). DMSO (at 1 %) did not cause changes in cell integrity, based on the PI exclusion assay, in the presence (data not shown) or absence of serum at 6 h (Fig 5C).

DMSO, in contrast to forskolin, does not induce morphological changes associated with S100B secretion. Phase-contrast microscopic images indicate that serum deprivation does not cause a significant stellation in primary astrocytes at 1 h after treatment. Cytochemical staining for actin confirmed typical flat and polygonal cells (Fig 6, panels A and D, respectively). The addition of DMSO (at 1 %) also did not alter this morphology (panels B and E); however, forskolin (10 μM) induced an intense stellation of cells (panels C and F).

DMSO induces an increase in cerebrospinal fluid S100B and in S100B secretion by acute hippocampal slices. Anesthetized rats received 2 μL ICV of DMSO or phosphate-buffered saline (control). Assuming a CSF volume of 200 μL (excluding subarachnoid space) (Huang *et al.* 1996), the final DMSO concentration is calculated to be approximately 1%. CSF was collected by cistern magna puncture at 1 h after and a significant increase in S100B was observed in this time (Fig 7A), without significant changes in S100B serum content (Fig 7B). Moreover, a significant increase in S100B secretion was found in *ex-vivo* hippocampal slices when exposed to 1% DMSO (Fig 7C).

Discussion

Astrocytes express and secrete S100B protein. There is much *in vitro* evidence to indicate that extracellular S100B plays a neurotrophic or toxic role in neural cells, depending on its concentration (Van Eldik & Wainwright 2003). The neurotoxic effect of S100B, particularly on neurons, appears to be dependent upon the redox environment (Donato et al. 2008) and does not necessarily involve Receptor for Advanced Glycation End products (RAGE), a well characterized receptor for S100B protein (Pichiule et al. 2007). Similarly, S100B secretion is affected by many conditions of metabolic stress or injury, but the underlying mechanism remains to be identified. A non-classical route for S100B release has been proposed, in which Ca^{2+} could be a mediator (Davey et al. 2001).

EGTA-induced S100B secretion has been previously observed in brains slices (Buyukuysal 2005) and in glioma cell cultures (Davey et al. 2001, Suzuki *et al.* 1987). Herein, using primary cortical astrocytes in culture, we found that S100B secretion negatively correlates with the external concentration of Ca^{2+}, i.e. S100B secretion increases proportionally with decreased external Ca^{2+} and this effect is dependent upon time. Removal of external Ca^{2+} in non-excitable cells, like astrocytes, induces mobilization of internal stores of Ca^{2+} (Verkhratsky 2002). In addition, herein, Ca^{2+}- ionophores also induced S100B secretion. In astrocytes, Ca^{2+}-entry (by channels in plasma membrane) occurs frequently for the replenishment of internal stores (Agulhon *et al.* 2008, Verkhratsky 2002) and this explains the blocking effect of Co^{2+} on S100B secretion. It is important to emphasize that basal S100B secretion in these experiments was induced by serum-deprivation, which *per se* mobilizes intracellular Ca^{2+} in astrocytes (Matsunaga & Ueda 2006).

DMSO is a common and widely-used solvent in biological studies and is often employed as a vehicle in pharmacological assays. This compound is often assumed to be a safe and inert

vehicle or is used in a determined safe concentration for specific assays. Concentrations of DMSO vehicle vary from low levels, such as 0.1% to higher levels, such as 20%. However, some effects (and side effects) of this agent are neglected. Due to its properties, particularly as a radical scavenger and hydrogen-bond disrupter, this membrane diffusible solvent can affect both *in vitro* and *in vivo* assays [See (Santos et al. 2003) for a review]. With regard to protein secretion, DMSO has been reported to increase the lipopolysaccharide (LPS)-induced secretion of interleukin-1 in human monocytes (Xing & Remick 2005), augment glucagon-like peptide1-induced secretion of insulin in a pancreatic beta-cell line (Kemp & Habener 2002) and decrease homocysteine-induced secretion of interleukin-8 in human monocytes (Zeng et al. 2003). In the latter case, this effect was attributed to the homocysteine scavenging property of this compound; however, the mechanisms of this effect remain unclear. It should be pointed out that, cAMP levels, investigated in the glucagon-like peptide1-induced secretion of insulin, were not affected by DMSO (Kemp & Habener 2002).

We found that DMSO increased S100B secretion, in a concentration- and time-dependent manner. The S100B secretion was robust when compared to a classical secretagogue, such as forskolin. Moreover, this increment was also robust even in the presence of serum. An effect of DMSO on S100B secretion involving Ca^{2+} release from internal stores is supported by the inhibitory effect observed with BAPTA-AM and thapsigargin (dissolved in 0.04 % DMSO). A very effective inhibition of DMSO-induced S100B secretion was obtained with Co^{2+} and verapamil. A significant inhibition was also obtained with U73122, an inhibitor of PLC, suggesting that IP_3 might be involved. These pharmacological agents were also effective in the S100B secretion induced by DMSO, in the presence of serum.

The next step was to investigate the intracellular calcium mobilization by DMSO. Indeed, DMSO (at 0.5% and 1%) induced an increase in intracellular calcium and the profile of this

effect (shape and amplitude of the responses) was more similar to that obtained with forskolin than that obtained by removal of external Ca^{2+} with EGTA. In addition, the response to DMSO was not altered in Ca^{2+}-free medium, indicating that this effect is dependent on intracellular calcium stores. In fact, this response was strongly modified when thapsigargin was added, suggesting that DMSO affects mobilization from the endoplasmic reticulum. It is important to note that TR1 was abolished by this treatment. On the other hand, the TR2 sustained response, was not completely inhibited by Ca^{2+}-free medium plus thapsigargin, indicating that DMSO may mobilize calcium from other intracellular stores not sensitive to thapsigargin. This source of calcium could be maintained in a different compartment to that of the endoplasmic reticulum, such as other important organelles, for instance the mitochondria or lysosomes, which may participate in calcium signalling and homeostasis in astrocytes (Simpson & Russell 1998, Heidemann *et al.* 2005, Li *et al.* 2008).

The secretagogue effects of forskolin on S100B are not fully understood, but could be explained by a positive modulation of IP_3-sensitive Ca^{2+} channels by Protein Kinase dependent on cAMP (PKA) (Bezprozvanny 2005). Accordingly, H89, an inhibitor of PKA, reduced the S100B secretion induced by serum deprivation (Goncalves et al. 2002); furthermore, thapsigargin, an inhibitor of endoplasmic reticulum Ca^{2+}-ATPase, reduced the S100B secretion induced by serum deprivation and forskolin (data not shown). However, astrocytes express primarily type 2 IP_3-channels (Agulhon et al. 2008), which apparently (at least in non-neural cells) are weakly sensitive to PKA phosphorylation (Soulsby & Wojcikiewicz 2007). An alternative explanation may be that the cAMP/PKA system induces cytoskeleton reorganization, which in turn remodels the space between the plasma membrane and ER, increasing calcium flow into the cells (Grimaldi *et al.* 1999). Moreover, cAMP itself could modulate capacitative Ca^{2+} entry (Wu *et al.* 1999). These possibilities require further investigation; however DMSO

induced a forskolin-like profile of Ca^{2+} intracellular increase, possibly also mobilizing Ca^{2+} from internal stores. Identification of any molecular target(s) is merely speculative at the moment.

Assuming that DMSO is a diffusible vehicle, a direct solvent effect on S100B secretion must be investigated, particularly when evaluating closer cell integrity and viability. No changes at 1h or 6 h in cell integrity (evaluated by LDH and PI assay) or cell viability (as evaluated by MTT reduction capacity assay), in the presence or absence of serum, were observed. It is important to mention that ethanol (at 1%) has no effect on S100B secretion (data not shown). Taken together, these data rule out a generic effect of this solvent on membrane permeability or alterations in cell viability that could explain S100B efflux.

Although DMSO and forskolin induced a similar profile of Ca^{2+} response, only forskolin caused a dramatic change in astrocyte morphology, as observed by phase-contrast microscopy or cytochemical staining for actin. Notice that S100B secretion induced by DMSO was not accompanied by cytoskeleton reorganization. It is important to emphasize that the effect of forskolin on cytoskeleton is reverted by lysophospatidic acid, which *per se* also caused an increase of S100B secretion (Pinto et al. 2000). These data, once more, suggest that S100B secretion profile in astrocytes is not necessarily associated with intense changes in cell morphology, at least in primary astrocyte cultures.

Finally, we investigated extracellular levels of S100B in the cerebrospinal fluid of rats after intracerebroventricular infusion of DMSO. Such findings are relevant because DMSO, at different concentrations, is commonly used as a vehicle (and control) for ICV drug administration. We found that 1% DMSO increased CSF S100B content, without affecting significantly the serum S100B content, suggesting that CSF changes in S100B content are not necessarily accompanied by serum variations in this protein. Moreover, acute hippocampal slices incubated with 1% DMSO secreted more S100B as cultured astrocytes do.

Our data consolidate a view that S100B secretion is triggered by agents that increase intracellular Ca^{2+} by mobilization from endoplasmic reticulum in astroglial cultures and brain slices; such triggering factors include serum-deprivation, ionophores, forskolin (Pinto et al. 2000), absence of external Ca^{2+} (Suzuki et al. 1987, Davey et al. 2001) and external low levels of K^{+} (Nardin et al. 2009). However, not all agents that mobilize intracellular calcium may necessarily result in S100B secretion and, furthermore, some S100B secretagogues act on downstream elements involved in the secretion triggered by Ca^{2+}.

In conclusion, whilst the molecular targets of DMSO are unknown, this agent (at concentration > 0.1%) increases intracellular Ca^{2+} and induces S100B secretion. This issue highlights the fact that secreted S100B is a possible mediator of the effects (and side-effects) of DMSO and that caution is necessary in the use of this vehicle, particularly assuming that many other secretable proteins, such as S100B, involved in intercellular communication, need to be investigated. Furthermore, the S100B secreting property of DMSO should be added to the list of therapeutic applications of this compound, particularly considering the neuroprotective effects of S100B that have been observed in traumatic brain damage (Kleindienst *et al.* 2005). In addition, the usefulness of DMSO as a tool to investigate intracellular calcium mobilization should be taken into consideration.

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References

Agulhon, C., Petravicz, J., McMullen, A. B., Sweger, E. J., Minton, S. K., Taves, S. R., Casper, K. B., Fiacco, T. A. and McCarthy, K. D. (2008) What is the role of astrocyte calcium in neurophysiology? *Neuron,* **59,** 932-946.

Bezprozvanny, I. (2005) The inositol 1,4,5-trisphosphate receptors. *Cell Calcium,* **38,** 261-272.

Buyukuysal, R. L. (2005) Protein S100B release from rat brain slices during and after ischemia: comparison with lactate dehydrogenase leakage. *Neurochem Int,* **47,** 580-588.

Carvalho, A. C., Sharpe, J., Rosenstock, T. R., Teles, A. F., Youle, R. J. and Smaili, S. S. (2004) Bax affects intracellular Ca2+ stores and induces Ca2+ wave propagation. *Cell Death Differ,* **11,** 1265-1276.

Davey, G. E., Murmann, P. and Heizmann, C. W. (2001) Intracellular Ca2+ and Zn2+ levels regulate the alternative cell density-dependent secretion of S100B in human glioblastoma cells. *J Biol Chem,* **276,** 30819-30826.

Donato, R., Sorci, G., Riuzzi, F., Arcuri, C., Bianchi, R., Brozzi, F., Tubaro, C. and Giambanco, I. (2008) S100B's double life: Intracellular regulator and extracellular signal. *Biochim Biophys Acta*.

Donato, R., Sorci, G., Riuzzi, F., Arcuri, C., Bianchi, R., Brozzi, F., Tubaro, C. and Giambanco, I. (2009) S100B's double life: intracellular regulator and extracellular signal. *Biochim Biophys Acta,* **1793,** 1008-1022.

Eriksen, J. L., Gillespie, R. and Druse, M. J. (2002) Effects of ethanol and 5-HT1A agonists on astroglial S100B. *Brain Res Dev Brain Res,* **139,** 97-105.

Goncalves, C. A., Concli Leite, M. and Nardin, P. (2008) Biological and methodological features of the measurement of S100B, a putative marker of brain injury. *Clin Biochem,* **41,** 755-763.

Goncalves, D., Karl, J., Leite, M., Rotta, L., Salbego, C., Rocha, E., Wofchuk, S. and Goncalves, C. A. (2002) High glutamate decreases S100B secretion stimulated by serum deprivation in astrocytes. *Neuroreport,* **13,** 1533-1535.

Gottfried, C., Cechin, S. R., Gonzalez, M. A., Vaccaro, T. S. and Rodnight, R. (2003) The influence of the extracellular matrix on the morphology and intracellular pH of cultured astrocytes exposed to media lacking bicarbonate. *Neuroscience,* **121,** 553-562.

Grimaldi, M., Favit, A. and Alkon, D. L. (1999) cAMP-induced cytoskeleton rearrangement increases calcium transients through the enhancement of capacitative calcium entry. *J Biol Chem,* **274,** 33557-33564.

Heidemann, A. C., Schipke, C. G. and Kettenmann, H. (2005) Extracellular application of nicotinic acid adenine dinucleotide phosphate induces Ca2+ signaling in astrocytes in situ. *J Biol Chem,* **280,** 35630-35640.

Huang, Y. L., Saljo, A., Suneson, A. and Hansson, H. A. (1996) Comparison among different approaches for sampling cerebrospinal fluid in rats. *Brain Res Bull,* **41,** 273-279.

Kemp, D. M. and Habener, J. F. (2002) Synergistic effect of dimethyl sulfoxide on glucagon-like peptide 1 (GLP-1)-stimulated insulin secretion and gene transcription in INS-1 cells: characterization and implications. *Biochem Pharmacol,* **64,** 689-697.

Kleindienst, A., McGinn, M. J., Harvey, H. B., Colello, R. J., Hamm, R. J. and Bullock, M. R. (2005) Enhanced hippocampal neurogenesis by intraventricular S100B infusion is associated with improved cognitive recovery after traumatic brain injury. *J Neurotrauma,* **22,** 645-655.

Leite, M., Frizzo, J. K., Nardin, P., de Almeida, L. M., Tramontina, F., Gottfried, C. and Goncalves, C. A. (2004) Beta-hydroxy-butyrate alters the extracellular content of S100B in astrocyte cultures. *Brain Res Bull,* **64,** 139-143.

Leite, M. C., Brolese, G., de Almeida, L. M., Pinero, C. C., Gottfried, C. and Goncalves, C. A. (2006) Ammonia-induced alteration in S100B secretion in astrocytes is not reverted by creatine addition. *Brain Res Bull,* **70,** 179-185.

Leite, M. C., Galland, F., Brolese, G., Guerra, M. C., Bortolotto, J. W., Freitas, R., Almeida, L. M., Gottfried, C. and Goncalves, C. A. (2008) A simple, sensitive and widely applicable ELISA for S100B: Methodological features of the measurement of this glial protein. *J Neurosci Methods,* **169,** 93-99.

Leite, M. C., Galland, F., de Souza, D. F., Guerra, M. C., Bobermin, L., Biasibetti, R., Gottfried, C. and Goncalves, C. A. (2009) Gap junction inhibitors modulate S100B secretion in astrocyte cultures and acute hippocampal slices. *J Neurosci Res*.

Li, D., Ropert, N., Koulakoff, A., Giaume, C. and Oheim, M. (2008) Lysosomes are the major vesicular compartment undergoing Ca2+-regulated exocytosis from cortical astrocytes. *J Neurosci,* **28,** 7648-7658.

Marenholz, I., Heizmann, C. W. and Fritz, G. (2004) S100 proteins in mouse and man: from evolution to function and pathology (including an update of the nomenclature). *Biochem Biophys Res Commun,* **322,** 1111-1122.

Matsunaga, H. and Ueda, H. (2006) Evidence for serum-deprivation-induced co-release of FGF-1 and S100A13 from astrocytes. *Neurochem Int,* **49,** 294-303.

Nardin, P., Tortorelli, L., Quincozes-Santos, A. et al. (2009) S100B Secretion in Acute Brain Slices: Modulation by Extracellular Levels of Ca(2+) and K (+). *Neurochem Res*.

Nardin, P., Tramontina, F., Leite, M. C., Tramontina, A. C., Quincozes-Santos, A., de Almeida, L. M., Battastini, A. M., Gottfried, C. and Goncalves, C. A. (2007) S100B content and secretion decrease in astrocytes cultured in high-glucose medium. *Neurochem Int,* **50,** 774-782.

Pichiule, P., Chavez, J. C., Schmidt, A. M. and Vannucci, S. J. (2007) Hypoxia-inducible factor-1 mediates neuronal expression of the receptor for advanced glycation end products following hypoxia/ischemia. *J Biol Chem,* **282,** 36330-36340.

Pinto, S. S., Gottfried, C., Mendez, A., Goncalves, D., Karl, J., Goncalves, C. A., Wofchuk, S. and Rodnight, R. (2000) Immunocontent and secretion of S100B in astrocyte cultures from different brain regions in relation to morphology. *FEBS Lett,* **486,** 203-207.

Rodrigues, L., Biasibetti, R., Swarowsky, A., Leite, M. C., Quincozes-Santos, A., Quilfeldt, J. A., Achaval, M. and Goncalves, C. A. (2009) Hippocampal alterations in rats submitted to streptozotocin-induced dementia model are prevented by aminoguanidine. *J Alzheimers Dis,* **17,** 193-202.

Sakatani, S., Seto-Ohshima, A., Shinohara, Y., Yamamoto, Y., Yamamoto, H., Itohara, S. and Hirase, H. (2008) Neural-activity-dependent release of S100B from astrocytes enhances kainate-induced gamma oscillations in vivo. *J Neurosci,* **28,** 10928-10936.

Santos, N. C., Figueira-Coelho, J., Martins-Silva, J. and Saldanha, C. (2003) Multidisciplinary utilization of dimethyl sulfoxide: pharmacological, cellular, and molecular aspects. *Biochem Pharmacol,* **65,** 1035-1041.

Simpson, P. B. and Russell, J. T. (1998) Role of mitochondrial Ca2+ regulation in neuronal and glial cell signalling. *Brain Res Brain Res Rev,* **26,** 72-81.

Smaili, S. S., Rosenstock, T. R. and Hsu, Y. T. (2008) Evaluation of some cell death features by real time real space microscopy. *Methods Enzymol,* **442,** 27-50.

Soulsby, M. D. and Wojcikiewicz, R. J. (2007) Calcium mobilization via type III inositol 1,4,5-trisphosphate receptors is not altered by PKA-mediated phosphorylation of serines 916, 934, and 1832. *Cell Calcium,* **42,** 261-270.

Steiner, J., Bernstein, H. G., Bielau, H., Berndt, A., Brisch, R., Mawrin, C., Keilhoff, G. and Bogerts, B. (2007) Evidence for a wide extra-astrocytic distribution of S100B in human brain. *BMC Neurosci,* **8,** 2.

Suzuki, F., Kato, K., Kato, T. and Ogasawara, N. (1987) S-100 protein in clonal astroglioma cells is released by adrenocorticotropic hormone and corticotropin-like intermediate-lobe peptide. *J Neurochem,* **49,** 1557-1563.

Tramontina, A. C., Tramontina, F., Bobermin, L. D., Zanotto, C., Souza, D. F., Leite, M. C., Nardin, P., Gottfried, C. and Goncalves, C. A. (2008) Secretion of S100B, an astrocyte-derived neurotrophic protein, is stimulated by fluoxetine via a mechanism independent of serotonin. *Prog Neuropsychopharmacol Biol Psychiatry,* **32,** 1580-1583.

Van Eldik, L. J. and Wainwright, M. S. (2003) The Janus face of glial-derived S100B: beneficial and detrimental functions in the brain. *Restor Neurol Neurosci,* **21,** 97-108.

Verkhratsky, A. S., N.; Toescu, E.C. (2002) *Calcium excitability of glial cells. In: The tripartite synapse. Glia in synaptic transmission*. Oxford University Press, NewYork.

Wu, M. L., Chen, W. H., Liu, I. H., Tseng, C. D. and Wang, S. M. (1999) A novel effect of cyclic AMP on capacitative Ca2+ entry in cultured rat cerebellar astrocytes. *J Neurochem,* **73,** 1318-1328.

Xing, L. and Remick, D. G. (2005) Mechanisms of dimethyl sulfoxide augmentation of IL-1 beta production. *J Immunol,* **174,** 6195-6202.

Zeng, X., Dai, J., Remick, D. G. and Wang, X. (2003) Homocysteine mediated expression and secretion of monocyte chemoattractant protein-1 and interleukin-8 in human monocytes. *Circ Res,* **93,** 311-320.

Figure Legends

Fig. 1. S100B secretion is modified by removal of external calcium. A, Rat cortical astrocytes were cultured in DMEM containing 10% FCS. After confluence, the medium was replaced by the DMEM without serum (open circles) or DMEM/10% FCS (black circles). S100B was measured by ELISA at 15 min, 1 h and 6 h. Statistical analysis was carried out by repeated measures ANOVA. B, The medium was replaced by the DMEM without serum in the presence or absence of EGTA (from 1 to 3 mM). S100B was measured by ELISA at 1 h. Means indicated with a different letter differ (one way ANOVA followed by Duncan's test, assuming $p< 0.05$). C, The medium was replaced by DMEM without serum in the presence or absence of 1 mM $CoCl_2$, 1 μM ionomycin, 1 μM A23187 or 10 μM forskolin. S100B was measured by ELISA at 1 h. For all these results, each value represents the mean (± standard error) of at least 5 independent experiments performed in triplicate. * Significantly different from basal secretion (Student t test followed by Bonferroni's adjustment, $p< 0.05$).

Fig. 2. DMSO increases S100B secretion in the presence or absence of serum. Rat cortical astrocytes were cultured in DMEM containing 10% FCS. After confluence, the medium was replaced by the DMEM without serum (A and C) or DMEM/10% FCS (B and D). In A and B, DMSO was added at a final concentration ranging from 0.1 to 1%. S100B was measured by ELISA at 15 min, 1 h and 6 h. Each value is the mean (± standard error) of at least 5 independent experiments performed in triplicate. Statistical analysis was carried out by repeated measures ANOVA. In C and D, cells were treated with 1 μM thapsigargin (TPG), 10 μM BAPTA-AM, 1 μM U73122, 1mM $CoCl_2$ or 50 μM verapamil (VER) for 15 min before media replacement. Media were then replaced by DMEM without serum (in C) or DMEM/10% FCS (in D),

containing 0.5% DMSO and the drug previously incubated. S100B was measured by ELISA after 1 h. Basal S100B secretion was assumed as 1 in each experiment. Each value is the mean (± standard error) of at least 5 independent experiments performed in triplicate. * Significantly different from basal secretion (one way ANOVA followed by Dunnett's test, assuming $p< 0.05$).

Fig 3. DMSO induces an increase in intracellular Ca^{2+}in astrocytes. Rat cortical astrocytes were cultured in DMEM containing 10% FCS. Intracellular calcium variations were evaluated in pre-confluent astrocytes loaded with Fura-2 by real time fluorescence microscopy. A, representative fluorescent images of cells loaded with Fura-2 before and after DMSO stimulation. B, representative graphs of the type 1 and type 2 responses (TR1 and TR2, respectively) induced by DMSO. C, percentage of responsive cells exposed to 0.5% DMSO, 2 mM EGTA or 10 µM forskolin and non-responsive cells: cells that exhibited type 1 response (TR1) are represented by *black* bars; cells that exhibited type 2 response (TR2) are represented by *dark-grey* bars; cells that exhibited type 1 and 2 responses (TR1/2) are represented by *grey* bars; cells that do not respond to DMSO or EGTA or forkolin stimulation are represented by *open* bars. D, intensity of cell response to 0.5% DMSO, 2 mM EGTA or 10 µM forskolin, compared with response induced by 0.1 mM digitonin (assumed as 100%): intensities of TR1 and TR2 are represented by *black* and *dark-grey* bars, respectively. Means, in each stimulating condition, indicated by different letters differ (one way ANOVA followed by Duncan's test in panel C and Student t test in panel D, assuming $p< 0.05$).

Fig. 4. DMSO induces intracellular calcium mobilization. Intracellular calcium variations were evaluated in pre-confluent astrocytes loaded with Fura-2 by real time fluorescence microscopy, stimulated by 0.5% DMSO (dotted black bars) or DMSO in a calcium-

free medium (DMSO/-Ca^{2+}, dotted grey bars) or DMSO in a calcium-free medium containing 1 µM thapsigargin (DMSO/-Ca^{2+}/TPG, dotted white bars). A, percentage of cells exhibiting only a type 1 response (TR1); exhibiting only a type 2 response (TR2); exhibiting both TR1 and TR2; and neither TR1 nor TR2 (none) were analysed separately, comparing the different stimulating conditions. B, intensities of type 1 and type 2 responses (TR1 and TR2, respectively) obtained under different stimulating conditions. Means, under each stimulating condition, indicated by a different letter differ (one way ANOVA followed by Duncan's test, assuming $p < 0.05$).

Fig. 5. DMSO does not affect cell integrity or viability. Rat cortical astrocytes were cultured in DMEM containing 10% FCS. Confluent astrocytes were exposed to DMSO at a final concentration of 0.1 or 0.5 or 1%, during 1 or 6 h. During this time astrocytes were incubated with propidium iodide (panel C) or were incubated with MTT at the end of this time period (panels A and B). A, MTT reduction assay performed after 1 h (black bars) or 6 h (grey bars) of incubation in DMEM without serum (in A) or with 10% FCS (in B). Statistical analysis was performed by one way ANOVA. C, representative propidium iodide fluorescent image of astrocytes after 6 h of incubation in DMEM with 10% FCS, in the absence (at left) or presence of 1% DMSO (at right). Each value is the mean (± standard error) of at least 4 independent experiments performed in triplicate. Statistical analysis was performed by Student t test. Scale bar: 50 µm.

Fig. 6. DMSO does not induce morphological changes associated with S100B secretion. Rat cortical astrocytes were cultured in DMEM containing 10% FCS. Confluent and pre-confluent cells in basal conditions (panels A and D, respectively) or incubated with 1% DMSO

(panels B and E) or incubated with 10 μM forskolin for 1 h (panels C and F) were analyzed by phase-contrast microscopy (A-C) and fluorescent microscopy after rhodamine-phalloidin staining for actin filaments (D-F). Images are representative of 4 independent experiments. Scale bar: 50 μm.

Fig. 7. *DMSO* increases cerebrospinal content of S100B and in vitro S100B secretion in brain slices. A and B, Intracerebroventricular injection of DMSO (2 μL) or saline solution (2 μL) in adult Wistar rats under anaesthesia. After 1 hour, cerebrospinal fluid and blood were collected by magna puncture and intracardic puncture, respectively. Cerebrospinal fluid (A) and serum (B) contents of S100B were measured by ELISA. Each value represents the mean (± standard error) of 5 rats per group. C, Adult Wistar rats were killed by decapitation and 0.3 mm hippocampal slices were obtained using a McIlwain chopper. After a metabolic recovery period, hippocampal slices were exposed to 1% DMSO and extracellular content of S100B was measured by ELISA. Each value is the mean (± standard error) of at least 4 independent experiments performed in triplicate. * Significantly different from the respective control (Student t test, $p < 0.05$).

Figure 1

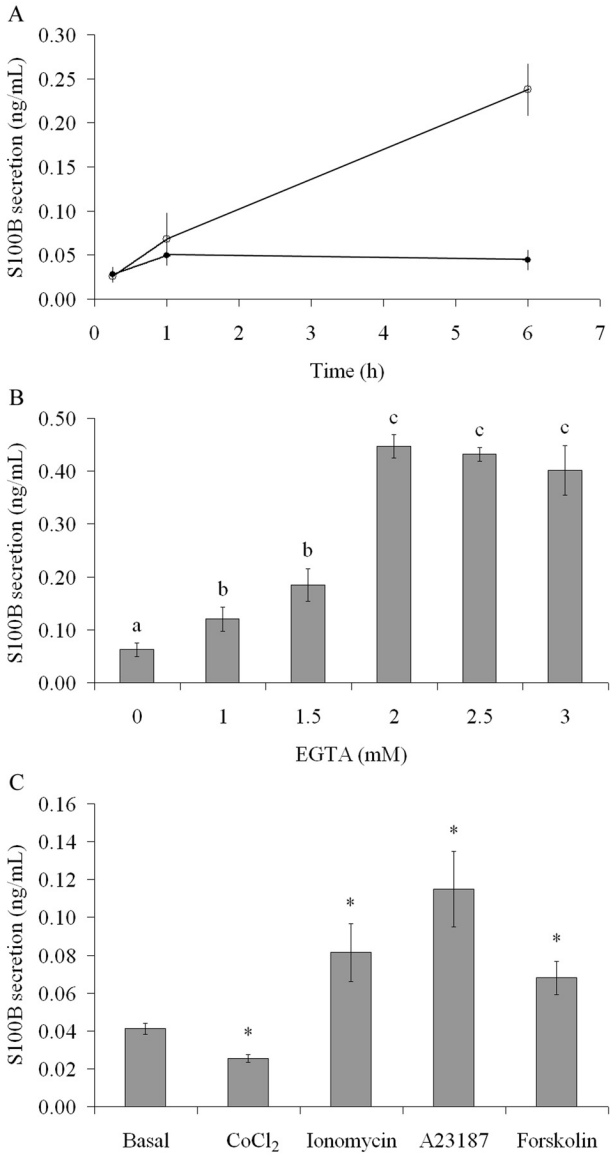

A
S100B secretion (ng/mL)
0.30
0.25
0.20
0.15
0.10
0.05
0.00
0
1
2
3
4
5
6
7
Time (h)
B
S100B secretion (ng/mL)
0.50
0.40
0.30
0.20
0.10
0.00
a
b
b
c
c
c
0
1
1.5
2
2.5
3
EGTA (mM)
C
S100B secretion (ng/mL)
0.16
0.14
0.12
0.10
0.08
0.06
0.04
0.02
0.00
*
*
*
*
Basal
CoCl2
Ionomycin
A23187
Forskolin

Figure 2

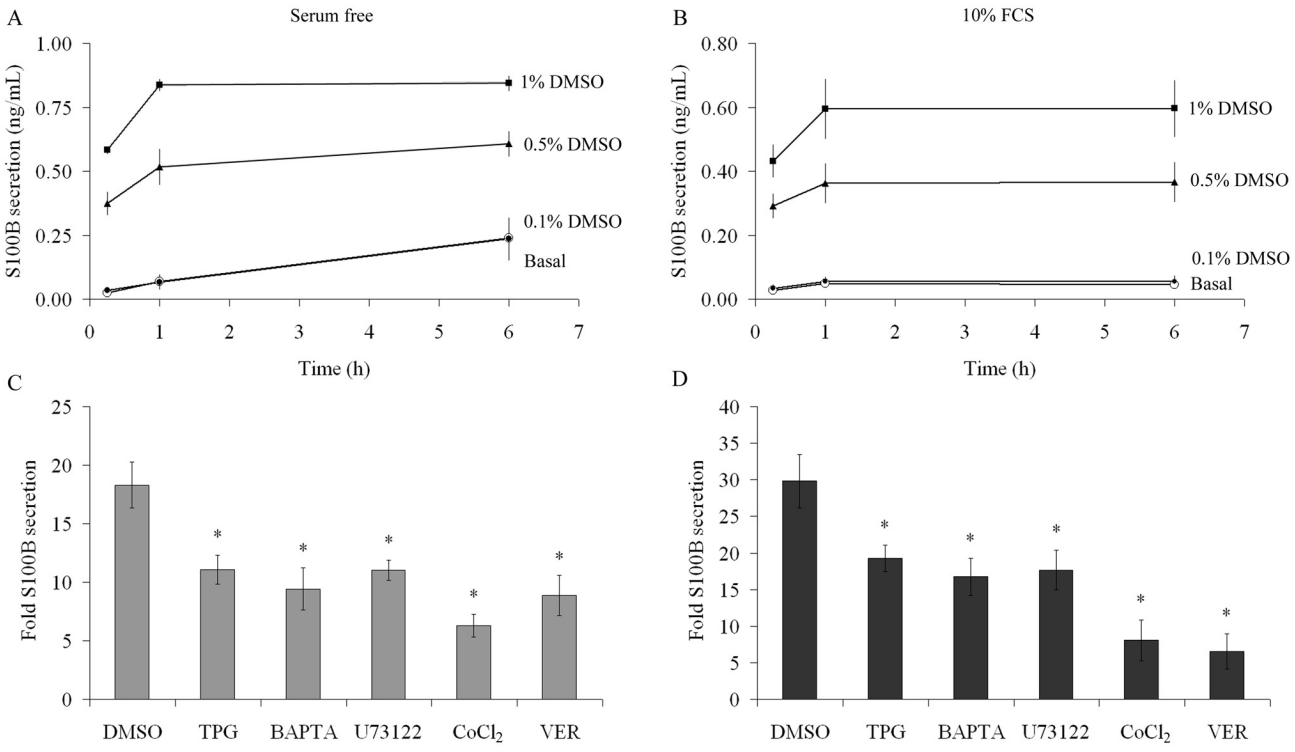

A
Serum free
S100B secretion (ng/mL)
1.00
0.75
0.50
0.25
0.00
0 1 2 3 4 5 6 7
Time (h)
1% DMSO
0.5% DMSO
0.1% DMSO
Basal
B
10% FCS
S100B secretion (ng/mL)
0.80
0.60
0.40
0.20
0.00
0 1 2 3 4 5 6 7
Time (h)
1% DMSO
0.5% DMSO
0.1% DMSO
Basal
C
Fold S100B secretion
25
20
15
10
5
0
DMSO TPG BAPTA U73122 CoCl2 VER
D
Fold S100B secretion
40
35
30
25
20
15
10
5
0
DMSO TPG BAPTA U73122 CoCl2 VER

Figure 3

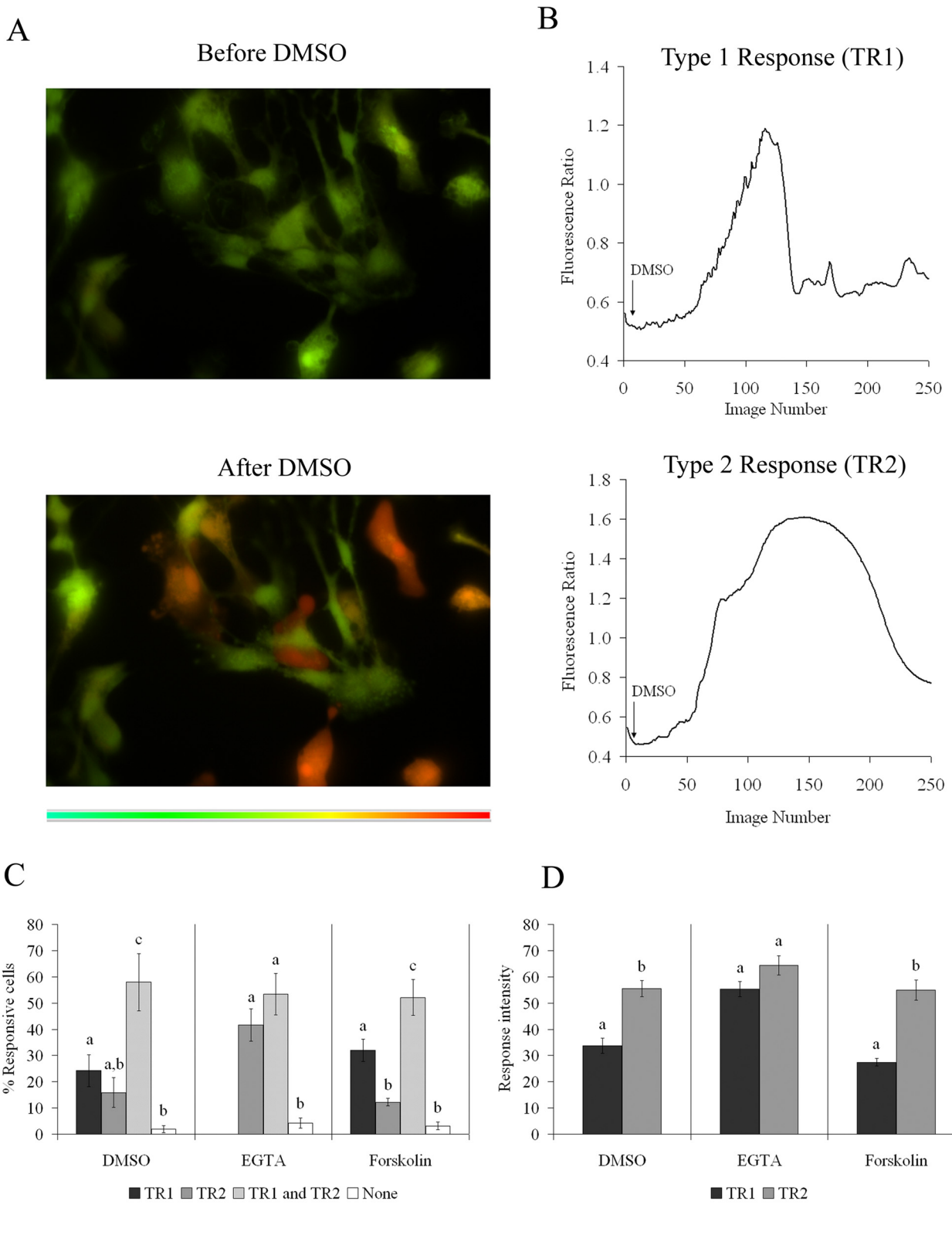

A
Before DMSO
After DMSO
B
Type 1 Response (TR1)
Fluorescence Ratio
DMSO
Image Number
Type 2 Response (TR2)
Fluorescence Ratio
DMSO
Image Number
C
% Responsive cells
DMSO
EGTA
Forskolin
TR1
TR2
TR1 and TR2
None
D
Response intensity
DMSO
EGTA
Forskolin
TR1
TR2

Figure 4

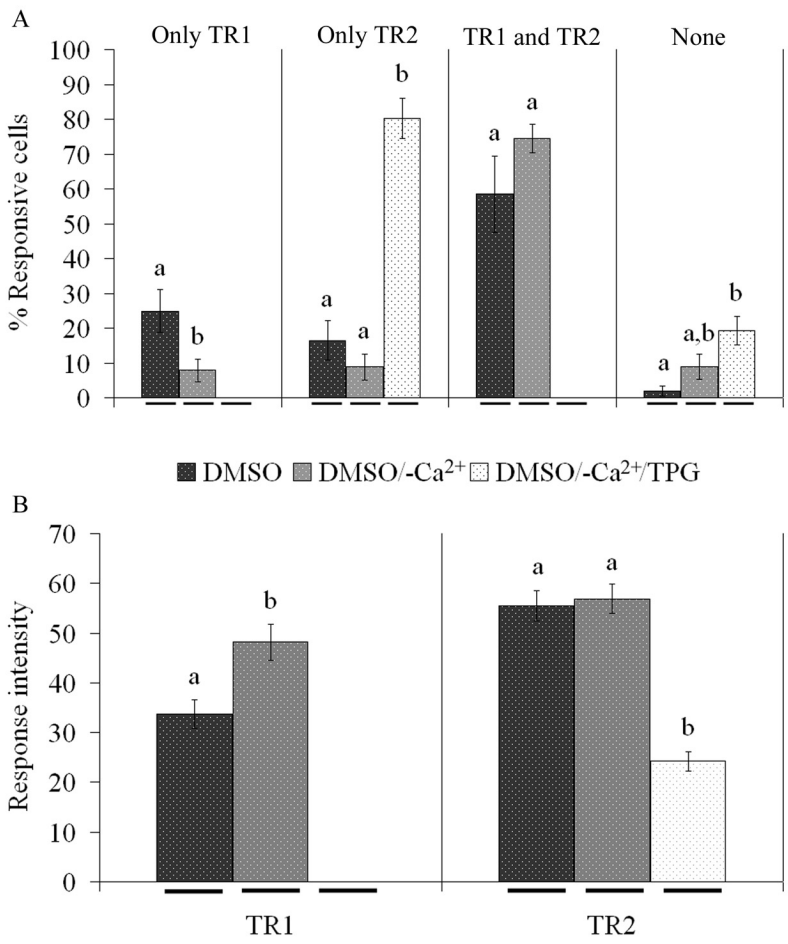

A
Only TR1
Only TR2
TR1 and TR2
None
% Responsive cells
100
90
80
70
60
50
40
30
20
10
0
a
b
a
a
b
a
a
a
b
DMSO
DMSO/-Ca2+
DMSO/-Ca2+/TPG
B
Response intensity
70
60
50
40
30
20
10
0
a
b
a
a
b
TR1
TR2

Figure 5

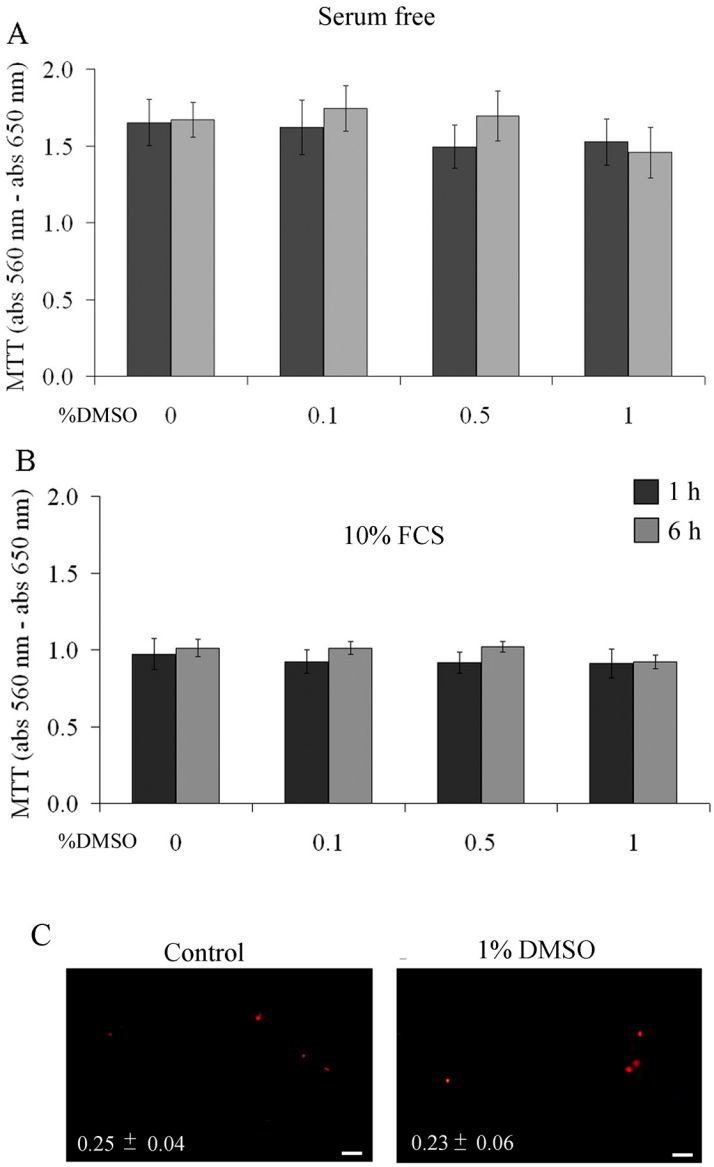

A
Serum free
MTT (abs 560 nm - abs 650 nm)
2.0
1.5
1.0
0.5
0.0
%DMSO 0 0.1 0.5 1
B
10% FCS
1 h
6 h
MTT (abs 560 nm - abs 650 nm)
2.0
1.5
1.0
0.5
0.0
%DMSO 0 0.1 0.5 1
C
Control
1% DMSO
0.25 ± 0.04
0.23 ± 0.06

Figure 6

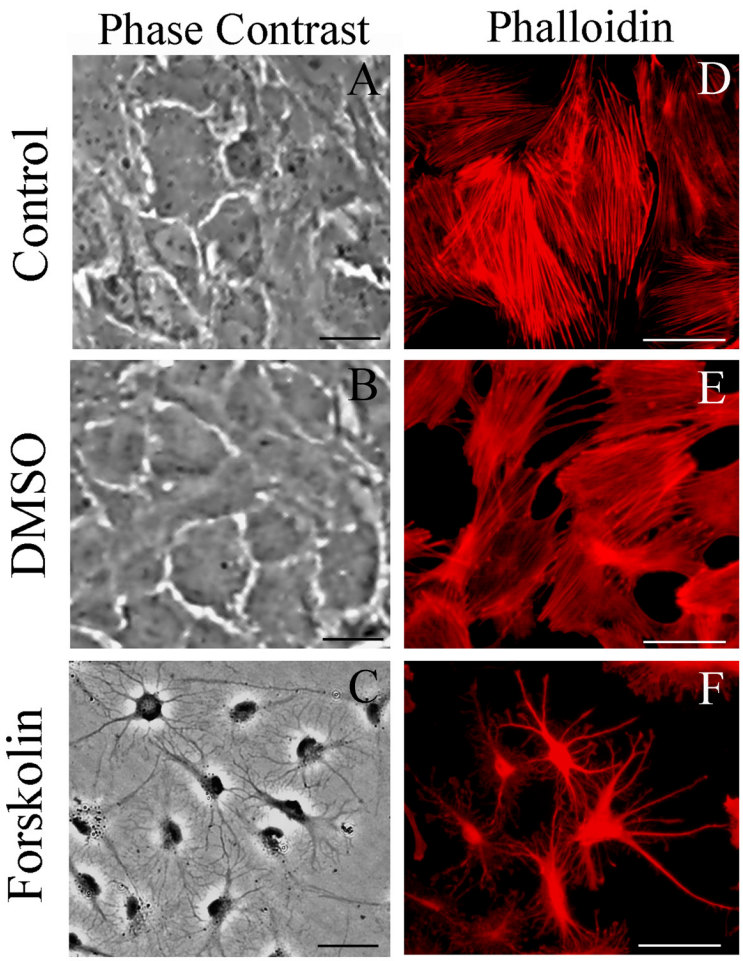
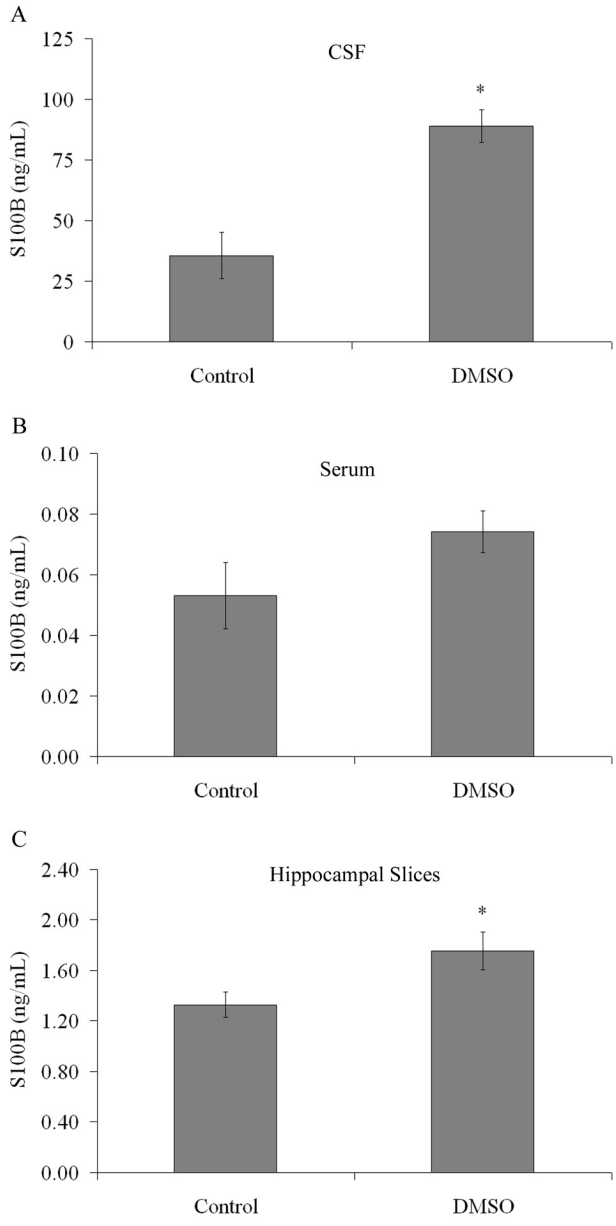


Figure 7


A
CSF
S100B (ng/mL)
125
100
75
50
25
0
*
Control
DMSO
B
Serum
S100B (ng/mL)
0.10
0.08
0.06
0.04
0.02
0.00
Control
DMSO
C
Hippocampal Slices
S100B (ng/mL)
2.40
2.00
1.60
1.20
0.80
0.40
0.00
*
Control
DMSO

Parte III

Discussão

1. Dosagem de S100B

1.1. Características do imunoensaio

Existem muitas técnicas diferentes disponíveis para a medida da S100B, o que muitas vezes provoca discrepâncias entre valores de concentração de S100B obtidos entre diferentes grupos de pesquisa, que acabam sendo discutidos sem levar em consideração essas diferenças metodológicas. Todas as metodologias disponíveis atualmente para a dosagem de S100B são imunoensaios, ou seja, são técnicas baseadas no reconhecimento da proteína por anticorpos comerciais específicos. Apesar de frequentemente diferentes técnicas utilizarem os mesmos anticorpos (Goncalves et al. 2008), o que deveria facilitar a comparação dos resultados de diferentes grupos, isso não acontece, devido a uma série de pequenas variações metodológicas que afetam a especificidade e a afinidade da detecção da S100B nos diferentes tipos de amostras biológicas.

Parece claro que pequenas alterações metodológicas podem causar grandes alterações na imunodetecção da S100B quando observamos os dois protocolos padronizados nesse trabalho. Ambos utilizam os mesmos anticorpos comerciais; entretanto, o protocolo 1 é dez vezes mais sensível que o protocolo 2, enquanto que este possui uma linearidade dez vezes maior. É importante ressaltar que a única diferença metodológica entre os dois protocolos está no tempo de incubação dos anticorpos e na forma como eles são incubados (em separado, no protocolo 1, ou simultaneamente, no protocolo 2). A sensibilidade e reprodutibilidade do protocolo 2 foi comparável a de kits comerciais para a dosagem de S100B, especialmente o da marca Sagtec (Tort *et al.*

2003), enquanto que o protocolo 1 apresentou sensibilidade maior do que a maioria das técnicas disponíveis para a dosagem de S100B (Goncalves et al. 2008).

O reconhecimento da S100B nas diferentes amostras biológicas testadas não foi alterado pelo congelamento e descongelamento das amostras. Nas amostras de tecido, inibidores de proteases como PMSF, EGTA e EDTA foram suficientes para preservar a estabilidade da proteína. Nas amostras de soro, líquor, urina e meio de cultura, não foi necessária a adição desses inibidores. Nos dois protocolos padronizados, o bloqueio com albumina de soro bovino pode ser substituído com a mesma eficiência no bloqueio de ligações inespecíficas, por um bloqueio com ovoalbumina ou PVP-40. O leite desnatado não pode ser usado como agente bloqueador, o que confirma a presença de S100B no leite (Gazzolo et al. 2003).

1.2. Interferentes na técnica

Sabe-se que um anticorpo específico reconhece um antígeno através de uma pequena parte de sua estrutura chamada epítopo. Esse reconhecimento é feito através de ligações não covalentes entre antígeno e anticorpo que, para se formarem, necessitam que antígeno e anticorpo estejam em uma conformação tridimensional específica. É por isso que o reconhecimento de uma proteína por seu anticorpo específico pode mudar se ela sofrer modificações pós-traducionais ou ainda se esse antígeno se ligar com algum ligante de forma não covalente. Essa especificidade dos anticorpos por uma estrutura tridimensional específica do antígeno é o que possibilita a produção de anticorpos que reconheçam apenas a forma fosforilada ou glicosilada de uma determinada proteína, por exemplo. Entretanto, esse tipo de alteração estrutural frequentemente não é levado em

consideração durante a elaboração e a interpretação dos imunoensaios (Tramontina *et al.* 2007).

A S100B é uma proteína ligante de cálcio, que pode mudar seu reconhecimento pelo anticorpo dependendo de seu estado de ligação ao cálcio (Goncalves *et al.* 1997). Trabalhos prévios demonstraram que o reconhecimento da S100B pelo anticorpo de captura utilizado nesse trabalho (clone SH-B1, da marca Sigma) não é alterado pela presença de cálcio (Goncalves et al. 1997). Entretanto, o reconhecimento da S100B por um anticorpo de detecção, conjugado com a peroxidase (da marca DAKO) é significativamente aumentado na presença de cálcio (Green et al. 1997, Tramontina *et al.* 2000). Inversamente, o uso de quelantes de cálcio aumentou o reconhecimento da proteína S100B pelo anticorpo em um imunoensaio comercial, da marca Sangtec (Tort et al. 2003). Em ambos os protocolos de ELISA para S100B demonstrados nesse trabalho, a presença de cálcio não modificou o reconhecimento da S100B por nenhum dos anticorpos utilizados (captura e detecção). É importante ressaltar que o anticorpo de detecção utilizado nesse trabalho foi o da maca DAKO, que apresentou alteração de reconhecimento da S100B na presença de cálcio em trabalhos prévios (Green et al. 1997, Tramontina et al. 2000). Nesses estudos anteriores, o anticorpo era conjugado à peroxidase. Recentemente a DAKO parou de produzir o anticorpo conjugado e o substituiu pela forma não conjugada, que foi utilizada no presente trabalho, o que sugere que pequenas modificações no anticorpo (como a conjugação com uma enzima) também possam causar modificações no reconhecimento de seu antígeno.

A S100B interfere no estado de fosforilação de muitas proteínas-alvo (Donato 2001), mas até agora não foi demonstrado que ela seja alvo de nenhuma proteína cinase

in vivo. Portanto, o estado de fosforilação da S100B não é relevante para os imunoensaios. No entanto, o estado de oxidação da S100B pode alterar o reconhecimento pelo anticorpo e também interfere na interação da S100B com proteínas-alvo específicas (Goncalves *et al.* 2000, Scotto *et al.* 1998). De fato, uma diminuição no reconhecimento da S100B foi observada quando ela foi oxidada através de um tratamento prévio com peróxido de hidrogênio ou com tetrationato de sódio. O DTT não foi capaz de reverter a diminuição do reconhecimento da S100B pelos anticorpos induzida por peróxido de hidrogênio, sugerindo que essa oxidação não é limitada à formação de ligações dissulfeto. Portanto, esse aspecto deve ser considerado na avaliação dos resultados, especialmente quando as amostras são expostas ao estresse oxidativo, inclusive durante a sua obtenção.

A S100B é uma proteína altamente solúvel e que pode interagir com muitos substratos diferentes (Donato 2001) e seu reconhecimento pelo anticorpo pode ser afetado pela interação com essas proteínas independentemente de ligações cruzadas. Nesse estudo, foi utilizado um anticorpo de captura monoclonal que não reconhece a S100A1, uma proteína abundante no tecido neural. Os anticorpos utilizados no imunoensaio comercial da marca Sangtec apresentam reação cruzada fraca com a S100A1 (Heizmann 2004) e que seu reconhecimento pode ser afetado na presença de outras proteínas da amostra (Fazio *et al.* 2004), o que demanda um cuidado maior na avaliação dos resultados quando a dosagem for realizada em homogeneizados de tecidos (Mazzini *et al.* 2005).

A alta concentração de lipídios no tecido adiposo se mostrou como um interferente nos dois protocolos padronizados nesse trabalho, diminuindo o reconhecimento da S100B pelos anticorpos. Esse fenômeno pode ser explicado pela

interferência dos lipídeos nas interações hidrofóbicas entre antígeno e anticorpo. Essa interferência pode ser eliminada através da delipidação prévia das amostras de tecido adiposo.

1.3. Conclusões

Dois protocolos para S100B foram padronizados, que devido a sua diferença de sensibilidade e linearidade são ideais para dosar uma diversidade de amostras. Dessa forma, amostras com baixas concentrações de S100B, como urina, soro, líquor e amostras de cultura de células podem ser dosadas utilizando o protocolo 1, de maior sensibilidade. Já amostras com maior concentração de S100B, como tecido adiposo e tecido cerebral, podem ser dosadas pelo protocolo 2, que apresenta maior linearidade, evitando o uso de diluições muito altas o que poderia causar um erro maior. O fato de serem técnicas muito parecidas facilita a comparação entre resultados obtidos em diferentes tipos de amostras biológicas. A técnica apresentou sensibilidade comparada à de imunoensaios comerciais, com um custo por amostra extremamente baixo.

O estado de oxidação da S100B afetou o reconhecimento pelo anticorpo. Esse aspecto é de extrema importância visto que as formas reduzida e oxidada da proteína podem coexistir *in vivo* e que o equilíbrio entre essas duas formas pode ser modificado pelo estresse oxidativo em situações fisiológicas ou patológicas ou ainda mesmo no momento da obtenção das amostras.

2. Comunicação por junção gap e secreção de S100B

2.1. Secreção de S100B na presença de inibidores de junção gap

Preparação / Tempo	Hal	Oct	Cbx	Gza	FFA	ET-1
Astrócitos 15 minutos	-	-	↑	-	-	↑
Astrócitos 1 h	↑	↑	↑	-	↑	↑
Astrócitos 6 h	-	↑	↑	-	-	↓
Fatias 1 h	-	-	↑	↑	-	↓
Glioma C6 1 h	-	-	-	-	-	-
Astrócitos pré-confluentes 1 h	-	-	-	-	-	-

2.2. Viabilidade celular – cultura de astrócitos e glioma C6

- Não foi observada alteração na integridade celular em cultura de astrócitos em 6 h de incubação com os bloqueadores de junção gap ou com ácido glicirrízico (Gza) pela técnica de captação do iodeto de propídio e liberação de LDH.
- Não foi observada alteração na viabilidade celular pela técnica de redução do MTT em astrócitos expostos aos bloqueadores de junção gap ou ao Gza durante 6 h. O FFA aumentou a redução de MTT.
- Apenas o FFA foi capaz de reduzir a captação do corante vermelho neutro em cultura de astrócitos, em 6 h de tratamento.

2.3. Viabilidade celular – fatias agudas hipocampais

- Não foi observado aumento de atividade de LDH no meio extracelular em 1 h de exposição aos bloqueadores de junção gap ou ao Gza.
- Todos os bloqueadores de junção gap, mas não o Gza, foram capazes de diminuir a redução do MTT em 1 h de exposição.

- Apenas a Cbx foi capaz de reduzir a captação do corante vermelho neutro durante 1 h de exposição.

2.4. Eficiência no bloqueio de junção gap em astrócitos

Todos os bloqueadores de junção gap testados foram capazes de bloquear a comunicação por junção gap, avaliada pela técnica de incorporação do corante "Lucifer Yellow".

2.5. Comentários

Os resultados mostram que a secreção de S100B aumenta com o bloqueio da comunicação por junção gap tanto em cultura de astrócitos quanto em fatias agudas hipocampais. Algumas variações foram observadas e podem ser justificadas pela natureza do bloqueador de junção gap, pelos seus diferentes alvos intracelulares e pelo tipo de preparação utilizada.

Não existe um bloqueador de junção gap altamente específico; a maioria deles possui outras atividades intracelulares. O halotano, por exemplo, além de bloquear junção gap, é um anestésico, e o efeito anestésico pode estar relacionado com o bloqueio da junção gap (Wentlandt *et al.* 2006). O ácido fluflenâmico, além de bloquear junção gap, é um composto com atividade anti-inflamatória. De todos os bloqueadores de junção gap, a carbenoxolona é considerada a mais específica.

Dessa forma, para confirmar se o efeito observado não é devido a um efeito inespecífico de um determinado bloqueador, utilizamos cinco diferentes bloqueadores. Todos os bloqueadores foram capazes de aumentar a secreção de S100B em culturas de

astrócitos no intervalo de 1 h, no qual o bloqueio de junção gap é efetivo. Os astrócitos pré-confluentes que ainda não estabeleceram comunicação por junção gap e as células C6 que praticamente não expressam conexinas (Tabernero et al. 2006) foram utilizadas como controle negativo. De fato, nenhum dos bloqueadores testados foi capaz de aumentar a secreção de S100B em cultura de astrócitos pré-confluentes e em células de glioma C6, mostrando que o aumento da secreção de S100B é devido ao bloqueio da junção gap.

O octanol também foi capaz de aumentar a secreção de S100B em culturas de astrócitos em 6 h de tratamento; entretanto, contrariamente aos resultados em astrócitos, nenhum dos agentes lipofílicos (halotano e octanol) foi capaz de modular a secreção de S100B em fatias agudas hipocampais durante 1 h de exposição. Essas moléculas atuam na bicamada lipídica e, aparentemente, têm alvos moleculares diferentes em cultura de células e em fatias. O ácido flufenâmico também não foi capaz de alterar a secreção de S100B em fatias agudas hipocampais e seu mecanismo de bloqueio de junção gap ainda é desconhecido. A carbenoxolona, que atua diretamente nas conexinas para bloquear a junção gap (Salameh & Dhein 2005), foi o único bloqueador capaz de aumentar rápida e persistentemente a secreção de S100B, tanto em cultura de astrócitos quanto em fatias agudas hipocampais.

O mecanismo de bloqueio de junção gap pela endotelina-1 envolve vias de sinalização intracelulares (Salameh & Dhein 2005) e contrariamente ao seu efeito em astrócitos em 1 h de exposição, em 6 h a endotelina-1 foi capaz de reduzir a secreção de S100B, bem como em fatias agudas hipocampais. Estudos com corantes fluorescentes demonstram que a endotelina-1 bloqueia rapidamente a comunicação por junção gap, mas a comunicação normal é restabelecida em 90 min (Blomstrand & Giaume 2006), o que

poderia explicar o efeito em longo prazo da endotelina-1 em cultura de astrócitos. Além disso, períodos prolongados de exposição à endotelina causam a diminuição de receptores de glutamato e da glutamina sintetase (Lehmann *et al.* 2008). Apesar de não se ter nenhuma informação a respeito da dessensibilização dos receptores de endotelina-1 em fatias agudas hipocampais, esse mecanismo não pode ser descartado. Outra explicação para a redução da secreção de S100B em fatias expostas à endotelina-1 pode ser o aumento da atividade neuronal mediada pelo glutamato (Goncalves et al. 2002, Shihara *et al.* 1998).

Outra diferença entre os modelos de cultura primária de astrócitos e preparações de fatias hipocampais ocorreu no que diz respeito ao uso do ácido glicirrízico. Esse composto é estruturalmente similar à cabenoxolona, mas não bloqueia junções gap. O ácido glicirrízico é um composto bastante utilizado como controle negativo. Nenhum efeito sobre a secreção de S100B foi observado em culturas de astrócitos expostas ao ácido glicirrízico; entretanto, em fatias agudas hipocampais, foi observado um aumento da secreção de S100B quando expostas a esse composto. Esse feito poderia ser explicado pela atividade do ácido glicirrízico em outras células presentes nas fatias que pudessem ter efeito secundário sobre a secreção de S100B. Sabe-se que o ácido glicirrízico tem efeito sobre a excitabilidade neuronal, principalmente sobre a permeabilidade do K^+ (Elsen *et al.* 2008).

A técnica de incorporação do corante fluorescente Lúcifer Yellow confirmou que o bloqueio da comunicação por junção gap foi efetivo em nossas preparações. Além disso, as técnicas de integridade ou viabilidade celular ajudaram a caracterizar o efeito

dos bloqueadores de junção gap sobre a secreção de S100B, bem como a identificar efeitos inespecíficos deles nas diferentes preparações.

O ensaio de redução do MTT em cultura primária de astrócitos mostrou que nenhum dos compostos utilizados foi capaz de diminuir a capacidade redutora das células. O ácido flufenâmico foi capaz até de aumentar a redução do MTT. Já em fatias agudas hipocampais, todos os bloqueadores de junção gap, mas não o ácido glicirrízico, foram capazes de diminuir a redução do MTT. Isso sugere que esse efeito em fatias, que são mais susceptíveis ao estresse oxidativo do que cultura de astrócitos, não envolve necessariamente o bloqueio da comunicação por junção gap. É importante mencionar que o ensaio de redução do MTT não envolve apenas a capacidade redutora da mitocôndria, mas também os processos de endocitose (para captar o MTT) e exocitose (para exportar o produto reduzido do MTT) (Liu *et al.* 1997). O ensaio de captação do corante vermelho neutro indicou que em geral os bloqueadores de junção gap não alteram a viabilidade celular em cultura primária de astrócitos ou em fatias agudas hipocampais, apesar de uma redução da captação do corante vermelho neutro ter sido observada durante a exposição ao ácido flufenâmico. Os ensaios de liberação LDH, incorporação de iodeto de propídio e redução do MTT não mostraram um efeito similar. A razão para essa discrepância nos ensaios não é clara, mas enfatiza a importância do uso de diferentes ensaios para a avaliação da viabilidade celular.

Estudos prévios mostraram uma redução da secreção basal de S100B quando células astrogliais atingem a confluência *in vitro* (Tramontina et al. 2002, Van Eldik & Zimmer 1987), o que pode ser modulado negativamente por mudanças na comunicação por junção gap nessas preparações. Os dados apresentados nesse trabalho permitem

formular a hipótese da existência de uma relação inversa entre a comunicação por junção gap e a secreção de S100B.

Atualmente sabe-se que os astrócitos sentem e respondem a estímulos gerados por neurônios e que essas alterações envolvem junções gap e hemicanais. Os inibidores de junção gap aumentam a vulnerabilidade neuronal induzida por estresse oxidativo em co-cultura de astrócitos e neurônios (Blanc et al. 1998) e também por altas concentrações de glutamato (Ozog et al. 2002). Por outro lado, considerando a rede formada por astrócitos interligados por junções gap, o bloqueio dessa comunicação pode limitar a extensão de uma lesão (Nodin et al. 2005). Portanto, com base na atividade neurotrófica da S100B observada em uma série de condições de injúria (Van Eldik & Wainwright 2003), pode-se inferir que a S100B secretada estimulada pelo bloqueio da comunicação por junção gap em astrócitos pode ser benéfica para a sobrevivência celular em uma área afetada por lesão. De fato, a sinalização alterada durante um dano (mediada por cálcio ou ATP, por exemplo) pode local e transitoriamente interromper a comunicação por junção gap e isso pode ser o gatilho para a sinalização para a sobrevivência celular, o que pode incluir a secreção de S100B.

Outros dados que reforçam essa relação inversa entre comunicação por junção gap e secreção de S100B é que altas concentrações de glutamato ou potássio (como as observadas em situações excitotóxicas) aumentam a comunicação por junção gap (Enkvist & McCarthy 1994) e diminuem a secreção de S100B em cultura de astrócitos (Goncalves et al. 2002) e fatias agudas hipocampais (Nardin *et al.* 2009).

2.6. Conclusões

Os dados indicam que o bloqueio da comunicação por junção gap aumenta a secreção de S100B em cultura primária de astrócitos, bem como em fatias agudas hipocampais. A secreção de S100B foi observada utilizando diferentes tipos de inibidores de junção gap e os efeitos observados foram dependentes do tempo, da natureza do inibidor e do tipo de preparação utilizada. Apenas a carbenoxolona foi capaz de produzir um aumento rápido e persistente na secreção de S100B nas duas preparações.

Fisiologicamente, um bloqueio local da comunicação por junção gap, associada com o aumento da secreção de S100B em uma condição de injúria, favorece a idéia da existência de um mecanismo comum para limitar a extensão da lesão e aumentar as chances de sobrevivência celular.

3. Mobilização de cálcio e secreção de S100B

3.1. Secreção basal de S100B

- Um aumento tempo dependente da secreção de S100B foi observado após a privação de soro fetal bovino (SFB).
- A redução do cálcio extracelular, quando as células foram expostas ao EGTA, foi capaz de aumentar a secreção de S100B estimulada pela ausência de SFB.
- O Co^{2+} diminuiu e os ionóforos de cálcio (ionomicina e A23187) aumentaram a secreção de S100B estimulada pela ausência de SFB. Esse aumento foi maior do que o observado com forscolina.

- A secreção de S100B foi estimulada na presença de concentrações de DMSO superiores a 0,1% tanto na presença quanto na ausência de SFB.
- Os moduladores de cálcio (tapsigargina, BAPTA-AM, U73122, $CoCl_2$ e verapamil) foram capazes de reduzir a secreção de S100B induzida por DMSO 0,5% tanto na presença quanto na ausência de SFB.

3.2. Medidas de cálcio intracelular

3.2.1. Estímulos com DMSO, forscolina e EGTA em astrócitos

- Dois tipos de resposta de aumento de cálcio foram observadas. Uma resposta rápida (tipo 1) e uma resposta sustentada (tipo 2).
- A exposição ao DMSO e à forscolina induziu uma porcentagem de 20-30% das células a responderem apenas com resposta de tipo 1, enquanto que a exposição ao EGTA não apresentou esse tipo de resposta.
- A exposição ao DMSO e à forscolina induziu menos de 20% das células à resposta do tipo 2, enquanto que o EGTA induziu 40% das células a apresentar esse tipo de resposta.
- Em todos os tratamentos (DMSO, forscolina e EGTA), mais de 50% das células apresentaram respostas do tipo 1 e do tipo 2 (na mesma célula) e menos de 5% das células não apresentaram nenhum tipo de resposta de aumento de cálcio intracelular.

- A intensidade das respostas tipo 1 e tipo 2 não foram diferentes quando as células foram estimuladas com EGTA, mas a resposta tipo 2 teve intensidade maior que a resposta tipo 1 nos tratamentos com DMSO e forscolina.

3.2.2. Variações de cálcio intra e extracelular

- A porcentagem de células que exibiram apenas resposta do tipo 1 induzida por DMSO (25%) caiu para 10% com a remoção do cálcio extracelular e foi abolida com a adição de tapsigargina.
- A porcentagem de células que apresentaram resposta do tipo 2 estimuladas com DMSO não foi alterada com a remoção do cálcio extracelular, mas aumentou quando as células foram expostas à tapsigargina.
- A porcentagem de células que apresentaram resposta do tipo 1 e do tipo 2 quando tratadas com DMSO não foi alterada com a remoção do cálcio extracelular, mas foi abolida com a exposição à tapsigargina.
- A intensidade da resposta tipo 1 induzida por DMSO foi aumentada com a remoção do cálcio e abolida com a tapsigargina.
- A intensidade da resposta tipo 2 induzida por DMSO não foi alterada pela remoção do cálcio extracelular, mas foi diminuída com a tapsigargina.

3.3. Comentários

Os resultados mostram um aumento da secreção de S100B estimulada por EGTA, sendo dessa forma a secreção de S100B negativamente correlacionada com a concentração de cálcio extracelular, e esse efeito foi depende do tempo. Estudos prévios já tinham observado um aumento de secreção de S100B induzido por EGTA em fatias cerebrais (Buyukuysal 2005) e em células de glioma C6 (Davey et al. 2001, Suzuki *et al.* 1987). Em células não excitáveis como os astrócitos, a remoção do cálcio externo provoca uma mobilização dos estoques de cálcio intracelular (Zanotti & Charles 1997). Além disso, o uso de ionóforos também aumentou a secreção de S100B. A entrada de cálcio do meio extra para o meio intracelular em astrócitos ocorre frequentemente para repor os estoques intracelulares de cálcio após um estímulo (Agulhon *et al.* 2008). Isso explica a redução da secreção de S100B em células expostas ao cobalto, que bloqueia transportadores de cálcio da membrana plasmática. É importante ressaltar que esses tratamentos foram realizados na ausência de soro fetal bovino que por si só mobiliza cálcio intracelular em astrócitos (Matsunaga & Ueda 2006).

O DMSO é um solvente comum e amplamente utilizado em estudos biológicos e também e em ensaios farmacológicos. Sua concentração de uso varia de 0,1% a 20% nesses estudos, onde é considerado como um solvente inerte. Entretanto, muitas vezes alguns efeitos do DMSO acabam sendo negligenciados. O DMSO é um solvente que se difunde facilmente por membranas e é capaz de seqüestrar radicais livres e romper pontes de hidrogênio, podendo assim interferir em ensaios *in vivo* e *in vitro* (Santos *et al.* 2003). Com relação à secreção protéica, já foi demonstrado que o DMSO aumenta a secreção de IL-1 induzida por LPS em monócitos humanos (Xing & Remick 2005), aumenta a

secreção de insulina induzida pelo peptídeo-1 análogo ao glucagon em uma linhagem de células beta de pâncreas (Kemp & Habener 2002) e diminui a secreção de IL-8 induzida por homocisteína em monócitos humanos (Zeng *et al.* 2003).

O DMSO foi capaz de aumentar a secreção de S100B de forma tempo e concentração dependente. Esse aumento foi maior do que o ocasionado pela forscolina, um secretagogo clássico de S100B, e também ocorreu na presença de soro fetal bovino, ou seja, sem nenhum outro estímulo. Um efeito do DMSO sobre a secreção de S100B envolvendo a liberação de cálcio dos estoques internos é reforçado pelo efeito inibitório do BAPTA-AM (um quelante de cálcio intracelular) e da tapsigargina (um bloqueador da SERCA). Uma inibição efetiva da secreção de S100B induzida por DMSO também foi obtida pelo tratamento com Co^{2+} e verapamil (bloqueadores de transportadores de cálcio da membrana plasmática). Uma inibição da secreção de S100B estimulada por DMSO também foi obtida no tratamento com U73122 (um inibidor da PLC), sugerindo o envolvimento do IP_3 como mensageiro intracelular responsável pela sinalização para essa liberação de cálcio dos estoques intracelulares de cálcio. Todos esses agentes farmacológicos foram efetivos em modular a secreção de S100B induzida por DMSO tanto na presença quanto na ausência de soro fetal bovino.

Para confirmar os dados obtidos com os agentes farmacológicos, foram realizadas medidas de cálcio intracelular em astrócitos estimulados com DMSO, forscolina e EGTA. De fato, o DMSO (0,5% e 1%) induziu um aumento de cálcio intracelular e o perfil desse efeito (tipo e intensidade de resposta) foi mais similar com o perfil induzido por forscolina do que com o perfil induzido por EGTA. A resposta ao DMSO não foi alterada quando o experimento foi realizado em um meio livre de cálcio, confirmando

que o aumento de cálcio intracelular é devido à mobilização de estoques internos e não a um influxo de cálcio do meio extracelular. Quando a tapsigargina foi adicionada (em um meio livre de cálcio), a resposta de aumento de cálcio intracelular estimulada por DMSO foi drasticamente alterada, sugerindo que o DMSO é capaz de mobilizar cálcio do retículo endoplasmático. É importante ressaltar que a resposta do tipo 1 foi abolida com tapsigargina, mas que a resposta sustentada do tipo 2 não foi completamente inibida. Entretanto, é importante observar que, apesar de uma porcentagem grande de células apresentarem resposta do tipo 2 quando expostas à tapsigargina e DMSO, a intensidade dessa resposta foi muito mais baixa que quando expostas apenas ao DMSO e um número maior de células não apresentou nenhum tipo de resposta. Sendo assim pode-se concluir que o principal mecanismo de mobilização de cálcio intracelular por DMSO envolve o retículo endoplasmático. Como a resposta não foi completamente abolida com o uso de tapsigargina, não se pode descartar o envolvimento da mobilização de cálcio de outros estoques intracelulares, como por exemplo, a mitocôndria ou os lisossomos, que também participam da sinalização por cálcio em astrócitos (Heidemann *et al.* 2005, Li *et al.* 2008, Simpson & Russell 1998).

O efeito secretagogo da forscolina ainda não é completamente entendido, mas poderia ser explicado pela modulação positiva dos canais de cálcio sensíveis a IP_3 pela PKA (Bezprozvanny 2005). De fato, o H89 (um inibidor de PKA) reduz a secreção de S100B estimulada pela ausência de soro fetal bovino (Goncalves et al. 2002). Por outro lado, astrócitos expressam primariamente canais sensíveis ao IP_3 do tipo 2 (Agulhon et al. 2008), que aparentemente (ao menos em células não neuronais) são pouco sensíveis à fosforilação por PKA (Soulsby & Wojcikiewicz 2007). Outra possível explicação é que o

cAMP e a PKA possam agir reorganizando o citoesqueleto de forma a remodelar o espaço entre a membrana plasmática e o retículo endoplasmático, aumentando assim a captação de cálcio pelo retículo (entrada capacitativa) (Grimaldi *et al.* 1999, Wu *et al.* 1999). Essas possibilidades requerem futuras investigações.

Como o DMSO é um solvente, uma possível liberação inespecífica de S100B foi investigada, particularmente no que se refere à integridade e viabilidade celular. Nenhuma mudança na integridade celular (avaliada pela liberação de LDH e pela captação de iodeto de propídio) ou na viabilidade celular (avaliada pela redução do MTT) foi observada. Além disso, a exposição das culturas de astrócitos a 1% de etanol não produziu uma aumento na secreção de S100B (dados não mostrados), tanto na presença quanto na ausência de soro fetal bovino. Dessa forma, não há evidências que apontem para uma liberação inespecífica de S100B por astrócitos, quando estimulados com DMSO.

Apesar de a forscolina causar um aumento na secreção de S100B similar ao observado com o DMSO, a secreção de S100B induzida por DMSO não é acompanhada por alteração morfológica. É importante enfatizar que o efeito da forscolina sobre o citoesqueleto é revertido com ácido lisofosfatídico, que por si só causa aumento na secreção de S100B (Pinto et al. 2000). Esses dados reforçam a idéia de que o perfil de secreção de S100B em astrócitos não está necessariamente associado com alterações morfológicas, pelo menos em cultura primária de células.

Finalmente, a concentração extracelular de S100B foi investigada no líquor de ratos que receberam uma infusão ICV de DMSO. Essa investigação se torna relevante, visto que o DMSO é comumente utilizado como veículo para a administração ICV de

fármacos. Os resultados mostram que uma concentração de 1% de DMSO aumentou significativamente o conteúdo de S100B no líquor sem, no entanto, aumentar a concentração de S100B no soro, sugerindo que aumentos na concentração de S100B no líquor não são necessariamente acompanhados de aumentos dessa proteína no soro. Além disso, fatias agudas hipocampais também mostraram um aumento na secreção de S100B quando estimuladas com 1% de DMSO.

3.4. Conclusões

Esses dados consolidam a visão de que a secreção de S100B é disparada por agentes que aumentam o cálcio intracelular por mobilização do retículo endoplasmático em culturas astrogliais e fatias agudas hipocampais. Esses agentes incluem a privação de soro fetal bovino (Goncalves et al. 2002), ionóforos, forscolina (Pinto et al. 2000), ausência de cálcio extracelular (Davey et al. 2001, Suzuki et al. 1987) e redução do K^+ extracelular (Nardin et al. 2009). Nem todos os agentes que mobilizam cálcio intracelular poderão necessariamente ser capazes de modular a secreção de S100B.

Apesar dos alvos moleculares do DMSO ainda serem desconhecidos, esse composto (em concentrações maiores que 0,1%) aumenta o cálcio intracelular e induz a secreção de S100B. Isso reforça o fato de que a S100B secretada seja um dos possíveis mediadores dos efeitos (e efeitos colaterais) do DMSO e que é necessária cautela na sua utilização como veículo. Esse fato é particularmente importante quando se considera proteínas secretadas envolvidas na comunicação intercelular, como a S100B, que também podem ser moduladas pelo DMSO. Além disso, a propriedade de aumentar a secreção de S100B do DMSO pode ser adicionada à lista de aplicações terapêuticas desse composto, especialmente considerando o efeito neuroprotetor da S100B que tem sido observado em

modelos de dano cerebral (Kleindienst *et al.* 2005). Além disso, o uso do DMSO como ferramenta para se estudar a mobilização de cálcio intracelular deve ser levado em consideração.

Considerações Finais

Apesar de muitos moduladores da secreção de S100B já terem sido identificados, pouco se sabe sobre os mecanismos de ativação e regulação dessa secreção. Nesse trabalho, algumas importantes questões para uma compreensão melhor dos mecanismos celulares que regulam a secreção de S100B foram abordadas.

Primeiramente, uma técnica para a dosagem da S100B foi padronizada. Isso nos possibilitou dosar essa proteína com uma maior sensibilidade e, consequentemente, a possibilidade de trabalhar com intervalos mais curtos de incubação, o que é fundamental no estudo da regulação da secreção. Essa técnica também se mostrou versátil, na medida em que foi aplicável para diversos tipos de amostras biológicas, o que facilita a comparação de valores de concentração de S100B obtidos em diferentes condições experimentais. Além disso, a técnica possui um custo muito baixo em relação aos kits comerciais disponíveis e é de fácil execução.

Considerando que a S100B é uma proteína com efeitos autócrinos e parácrinos, podendo ser trófica ou tóxica dependendo de sua concentração, e ainda que as junções gap em astrócitos estão intimamente relacionadas com a comunicação entre astrócitos e neurônios, o efeito do bloqueio das junções gap sobre a secreção de S100B foi investigado. O aumento da secreção de S100B observado pode indicar uma resposta trófica dos astrócitos a uma situação de dano, em que a comunicação por junção gap é bloqueada para limitar a extensão da lesão. Ainda não foi demonstrado um efeito do bloqueio das junções gap sobre a mobilização de cálcio intracelular; entretanto, as junções gap podem ter alvos protéicos envolvidos diretamente na maquinaria de secreção da S100B, que ainda é desconhecida.

Finalmente, visto que aumentos de cálcio intracelular parecem estar envolvidos na secreção de S100B em linhagens celulares e que também é capaz de regular junções gap, um possível envolvimento da mobilização de cálcio intracelular em astrócitos na secreção de S100B estimulada por secretagogos conhecidos (como a forscolina) foi investigado. Durante esse estudo, nós descobrimos que o DMSO é um secretagogo mais potente do que a forscolina e por isso ele foi utilizado nesse estudo. De fato, o DMSO é capaz de mobilizar cálcio do retículo endoplasmático e essa mobilização está diretamente relacionada com o aumento da secreção de S100B observado. Dessa forma, deve se ter cautela com o uso do DMSO como veículo em ensaios bioquímicos e farmacológicos e mais estudos devem ser realizados sobre a possibilidade desse composto ter atividade como secretagogo de outras proteínas envolvidas na sinalização celular.

Perspectivas

- Investigar se os moduladores da secreção de S100B conhecidos podem ter efeito sobre a comunicação por juncão gap em astrócitos, bem como se a carbenoxolona é capaz de mobilizar cálcio intracelular.
- Investigar a participação de outras organelas, que não o retículo endoplasmático, no aumento de cálcio intracelular estimulado por DMSO em astrócitos.
- Investigar um possível mecanismo vesicular da secreção de S100B em cultura primária de astrócitos, utilizando o DMSO como estímulo.

Referências Bibliográficas

Adami, C., Sorci, G., Blasi, E., Agneletti, A. L., Bistoni, F. and Donato, R. (2001) S100B expression in and effects on microglia. *Glia,* **33,** 131-142.

Agulhon, C., Petravicz, J., McMullen, A. B., Sweger, E. J., Minton, S. K., Taves, S. R., Casper, K. B., Fiacco, T. A. and McCarthy, K. D. (2008) What is the role of astrocyte calcium in neurophysiology? *Neuron,* **59,** 932-946.

Anderson, R. E., Hansson, L. O., Nilsson, O., Liska, J., Settergren, G. and Vaage, J. (2001) Increase in serum S100A1-B and S100BB during cardiac surgery arises from extracerebral sources. *Ann Thorac Surg,* **71,** 1512-1517.

Andreazza, A. C., Cassini, C., Rosa, A. R. et al. (2007) Serum S100B and antioxidant enzymes in bipolar patients. *J Psychiatr Res,* **41,** 523-529.

Araque, A., Carmignoto, G. and Haydon, P. G. (2001) Dynamic signaling between astrocytes and neurons. *Annu Rev Physiol,* **63,** 795-813.

Araque, A., Parpura, V., Sanzgiri, R. P. and Haydon, P. G. (1998a) Glutamate-dependent astrocyte modulation of synaptic transmission between cultured hippocampal neurons. *Eur J Neurosci,* **10,** 2129-2142.

Araque, A., Sanzgiri, R. P., Parpura, V. and Haydon, P. G. (1998b) Calcium elevation in astrocytes causes an NMDA receptor-dependent increase in the frequency of miniature synaptic currents in cultured hippocampal neurons. *J Neurosci,* **18,** 6822-6829.

Bennett, M. V., Contreras, J. E., Bukauskas, F. F. and Saez, J. C. (2003) New roles for astrocytes: gap junction hemichannels have something to communicate. *Trends Neurosci,* **26,** 610-617.

Berridge, M. (2004) Conformational coupling: a physiological calcium entry mechanism. *Sci STKE,* **2004,** pe33.

Berridge, M. J. (2002) The endoplasmic reticulum: a multifunctional signaling organelle. *Cell Calcium,* **32,** 235-249.

Berridge, M. J. (2009) Inositol trisphosphate and calcium signalling mechanisms. *Biochim Biophys Acta,* **1793,** 933-940.

Bezprozvanny, I. (2005) The inositol 1,4,5-trisphosphate receptors. *Cell Calcium,* **38,** 261-272.

Bianchi, R., Giambanco, I. and Donato, R. (2008) S100B/RAGE-dependent activation of microglia via NF-kappaB and AP-1 Co-regulation of COX-2 expression by S100B, IL-1beta and TNF-alpha. *Neurobiol Aging*.

Blanc, E. M., Bruce-Keller, A. J. and Mattson, M. P. (1998) Astrocytic gap junctional communication decreases neuronal vulnerability to oxidative stress-induced disruption of Ca2+ homeostasis and cell death. *J Neurochem,* **70,** 958-970.

Blomstrand, F. and Giaume, C. (2006) Kinetics of endothelin-induced inhibition and glucose permeability of astrocyte gap junctions. *J Neurosci Res,* **83,** 996-1003.

Bowser, D. N. and Khakh, B. S. (2007) Vesicular ATP is the predominant cause of intercellular calcium waves in astrocytes. *J Gen Physiol,* **129,** 485-491.

Buyukuysal, R. L. (2005) Protein S100B release from rat brain slices during and after ischemia: comparison with lactate dehydrogenase leakage. *Neurochem Int,* **47,** 580-588.

Celsi, F., Pizzo, P., Brini, M., Leo, S., Fotino, C., Pinton, P. and Rizzuto, R. (2009) Mitochondria, calcium and cell death: a deadly triad in neurodegeneration. *Biochim Biophys Acta,* **1787,** 335-344.

Ciccarelli, R., Di Iorio, P., Bruno, V., Battaglia, G., D'Alimonte, I., D'Onofrio, M., Nicoletti, F. and Caciagli, F. (1999) Activation of A(1) adenosine or mGlu3 metabotropic glutamate receptors enhances the release of nerve growth factor and S-100beta protein from cultured astrocytes. *Glia,* **27,** 275-281.

Davey, G. E., Murmann, P. and Heizmann, C. W. (2001) Intracellular Ca2+ and Zn2+ levels regulate the alternative cell density-dependent secretion of S100B in human glioblastoma cells. *J Biol Chem,* **276,** 30819-30826.

de Almeida, L. M., Pineiro, C. C., Leite, M. C., Brolese, G., Tramontina, F., Feoli, A. M., Gottfried, C. and Goncalves, C. A. (2007) Resveratrol increases glutamate uptake, glutathione content, and S100B secretion in cortical astrocyte cultures. *Cell Mol Neurobiol,* **27,** 661-668.

de Souza, D. F., Leite, M. C., Quincozes-Santos, A., Nardin, P., Tortorelli, L. S., Rigo, M. M., Gottfried, C., Leal, R. B. and Goncalves, C. A. (2009) S100B secretion is stimulated by IL-1beta in glial cultures and hippocampal slices of rats: Likely involvement of MAPK pathway. *J Neuroimmunol,* **206,** 52-57.

Deloulme, J. C., Raponi, E., Gentil, B. J., Bertacchi, N., Marks, A., Labourdette, G. and Baudier, J. (2004) Nuclear expression of S100B in oligodendrocyte progenitor cells correlates with differentiation toward the oligodendroglial lineage and modulates oligodendrocytes maturation. *Mol Cell Neurosci,* **27,** 453-465.

Donato, R. (2001) S100: a multigenic family of calcium-modulated proteins of the EF-hand type with intracellular and extracellular functional roles. *Int J Biochem Cell Biol,* **33,** 637-668.

Donato, R. (2003) Intracellular and extracellular roles of S100 proteins. *Microsc Res Tech,* **60,** 540-551.

Donato, R. (2007) RAGE: a single receptor for several ligands and different cellular responses: the case of certain S100 proteins. *Curr Mol Med,* **7,** 711-724.

Donato, R., Sorci, G., Riuzzi, F., Arcuri, C., Bianchi, R., Brozzi, F., Tubaro, C. and Giambanco, I. (2009) S100B's double life: intracellular regulator and extracellular signal. *Biochim Biophys Acta,* **1793,** 1008-1022.

Elsen, F. P., Shields, E. J., Roe, M. T., Vandam, R. J. and Kelty, J. D. (2008) Carbenoxolone induced depression of rhythmogenesis in the pre-Botzinger Complex. *BMC Neurosci,* **9,** 46.

Enkvist, M. O. and McCarthy, K. D. (1994) Astroglial gap junction communication is increased by treatment with either glutamate or high K+ concentration. *J Neurochem,* **62,** 489-495.

Fazio, V., Bhudia, S. K., Marchi, N., Aumayr, B. and Janigro, D. (2004) Peripheral detection of S100beta during cardiothoracic surgery: what are we really measuring? *Ann Thorac Surg,* **78,** 46-52; discussion 52-43.

Fellin, T. and Carmignoto, G. (2004) Neurone-to-astrocyte signalling in the brain represents a distinct multifunctional unit. *J Physiol,* **559,** 3-15.

Frizzo, J. K., Tramontina, F., Bortoli, E., Gottfried, C., Leal, R. B., Lengyel, I., Donato, R., Dunkley, P. R. and Goncalves, C. A. (2004) S100B-mediated inhibition of the

phosphorylation of GFAP is prevented by TRTK-12. *Neurochem Res,* **29,** 735-740.

Froes, M. M., Correia, A. H., Garcia-Abreu, J., Spray, D. C., Campos de Carvalho, A. C. and Neto, M. V. (1999) Gap-junctional coupling between neurons and astrocytes in primary central nervous system cultures. *Proc Natl Acad Sci U S A,* **96,** 7541-7546.

Gazzolo, D., Frigiola, A., Bashir, M. et al. (2009) Diagnostic accuracy of S100B urinary testing at birth in full-term asphyxiated newborns to predict neonatal death. *PLoS One,* **4,** e4298.

Gazzolo, D., Monego, G., Corvino, V., Bruschettini, M., Bruschettini, P., Zelano, G. and Michetti, F. (2003) Human milk contains S100B protein. *Biochim Biophys Acta,* **1619,** 209-212.

Gerlach, R., Demel, G., Konig, H. G., Gross, U., Prehn, J. H., Raabe, A., Seifert, V. and Kogel, D. (2006) Active secretion of S100B from astrocytes during metabolic stress. *Neuroscience,* **141,** 1697-1701.

Goncalves, C. A., Gottfried, C., Kommers, T. and Rodnight, R. (1997) Calcium-modulated proteins change their immunoreactivity in the presence of Ca2+: a study of antibody recognition in a dot immunoassay for calmodulin, calcineurin (beta-subunit), and S100B. *Anal Biochem,* **253,** 127-130.

Goncalves, C. A., Leite, M. C. and Nardin, P. (2008) Biological and methodological features of the measurement of S100B, a putative marker of brain injury. *Clin Biochem,* **41,** 755-763.

Goncalves, D., Karl, J., Leite, M., Rotta, L., Salbego, C., Rocha, E., Wofchuk, S. and Goncalves, C. A. (2002) High glutamate decreases S100B secretion stimulated by serum deprivation in astrocytes. *Neuroreport,* **13,** 1533-1535.

Goncalves, D. S., Lenz, G., Karl, J., Goncalves, C. A. and Rodnight, R. (2000) Extracellular S100B protein modulates ERK in astrocyte cultures. *Neuroreport,* **11,** 807-809.

Green, A. J., Keir, G. and Thompson, E. J. (1997) A specific and sensitive ELISA for measuring S-100b in cerebrospinal fluid. *J Immunol Methods,* **205,** 35-41.

Grimaldi, M., Favit, A. and Alkon, D. L. (1999) cAMP-induced cytoskeleton rearrangement increases calcium transients through the enhancement of capacitative calcium entry. *J Biol Chem,* **274,** 33557-33564.

Haghikia, A., Ladage, K., Lafenetre, P., Hinkerohe, D., Smikalla, D., Haase, C. G., Dermietzel, R. and Faustmann, P. M. (2008) Intracellular application of TNF-alpha impairs cell to cell communication via gap junctions in glioma cells. *J Neurooncol,* **86,** 143-152.

Harpio, R. and Einarsson, R. (2004) S100 proteins as cancer biomarkers with focus on S100B in malignant melanoma. *Clin Biochem,* **37,** 512-518.

Heidemann, A. C., Schipke, C. G. and Kettenmann, H. (2005) Extracellular application of nicotinic acid adenine dinucleotide phosphate induces Ca2+ signaling in astrocytes in situ. *J Biol Chem,* **280,** 35630-35640.

Heizmann, C. W. (2004) S100B protein in clinical diagnostics: assay specificity. *Clin Chem,* **50,** 249-251.

Hofmann, M. A., Drury, S., Fu, C. et al. (1999) RAGE mediates a novel proinflammatory axis: a central cell surface receptor for S100/calgranulin polypeptides. *Cell,* **97,** 889-901.

Holtkamp, K., Buhren, K., Ponath, G., von Eiff, C., Herpertz-Dahlmann, B., Hebebrand, J. and Rothermundt, M. (2008) Serum levels of S100B are decreased in chronic starvation and normalize with weight gain. *J Neural Transm,* **115,** 937-940.

Jonsson, H., Johnsson, P., Alling, C., Backstrom, M., Bergh, C. and Blomquist, S. (1999) S100beta after coronary artery surgery: release pattern, source of contamination, and relation to neuropsychological outcome. *Ann Thorac Surg,* **68,** 2202-2208.

Kemp, D. M. and Habener, J. F. (2002) Synergistic effect of dimethyl sulfoxide on glucagon-like peptide 1 (GLP-1)-stimulated insulin secretion and gene transcription in INS-1 cells: characterization and implications. *Biochem Pharmacol,* **64,** 689-697.

Kielian, T. (2008) Glial connexins and gap junctions in CNS inflammation and disease. *J Neurochem,* **106,** 1000-1016.

Kielian, T. and Esen, N. (2004) Effects of neuroinflammation on glia-glia gap junctional intercellular communication: a perspective. *Neurochem Int,* **45,** 429-436.

Kimelberg, H. K. and Norenberg, M. D. (1989) Astrocytes. *Sci Am,* **260,** 66-72, 74, 76.

Kirchhoff, F., Dringen, R. and Giaume, C. (2001) Pathways of neuron-astrocyte interactions and their possible role in neuroprotection. *Eur Arch Psychiatry Clin Neurosci,* **251,** 159-169.

Kleindienst, A., McGinn, M. J., Harvey, H. B., Colello, R. J., Hamm, R. J. and Bullock, M. R. (2005) Enhanced hippocampal neurogenesis by intraventricular S100B

infusion is associated with improved cognitive recovery after traumatic brain injury. *J Neurotrauma,* **22,** 645-655.

Leal, R. B., Frizzo, J. K., Tramontina, F., Fieuw-Makaroff, S., Bobrovskaya, L., Dunkley, P. R. and Goncalves, C. A. (2004) S100B protein stimulates calcineurin activity. *Neuroreport,* **15,** 317-320.

Lehmann, C., Eisner, F. and Engele, J. (2008) Role of endothelins as mediators of injury-induced alterations of glial glutamate turnover. *J Neurosci Res,* **86,** 660-667.

Leite, M., Frizzo, J. K., Nardin, P., de Almeida, L. M., Tramontina, F., Gottfried, C. and Goncalves, C. A. (2004) Beta-hydroxy-butyrate alters the extracellular content of S100B in astrocyte cultures. *Brain Res Bull,* **64,** 139-143.

Leite, M. C., Brolese, G., de Almeida, L. M., Pinero, C. C., Gottfried, C. and Goncalves, C. A. (2006) Ammonia-induced alteration in S100B secretion in astrocytes is not reverted by creatine addition. *Brain Res Bull,* **70,** 179-185.

Leite, M. C., Galland, F., Brolese, G., Guerra, M. C., Bortolotto, J. W., Freitas, R., Almeida, L. M., Gottfried, C. and Goncalves, C. A. (2008) A simple, sensitive and widely applicable ELISA for S100B: Methodological features of the measurement of this glial protein. *J Neurosci Methods,* **169,** 93-99.

Leite, M. C., Galland, F., de Souza, D. F., Guerra, M. C., Bobermin, L., Biasibetti, R., Gottfried, C. and Goncalves, C. A. (2009) Gap junction inhibitors modulate S100B secretion in astrocyte cultures and acute hippocampal slices. *J Neurosci Res,* **87,** 2439-2446.

Li, D., Ropert, N., Koulakoff, A., Giaume, C. and Oheim, M. (2008) Lysosomes are the major vesicular compartment undergoing Ca2+-regulated exocytosis from cortical astrocytes. *J Neurosci,* **28,** 7648-7658.

Liu, L., Zheng, C. X., Peng, S. F., Zhou, H. Y., Su, Z. Y., He, L. and Ai, T. (2010) Evaluation of Urinary S100B Protein Level and Lactate/Creatinine Ratio for Early Diagnosis and Prognostic Prediction of Neonatal Hypoxic-Ischemic Encephalopathy. *Neonatology,* **97,** 41-44.

Liu, Y., Peterson, D. A., Kimura, H. and Schubert, D. (1997) Mechanism of cellular 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) reduction. *J Neurochem,* **69,** 581-593.

Magistretti, P. J. (2006) Neuron-glia metabolic coupling and plasticity. *J Exp Biol,* **209,** 2304-2311.

Magistretti, P. J. and Pellerin, L. (1999) Cellular mechanisms of brain energy metabolism and their relevance to functional brain imaging. *Philos Trans R Soc Lond B Biol Sci,* **354,** 1155-1163.

Marchi, N., Cavaglia, M., Fazio, V., Bhudia, S., Hallene, K. and Janigro, D. (2004) Peripheral markers of blood-brain barrier damage. *Clin Chim Acta,* **342,** 1-12.

Matsunaga, H. and Ueda, H. (2006) Evidence for serum-deprivation-induced co-release of FGF-1 and S100A13 from astrocytes. *Neurochem Int,* **49,** 294-303.

Mazzini, G. S., Schaf, D. V., Oliveira, A. R. et al. (2005) The ischemic rat heart releases S100B. *Life Sci,* **77,** 882-889.

Meme, W., Calvo, C. F., Froger, N., Ezan, P., Amigou, E., Koulakoff, A. and Giaume, C. (2006) Proinflammatory cytokines released from microglia inhibit gap junctions in astrocytes: potentiation by beta-amyloid. *FASEB J,* **20,** 494-496.

Mese, G., Richard, G. and White, T. W. (2007) Gap junctions: basic structure and function. *J Invest Dermatol,* **127,** 2516-2524.

Michetti, F. and Gazzolo, D. (2002) S100B protein in biological fluids: a tool for perinatal medicine. *Clin Chem,* **48,** 2097-2104.

Missler, U., Walek, T. and Stange, E. (1995) Time-resolved immunofluorometric assay for the quantification of lipoprotein(a) in serum. *Eur J Clin Chem Clin Biochem,* **33,** 805-812.

Moore, B. W. (1965) A soluble protein characteristic of the nervous system. *Biochem Biophys Res Commun,* **19,** 739-744.

Nardin, P., Tortorelli, L., Quincozes-Santos, A. et al. (2009) S100B secretion in acute brain slices: modulation by extracellular levels of Ca(2+) and K (+). *Neurochem Res,* **34,** 1603-1611.

Nardin, P., Tramontina, F., Leite, M. C., Tramontina, A. C., Quincozes-Santos, A., de Almeida, L. M., Battastini, A. M., Gottfried, C. and Goncalves, C. A. (2007) S100B content and secretion decrease in astrocytes cultured in high-glucose medium. *Neurochem Int,* **50,** 774-782.

Netto, C. B., Conte, S., Leite, M. C., Pires, C., Martins, T. L., Vidal, P., Benfato, M. S., Giugliani, R. and Goncalves, C. A. (2006) Serum S100B protein is increased in fasting rats. *Arch Med Res,* **37,** 683-686.

Netto, C. B., Portela, L. V., Ferreira, C. T. et al. (2005) Ontogenetic changes in serum S100B in Down syndrome patients. *Clin Biochem,* **38,** 433-435.

Nickel, W. (2003) The mystery of nonclassical protein secretion. A current view on cargo proteins and potential export routes. *Eur J Biochem,* **270,** 2109-2119.

Nickel, W. and Rabouille, C. (2009) Mechanisms of regulated unconventional protein secretion. *Nat Rev Mol Cell Biol,* **10,** 148-155.

Nodin, C., Nilsson, M. and Blomstrand, F. (2005) Gap junction blockage limits intercellular spreading of astrocytic apoptosis induced by metabolic depression. *J Neurochem,* **94,** 1111-1123.

Nowycky, M. C. and Thomas, A. P. (2002) Intracellular calcium signaling. *J Cell Sci,* **115,** 3715-3716.

Orthmann-Murphy, J. L., Abrams, C. K. and Scherer, S. S. (2008) Gap junctions couple astrocytes and oligodendrocytes. *J Mol Neurosci,* **35,** 101-116.

Ozog, M. A., Siushansian, R. and Naus, C. C. (2002) Blocked gap junctional coupling increases glutamate-induced neurotoxicity in neuron-astrocyte co-cultures. *J Neuropathol Exp Neurol,* **61,** 132-141.

Pedersen, A., Diedrich, M., Kaestner, F. et al. (2008) Memory impairment correlates with increased S100B serum concentrations in patients with chronic schizophrenia. *Prog Neuropsychopharmacol Biol Psychiatry,* **32,** 1789-1792.

Pellerin, L. (2008) Brain energetics (thought needs food). *Curr Opin Clin Nutr Metab Care,* **11,** 701-705.

Pellerin, L., Bouzier-Sore, A. K., Aubert, A., Serres, S., Merle, M., Costalat, R. and Magistretti, P. J. (2007) Activity-dependent regulation of energy metabolism by astrocytes: an update. *Glia,* **55,** 1251-1262.

Perea, G. and Araque, A. (2006) Synaptic information processing by astrocytes. *J Physiol Paris,* **99,** 92-97.

Perea, G., Navarrete, M. and Araque, A. (2009) Tripartite synapses: astrocytes process and control synaptic information. *Trends Neurosci,* **32,** 421-431.

Peskind, E. R., Griffin, W. S., Akama, K. T., Raskind, M. A. and Van Eldik, L. J. (2001) Cerebrospinal fluid S100B is elevated in the earlier stages of Alzheimer's disease. *Neurochem Int,* **39,** 409-413.

Petrova, T. V., Hu, J. and Van Eldik, L. J. (2000) Modulation of glial activation by astrocyte-derived protein S100B: differential responses of astrocyte and microglial cultures. *Brain Res,* **853,** 74-80.

Petzold, A., Jenkins, R., Watt, H. C., Green, A. J., Thompson, E. J., Keir, G., Fox, N. C. and Rossor, M. N. (2003) Cerebrospinal fluid S100B correlates with brain atrophy in Alzheimer's disease. *Neurosci Lett,* **336,** 167-170.

Pinto, S. S., Gottfried, C., Mendez, A., Goncalves, D., Karl, J., Goncalves, C. A., Wofchuk, S. and Rodnight, R. (2000) Immunocontent and secretion of S100B in astrocyte cultures from different brain regions in relation to morphology. *FEBS Lett,* **486,** 203-207.

Pizzo, P. and Pozzan, T. (2007) Mitochondria-endoplasmic reticulum choreography: structure and signaling dynamics. *Trends Cell Biol,* **17,** 511-517.

Portela, L. V., Tort, A. B., Schaf, D. V. et al. (2002) The serum S100B concentration is age dependent. *Clin Chem,* **48,** 950-952.

Prudovsky, I., Tarantini, F., Landriscina, M. et al. (2008) Secretion without Golgi. *J Cell Biochem,* **103,** 1327-1343.

Quincozes-Santos, A., Abib, R. T., Leite, M. C., Bobermin, D., Bambini-Junior, V., Goncalves, C. A., Riesgo, R. and Gottfried, C. (2008) Effect of the atypical neuroleptic risperidone on morphology and S100B secretion in C6 astroglial lineage cells. *Mol Cell Biochem,* **314,** 59-63.

Rana, S. and Dringen, R. (2007) Gap junction hemichannel-mediated release of glutathione from cultured rat astrocytes. *Neurosci Lett,* **415,** 45-48.

Rizzuto, R. and Pozzan, T. (2006) Microdomains of intracellular Ca2+: molecular determinants and functional consequences. *Physiol Rev,* **86,** 369-408.

Rodrigues, L., Biasibetti, R., Swarowsky, A., Leite, M. C., Quincozes-Santos, A., Quilfeldt, J. A., Achaval, M. and Goncalves, C. A. (2009) Hippocampal alterations in rats submitted to streptozotocin-induced dementia model are prevented by aminoguanidine. *J Alzheimers Dis,* **17,** 193-202.

Rouach, N., Avignone, E., Meme, W., Koulakoff, A., Venance, L., Blomstrand, F. and Giaume, C. (2002) Gap junctions and connexin expression in the normal and pathological central nervous system. *Biol Cell,* **94,** 457-475.

Rouach, N., Glowinski, J. and Giaume, C. (2000) Activity-dependent neuronal control of gap-junctional communication in astrocytes. *J Cell Biol,* **149,** 1513-1526.

Rouach, N., Koulakoff, A., Abudara, V., Willecke, K. and Giaume, C. (2008) Astroglial metabolic networks sustain hippocampal synaptic transmission. *Science,* **322,** 1551-1555.

Rouach, N., Koulakoff, A. and Giaume, C. (2004) Neurons set the tone of gap junctional communication in astrocytic networks. *Neurochem Int,* **45,** 265-272.

Rozental, R., Giaume, C. and Spray, D. C. (2000) Gap junctions in the nervous system. *Brain Res Brain Res Rev,* **32,** 11-15.

Saez, J. C., Contreras, J. E., Bukauskas, F. F., Retamal, M. A. and Bennett, M. V. (2003) Gap junction hemichannels in astrocytes of the CNS. *Acta Physiol Scand,* **179,** 9-22.

Salameh, A. and Dhein, S. (2005) Pharmacology of gap junctions. New pharmacological targets for treatment of arrhythmia, seizure and cancer? *Biochim Biophys Acta,* **1719,** 36-58.

Santos, N. C., Figueira-Coelho, J., Martins-Silva, J. and Saldanha, C. (2003) Multidisciplinary utilization of dimethyl sulfoxide: pharmacological, cellular, and molecular aspects. *Biochem Pharmacol,* **65,** 1035-1041.

Scemes, E. and Giaume, C. (2006) Astrocyte calcium waves: what they are and what they do. *Glia,* **54,** 716-725.

Schulpis, K. H., Moukas, M., Parthimos, T., Tsakiris, T., Parthimos, N. and Tsakiris, S. (2007) The effect of alpha-Tocopherol supplementation on training-induced elevation of S100B protein in sera of basketball players. *Clin Biochem,* **40,** 900-906.

Scotto, C., Mely, Y., Ohshima, H., Garin, J., Cochet, C., Chambaz, E. and Baudier, J. (1998) Cysteine oxidation in the mitogenic S100B protein leads to changes in phosphorylation by catalytic CKII-alpha subunit. *J Biol Chem,* **273,** 3901-3908.

Shihara, M., Hirooka, Y., Hori, N., Matsuo, I., Tagawa, T., Suzuki, S., Akaike, N. and Takeshita, A. (1998) Endothelin-1 increases the neuronal activity and augments the responses to glutamate in the NTS. *Am J Physiol,* **275,** R658-665.

Simpson, P. B. and Russell, J. T. (1998) Role of mitochondrial Ca2+ regulation in neuronal and glial cell signalling. *Brain Res Brain Res Rev*, **26,** 72-81.

Soulsby, M. D. and Wojcikiewicz, R. J. (2007) Calcium mobilization via type III inositol 1,4,5-trisphosphate receptors is not altered by PKA-mediated phosphorylation of serines 916, 934, and 1832. *Cell Calcium,* **42,** 261-270.

Steiner, J., Bernstein, H. G., Bogerts, B., Gos, T., Richter-Landsberg, C., Wunderlich, M. T. and Keilhoff, G. (2008) S100B is expressed in, and released from, OLN-93 oligodendrocytes: Influence of serum and glucose deprivation. *Neuroscience,* **154,** 496-503.

Steiner, J., Schiltz, K., Walter, M. et al. (2009a) S100B serum levels are closely correlated with body mass index: An important caveat in neuropsychiatric research. *Psychoneuroendocrinology*.

Steiner, J., Walter, M., Wunderlich, M. T. et al. (2009b) A new pathophysiological aspect of S100B in schizophrenia: potential regulation of S100B by its scavenger soluble RAGE. *Biol Psychiatry,* **65,** 1107-1110.

Stridh, M. H., Tranberg, M., Weber, S. G., Blomstrand, F. and Sandberg, M. (2008) Stimulated efflux of amino acids and glutathione from cultured hippocampal

slices by omission of extracellular calcium: likely involvement of connexin hemichannels. *J Biol Chem,* **283,** 10347-10356.

Suadicani, S. O., Flores, C. E., Urban-Maldonado, M., Beelitz, M. and Scemes, E. (2004) Gap junction channels coordinate the propagation of intercellular Ca2+ signals generated by P2Y receptor activation. *Glia,* **48,** 217-229.

Suzuki, F., Kato, K., Kato, T. and Ogasawara, N. (1987) S-100 protein in clonal astroglioma cells is released by adrenocorticotropic hormone and corticotropin-like intermediate-lobe peptide. *J Neurochem,* **49,** 1557-1563.

Swarowsky, A., Rodrigues, L., Biasibetti, R. et al. (2008) Glial alterations in the hippocampus of rats submitted to ibotenic-induced lesion of the nucleus basalis magnocellularis. *Behav Brain Res,* **190,** 206-211.

Tabernero, A., Medina, J. M. and Giaume, C. (2006) Glucose metabolism and proliferation in glia: role of astrocytic gap junctions. *J Neurochem,* **99,** 1049-1061.

Tort, A. B., Dietrich, M. O., Goncalves, C. A., Souza, D. O. and Portela, L. V. (2003) Influence of anticoagulants on the measurement of S100B protein in blood. *Clin Biochem,* **36,** 629-632.

Tramontina, A. C., Tramontina, F., Bobermin, L. D., Zanotto, C., Souza, D. F., Leite, M. C., Nardin, P., Gottfried, C. and Goncalves, C. A. (2008) Secretion of S100B, an astrocyte-derived neurotrophic protein, is stimulated by fluoxetine via a mechanism independent of serotonin. *Prog Neuropsychopharmacol Biol Psychiatry,* **32,** 1580-1583.

Tramontina, F., Conte, S., Goncalves, D., Gottfried, C., Portela, L. V., Vinade, L., Salbego, C. and Goncalves, C. A. (2002) Developmental changes in S100B content in brain tissue, cerebrospinal fluid, and astrocyte cultures of rats. *Cell Mol Neurobiol,* **22,** 373-378.

Tramontina, F., Karl, J., Gottfried, C., Mendez, A., Goncalves, D., Portela, L. V. and Goncalves, C. A. (2000) Digitonin-permeabilization of astrocytes in culture monitored by trypan blue exclusion and loss of S100B by ELISA. *Brain Res Brain Res Protoc,* **6,** 86-90.

Tramontina, F., Leite, M. C., Cereser, K. et al. (2007) Immunoassay for glial fibrillary acidic protein: antigen recognition is affected by its phosphorylation state. *J Neurosci Methods,* **162,** 282-286.

Tramontina, F., Tramontina, A. C., Souza, D. F., Leite, M. C., Gottfried, C., Souza, D. O., Wofchuk, S. T. and Goncalves, C. A. (2006) Glutamate uptake is stimulated by extracellular S100B in hippocampal astrocytes. *Cell Mol Neurobiol,* **26,** 81-86.

Van Eldik, L. J. and Griffin, W. S. (1994) S100 beta expression in Alzheimer's disease: relation to neuropathology in brain regions. *Biochim Biophys Acta,* **1223,** 398-403.

Van Eldik, L. J., Hertzberg, E. L., Berdan, R. C. and Gilula, N. B. (1985) Interaction of calmodulin and other calcium-modulated proteins with mammalian and arthropod junctional membrane proteins. *Biochem Biophys Res Commun,* **126,** 825-832.

Van Eldik, L. J. and Wainwright, M. S. (2003) The Janus face of glial-derived S100B: beneficial and detrimental functions in the brain. *Restor Neurol Neurosci,* **21,** 97-108.

Van Eldik, L. J. and Zimmer, D. B. (1987) Secretion of S-100 from rat C6 glioma cells. *Brain Res,* **436,** 367-370.

Vicente, E., Boer, M., Leite, M., Silva, M., Tramontina, F., Porciuncula, L., Dalmaz, C. and Goncalves, C. A. (2004) Cerebrospinal fluid S100B increases reversibly in neonates of methyl mercury-intoxicated pregnant rats. *Neurotoxicology,* **25,** 771-777.

Vicente, E., Degerone, D., Bohn, L. et al. (2009) Astroglial and cognitive effects of chronic cerebral hypoperfusion in the rat. *Brain Res,* **1251,** 204-212.

Vives, V., Alonso, G., Solal, A. C., Joubert, D. and Legraverend, C. (2003) Visualization of S100B-positive neurons and glia in the central nervous system of EGFP transgenic mice. *J Comp Neurol,* **457,** 404-419.

Wang, D. D. and Bordey, A. (2008) The astrocyte odyssey. *Prog Neurobiol,* **86,** 342-367.

Wentlandt, K., Samoilova, M., Carlen, P. L. and El Beheiry, H. (2006) General anesthetics inhibit gap junction communication in cultured organotypic hippocampal slices. *Anesth Analg,* **102,** 1692-1698.

Whitaker-Azmitia, P. M., Murphy, R. and Azmitia, E. C. (1990) Stimulation of astroglial 5-HT1A receptors releases the serotonergic growth factor, protein S-100, and alters astroglial morphology. *Brain Res,* **528,** 155-158.

Wolff, J. R., Stuke, K., Missler, M., Tytko, H., Schwarz, P., Rohlmann, A. and Chao, T. I. (1998) Autocellular coupling by gap junctions in cultured astrocytes: a new view on cellular autoregulation during process formation. *Glia,* **24,** 121-140.

Wu, M. L., Chen, W. H., Liu, I. H., Tseng, C. D. and Wang, S. M. (1999) A novel effect of cyclic AMP on capacitative Ca2+ entry in cultured rat cerebellar astrocytes. *J Neurochem,* **73,** 1318-1328.

Xing, L. and Remick, D. G. (2005) Mechanisms of dimethyl sulfoxide augmentation of IL-1 beta production. *J Immunol,* **174,** 6195-6202.

Yang, Q., Hou, X., Hamberger, A., Wang, S., Dahlstrom, A. and Haglid, K. G. (1995) S-100 beta immunoreactivity in neurones of the rat peripheral sensory ganglia. *Neuroreport,* **6,** 2005-2009.

Ye, Z. C., Wyeth, M. S., Baltan-Tekkok, S. and Ransom, B. R. (2003) Functional hemichannels in astrocytes: a novel mechanism of glutamate release. *J Neurosci,* **23,** 3588-3596.

Yeager, M. and Harris, A. L. (2007) Gap junction channel structure in the early 21st century: facts and fantasies. *Curr Opin Cell Biol,* **19,** 521-528.

Zanotti, S. and Charles, A. (1997) Extracellular calcium sensing by glial cells: low extracellular calcium induces intracellular calcium release and intercellular signaling. *J Neurochem,* **69,** 594-602.

Zeng, X., Dai, J., Remick, D. G. and Wang, X. (2003) Homocysteine mediated expression and secretion of monocyte chemoattractant protein-1 and interleukin-8 in human monocytes. *Circ Res,* **93,** 311-320.

Zhang, Y., Rothermundt, M., Peters, M., Wiesmann, M., Hoy, L., Arolt, V., Emrich, H. M. and Dietrich, D. E. (2009) S100B serum levels and word memory processing in remitted major depression as reflected by brain potentials. *Neuropsychobiology,* **59,** 172-177.

Ziegler, D. R., Innocente, C. E., Leal, R. B., Rodnight, R. and Goncalves, C. A. (1998) The S100B protein inhibits phosphorylation of GFAP and vimentin in a cytoskeletal fraction from immature rat hippocampus. *Neurochem Res,* **23,** 1259-1263.

Ziegler, D. R., Oliveira, D. L., Pires, C. et al. (2004) Ketogenic diet fed rats have low levels of S100B in cerebrospinal fluid. *Neurosci Res,* **50,** 375-379.