UNIVERSIDADE FEDERAL DO RIO GRANDE DO SUL
INSTITUTO DE CIÊNCIAS BÁSICAS DA SAÚDE
PROGRAMA DE PÓS-GRADUAÇÃO EM BIOQUÍMICA
TESE DE DOUTORADO

Avaliação cognitiva e de parâmetros bioquímicos astrogliais em modelos *in vitro* e *in vivo* de desordens do metabolismo da glicose

Ana Carolina Tramontina

Porto Alegre, novembro de 2011

UNIVERSIDADE FEDERAL DO RIO GRANDE DO SUL
INSTITUTO DE CIÊNCIAS BÁSICAS DA SAÚDE
PROGRAMA DE PÓS-GRADUAÇÃO EM BIOQUÍMICA

Avaliação cognitiva e de parâmetros bioquímicos astrogliais em modelos *in vitro* e *in vivo* de desordens do metabolismo da glicose

Ana Carolina Tramontina
Orientador: Carlos Alberto Gonçalves

Tese apresentada ao Programa de Pós-Graduação em Bioquímica da Universidade Federal do Rio Grande do Sul como requisito parcial para obtenção do título de doutor em Bioquímica

Porto Alegre, novembro de 2011.

Dedico à minha família, meu porto seguro...

Agradecimentos

Em primeiro lugar eu gostaria de agradecer a duas pessoas, que são exemplos de pesquisadores a seguir: CA e minha irmã, Francine.

Ao CA, por ter me recebido no laboratório, por ter acreditado no meu potencial, por ter aceitado a minha ausência no laboratório nesses últimos meses, e principalmente por ter me dado a oportunidade de continuar no lab 33!!

À Fran, por ter me apresentado ao mundo da pesquisa, por ter me trazido ao lab 33, por toda a ajuda desde o tempo da faculdade, e por ter trazido ao mundo a minha Jujuzinha!!

À Juju, que torna meus dias mais felizes, pelo simples fato de existir!

Ao Márcio, por ter aceitado a minha ausência, por ter me apoiado nas minhas decisões e por fazer parte da minha vida!!

Aos meus pais, Valmor e Carmem, pelo amor incondicional e pelo apoio a todas as minhas decisões!!

Agora os colegas do lab, agradeço a ajuda e disponibilidade de todos que participaram dos meus experimentos!!

Nina, Lets, André, Larica, Anita, Caren, Lucas, Núbia, Cris, Pati, Paulinha. Sem vocês essa tese não seria possível!!!

Minha querida IC Krista, as palavras não são suficientes para agradecer toda a tua dedicação!! Tenho certeza que tua carreira de pesquisadora está só começando!! Tu serás uma ótima mestranda!!

Aos colegas do lab 33, pelos momentos e risadas compartilhadas!!! Mamãe Dani, Carol Zanotto, Carol, Elisa, Paulo, Fafá, Fê, Jô, Maria, Jaque, Beta, Tamara, Adri, Márcio, enfim, a todos (desculpem se esqueci de alguém, mas são tantos!!)

À professora Marina, que está sempre pronta pra tirar dúvidas, dar uma mãozinha e até arrumar umas figuras...

Obrigada aos funcionários da Universidade, da secretaria ao biotério, por tornarem o trabalho possível.

Muito obrigada, PPG Bioquímica, CNPq e UFRGS.

“Eis o meu segredo: só se vê bem o coração.
O essencial é invisível aos olhos...
... tu te tornas eternamente responsável por aquilo que cativas.
Antoine de Saint-Exupéry

Sumário

PARTE I

Lista de Abreviaturas

AChE – acetilcolinesterase
AG – aminoguanidina
AGE – produto final de glicação avançada
ApoE – apolipoproteína E
APP – proteína precursora amilóide
βA – peptídeo beta amilóide
BHE – barreira hematoencefálica
DA – doença de Alzheimer
DM – diabetes mellitus
eNOS – óxido nítrico sintase endotelial
GFAP – proteína ácida fibrilar glial
GS – glutamina sintetase
GSH – glutationa
HO-1 – heme oxigenase-1
HMG-CoA – 3-hidroxi-3-metilglutaril CoA
ICV – intracerebroventricular
IGF – fator de crescimento semelhante à insulina
IL-1β – interleucina 1 beta
iNOS – óxido nítrico sintase induzível
NAC – n-acetil-cisteína
NO – óxido nítrico
Nrf2 – fator eritróide nuclear 2
PV – pravastatina
RAGE – receptor para produtos de glicação avançada
RI – receptor de insulina
SNC – sistema nervoso central
STZ – estreptozotocina
SV – sinvastatina
TNF-α – fator de necrose tumoral alfa

Resumo

A Doença de Alzheimer é uma desordem que acomete milhões de indivíduos por todo o mundo, e a sua incidência tem aumentado muito nas últimas décadas. A doença é caracterizada por diversas alterações no sistema nervoso central, e dentre elas ocorrem alterações importantes no metabolismo da glicose, com redução da utilização desse composto, levando a um importante déficit energético. A Doença de Alzheimer está intimamente relacionada com o Diabetes Mellitus, pois além de ocorrerem alterações semelhantes nas duas desordens, pacientes com diabetes têm risco aumentado de desenvolver Alzheimer. Além da correlação com Diabetes Mellitus, existem evidências de que a hipercolesterolemia também está associada ao maior risco de desenvolver Doença de Alzheimer, o que nos levou a investigar o potencial neuroprotetor das estatinas. Esse trabalho tem como objetivo avaliar alterações comportamentais e bioquímicas em dois modelos de desordens do metabolismo da glicose, bem como avaliar o possível papel neuroprotetor das estatinas, aminoguanidina e n-acetil-cisteína.

Abstract

Alzheimer's disease is a disorder that affects millions of individuals around the world, and its incidence has increased in recent decades. Alzheimer's disease is characterized by several alterations in the central nervous system, and changes in glucose metabolism is among the most important, reducing the uptake of this compound, leading to a significant energy deficit. Alzheimer's disease is closely related to Diabetes Mellitus, and similar metabolic changes occur in both disorders. Besides, diabetic patients present increased risk for Alzheimer's disease. In addition, another risk factor to Alzheimer's is high levels of plasma cholesterol, which lead some research groups to evaluate the potential neuroprotective effect of statins. The aim of this study is to asses behavioral and biochemical changes in two models of glucose metabolism disorders, and evaluate the possible neuroprotective role of statins, aminoguanidine and N-acetyl-cysteine

Introdução

1. Doença de Alzheimer

Estima-se que 10% dos adultos com idade acima de 65 anos apresenta sintomas de demência [1]. Os sintomas incluem perdas de memória e de raciocínio, decorrentes do próprio envelhecimento ou associados a patologias. O déficit cognitivo apresentado em um quadro de demência é mais pronunciado do que aquele decorrente do avanço normal da idade.

Existem diversos tipos de demência, as quais podem ser primárias ou seja, a causa base da própria demência, ou secundárias, quando decorrentes a determinadas patologias[2]. Entre os tipos mais incidentes está a doença de Alzheimer (DA), compreendendo 50 a 70% de todos os casos, afetando 35% dos indivíduos com mais de 85 anos [3].

Nas últimas décadas, a incidência de DA e de outras desordens neurodegenerativas tem aumentado muito, principalmente nos países desenvolvidos [3]. Esse crescimento no número de pacientes com desordens como a DA está diretamente associado com o aumento da expectativa de vida, e acredita-se que irá ocorrer um aumento sem proporções nos casos de demência, principalmente em indivíduos com mais de 70 anos [4, 5]. Desta forma, torna-se essencial e urgente a busca por alternativas neuroprotetoras e mecanismos para prevenção da DA.

1.2. Alterações clínicas e patofisiológicas da DA

Os indivíduos com DA apresentam progressivas alterações de memória e da cognição, perda das habilidades sociais e das habilidades de linguagem,

alterações comportamentais e emocionais, e marcadores histopatológicos da desordem já são conhecidos [3, 6].

Histologicamente, a DA caracteriza-se por uma extensa perda neuronal e gliose, além de duas alterações clássicas: as placas neuríticas, que são depósitos extracelulares de peptídeo β-amilóide (βA), e emaranhados neurofibrilares intracelulares, decorrentes da hiperfosforilação da proteína tau, uma proteína associada aos microtúbulos [7-9].

Acompanhando as alterações histológicas citadas, há uma significativa redução da atividade colinérgica, com acentuada perda de neurônios colinérgicos, principalmente nas regiões cerebrais relacionadas com aprendizado e memória [10-12].

O quadro geral que caracteriza a DA inclui, além do déficit colinérgico, alterações no metabolismo do glutamato, estresse oxidativo, neuroinflamação, alterações na homeostase do cálcio e alterações mitocondriais, causando prejuízo na transmissão sináptica e alterando o equilíbrio de diversos neurotransmissores [6, 13-15].

A DA pode ser hereditária (tipo I), quando relacionada a alguns genes, como o da proteína precursora amilóide (APP), das presenilinas e da apolipoproteína E (ApoE), ou esporádica (tipo II), a qual todos os indivíduos estão sujeitos à medida que envelhecem, sendo essa última a mais comum [8, 16]. Alguns fatores de risco estão associados ao desenvolvimento da DA esporádica, e em especial podemos citar o Diabetes *Mellitus* (DM) tipo II, e a hipercolesterolemia [3, 17, 18].

1.2. Colesterol e Doença de Alzheimer

Além das mutações nos genes que codificam as proteínas envolvidas na geração do βA (APP, presenilinas), o maior fator de risco para o desenvolvimento da DA é o genótipo para a ApoE, o maior carreador de colesterol do organismo [18]. Recentemente, estudos têm indicado que há uma correlação entre altos níveis de colesterol plasmático e desenvolvimento da DA [19-21], e acúmulo de colesterol tem sido observado em associação com placas neuríticas no cérebro de pacientes com DA e em modelos de animais transgênicos da doença [22].

O colesterol é um componente essencial da membrana plasmática de todas as células, onde atua afetando a fluidez e a permeabilidade da mesma [3]. O colesterol de membrana é encontrado principalmente nas *rafts* lipídicas [23], microdomínios que atuam na sinalização celular, promovendo interações entre proteínas, e entre proteínas e lipídios [24].

O colesterol é originado em parte da alimentação, e em parte pela síntese *de novo*, principalmente no fígado e no intestino, sendo que a via de biossíntese também gera intermediários para a síntese de ubiquinona e isoprenilação de proteínas [18].

O sistema nervoso central (SNC) é particularmente rico em colesterol, ocorrendo síntese no cérebro, sendo este independente do colesterol sérico. As células neuronais parecem sintetizar colesterol durante o desenvolvimento, porém os neurônios maduros perdem a capacidade de síntese, obtendo então o colesterol de astrócitos [25, 26].

Muitos estudos têm apontado os efeitos deletérios do colesterol no desenvolvimento da DA, porém não se sabe exatamente até que ponto os altos níveis de colesterol circulantes estão diretamente associados ao desenvolvimento da doença, ou se a aterosclerose decorrente da hipercolesterolemia causa redução da circulação e suprimento de oxigênio ao SNC, resultando em susceptibilidade aumentada ao desenvolvimento da DA [27, 28].

Sabe-se que pelo menos uma fração da APP, bem como as β e γ-secretases encontram-se nas *rafts* lipídicas [29], e variações no colesterol da membrana são acompanhadas de alterações na atividade das secretases *in vitro* [30, 31]. Acredita-se que aumento do colesterol cause um aumento da clivagem da APP com produção aumentada de βA, enquanto a redução de colesterol causa uma redução da atividade da γ-secretase, reduzindo a deposição do peptídeo e a formação de placas [9, 32, 33].

Além das evidências sugerindo o efeito direto do colesterol no desenvolvimento da DA, muitos estudos sugerem um possível efeito protetor das estatinas, drogas que atuam na redução dos níveis de colesterol, na prevenção da DA [34-38].

1.3. Diabetes Mellitus e Doença de Alzheimer

O DM é uma desordem metabólica causada por uma deficiência relativa ou absoluta do hormônio insulina [39]. O DM tipo 1 é uma doença auto-imune que ocorre em conseqüência da destruição das células β pancreáticas, o que leva a ausência da produção de insulina. O DM tipo 2, forma mais freqüente, é

caracterizado por redução parcial na produção ou resistência à insulina, e está associado com envelhecimento, histórico familiar e obesidade [40].

O DM está frequentemente associado com complicações severas, como doença cardiovascular, nefro e retinopatia, bem como neuropatia periférica [41]. Com o aumento da sobrevida dos pacientes diabéticos, se observa que esta doença está associada a déficits cognitivos, alterações estruturais e neurofisiológicas no cérebro, uma condição referida como encefalopatia diabética [42], indicando que o DM também afeta o SNC.

Além das complicações já citadas, inúmeros estudos correlacionam o DM, principalmente do tipo 2, com risco aumentado de desenvolvimento de DA, [43-45].

Sabe-se que o DM e a DA possuem algumas características patológicas em comum, e entre elas podemos citar: alterações do metabolismo energético e da glicose; resistência à insulina, desenvolvimento de estresse oxidativo e aumento da geração de produtos finais de glicação avançada (AGEs) [46].

Os AGEs são produtos de glicação de proteínas e lipídeos, que se acumulam no DM, bem como em doenças inflamatórias e neurodegenerativas como a DA, possuindo múltiplos e potentes efeitos sobre tecidos e vasos [47].

Os AGEs atuam por ligação com os receptores para produtos finais de glicação avançada (RAGEs), que são membros da superfamília das imunoglobulinas. Embora se liguem aos AGEs, outros ligantes pra os RAGEs já foram demonstrados, como a S100B, e o próprio peptídeo βA, aumentado na DA [48, 49]. A ativação do RAGE por seus ligantes estimula a auto-expressão do receptor juntamente com a produção de citocinas pró-inflamatórias e estresse oxidativo [50].

Além do envolvimento dos AGEs na patofisiologia da DA e do DM, dados de estudos *in vivo* e *in vitro* têm revelado uma relação entre insulina e metabolismo do peptídeo βA, demonstrando que a insulina afeta tanto a produção quanto a degradação do mesmo [51-53]. Em adição a esse fato, algumas moléculas da via de sinalização da insulina possuem papel central na fosforilação da proteína tau, processo importante na neurodegeneração que ocorre na DA [54].

Alterações cerebrovasculares também podem ser um possível mecanismo correlacionando DM e DA, visto que é uma alteração comum às duas doenças. O βA pode estar envolvido nas alterações vasculares que ocorrem na DA, e no DM as complicações vasculares já estão bem descritas. A inflamação vascular que ocorre em ambas as desordens pode estar relacionada também à ativação dos RAGEs [55]. Alguns trabalhos, no entanto, sugerem que a correlação entre DM e DA é independente de lesões vasculares, estando diretamente relacionada à resistência insulínica e hiperglicemia [45].

2. Astrócitos na organização do Sistema Nervoso Central

Os neurônios foram por muito tempo considerados os elementos celulares responsáveis pelo processamento da informação, enquanto as células gliais eram reconhecidas apenas pelo seu papel de suporte no SNC [56]. Nas últimas décadas um grande número de pesquisas demonstra que as células da glia não atuam apenas como um elemento de suporte neuronal, mas sim como um componente ativo em funções cerebrais essenciais [57].

As células da glia são divididas em dois grupos principais, a macroglia, composta de astrócitos, oligodendrócitros e células ependimais, e a microglia, composta de células fagocíticas envolvidas em respostas inflamatórias. Os astrócitos compreendem 50% da massa cerebral, sendo assim, as células gliais mais abundantes no SNC. Entre os diferentes tipos de células gliais, os astrócitos têm recebido atenção especial, provavelmente pela sua íntima relação com neurônios e sinapses [56].

Os astrócitos constituem o terceiro elemento da sinapse tripartida, e possuem papel-chave no desenvolvimento e funções do cérebro, como no metabolismo neuronal, sinaptogênese, homeostase do meio extracelular e microcirculação cerebral [58]. Essas células atuam desde o controle da diferenciação e migração neuronal, à formação e manutenção da barreira hematoencefálica (BHE), tamponamento de potássio, captação e conversão do glutamato em glutamina, defesa antioxidante, homeostase do Ca^{2+} até a liberação de gliotransmissores, e na própria modulação sináptica [56, 59].

Os astrócitos possuem três proteínas específicas usadas como marcadoras: a GFAP (proteína fibrilar ácida glial), a glutamina sintetase (GS) e a S100B. A GFAP é o principal filamento intermediário de astrócitos maduros do SNC, e é considerada como um dos principais antígenos utilizados para a identificação e estudo do comportamento astrocítico [60].

A S100B é uma proteína ligante de cálcio, expressa e secretada pelos astrócitos, que possui efeitos parácrinos sobre neurônios e microglia, e autócrinos sobre astrócitos [61]. Dentre estas ações, estão a regulação da proliferação, diferenciação e morfologia celular, homeostase do Ca^{2+},

fosforilação e transcrição de proteínas, atividade enzimática e metabolismo [62, 63].

A S100B exerce efeitos extracelulares *in vitro*, tróficos ou tóxicos, dependendo de sua concentração. Níveis extracelulares na ordem de nanomolar têm efeito neurotrófico, induzindo o crescimento de neuritos, aumentando a sobrevivência neuronal e servindo como molécula-guia para o desenvolvimento de neurônios, além de possuir atividade neurotrófica ou neuroprotetora após dano cerebral. Em concentrações micromolares, a S100B exerce efeitos neurotóxicos por mecanismo dependente da indução de liberação de citocinas pró-inflamatórias [57] e ao estimular a secreção de óxido nítrico por astrócitos e microglia [61].

Os astrócitos são as células responsáveis pela manutenção dos níveis extracelulares de glutamato, através da captação de glutamato, mecanismo de importância vital na prevenção da excitotoxicidade [64]. O glutamato captado pelos astrócitos sofre ação da enzima GS, que é a responsável pela conversão do glutamato em glutamina. Essa conversão é um mecanismo de extrema importância para o *turnover* do neurotransmissor no SNC. A glutamina gerada pela ação da GS é liberada para o meio extracelular, sendo captada pelos neurônios, onde irá regenerar o glutamato [65].

Os astrócitos também provêem um importante sistema antioxidante para o SNC, a partir da síntese e liberação de glutationa (GSH), que é essencial para a remoção de espécies reativas de oxigênio (ERO), continuamente geradas pelo metabolismo oxidativo no SNC [66].

Os astrócitos atuam na síntese do colesterol cerebral, e também internalizam e reciclam o colesterol liberado de terminais nervosos em degeneração, devolvendo o colesterol aos neurônios, processos estes que requerem a ligação do colesterol com a ApoE, principal apolipoproteína do SNC [3, 26].

2.1. Astrócitos e neurodegeneração

Os processos neurodegenerativos iniciam com alterações na conectividade dos circuitos cerebrais, resultando em morte neuronal e atrofia cerebral. Sendo os astrócitos essenciais para a manutenção da homeostasia do SNC, estão envolvidos no desenvolvimento de várias desordens neurodegenerativas, ocorrendo extensa remodelação na DA [64, 67].

Astrócitos reativos, caracterizados por hipertrofia e aumento da expressão da GFAP e S100B, são frequentemente encontrados em tecido cerebral de pacientes com DA. Esses astrócitos ativados são encontrados principalmente associados às placas amilóides, e o grau de astrocitose pode ser correlacionado à extensão da deposição de βA [68].

Os astrócitos ativados encontrados no cérebro de pacientes com DA têm expressão aumentada de S100B, e a maioria dessas células está em associação com as placas amilóides [69], o que sugere que a proteína S100B pode ser um importante fator na gênese e evolução das placas amilóides na DA [70]. Estudos também demonstraram que pacientes nos estágios iniciais da DA têm um alto número de astrócitos associados às placas amilóides, enquanto aqueles em estágio mais avançado da doença apresentam uma menor concentração de astrócitos na periferia das placas, o que corrobora com

a teoria de que os astrócitos ativados, com maior expressão de S100B possuem papel chave no desenvolvimento da DA [71].

Acredita-se que a ativação astrocitária ocorra em resposta à liberação de interleucina-1 (IL-1) pela microglia ativada (ativação por depósito amilóide), e que essa citocina, junto com a S100B, atue na transformação de depósitos amilóides difusos em placas neuríticas, causando toxicidade aos neurônios [68].

Além do aumento da expressão da proteína S100B, os astrócitos reativos também atuam produzindo e liberando citocinas pró-inflamatórias, aumentam a produção de ERO e espécies reativas de nitrogênio, contribuindo para o dano oxidativo, recrutando mais astrócitos e agravando o quadro inflamatório, com consequente aumento do depósito de proteínas, em um ciclo vicioso [72, 73].

Alterações astrocíticas também têm sido associadas ao DM. Pacientes com DM tipo 2 apresentam uma menor concentração sérica de S100B [74], e modelos animais de diabetes têm provido importantes resultados quanto aos efeitos da desordem sobre os astrócitos. Modelo de DM com estreptozotocina (STZ) tem demonstrado uma redução da expressão de GFAP [75], e aumento de enzimas associadas ao estresse oxidativo [76].

3. Neuroproteção

A DA afeta milhões de pessoas no mundo todo, e até o momento não existe cura ou um tratamento específico para a doença [77], e por esse motivo, inúmeras alternativas terapêuticas têm sido avaliadas para a redução dos danos causados pela doença e para neuroproteção. Dentre as substâncias

testadas, podemos citar a aminoguanidina (AG) [78], as estatinas [79] e a n-acetil-cisteína (NAC) [80].

3.1. Aminoguanidin

Os compostos guanidínicos como a AG, a creatinina e a arginina estão presentes no encéfalo de mamíferos. Esses compostos estão envolvidos em processos de indução de convulsões, geração de radicais livres até geração de energia e prevenção de apoptose [81].

A AG é um composto guanidídico sintético que tem recebido maior atenção pelo seu potencial como droga de ação anti-glicação. Ela atua reagindo com os grupos carbonila de açúcares reduzidos ou com o 3-diacilglicerol, prevenindo a formação de AGEs [82].

A AG também age no combate ao estresse oxidativo através da ligação e neutralização do peroxinitrito [83], e como agente quelante de íons de metais de transição [84]. Clinicamente, sabe-se que a AG previne o estresse tecidual crônico decorrente da formação de AGEs, e, por esse motivo, tem sido testada no tratamento do DM. Porém, na forma como vem sendo apresentada, tem causado uma série de efeitos colaterais na fase III das triagens clínicas, como, por exemplo, a deficiência de vitamina B6 pelo sequestro do piridoxal [85].

Sobre a atuação da AG no SNC, alguns estudos apontam para as propriedades neuroprotetoras da aminoguanidina em modelos animais de danos corticais e isquemia [86]. Os mecanismos de tal efeito não estão totalmente esclarecidos, porém se sabe que além de diminuir a formação de AGEs e outras ações citadas acima, este composto atua como inibidor seletivo da enzima óxido nítrico sintase induzível (iNOS) [81].

3.2. Estatinas

As estatinas são drogas que atuam inibindo competitivamente a enzima chave da síntese de colesterol, a 3-hidroxi-3-metilglutaril-CoA redutase (HMG-CoA redutase), e são amplamente utilizadas em terapias de redução de colesterol, além de estarem implicadas na redução da incidência de doença coronariana em testes clínicos [87].

A inibição da HMG-CoA redutase reduz não apenas a síntese do colesterol, mas também a produção de isoprenóides como o farnesil pirofosfato e geranilgeranil pirofosfato [79]. Esses compostos atuam na modificação pós-traducional das proteínas, e podem estar relacionados aos efeitos pleiotrópicos das estatinas, não correlacionados diretamente com a redução do colesterol [88]. Entre as proteínas-alvo da isoprenilação, estão as GTPases, que estão envolvidas em diferentes processos celulares, incluindo rearranjo do citoesqueleto, fagocitose, regulação da transcrição, crescimento e desenvolvimento celular [89]. A figura 1 ilustra os efeitos das estatinas sobre a via de síntese do colesterol.

As estatinas são utilizadas na terapêutica como lactonas ou como ácidos livres. As lactonas são pró-drogas que necessitam da transformação hepática para a forma ácida, que representa a forma farmacologicamente ativa. A principal diferença entre as lactonas e os ácidos é a solubilidade em água. As lactonas são mais lipofílicas, e incluem a lovastatina, sinvastatina (SV) e cerivastatina, enquanto os ácidos são mais hidrofílicos, e incluem a pravastatina(PV) e fluvastatina [17].

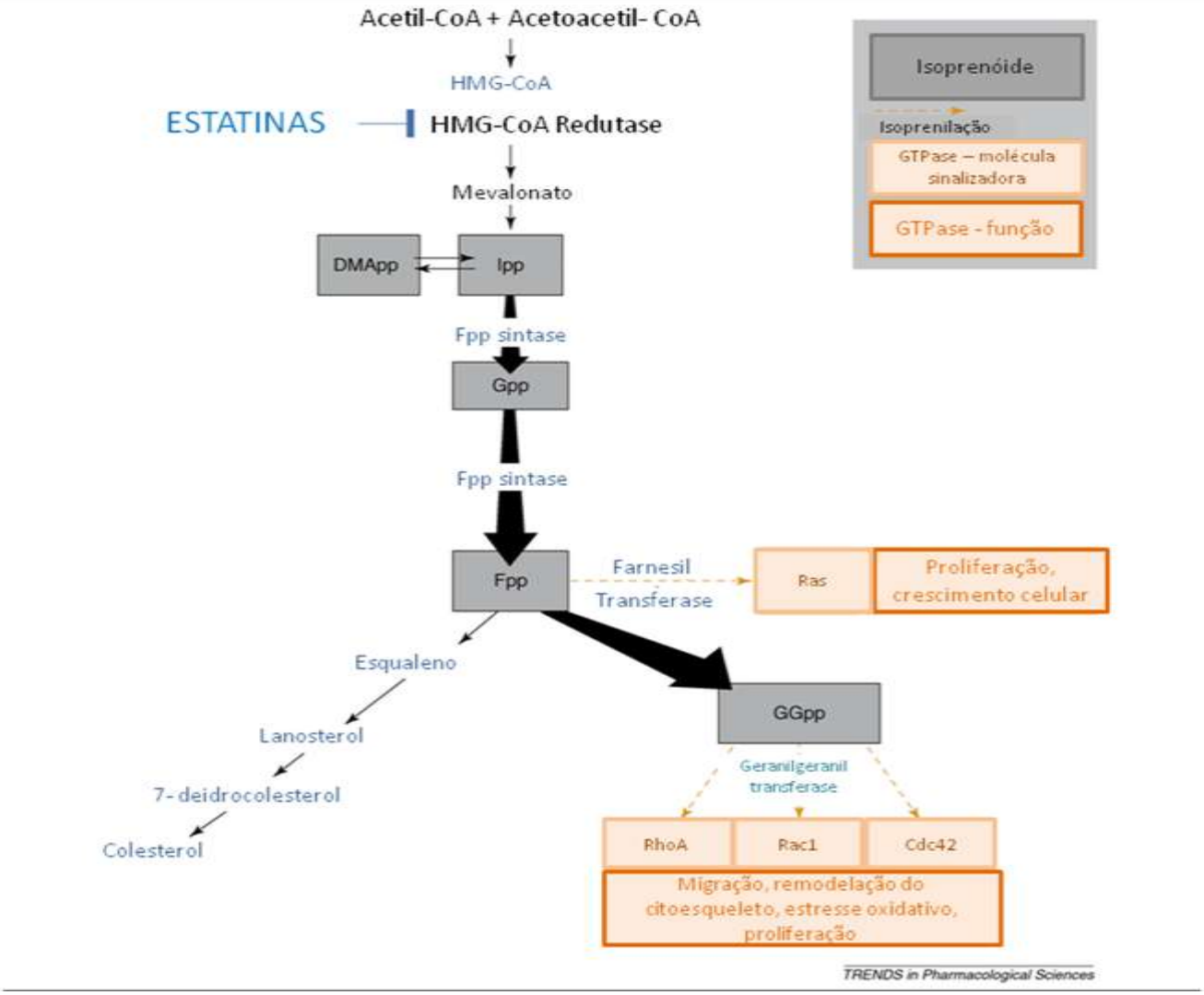


Fig. 1 : Via da HMG-CoA redutase, levando à síntese de colesterol e isoprenóides. HMG-CoA redutase (HMGCR) é a enzima limitante na via, que inicia a partir de acetil-CoA e acetoacetil-CoA, que são convertidos em HMG-CoA. Esse substrato é então convertido a mevalonato, que por sua vez é convertido em isopentenil pirofosfato (Ipp), que pode ser isomerizado a dimetilalil difosfato (DMApp). Os dois isômeros atuam síntese dos isoprenóides geranil pirofosfato (Gpp), farnesil pirofosfato (Fpp) e geranilgeranil pirofosfato (GGpp). As estatinas atuam inibindo a enzima HMG-CoA redutase, reduzindo os níveis de colesterol e isoprenóides.
Adaptada de Zipp *et al.*(2007). Impact of HMG-CoA reductase inhibition on brain pathology. TRENDS in Pharmacological Sciences 28 (7), 342-349.

Os efeitos pleiotrópicos das estatinas incluem propriedade antiinflamatórias [90], vasculares e imunomodulatórias [91]. Essa gama de

efeitos pleiotrópicos sugere que esses compostos podem ser utilizados em inúmeras desordens, desde demência até doenças auto-imunes [92].

Estudos recentes têm demonstrado que as estatinas podem conferir proteção contra a demência [17], e a conexão entre DA e colesterol tem dado fundamento para a utilização desses fármacos na DA.

Os efeitos benéficos das estatinas na proteção contra a DA podem estar relacionados diretamente à redução dos depósitos amilóides. Estudos recentes têm demonstrado que as estatinas causam uma redução das placas amilóides em modelos animais de demência [93, 94] e também *in vitro* [93]. Estudos clínicos também têm demonstrado redução de βA em soro de pacientes tratados com estatinas [95], e em líquor de pacientes com DA em estágio inicial, e tratados com SV [96].

A resposta imune possui papel chave na neurodegeneração, e as estatinas exercem uma variedade de efeitos sobre o sistema imune. A maioria dos estudos demonstra que as estatinas atuam inibindo a ativação e resposta do sistema imune, bem como a infiltração das células imunológicas nos tecidos [91, 97]. O mecanismo pelo qual esses compostos afetam a resposta imune ainda não está claro, mas parece envolver a redução dos isoprenóides [79].

As estatinas podem ainda proteger as células e tecidos do dano oxidativo. Essas drogas apresentam efeitos em tecidos e modelos de demência *in vivo* [98] [99, 100], e também em estudos clínicos [101].

Ainda há muita controvérsia nos dados referentes à utilização das estatinas na prevenção da DA, e devido à capacidade antiinflamatória, imunomodulatória e sobre o depósito amilóide, as estatinas devem ter seu potencial neuroprotetor avaliado.

3.3. N-acetil-cisteína

A NAC é um composto que possui ação antioxidante, e é aprovado para utilização em intoxicação por paracetamol e intoxicação por metais pesados. O efeito antioxidante da NAC pode ser direto, pela sua ação na inativação de radicais livres, ou indireto, por aumentar os níveis intracelulares de GSH[102].

Na DA ocorre redução dos níveis de GSH, acarretando em dano oxidativo aumentado. A NAC poderia ser uma alternativa para a redução do estresse oxidativo que ocorre na doença. De fato, o aumento de GSH endógena e a redução do estresse oxidativo no cérebro através da administração de NAC já foram demonstrados [103], e esse composto também foi capaz de reduzir o estresse oxidativo e melhorar a memória de ratos senescentes[104].

4. Modelo de demência por infusão intracerebroventricular (ICV) de estreptozotocina (STZ)

Diversos modelos animais não-transgênicos para a DA são utilizados, e visam mimetizar algumas das características neuroquímicas e comportamentais apresentadas na DA do tipo esporádica. Dentre estes, tem-se a infusão ICV direta do peptídeo βA[105], a destruição neurotóxica do núcleo basal magnocelular e suas eferências colinérgicas corticais[106], a oclusão permanente e bilateral das carótidas comuns e o modelo por infusão ICV da STZ, nosso objeto de estudo, entre outros.

A STZ é uma droga de ação antibiótica utilizada experimentalmente para induzir diabetes, principalmente em roedores. Para isso, é administrada por via sistêmica e age no pâncreas destruindo permanentemente as células

produtoras de insulina, originando um quadro de diabetes [107]. O mecanismo de ação da droga no pâncreas consiste na captação da mesma pelas células β, através de transportadores de glicose do tipo 2. No interior das células, a STZ gera compostos reativos que causam a fragmentação do DNA, ocorrendo a formação de radicais livres, que acabam promovendo a morte celular[108].

Quando administrada via ICV, a STZ causa uma espécie de dessensibilização dos receptores de insulina (RI) e do IGF com consequentes alterações bioquímicas e fisiopatológicas semelhantes às encontradas na demência do tipo Alzheimer. Por esta razão, tem sido amplamente aceita para produzir um modelo experimental para a DA [109]. É importante ressaltar que a STZ não ultrapassa a BHE e a administração ICV não causa comprometimento sistêmico.

O mau funcionamento do RI resulta também em redução do metabolismo energético cerebral com conseqüente estresse oxidativo, o que se reflete em disfunções cognitivas devido, entre outros fatores, à inibição da formação de ATP e acetil-CoA [110]. Trabalhos demonstram que a injeção ICV de SZT em dose subdiabetogênica reduz de 10 a 30% os níveis de glicose no córtex cerebral e hipocampo, com diminuição significativa das principais enzimas da via glicolítica[111]. Além disso, estudos feitos por Duelli e colaboradores[112] mostraram redução na utilização da glicose em inúmeras áreas encefálicas. Também foram relatados comprometimentos de aprendizagem e memória, bem como redução dos níveis da enzima colina acetiltransferase, também no hipocampo, o que interfere na neurotransmissão colinérgica [110, 113].

Objetivos

Tendo em vista a forte correlação entre DM e demência, o fato de que os efeitos da resposta glial na patofisiologia dessas duas desordens necessitam ser melhor estudados, bem como o fato de que os efeitos de moléculas neuroprotetoras como as estatinas não são totalmente conhecidos, delineamos os seguintes objetivos para a tese:

Objetivos Gerais

1) Avaliar o efeito da alta concentração de glicose sobre cultura astroglial, avaliando possíveis efeitos benéficos da aminoguanidina e N-acetil-cisteína;
2) Avaliar o efeito da pravastatina e sinvastatina em modelo de demência, observando parâmetros comportamentais e bioquímicos.

Objetivos Específicos

1) Em culturas de glioma C6 expostas à alta concentração de glicose, avaliar:
   a) Captação de glutamato e secreção de S100B;
   b) Ocorrência de estresse oxidativo e dano ao DNA;
   c) Possível efeito protetor da aminoguanidina e N-acetil-cisteína sobre a captação de glutamato e secreção de S100B;
2) Em animais expostos a modelo de demência por injeção intracerebroventricular de estreptozotocina, avaliar:
   a) Déficit cognitivo;
   b) Estresse oxidativo e nitrosativo, dano a proteínas e lipídeos;
   c) Captação de glutamato;

d) Captação de glicose;

e) Expressão de S100B e GFAP;

f) Atividade da enzima acetilcolinesterase ;

g) Possível efeito da sinvastatina e pravastatina sobre todos os parâmetros acima citados.

PARTE II

CAPÍTULO I

High-glucose and S100B stimulate glutamate uptake in C6 glioma cells.

Artigo publicado no periódico Neurochemical Research

DOI: 10.1007/s11064-012-0722-4

High-glucose and S100B stimulate glutamate uptake in C6 glioma cells

Ana Carolina Tramontina, Patricia Nardin, André Quincozes-Santos, Lucas Tortorelli, Krista Minéia Wartchow, Ana Cristina Andreazza, Elizandra Braganhol, Diogo Onofre Souza, Carlos-Alberto Gonçalves*

Departamento de Bioquímica, Instituto de Ciências Básicas da Saúde, Universidade Federal do Rio Grande do Sul, Porto Alegre, Brazil

* Corresponding author: Carlos-Alberto Gonçalves
Depto Bioquímica, ICBS, UFRGS
Ramiro Barcelos, 2600-anexo
Porto Alegre, RS, Brazil
90035-003
Fax: 55-51-3308 5535
E-mail: casg@ufrgs.br

Abstract

Diabetes mellitus is a disease associated with several changes in the central nervous system, including oxidative stress and abnormal glutamatergic neurotransmission, and the astrocytes play an essential role in these alterations. *In vitro* studies of astroglial function have been performed using cultures of primary astrocytes or C6 glioma cells. Herein, we investigated glutamate uptake, glutamine synthetase and S100B secretion in C6 glioma cells cultured in a high-glucose environment, as well as some parameters of oxidative stress and damage. C6 glioma cells, cultured in high-glucose medium, exhibited signals of oxidative and nitrosative stress similar to those found in diabetes mellitus and other models of diabetic disease (decrease in glutathione, elevated nitric oxide, DNA damage). Interestingly, we found an increase in glutamate uptake, which might be linked to the altered glutamatergic communication in diabetes mellitus. Moreover, glutamate uptake in C6 glioma cells, like primary astrocytes, was stimulated by extracellular S100B. Together, these data emphasize the relevance of astroglia in diabetes mellitus, as well as the importance of glial parameters (e.g. glutamine synthetase) in the evaluation of diabetic disease progression and treatment.

Keywords: astrocyte; C6 glioma; diabetes mellitus; glutamate; oxidative stress; S100B

1. Introduction

Diabetes mellitus (DM) is a disease associated with several changes in the central nervous system (CNS), including oxidative stress, changes in glutamatergic neurotransmission, and elevated risk of cognitive dysfunction and dementia [1-3], possibly due to the absence of insulin, insulin resistance or elevated levels of circulating glucose.

Astrocytes are the most abundant cells in CNS, and play a crucial role in antioxidant defense [4], in the prevention of excitotoxicity by removal of glutamate from the synaptic cleft [5], and in the secretion of trophic factors, including S100B. *In vitro* extracellular S100B effects include proliferation of C6 cells, neurite outgrowth and protection against glutamate-induced damage (see [6] for a review).

In vitro studies of astrocyte function have been performed using cultures of primary astrocytes or C6 gliomas cells. In fact, these cells have been used almost indistinctly to study many biochemical parameters and signal transduction, including expression and secretion of S100B [7, 8]. However, these cells have different regulatory mechanisms for the gene expression of S100B [9] and oxidative metabolism of glucose [10, 11]. We observed that S100B secretion is reduced in astrocytes growing up in a high-glucose environmental, but is increased in C6 glioma cells under similar conditions [12].

One extracellular activity of S100B is the stimulation of glutamate uptake by astrocytes [13] that putatively contribute to neuroprotection against glutamate-induced damage. Brain glutamate uptake is mainly carried out by the sodium-dependent glutamate transporters, GLAST/EAAT1 and GLT-1/EAAT2, located on astrocytes [5]. These transporters are sensitive to oxidative stress [14, 15].

Therefore, high-glucose medium should induce a decrease in glutamate uptake activity and this, in turn, may partially explain the elevated glutamate levels in diabetic individuals [16].

However, in contrast, glutamate uptake does not change in primary astrocytes cultured under high-glucose, pro-oxidant conditions [12] nor in retinal glial cells in STZ-induced diabetes in rats [17] or in synaptosomes of Goto-Kakizaki diabetic rats [18]. On the other hand, glutamate uptake was found to be increased in glial plasmalemmal vesicle preparations from STZ-induced diabetic rats [19].

Previous studies from our group, using C6 glioma cells and astrocytes, suggest that antioxidants could stimulate S100B secretion and glutamate uptake [20-22]. C6 glioma cells, in contrast to primary astrocyte cultures, predominantly express the glutamate transporter, EAAT3 [5, 23]. There is no information in the literature, at least to our knowledge, as to whether high-glucose conditions affect glutamate uptake in these cells and as to whether S100B also is able to stimulate glutamate uptake.

Herein, we investigated the glutamate uptake in C6 glioma cells cultured in a 12mM glucose medium, as well as some parameters of oxidative stress (DCF assay, nitric oxide (NO) and glutathione contents), oxidative damage (COMET assay and glutamine synthetase (GS) activity). We also investigated whether S100B stimulates glutamate uptake, since a previous study indicated that high-glucose conditions cause a higher secretion of S100B [12].

2. Material and Methods

2.1. Materials

Dulbecco's modified Eagle's medium (DMEM) and other material for cell culture were purchased from Gibco. S100B protein, standard glutathione, o-phthaldialdehyde, c-glutamylhydroxamate and dichlorofluorescein diacetate (DCF-DA), aminoguanidine and N-acetyl-cysteine were purchased from Sigma. $_{L}$-[2,3-^{3}H] glutamate was purchased from Perkin Elmer. The ECL kit was purchased from Amersham International. Anti-EAAT3 was purchased from Novus Biologicals. Anti-rabbit immunoglobulin was bought from Dako. Other reagents were purchased from local commercial suppliers (Sulquímica, Labsul and Biogen, Porto Alegre, Brazil)

2.2. Maintenance of cell lineage

The C6 glioma cell line was obtained from the American Type Culture Collection (Rockville, Maryland, USA) and was maintained essentially according to the procedure previously described by [20]. The cells were seeded in flasks and cultured in DMEM (pH 7.4) containing 5% fetal bovine serum (FBS). Cells were kept at a temperature of 37°C in an atmosphere of 5% CO_2/95% air. Exponentially-growing cells were detached from the culture flasks using 0.05% trypsin/ EDTA and seeded in 96-, 24- or 6-well plates, and cultured in 6 mM glucose or 12 mM glucose DMEM, containing 5% FBS. C6 cells exposed to different media were cultured in the same plate; confluence of cells in 6 mM glucose DMEM occurred in approximately 4 days, and in 12mM glucose, confluence occurred in 5 days

2.3. *Incubation with aminoguanidine or N-acetyl-cysteine*

C6 glioma cells were cultured with 12mM glucose medium and incubated with 250 µM aminoguanidine or 100 µM N-acetyl-cysteine. The drugs were incubated in two different manners; firstly, after C6 cell confluence, for 24 h without FBS; secondly, the cells were cultured with 12 mM glucose medium in the presence of aminoguanidine or N-acetyl-cysteine. At confluence, the media were replaced to a high-glucose FBS-free medium, with aminoguanidine or N-acetyl-cysteine, for 24h. The total time of incubation was 96 h in the second set of experiments.

2.4. Glutamate uptake assay

Glutamate uptake was performed, as previously described [24]. Briefly, C6 glioma cells were incubated at 37°C in a Hank's balanced salt solution (HBSS) containing (in mM): 137 NaCl, 5.36 KCl, 1.26 $CaCl_2$, 0.41 $MgSO_4$, 0.49 $MgCl_2$, 0.63 $Na_2HPO_4.7H_2O$, 0.44 KH_2PO_4, 4.17 $NaHCO_3$ and 5.6 glucose, adjusted to pH 7.4. The assay was started by the addition of 0.1 mM L-glutamate and 0.33 µCi/ml L-[2,3-^{3}H] glutamate. The incubation was stopped after 10 min by removal of the medium and rinsing the cells twice with ice-cold HBSS. The cells were then lysed in a solution containing 0.5 M NaOH. Radioactivity was measured in a scintillation counter. Sodium independent uptake was determined using N-methyl-D-glucamine instead of NaCl. Sodium-dependent glutamate uptake was obtained by subtracting the non-specific uptake from the total uptake to obtain specific uptake. In another set of experiments, S100B was added in three different concentrations (0.01, 0.05 and 0.1 ng/ml) immediately after the serum removal, in order to evaluate the influence of exogenous S100B on glutamate uptake in C6 glioma cells cultured in 6 mM glucose medium

2.5. ELISA for S100B

The S100B concentration was measured, as described previously [25]. Briefly, 50 µL of sample plus 50 µL of Tris buffer were incubated for 2 h in 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 o-phenylenediamine was measured at 492 nm. The standard S100B curve ranged from 0.002 to 1 ng/mL.

2.6. Glutamine synthetase (GS) activity

The enzymatic assay was performed, as previously described [20]. Briefly, homogenate (0.1 mL) was added to 0.1 mL of reaction mixture containing (in mM): 10 $MgCl_2$; 50 L-glutamate; 100 imidazole-HCl buffer (pH 7.4); 10 2-mercaptoethanol; 50 hydroxylamine-HCl; 10 ATP and incubated for 15 min at 37ºC. The reaction was stopped by the addition of 0.4 mL of a solution containing (in mM): 370 ferric chloride; 670 HCl; 200 trichloroacetic acid. After centrifugation, the supernatant was measured at 530 nm and compared to the absorbance generated by standard quantities of γ-glutamylhydroxamate, treated with ferric chloride reagent.

2.7. Glutathione (GSH) Content Assay

GSH levels (nmol/mg protein) were measured, as described previously [26]. C6 glioma cell homogenates were diluted in 10 volumes of 100 mM sodium phosphate buffer pH 8.0 containing 5 mM EDTA and protein was precipitated with 1.7% meta-phosphoric acid. Supernatant was assayed with *o*-phthaldialdeyde (1mg/mL methanol) at room temperature for 15 min. Fluorescence was measured using excitation and emission wavelengths of 350

and 420 nm, respectively. A calibration curve was performed with standard GSH solutions (0-500 µM).

2.8. Evaluation of intracellular reactive oxygen species(ROS) production

Intracellular ROS production was detected using the nonfluorescent cell permeating compound, 2'–7'-dichlorofluorescein diacetate (DCF-DA). DCF-DA is hydrolyzed by intracellular esterases and then oxidized by ROS to a fluorescent compound, 2'–7'-dichlorofluorescein (DCF). C6 cells were treated with DCF-DA (10 µM) for 30 min at 37°C and rinsed with DMEM without serum. Following DCF-DA exposure, the cells were rinsed and then scraped into PBS with 0.2% Triton X-100. The fluorescence was measured in a plate reader (Spectra Max GEMINI XPS, Molecular Devices, USA) with excitation at 485 nm and emission at 520 nm [22].

2.9. Nitric oxide (NO) production

NO was determined by measurement of nitrite (a stable oxidation product of NO), based on the Griess reaction. The Griess reagent was prepared by mixing equal volumes of 1% sulfanilamide in 0.5 N HCl and 0.1% N-(1-naphthyl) ethylenediamine in deionized water. The assay was performed as described [27] with modifications. Briefly, cells were cultured on 96-well plates and, after treatment, the Griess reagent was added directly to the cell culture and the incubation was maintained under reduced light conditions at room temperature for 15 min. Samples were analyzed at 550 nm on a microplate spectrophotometer. Controls and blanks were run simultaneously. Nitrite concentrations were calculated using a standard curve prepared with sodium nitrite (0–50 µM).

2.10. COMET Assay

A standard protocol for COMET assay preparation and analysis was based on references [28, 29]. C6 glioma cells were detached by incubating in the presence of trypsin /EDTA 0.05% (100 µl) for 5 min. During trypsinization, cells were carefully manipulated to avoid mechanical stress. Once detached, 100 µl of DMEM supplemented with 5% FBS were added. Slides were prepared by mixing 50 µl of C6 glioma suspension with 70 µl of low melting point agarose (0.75%). The mixture (cells/agarose) was added to a fully frosted microscope slide coated with a layer of 300 µl of normal melting agarose (1%). After solidification, the cover slip was gently removed and the slides were placed in lysing solution (2.5 M NaCl, 100 mM disodium EDTA and 10 mM Tris, pH 10.0, with freshly added 1% Triton X-100 and 10% dimethyl sulfoxide) for up to 24 h. Subsequently, the slides were incubated in freshly prepared alkaline buffer (300 mM NaOH and 1 mM EDTA, pH 12.6) for 10 min. The DNA was electrophoresed for 20 min at 25 V (0.90 V/cm^2) and 300 mA. Following electrophoresis, slides were immersed in neutralizing buffer (0.4 M Tris-HCl, PH 7.5, 4^{o}C) for 5 min, before finally applying 50 µL of 5 µg/mL ethidium bromide to them and leaving in the dark for 20 min to stain the DNA. Negative and positive controls were used for each electrophoresis assay in order to ensure the reliability of the procedure. Images of 100 randomly selected nuclei (50 nuclei from two replicated slides) were analyzed for each treatment. Nuclei were scored visually for comet tail size, based on an arbitrary scale of 0-4 [30].

2.11. EAAT3 immunocontent determination

The C6 glioma cells were scraped, transferred to Eppendorf tubes and equal amounts (30 µg) of proteins from each sample were boiled in sample buffer (0.0625MTris–HCl, pH 6.8, 2% (w/v) SDS, 5% (w/v) β-mercaptoethanol, 10%

(v/v) glycerol, 0.002% (w/v) bromphenol blue) and electrophoresed in 10% (w/v) SDS-polyacrylamide gel. The separated proteins were blotted onto a nitrocellulose membrane. Equivalent loading of each sample was confirmed with Ponceau S staining. Anti-EAAT3 was used at a dilution of 1:1000. After incubating with the primary antibody for 24 h at room temperature, filters were washed and incubated with peroxidase-conjugated anti-rabbit immunoglobulin (IgG) at a dilution of 1:2000. The chemiluminescence signal was detected using an ECL kit from Amersham.

2.12. Protein determination

Protein content was measured by Lowry's method using bovine serum albumin as standard [31].

2.13. Statistical analysis

Data from the experiments are presented as mean ± standard error (SE) and were analyzed statistically by Student's *t test*. Values of $p < 0.05$ were considered to be significant. For glutamate uptake with exogenous S100B (Fig 1B), glutamate uptake with aminoguanidine/ N-acetyl-cysteine (Fig 5) and S100B secretion with aminoguanidine/ N-acetyl-cysteine (Fig 6), the data are presented as mean ± SE and were analyzed statistically by one-way ANOVA followed by Tukey's test.

3. Results

3.1 Glutamate uptake is increased in C6 glioma cells cultured in 12 mM glucose medium.

C6 cells cultured in 12 mM or 6mM glucose medium showed a different profile of glutamate uptake. Basal levels of glutamate uptake changed from 0.40 to 0.60 nmol/microgram of protein/min (Fig 1A). Importantly, glutamate uptake in C6 cells was lower than that observed in primary astrocyte cultures, independently of whether normal or high-glucose culture medium was used [12]. This difference in glutamate uptake in C6 cells, induced by 12 mM glucose medium (not observed in astrocyte cultures) [12], could be due to the presence of EAAT3. However, no change in the content of this transporter was observed in C6 cells cultured in 12 mM glucose medium (insert Fig 1A).

3.2 S100B stimulates glutamate uptake in C6 glioma cells

In order to evaluate the influence of S100B on C6 glutamate uptake, we added different concentrations of S100B (from 0.01 to 0.1ng/ml) to C6 cells cultured in 6mM glucose medium (Fig 1B). S100B was able to increase glutamate uptake; a linear correlation analysis indicates a strong ($R = 0.63$) and significant ($p = 0.005$) effect. A similar increment was found in primary astrocyte cultures [13].

3.3 12 mM glucose medium increased free radical levels in C6 cells.

To evaluate the development of oxidative and nitrosative stress, intracellular ROS production was measured (based on the oxidation of DCF), as well as NO production (based on nitrite content), respectively. ROS increased by approximately 19% in C6 cells cultured in 12 mM glucose medium (Fig 2A), while NO content increased by about 56% (Fig 2B).

3.4 DNA damage was increased in 12 mM glucose treated C6 cells

To evaluate the consequences of oxidative stress, DNA damage was measured using the COMET assay. The total index of DNA damage increased by about 150% in C6 glioma cells (Fig. 3A), particularly due to an increase in DNA

damage to levels 2, 3 and 4, when compared with cells cultured in 6 mM glucose medium (Fig. 3B).

3.5 12 mM glucose medium decreased the glutathione content and glutamine synthetase activity in C6 glioma cells

Two astroglial-specific parameters of oxidative damage were analyzed: GSH content and GS activity, which is sensitive to nitrosative stress (Fig 4). Total GSH content and GS activity decreased by about 25% and 20%, respectively, in C6 glioma cells cultured in 12 mM glucose medium.

3.6. Aminoguanidine, but not N-acetyl-cysteine, prevented the effect of 12 mM glucose medium on glutamate uptake in C6 glioma cells

In order to evaluate the effects of oxidative stress and glycation on glutamate uptake in 12 mM glucose cultivated C6 cells, we incubated the C6 cells with aminoguanidine or N-acetyl-cysteine. N-acetyl-cysteine was not able to prevent or reverse the effect of 12 mM glucose (Fig. 5A and 5B). Conversely, aminoguanidine prevented the effect of 12 mM glucose when the cells were cultured for 4 days with 12 mM glucose medium and aminoguanidine (Fig. 5B). When the cells were incubated for 24 hours with aminoguanidine, there was no effect on glutamate uptake (Fig. 5A).

3.7. *Aminoguanidine and N-acetyl-cysteine did not alter S100B secretion in 12 mM glucose C6 glioma cells*

In this set of experiments, we evaluated the effect of oxidative stress and glycation on S100B secretion in 12 mM glucose cultured cells. Neither the incubation with N-acetyl-cysteine or aminoguanidine for 24h, nor the culture in the presence of these drugs were capable of reversing the effect of 12 mM glucose on S100B secretion (Fig.6A and 6B).

4. Discussion

Metabolic changes and oxidative stress in diabetic individuals are associated with the incidence of depression, stroke, cognitive dysfunction and dementia [32-34]. Streptozotocin (STZ)-induced diabetic rodents and cultured neural cells are models for investigating the mechanisms of DM and the efficacy of therapeutic agents for the prevention and treatment of this condition [35].

Elevated extracellular levels of glutamate have been described in these patients [16] and such alterations could be due to oxidative stress, and also contribute to the production of reactive species. Altered glutamate levels and other evidence indicate an impairment of the glutamatergic system in DM [3]. Both receptors and transporters of glutamate have been investigated in patients and diabetic models. Some of the alterations in glutamatergic communication observed may involve changes in the expression and affinity of glutamate receptors [1]. No changes in the expression of astroglial glutamate transporters have been observed in diabetic rat models [36]. However, data demonstrating changes in the activities of these transporters are controversial. No changes in the activities were observed in retinal glial cells in STZ-induced diabetes in rats [17] or in synaptosomes of Goto-Kakizaki diabetic rats [18]. Recently, however, an increase in glutamate uptake activity (possibly involving EAAT2/GLT-1) was reported in glial plasmalemmal vesicle preparations from STZ-induced diabetic rats [19], although no changes in the expression of glutamate transporters were observed.

Astroglial alterations observed in diabetic rat models have been attributed primarily to insulin misbalance. We investigated the effect of 12mM glucose incubation on glial cells, *in vitro*. The oxidative stress caused by elevated

glucose affects many cell types and involves different mechanisms, including glucose auto-oxidation, alteration of the mitochondrial metabolism and formation of advanced glycated end products (AGE) [37].

Aminoguanidine has been proposed as a neuroprotective agent in many conditions of brain injury [38, 39], and it is known that the aminoguanidine is able to prevent the formation of AGEs and the development of oxidative stress [40]. We found that aminoguanidine was able to prevent the effect of 12 mM glucose on glutamate uptake increase, but not on S100B secretion. In addition, this drug only prevented the effect of 12 mM glucose, but was not able to reverse this effect. As N-acetyl-cysteine is a compound that exerts antioxidant effects [41], and did not demonstrate any effect, we believe that the increase in glutamate uptake due to 12 mM levels of glucose is dependent upon glycation and that aminoguanidine prevents this phenomenon.

No changes in glutamate uptake were observed in primary astrocyte cultures cultured in 12 mM glucose [12], however, we found an increase in glutamate uptake in C6 glioma cells. Again, this finding reinforces the idea of a metabolic difference between these cells when exposed to a high-glucose environment. C6 cells also express the glutamate transporter, EAAT3, the major neuronal glutamate transporter, while primary astrocytes express only GLAST/EAAT1 and GLT-1/EAAT2 [23]. Although, in general, glutamate transporters are sensitive to oxidants [5], it is possible that each type exhibits a different sensitivity and response to oxidative stress, resulting in specific changes, if not in their expression, in their location and affinity. Moreover, EAAT3 may also contribute to the increase in glutamate uptake observed in glial plasmalemmal vesicle preparations from STZ-induced diabetic rats [19]. The increase in

glutamate uptake observed in STZ-diabetic animals and C6 glioma cells could represent a compensatory mechanism to prevent an imminent risk of excitotoxity in DM. However, in this study, we did not observe any changes in the content of EAAT3 in C6 cells exposed to 12 mM glucose. Interestingly, aminoguanidine prevented the augment in glutamate uptake, suggesting a phenomenon mediated by glycation.

An interaction between gap junction communication, glucose uptake and rate of proliferation has been suggested in astroglial cells (see [10] for a review), involving an increase in ribose levels by activation of the pentose phosphate pathway (PPP), which is necessary for DNA duplication. C6 cells, which have few gap junctions, would therefore present an increased proliferation. Possibly, the activation of the PPP contributes to the higher resistance to oxidative-induced apoptosis, due to NADPH production. However, we noted that the 12 mM glucose environment caused oxidative stress (based on the DCF parameter) and oxidative damage (based on the COMET assay and decreased glutathione content). A number of previous studies have reported the influence of high glucose concentration and oxidative stress on DNA damage [42], where a decrease in the content of GSH has been proposed as one of the mechanisms that induces apoptosis in cells exposed to high glucose levels [43]. One simple explanation for this decrease in GSH is the increased flux of the polyol pathway. This pathway reduces glucose to sorbitol, which is later oxidized to fructose. The increased flux of the polyol pathway occurs in cases of high glucose concentrations, and consumes NADPH, which is necessary for the regeneration of oxidized GSH [44].

We have observed that S100B secretion and glutamate uptake are somehow functionally connected; for example, antioxidants such as resveratrol and epicathechinas stimulate both S100B secretion and glutamate uptake in astrocytes and C6 glioma cells [20-22]. In addition, decreased S100B secretion and glutamate uptake were observed in aged astrocyte cultures [24]. In support of these findings, very low levels of S100B were seen to stimulate glutamate uptake in astrocytes [13]. The present study found a similar effect in C6 glioma cells, which express EAAT3, the major neuronal glutamate transporter. However, in spite of this putative positive connection between S100B secretion and glutamate uptake, it is important to keep in mind that excitotoxic conditions could reduce S100B secretion [45, 46], and that elevated S100B secretion, in turn, may induce neuronal apoptosis [47].

A high-glucose environment induces oxidative and nitrosative stress in neural and non-neural cells, generating species such as superoxide and NO [48]. Confirming this, we found elevated levels of nitrites in C6 glioma cell cultures in the 12 mM glucose medium. In order to evaluate a consequence of this alteration, we evaluated GS activity, a specific astroglial enzyme responsible for the synthesis of glutamine from glutamate and negatively modulated by NO [49]. In fact, we observed a significant decrease in the activity of this enzyme.

5. Conclusions

C6 glioma cells, when cultured in 12 mM glucose medium, exhibited signals of oxidative and nitrosative stress similar to those observed in diabetic patients and other models of diabetic disease (decreased GSH, elevated NO, DNA damage). Interestingly, we found an increase in glutamate uptake, which might

be linked to the altered glutamatergic communication associated with DM. Moreover, glutamate uptake in C6 glioma cells, like primary astrocytes [13], was stimulated by extracellular S100B. Aminoguanidine partially prevented the glial alterations induced by 12 mM glucose medium. Together, these data emphasize the relevance of astroglia in DM, as well as the importance of glial parameters (e.g. GS) in the evaluation of diabetic disease progression and treatment.

Acknowledgements. This work was supported by the Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq), Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES**),** FINEP (IBN 01.06.0842-00) and INCT-National Institute of Science and Technology for Excitotoxicity and Neuroprotection.

Figure legends

Figure 1. *Effect of 12 mM glucose and S100B medium on basal glutamate uptake by C6 glioma cells..* (A) C6 glioma cells were cultured in 6 mM glucose or 12 mM glucose DMEM. After confluence, media were replaced with HBSS containing [^{3}H]-glutamate and incubated for 10 min. Each value is the mean ± SE of 4 independent experiments performed in triplicate. EAAT3 expression was evaluated by immunodetection. * Significantly different from basal by Student's T test ($p< 0.05$). (B) C6 glioma cells were cultured in 6 mM glucose DMEM. After confluence, cell culture media were replaced with HBSS containing exogenous S100B (from 0.01 to 0.1 ng/mL) and incubated with [^{3}H]-glutamate for 10 min. Each value is the mean ± SE of 4 independent experiments performed in triplicate. * Significantly different from basal by one-way ANOVA followed by Tukey's test ($p< 0.05$). Values of basal and 0.1 ng/mL S100B-stimulated glutamate uptake in primary astrocytes are indicated in the table.

Figure 2. *Oxidative and nitrosative stress in C6 glioma cells cultured in 12 mM glucose medium*. (A) ROS content was evaluated by DCF-DA assay. Values were measured in a fluorescence microplate reader (excitation 485 nm and emission 520 nm) and cells cultured in 6 mM glucose medium were assumed as being 100%. Each value is the mean ± SE of 4 independent experiments performed in triplicate. (B) NO levels were evaluated by nitrite content, measured by the Griess reaction. * Significantly different from basal by Student'sT test ($p< 0.05$).

Figure 3. *DNA damage in 12 mM -glucose cultured C6 cells*. The C6 cell lineage was cultured in 12 mM glucose medium or 6 mM glucose medium.The

extent of DNA damage was measured by COMET assay. (A) Total index DNA damage. (B) Index of DNA damage in each different type of damage, as described in the Material and Methods, section. Each value is the mean of three independent experiments performed in triplicate. * Significant difference from 6 mM glucose cultured cells by the Student's t test ($p<0.05$).

Figure 4. *Glutathione content and glutamine synthetase activity C6 glioma cells cultured in 12 mM glucose medium*. The C6 cell lineage was cultured in 12mM glucose medium or 6 mM glucose medium. (A) GSH was measured by a colorimetric reaction with o-phthaldialdeyde. (B) GS activity was determined by a colorimetric assay using glutamate and hydroxylamine as substrates, as described in the Methods section. Each value is the mean (± standard error) of 3 independent experiments performed in triplicate. * Significantly different from cells cultured in 6 mM glucose medium, by Student's t test ($p< 0.05$).

Figure 5. *Effect of aminoguanidine or N-acetyl-cysteine on glutamate uptake oby C6 glioma cells cultured in 12 mM glucose medium.* (A) C6 glioma cells were cultured in 6 mM or 12 mM glucose medium. After confluence, the medium was replaced by a FBS-free medium, containing 250 μM aminoguanidine or 100 μM N-acetyl-cysteine, and maintained for 24h. Afterwards, media were replaced with HBSS containing [^{3}H]-glutamate and incubated for 10 min. Each value is the mean ± SE of 4 independent experiments performed in triplicate. [a] Significantly different from respective basal glutamate uptake(6 mM glucose content) by one way ANOVA followed by Tukey's test ($p<0.05$). (B) C6 glioma cells were cultivated in 6 mM or 12 mM glucose medium in the presence of 250 μM aminoguanidine or 100 μM N-acetyl-cysteine At confluence, the media were replaced by a FBS-free medium,

containing 250 μM aminoguanidine or 100 μM N-acetyl-cysteine, and maintained for 24h. The total time of incubation was 96 h. Afterwards, media were replaced with HBSS containing [^{3}H]-glutamate and incubated for 10 min. Each value is the mean ± SE of 4 independent experiments performed in triplicate. [a] Significantly different from respective basal S100B secretion (6 mM glucose content) by one way ANOVA followed by Tukey's test ($p<0.05$).

Figure 6. *Effect of aminoguanidine or N-acetyl-cysteine on S100B secretion byC6 glioma cells cultured in 12 mM glucose medium.* (A) C6 glioma cells were cultured in 6 mM or 12 mM glucose medium. At confluence, the media were replaced by a FBS-free medium, containing 250 μM aminoguanidine or 100 μM N-acetyl-cysteine, and maintained for 24h. S100B was measured by ELISA. Each value is the mean ± SE of 4 independent experiments performed in triplicate. [a] Significantly different from respective basal S100B secretion (6 mM glucose content) by one way ANOVA followed by Tukey's test ($p<0.05$). (B) C6 glioma cells were cultured in 6 mM or 12 mM glucose medium in the presence of 250 μM AG or 100 μM NAC. At confluence, the media were replaced with a 12 mM glucose FBS-free medium, with 250 μM aminoguanidine or 100 μM N-acetyl-cysteine C, for 24h. The total time of incubation was 96 h, and S100B was measured by ELISA. Each value is the mean ± SE of 4 independent experiments performed in triplicate. [a] Significantly different from respective basal S100B secretion (6 mM glucose content) by one way ANOVA followed by Tukey's test ($p<0.05$).

References

1. Gardoni F, Kamal A, Bellone C, Biessels GJ, Ramakers GM, Cattabeni F, Gispent WH, Di Luca M (2002) Effects of streptozotocin-diabetes on the hippocampal NMDA receptor complex in rats. J Neurochem 80:438-447.
2. Kroner Z (2009) The relationship between Alzheimer's disease and diabetes: Type 3 diabetes? Altern Med Rev 14:373-379.
3. Biessels GJ, Gispen WH (2005) The impact of diabetes on cognition: what can be learned from rodent models? Neurobiol Aging 26 Suppl 1:36-41.
4. Dringen R, Hirrlinger J (2003) Glutathione pathways in the brain. Biol Chem 384:505-516.
5. Danbolt NC (2001) Glutamate uptake. Prog Neurobiol 65:1-105.
6. Donato R, Sorci G, Riuzzi F, Arcuri C, Bianchi R, Brozzi F, Tubaro C, Giambanco I (2009) S100B's double life: intracellular regulator and extracellular signal. Biochim Biophys Acta 1793:1008-1022.
7. 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.
8. Davey GE, Murmann P, Heizmann CW (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.
9. Castets F, Griffin WS, Marks A, Van Eldik LJ (1997) Transcriptional regulation of the human S100 beta gene. Brain Res Mol Brain Res 46:208-216.
10. Tabernero A, Medina JM, Giaume C (2006) Glucose metabolism and proliferation in glia: role of astrocytic gap junctions. J Neurochem 99:1049-1061.
11. Haghighat N, McCandless DW (1997) Effect of ammonium chloride on energy metabolism of astrocytes and C6-glioma cells in vitro. Metab Brain Dis 12:287-298.
12. Nardin P, Tramontina F, Leite MC, Tramontina AC, Quincozes-Santos A, de Almeida LM, Battastini AM, Gottfried C, Goncalves CA (2007) S100B content and secretion decrease in astrocytes cultured in high-glucose medium. Neurochem Int 50:774-782.
13. Tramontina F, Tramontina AC, Souza DF, Leite MC, Gottfried C, Souza DO, Wofchuk ST, Goncalves CA (2006) Glutamate uptake is stimulated by extracellular S100B in hippocampal astrocytes. Cell Mol Neurobiol 26:81-86.
14. Miralles VJ, Martinez-Lopez I, Zaragoza R, Borras E, Garcia C, Pallardo FV, Vina JR (2001) Na+ dependent glutamate transporters (EAAT1, EAAT2, and EAAT3) in primary astrocyte cultures: effect of oxidative stress. Brain Res 922:21-29.
15. Matos M, Augusto E, Oliveira CR, Agostinho P (2008) Amyloid-beta peptide decreases glutamate uptake in cultured astrocytes: involvement of oxidative stress and mitogen-activated protein kinase cascades. Neuroscience 156:898-910.

16. Hansson E, Muyderman H, Leonova J, Allansson L, Sinclair J, Blomstrand F, Thorlin T, Nilsson M, Ronnback L (2000) Astroglia and glutamate in physiology and pathology: aspects on glutamate transport, glutamate-induced cell swelling and gap-junction communication. Neurochem Int 37:317-329.
17. Ward MM, Jobling AI, Kalloniatis M, Fletcher EL (2005) Glutamate uptake in retinal glial cells during diabetes. Diabetologia 48:351-360.
18. Duarte AI, Santos MS, Seica R, Oliveira CR (2004) Oxidative stress affects synaptosomal gamma-aminobutyric acid and glutamate transport in diabetic rats: the role of insulin. Diabetes 53:2110-2116.
19. Coleman ES, Dennis JC, Braden TD, Judd RL, Posner P Insulin treatment prevents diabetes-induced alterations in astrocyte glutamate uptake and GFAP content in rats at 4 and 8 weeks of diabetes duration. Brain Res 1306:131-141.
20. dos Santos AQ, Nardin P, Funchal C, de Almeida LM, Jacques-Silva MC, Wofchuk ST, Goncalves CA, Gottfried C (2006) Resveratrol increases glutamate uptake and glutamine synthetase activity in C6 glioma cells. Arch Biochem Biophys 453:161-167.
21. Abib RT, Quincozes-Santos A, Nardin P, Wofchuk ST, Perry ML, Goncalves CA, Gottfried C (2008) Epicatechin gallate increases glutamate uptake and S100B secretion in C6 cell lineage. Mol Cell Biochem 310:153-158.
22. de Almeida LM, Pineiro CC, Leite MC, Brolese G, Tramontina F, Feoli AM, Gottfried C, Goncalves CA (2007) Resveratrol increases glutamate uptake, glutathione content, and S100B secretion in cortical astrocyte cultures. Cell Mol Neurobiol 27:661-668.
23. Palos TP, Ramachandran B, Boado R, Howard BD (1996) Rat C6 and human astrocytic tumor cells express a neuronal type of glutamate transporter. Brain Res Mol Brain Res 37:297-303.
24. Gottfried C, Tramontina F, Goncalves D, Goncalves CA, Moriguchi E, Dias RD, Wofchuk ST, Souza DO (2002) Glutamate uptake in cultured astrocytes depends on age: a study about the effect of guanosine and the sensitivity to oxidative stress induced by H(2)O(2). Mech Ageing Dev 123:1333-1340.
25. 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.
26. Browne RW, Armstrong D (1998) Reduced glutathione and glutathione disulfide. Methods Mol Biol 108:347-352.
27. Hu J, Castets F, Guevara JL, Van Eldik LJ (1996) S100 beta stimulates inducible nitric oxide synthase activity and mRNA levels in rat cortical astrocytes. J Biol Chem 271:2543-2547.
28. Singh NP, McCoy MT, Tice RR, Schneider EL (1988) A simple technique for quantitation of low levels of DNA damage in individual cells. Exp Cell Res 175:184-191.
29. Hartmann A, Agurell E, Beevers C, Brendler-Schwaab S, Burlinson B, Clay P, Collins A, Smith A, Speit G, Thybaud V, Tice RR (2003) Recommendations for conducting the in vivo alkaline Comet assay. 4th International Comet Assay Workshop. Mutagenesis 18:45-51.

30. Quincozes-Santos A, Andreazza AC, Nardin P, Funchal C, Goncalves CA, Gottfried C (2007) Resveratrol attenuates oxidative-induced DNA damage in C6 Glioma cells. Neurotoxicology 28:886-891.
31. Lowry OH, Rosebrough NJ, Farr AL, Randall RJ (1951) Protein measurement with the Folin phenol reagent. J Biol Chem 193:265-275.
32. Bauduceau B, Bourdel-Marchasson I, Brocker P, Taillia H (2005) The brain of the elderly diabetic patient. Diabetes Metab 31 Spec No 2:5S92-95S97.
33. Gispen WH, Biessels GJ (2000) Cognition and synaptic plasticity in diabetes mellitus. Trends Neurosci 23:542-549.
34. Biessels GJ, Deary IJ, Ryan CM (2008) Cognition and diabetes: a lifespan perspective. Lancet Neurol 7:184-190.
35. Sango K, Saito H, Takano M, Tokashiki A, Inoue S, Horie H (2006) Cultured adult animal neurons and schwann cells give us new insights into diabetic neuropathy. Curr Diabetes Rev 2:169-183.
36. Coleman E, Judd R, Hoe L, Dennis J, Posner P (2004) Effects of diabetes mellitus on astrocyte GFAP and glutamate transporters in the CNS. Glia 48:166-178.
37. Tan AL, Forbes JM, Cooper ME (2007) AGE, RAGE, and ROS in diabetic nephropathy. Semin Nephrol 27:130-143.
38. Wilkinson-Berka JL, Kelly DJ, Koerner SM, Jaworski K, Davis B, Thallas V, Cooper ME (2002) ALT-946 and aminoguanidine, inhibitors of advanced glycation, improve severe nephropathy in the diabetic transgenic (mREN-2)27 rat. Diabetes 51:3283-3289.
39. Cockroft KM MMr, Zimmerman GA, Risucci D, Bloom O, Cerami A, Tracey KJ (1996) Cerebroprotective effects of aminoguanidine in a rodent model of stroke. Stroke 27:6.
40. Schimchowitsch S CJ (2006) Polyamine and aminoguanidine treatments to promote structural and functional recovery in the adult mammalian brain after injury: a brief literature review and preliminary data about their combined administration. Journal of physiology, Paris 99:11.
41. Kamboj SS CK, Sandhir R. (2008) Neuroprotective effect of N-acetylcysteine in the development of diabetic encephalopathy in streptozotocin-induced diabetes. Metabolic Brain Disease 23:16.
42. Goodarzi MT, Navidi AA, Rezaei M, Babahmadi-Rezaei H Oxidative damage to DNA and lipids: correlation with protein glycation in patients with type 1 diabetes. J Clin Lab Anal 24:72-76.
43. Powell LA, Nally SM, McMaster D, Catherwood MA, Trimble ER (2001) Restoration of glutathione levels in vascular smooth muscle cells exposed to high glucose conditions. Free Radic Biol Med 31:1149-1155.
44. Brownlee M (2005) The pathobiology of diabetic complications: a unifying mechanism. Diabetes 54:1615-1625.
45. 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.
46. Buyukuysal RL (2005) Protein S100B release from rat brain slices during and after ischemia: comparison with lactate dehydrogenase leakage. Neurochem Int 47:580-588.

47. Hu J, Ferreira A, Van Eldik LJ (1997) S100beta induces neuronal cell death through nitric oxide release from astrocytes. J Neurochem 69:2294-2301.
48. Allen DA, Yaqoob MM, Harwood SM (2005) Mechanisms of high glucose-induced apoptosis and its relationship to diabetic complications. J Nutr Biochem 16:705-713.
49. Kosenko E, Llansola M, Montoliu C, Monfort P, Rodrigo R, Hernandez-Viadel M, Erceg S, Sanchez-Perez AM, Felipo V (2003) Glutamine synthetase activity and glutamine content in brain: modulation by NMDA receptors and nitric oxide. Neurochem Int 43:493-499.

Figure1

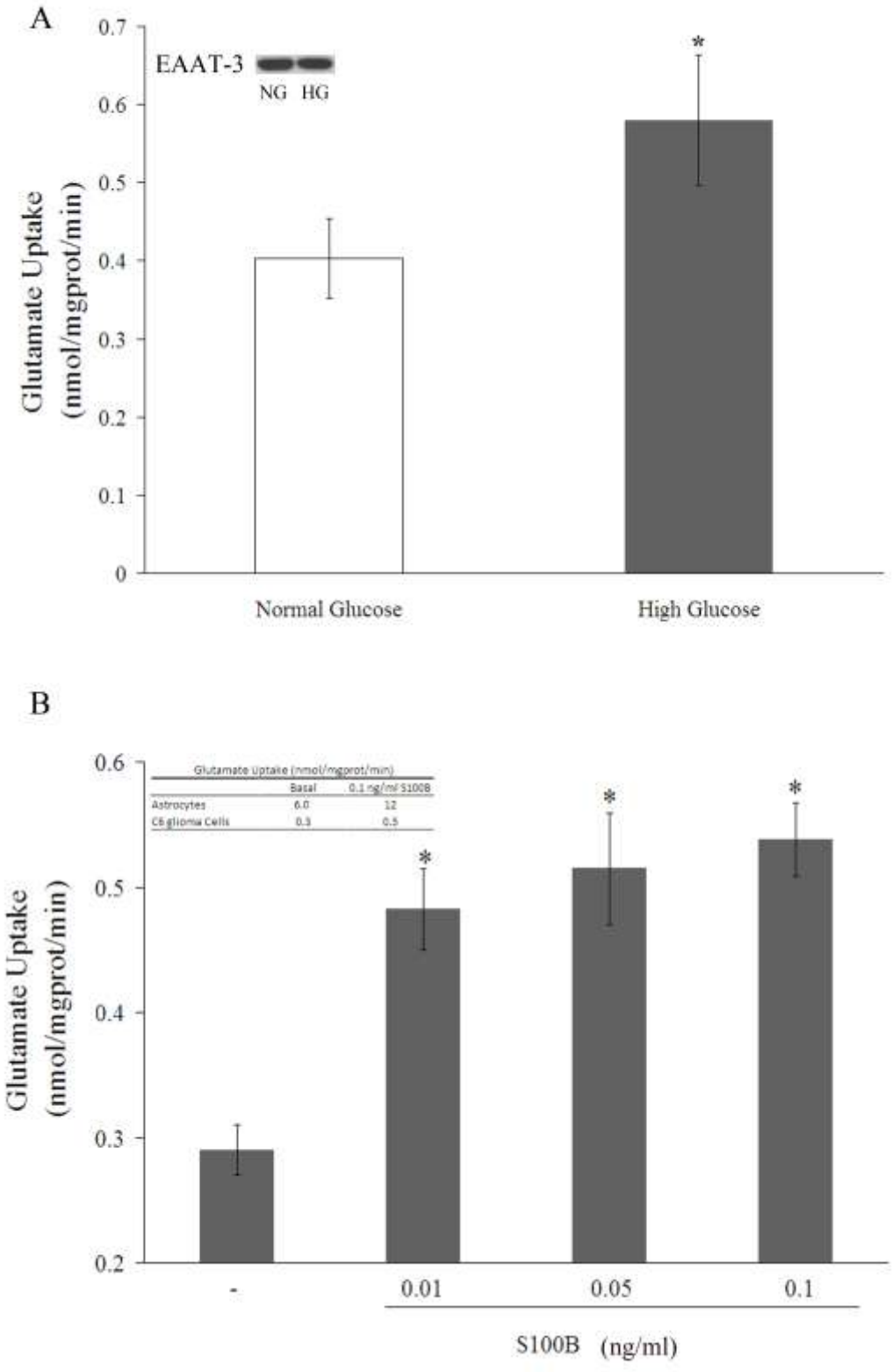

A
EAAT-3
NG HG
Glutamate Uptake (nmol/mgprot/min)
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
*
Normal Glucose
High Glucose
B
Glutamate Uptake (nmol/mgprot/min)
Basal
0.1 ng/ml S100B
Astrocytes
6.0
12
C6 glioma Cells
0.3
0.5
Glutamate Uptake (nmol/mgprot/min)
0.6
0.5
0.4
0.3
0.2
*
*
*
-
0.01
0.05
0.1
S100B (ng/ml)

Figure 2

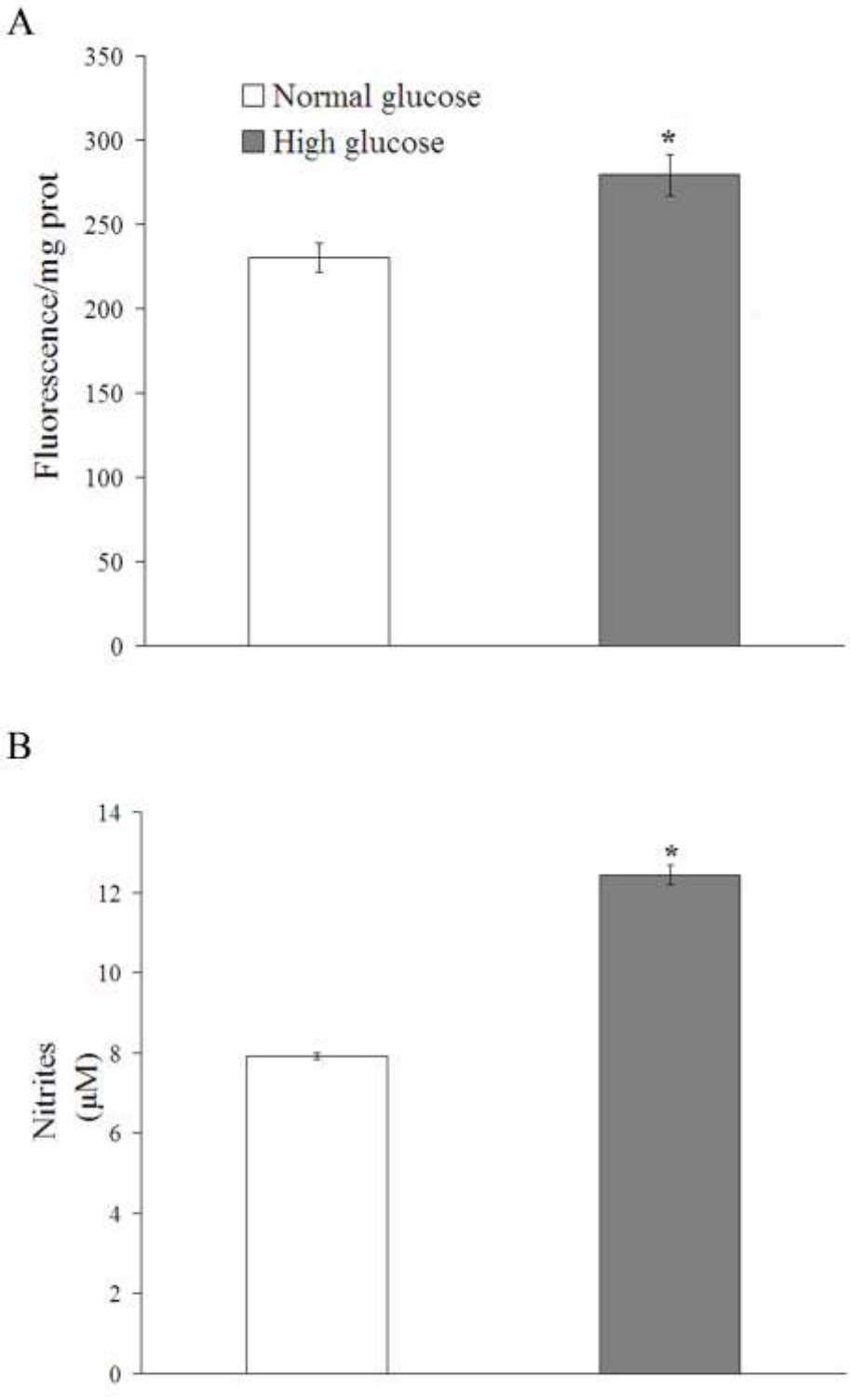

A
Normal glucose
High glucose
Fluorescence/mg prot
350
300
250
200
150
100
50
0
*
B
Nitrites (µM)
14
12
10
8
6
4
2
0
*

Figure 3

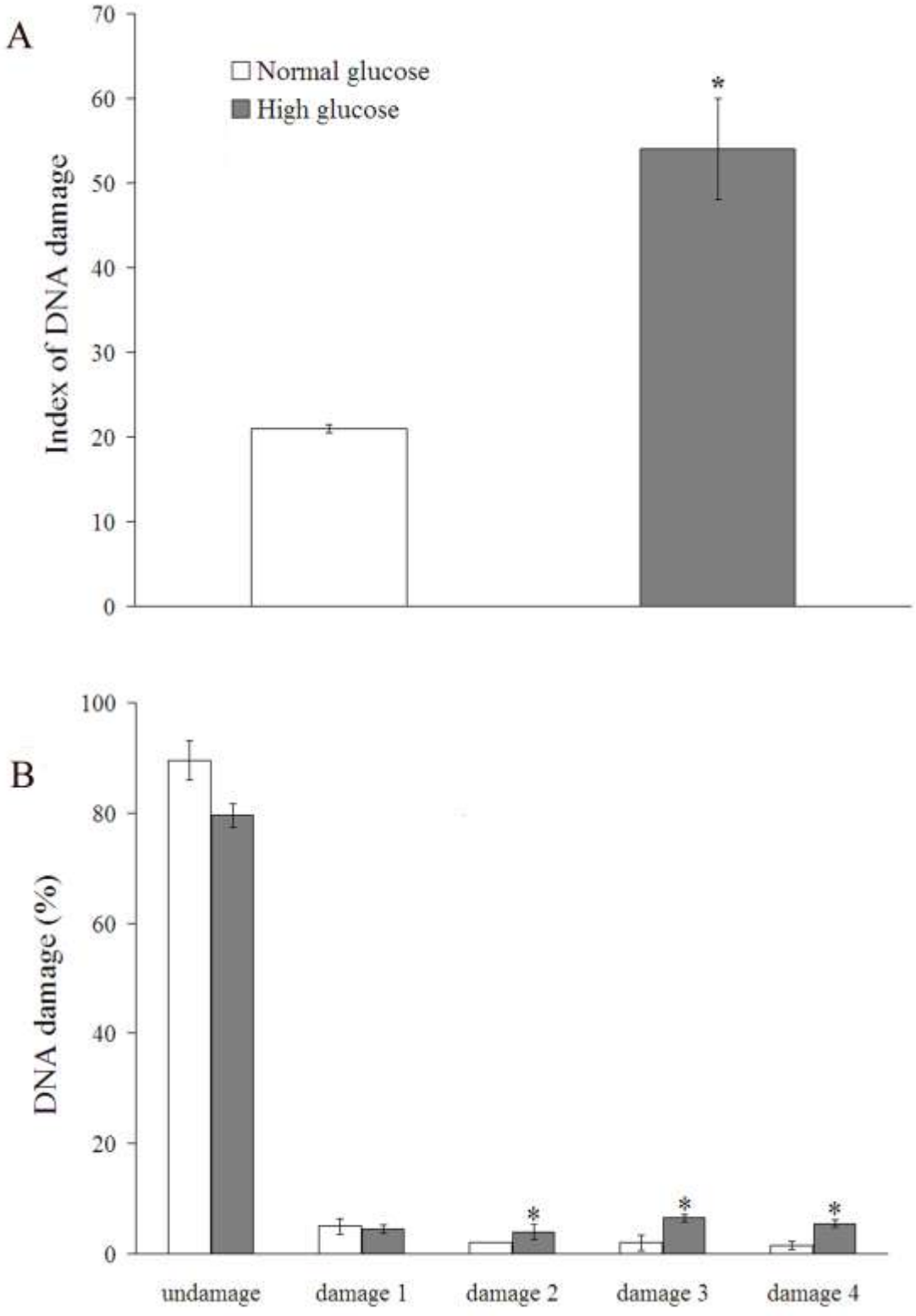

A
Normal glucose
High glucose
Index of DNA damage
70
60
50
40
30
20
10
0
*
B
DNA damage (%)
100
80
60
40
20
0
*
*
*
undamage
damage 1
damage 2
damage 3
damage 4

Figure 4

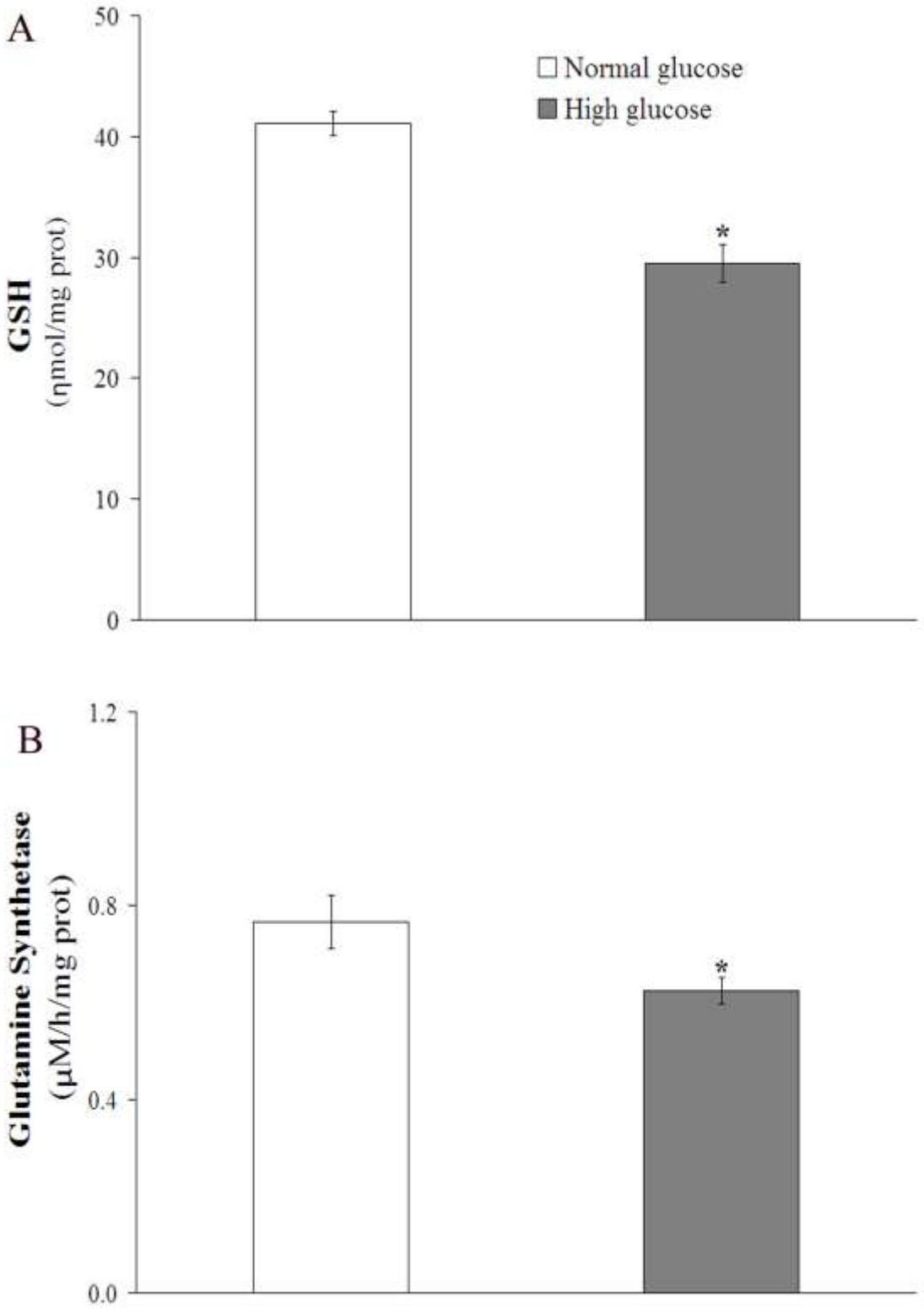

A
50
40
30
20
10
0
GSH
(ηmol/mg prot)
Normal glucose
High glucose
*
B
1.2
0.8
0.4
0.0
Glutamine Synthetase
(μM/h/mg prot)
*

Figure 5

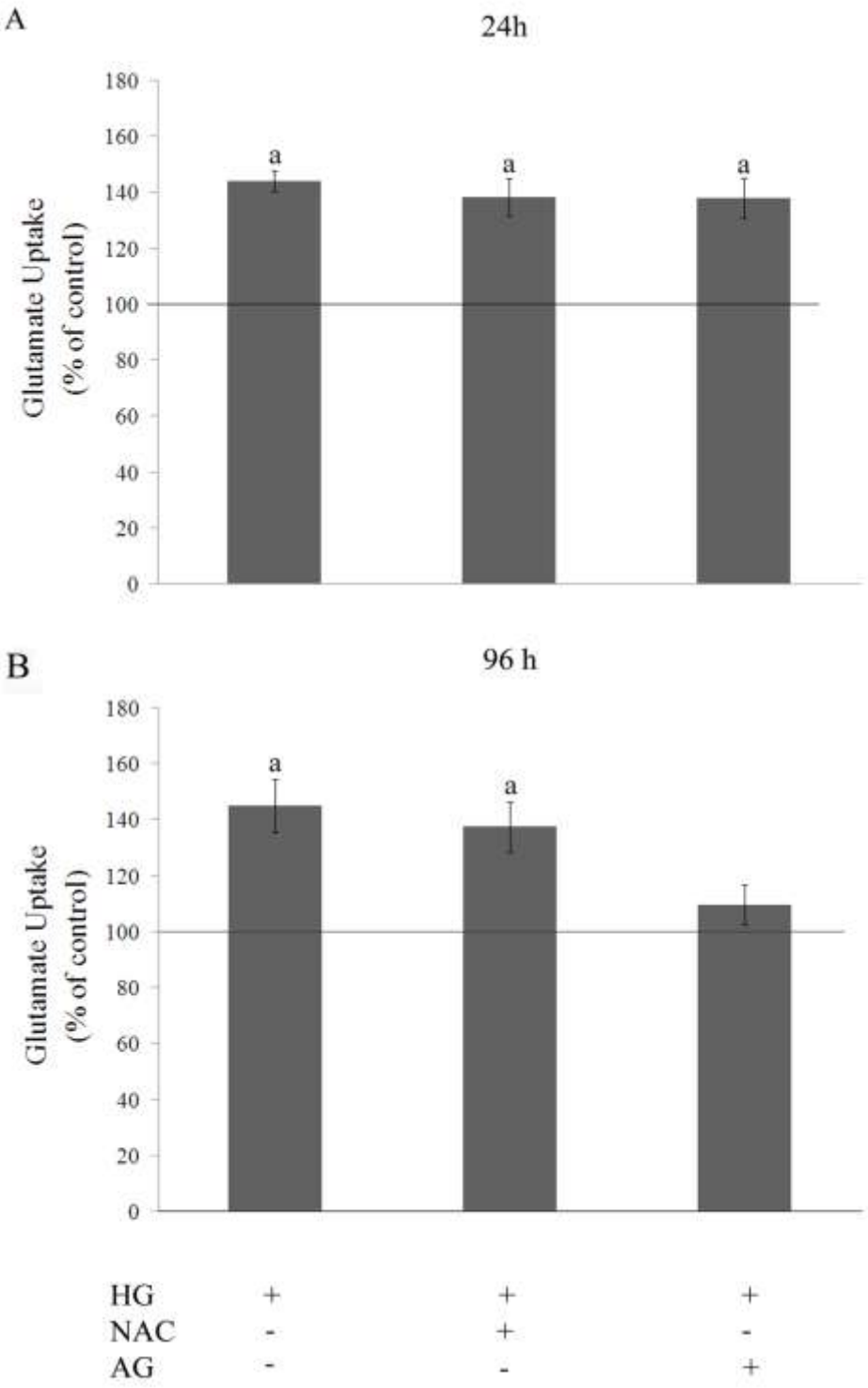
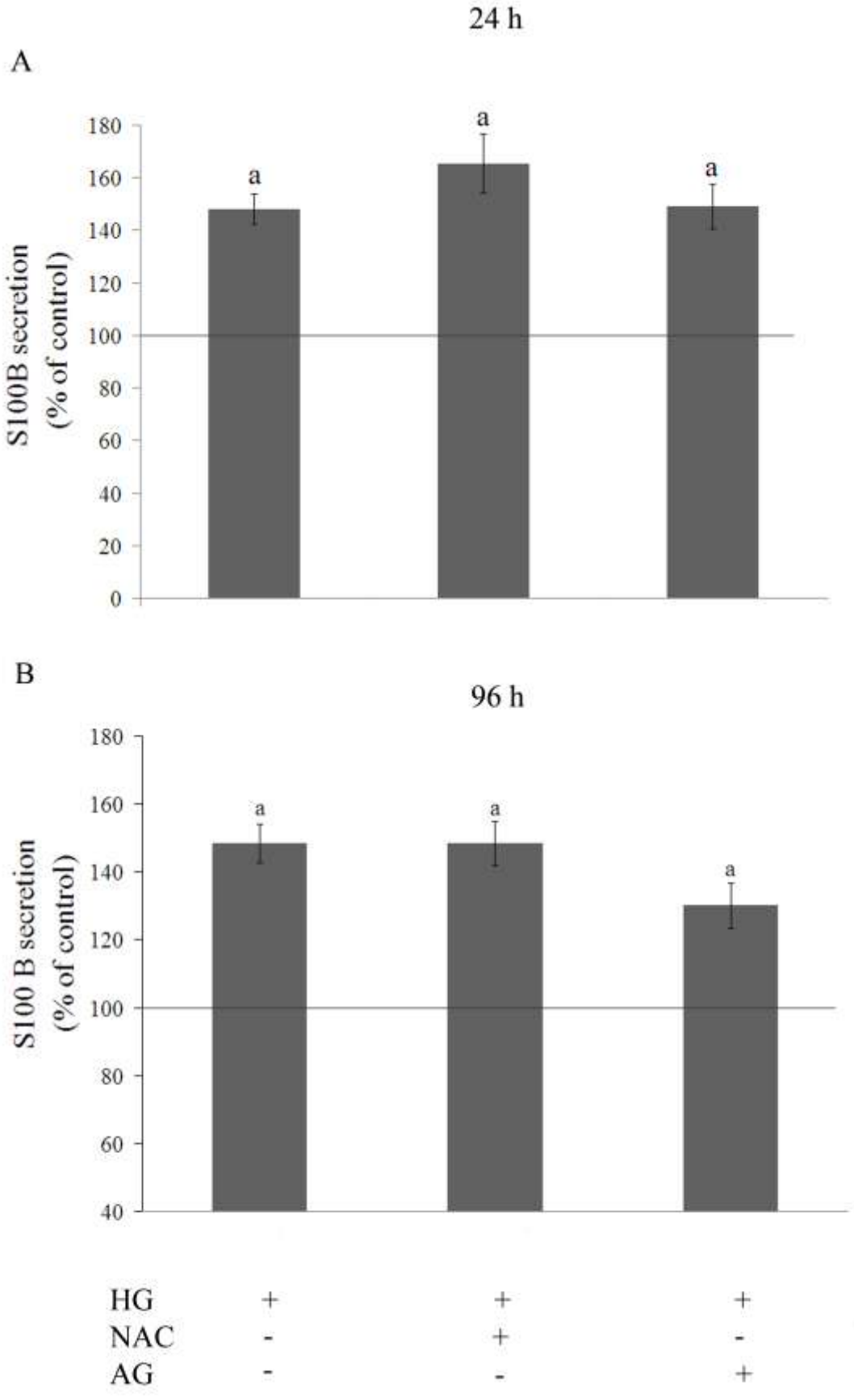

A
24h
Glutamate Uptake
(% of control)
180
160
140
120
100
80
60
40
20
0
a
a
a
B
96 h
Glutamate Uptake
(% of control)
180
160
140
120
100
80
60
40
20
0
a
a
HG + + +
NAC - + -
AG - - +

Figure 6


24 h
A
S100B secretion
(% of control)
180
160
140
120
100
80
60
40
20
0
a
a
a
B
96 h
S100 B secretion
(% of control)
180
160
140
120
100
80
60
40
a
a
a
HG + + +
NAC - + -
AG - - +

CAPÍTULO II

The neuroprotective effect of two statins: simvastatin and pravastatin on a streptozotocin-induced model of Alzheimer's disease in rats

Artigo publicado no periódico Journal of Neural Transmission

DOI : 10.1007/s00702-011-0680-z

The neuroprotective effect of two statins: simvastatin and pravastatin on a streptozotocin-induced model of Alzheimer's disease in rats

Ana Carolina Tramontina[1], Krista Minéia Wartchow[1], Letícia Rodrigues[1], Regina Biasibetti[1], André Quincozes-Santos[1], Larissa Bobermin[1], Francine Tramontina[2], Carlos-Alberto Gonçalves[1,*]

[1] Departamento de Bioquímica, Instituto de Ciências Básicas da Saúde, Universidade Federal do Rio Grande do Sul, Porto Alegre, Brazil; [2] Universidade Estadual do Rio Grande do Sul, Bento Gonçalves, Brazil

* Corresponding author: Carlos-Alberto Gonçalves

Depto Bioquímica, ICBS, UFRGS

Ramiro Barcelos, 2600-anexo

Porto Alegre, RS, Brazil

90035-003

Fax: 55-51-3308 5535

E-mail: casg@ufrgs.br

Abstract

Astrocytes play a fundamental role in glutamate metabolism by regulating the extracellular levels of glutamate and intracellular levels of glutamine. They also participate in antioxidant defenses, due to the synthesis of glutathione, coupled to glutamate metabolism. Although the cause of Alzheimer's disease (AD) remains elusive, some changes in neurochemical parameters, such as glutamate uptake, glutamine synthetase activity and glutathione have been investigated in this disease. A possible neuroprotective effect of two statins, simvastatin and pravastatin (administered p.o.), was evaluated using a model of dementia, based on the intracerebroventricular (ICV) administration of streptozotocin (STZ), and astrocyte parameters were determined. We confirmed a cognitive deficit in rats submitted to ICV-STZ, and a prevention of this deficit by statin administration. Moreover, both statins were able to prevent the decrease in glutathione content and glutamine synthetase activity in this model of AD. Interestingly, simvastatin increased *per se* glutamate uptake activity, while both statins increased glutamine synthetase activity *per se*. These results support the idea that these drugs could be effective for the prevention of alterations observed in the STZ dementia model, and may contribute to reduce the cognitive impairment and brain damage observed in AD patients.

Key words: astrocyte, glutamate, dementia, statins, streptozotocin.

Introduction

Alzheimer's disease (AD) is the most common form of dementia and affects nearly 35% of the population of over 85 years (Stefani and Liguri 2009). Dementia is characterized by a decline in cognitive and social functions and severe memory loss (M. Weinstock 2004; Weinstock and Shoham 2004). The histopathological marks of AD are the formation of senile plaques, caused by the extracellular accumulation of amyloid fibrils in the brain, and also by the intraneuronal aggregates of neurofibrillary tangles, which leads to progressive brain dysfunction (Silvestrelli et al. 2006).

Astrocytes are closely linked to neurons, and play an active role in the development of AD (Maccioni et al. 2001). In fact, activated astrocytes are found in association with senile plaques (Mrak and Griffinbc 2001). Physiologically, astrocytes play a fundamental role in glutamate metabolism by regulating the extracellular levels of glutamate and intracellular levels of glutamine (Danbolt 2001). Coupled to this function, astrocytes are also responsible for the synthesis and secretion of glutathione (GSH), the main non-enzymatic antioxidant defense in the central nervous system (Dringen and Hirrlinger 2003).

The exact cause of AD remains elusive. However, several risk factors seem to be involved in the development of this condition. Type II diabetes mellitus, the apolipoprotein ε4 allele and high levels of plasma cholesterol are some of these factors (Candore et al. 2010; Pasquier et al. 2006). The mechanism by which cholesterol is involved in the development of AD is still unclear, but, indeed accumulation of cholesterol has been found in association

with senile plaques and in transgenic models of AD (Mori et al. 2001). Taking into account the contribution of cholesterol to the development of AD, a range of studies suggest that statins, a class of lipid-lowering drugs, can confer protection against this dementia (Hoglund and Blennow 2007; Jick et al. 2000; Wolozin et al. 2007)

Statins are a class of cholesterol-lowering drugs that act by inhibiting the rate-limiting enzyme in cholesterol biosynthesis, 3-hydroxy-3-methylglutaryl coenzyme A (Zipp et al. 2007). In addition to this effect, statins also reduce the formation of isoprenoid intermediates in the cholesterol pathway (Liao and Laufs 2005), which can confer anti-inflammatory (Li et al. 2009; Yoshida 2003) and immunomodulatory properties to statins (Adamson and Greenwood 2003; Kuipers and van den Elsen 2007). The action of statins in cardiovascular disease is well known (Schonbeck and Libby 2004), however their neuroprotective effect demands further characterization. We chose two statins, based on their difference abilities to cross the blood-brain-barrier and potential neuroprotector activities (Ramirez et al. 2011; Sierra et al. 2010).

There are a few experimental models to study AD, but the intracerebroventricular (ICV) streptozotocin (STZ) model demonstrates metabolic changes that are very similar to those found in the sporadic form of AD. In this model, deficits in learning, memory and cognitive behavior are reported (Lannert and Hoyer 1998). In addition, increased oxidative damage (Sharma and Gupta 2001; Sharma and Gupta 2002), alterations in glucose utilization and neuronal damage have been described (Grunblatt et al. 2007; Weinstock and Shoham 2004).

The aim of this study was to evaluate the spatial cognitive deficit in animals exposed to ICV-STZ, and hippocampal changes in glutamate uptake, GSH content and the activity of glutamine synthetase (GS), as well as to evaluate a possible neuroprotective effect of two statins: simvastatin and pravastatin in this model of dementia.

Material and methods

Chemicals

Streptozotocin, simvastatin, pravastatin, albumin, standard GSH, γ-glutamylhydroxamate were purchased from Sigma. L-[H^3]-glutamate were purchased from AmershamInternational (UK) and HBSS from Gibco. All other chemicals were purchased from local commercial suppliers.

Animals

Forty-two male Wistar rats (90 days old, weighing 250–320 g) were obtained from our breeding colony (at the Department of Biochemistry, Universidade Federal do Rio Grande do Sul), and were maintained under controlled light and environmental conditions (12 hour light/12 hour dark cycle at a constant temperature of 22 ± 1◦C) with free access to food and water. All animal experiments were carried out in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals (NIH Publications No. 80-23) revised 1996, and following the regulations of the local animal house authorities.

Experimental groups

Rats were divided into 6 groups of 7 rats: sham, sham-simvastatin, sham-pravastatin, STZ, STZ-simvastatin, and STZ-pravastatin. (a) Sham group: the animals received ICV injection of STZ vehicle (HBSS). On day two after the

surgery and every two days they received the statin vehicle, p.o.; (b) Sham-simvastatin group: the animals received an ICV injection of STZ vehicle (HBSS, detailed further). On day two after the surgery, and every two days, they received simvastatin (5 mg/kg) p.o.; (c) Sham-pravastatin group: Sham-simvastatin group: the animals received an ICV injection of the STZ vehicle (HBSS). On day two after the surgery and every two days they received pravastatin (5 mg/kg) p.o.; (d) STZ group: the animals received an ICV injection of STZ (3mg/kg). On day two after the surgery and each two days they received the statins vehicle, p.o.; (e) STZ-simvastatin group: the animals received an ICV injection of STZ (3 mg/kg). On day two after the surgery and every two days they received simvastatin (5 mg/kg) p.o.; and (f) STZ-pravastatin group: the animals received an ICV injection of STZ (3 mg/kg). On day two after the surgery and every two days they received pravastatin (5 mg/kg) p.o.

Surgical procedure for ICV administration of STZ

STZ was ICV infused, as previously described (Rodrigues et al. 2009; Sharma and Gupta 2001). Briefly, on the day of the surgery, animals were anesthetized with ketamine/xylazine (75 and 10 mg/Kg, respectively, i.p.) and placed in a stereotaxic apparatus. A midline sagittal incision was made in the scalp. Burr holes were drilled in the skull on both sides over the lateral ventricles. The lateral ventricles were accessed using the following coordinates (Paxinos 1997): 0.9 mm posterior to bregma; 1.5 mm lateral to sagittal suture; 3.6mm beneath the surface of the brain. Rats received a single bilateral infusion of 5 μL STZ (3 mg/Kg) or vehicle (Hank's balanced salt solution – HBSS – containing in mM: 137 NaCl; 0.63 Na_2HPO_4; 4.17 $NaHCO_3$; 5.36 KCl; 0.44 KH_2PO_4; 1.26 $CaCl_2$; 0.41 $MgSO_4$; 0.49 $MgCl_2$ and 10 glucose, in pH 7.4) using

a 10 μL Hamilton syringe. After the surgical procedure, rats were placed on a heating pad to maintain body temperature at 37.5 ± 0.5◦C and were kept there until recovery from anesthesia. The animals were submitted to evaluation and biochemical analysis 3 weeks after SZT-injection.

Statins administration

Simvastatin and pravastatin were dissolved in 50% ethanol, and administered per gavage. Rats received 0.1 mL, every two days, of simvastatin (5mg/kg), pravastatin (5mg/kg) or vehicle. The treatment lasted four weeks (three weeks after the surgical procedure and one week during the cognitive evaluation).

Cognitive evaluation

Three weeks after surgery, rats were submitted to training in the Morris water maze (Morris 1984; Silva et al. 2005). The apparatus consisted of a circular pool (180 cm diameter, 60 cm high) filled with water (depth 30 cm; 24± 1◦C), placed in a room with consistently located spatial cues. An escape platform (10 cm diameter) was placed in the middle of one of the quadrants, 1.5 cm below the water surface, equidistant from the sidewall and the middle of the pool. The platform provided the only escape from the water and was located in the same quadrant every trial. Four different starting positions were equally spaced around the perimeter of the pool. On each training day, all four start positions were used once in a random sequence, i.e., four training trials per day. A trial began by placing the animal in the water, facing the wall of the pool at one of the starting points. If the animal failed to escape within 60 s it was gently conducted to the platform by the experimenter. The rat was allowed to stay there for 20 s. The inter-trial interval was 10 min. After each trial, the rats

were dried, and returned to their cages at the end of the session. Animals were trained for 5 days. 24 h after the last training session, the rats were submitted to a test session (four weeks after surgery). Before this session, the submerged platform was removed. The retention test consisted of placing the animals in the water for 1 min. The number of crossings over the original position of the platform and time spent in the target quadrant compared to the opposite quadrant were measured. After cognitive evaluation, rats were anaesthetized, as subsequently described, for CSF obtaining and brain slice preparation.

Serum biochemical measurements

Animals were anesthetized as described above and blood was obtained by cardiac puncture. Serum contents of cholesterol, triglycerides, glucose, glutamic oxalacetic transaminase (GOT) and glutamic pyruvic transaminase (GPT) levels were analyzed using commercial kits (Human do Brasil, Itabira, Brazil).

Hippocampal tissue samples

Anesthetized animals were killed by decapitation, and the brains were removed and placed in cold 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 homogenized for measurement of glutamine synthetase activity and glutathione content or cut into transverse slices of 0.3 mm using a McIlwain tissue chopper. Slices were transferred immediately to 24-well culture plates, each well containing 0.3 mL of HBSS for measuring glutamate uptake.

Glutamine synthetase activity

The enzymatic assay was performed as previously described (dos Santos et al. 2006). Briefly, hipocampal homogenates (0.1 mL) were added to 0.1 mL of reaction mixture containing (in mM): 10 $MgCl_2$; 50 L-glutamate; 100 imidazole-HCl buffer (pH 7.4); 10 2-mercaptoethanol; 50 hydroxylamine-HCl; 10 ATP and incubated for 15 min at 37ºC. The reaction was stopped by the addition of 0.4 mL of a solution containing (in mM): 370 ferric chloride; 670 HCl; 200 trichloroacetic acid. After centrifugation, the supernatant was measured at 530 nm and compared to the absorbance generated by standard quantities of γ-glutamylhydroxamate treated with ferric chloride reagent.

Glutathione content Assay

GSH levels (nmol/mg protein) were measured as described previously (Browne and Armstrong 1998). Hipocampal homogenates were diluted in 10 volumes of 100 mM sodium phosphate buffer, pH 8.0, containing 5 mM EDTA and protein was precipitated with 1.7% meta-phosphoric acid. Supernatant was assayed with *o*-phthaldialdeyde (1mg/mL methanol) at room temperature for 15 min. Fluorescence was measured using excitation and emission wavelengths of 350 and 420 nm, respectively. A calibration curve was performed with standard GSH solutions (0-500 µM).

Glutamate uptake assay

Glutamate uptake was performed, as previously described (Gottfried et al. 2002) with some modifications (Thomazi et al. 2004). Briefly, hipocampal slices were transferred to 24-well plates and incubated for 23min at 37°C in a Hank's balanced salt solution (HBSS) containing (in mM): 137 NaCl, 5.36 KCl, 1.26 $CaCl_2$, 0.41 $MgSO_4$, 0.49 $MgCl_2$, 0.63 $Na_2HPO_4.7H_2O$, 0.44 KH_2PO_4, 4.17 $NaHCO_3$ and 5.6 glucose, adjusted to pH 7.4. The assay was started by the

addition of 0.1 mM L-glutamate and 0.33 µCi/ml L-[2,3-^{3}H] glutamate. Incubation was stopped after 5 min by removal of the medium and rinsing the cells twice with ice-cold HBSS. The slices were then lysed in a solution containing 0.5 M NaOH. Radioactivity was measured in a scintillation counter. Sodium-independent uptake was determined using N-methyl-D-glucamine instead of NaCl. Sodium-dependent glutamate uptake was obtained by subtracting the non-specific uptake of the total uptake to obtain the specific uptake.

Protein determination

Protein content was measured by Lowry's method using bovine serum albumin as standard (Lowry et al. 1951).

Statistical analysis

Parametric data from the experiments are presented as means±standard error and statistically evaluated by two-way analysis of variance, followed by the Tukey's test, assuming $p < 0.05$. The escape latency parameter in the water maze task was evaluated by repeated measures analysis of variance, assuming $p < 0.05$.

Results

Cognitive evaluation

The Morris water maze was used to evaluate reference memory in the six groups: sham, sham-simvastatin, sham-pravastatin, STZ, STZ-simvastatin and STZ-pravastatin. In the training sessions, from day 1 onwards, there was a significant increase in the average time to find the platform in the STZ group (escape latency), when compared to the sham group (Fig. 1A) ($F_{(5-45)} = 1.497$, *p*

< *0.05*). In the trial session, STZ rats (without statins) took more time to find the platform ($F_{(5-45)}$= 3.836, $p < 0.05$), when compared to the other groups, including the STZ + statins group (Fig. 1B). The number of crossings over the platform location was lower in the STZ group, compared to the other groups (Fig. 1C) ($F_{(5-45)} = 4.256$, $p < 0.05$). Moreover, the STZ group (without statins) also spent less time in the target quadrant than all the other groups (Fig.1D), ($F_{(5, 45)} = 2.464$, $p < 0.05$). No differences were observed in the time spent in the opposite quadrant ($F(5,45) = 1.211$, $p > 0.05$). Note that simvastatin and pravastatin "per se" had no effect on the cognitive task.

Glutamate uptake

The treatment with STZ did not affect the glutamate uptake activity in the hippocampus. However, simvastatin *per se* increased glutamate uptake (about 30%) ($F_{(5,32)} = 2.364$, $p < 0.05$) when compared to Sham group (Fig. 2). However, the simvastatin group did not differ from the STZ groups. Pravastatin *per se* did not alter glutamate uptake

Glutathione (GSH) content

In order to evaluate a possible development of oxidative stress, a measurement of reduced GSH was performed. GSH content was reduced in the STZ-treated animals by about 30% when compared to the sham groups (with and without statins) ($F_{(5,36)} = 2.702$, $p < 0.05$) (Fig. 3). However, administration of simvastatin and pravastatin was able to prevent the effect of STZ on GSH content.

Glutamine synthetase (GS) activity

Hippocampal GS activity was reduced in the STZ-treated group, and both pravastatin and simvastatin were able to prevent this decrease ($F_{(5,36)}$ = 13.218, $p < 0.05$) (Fig. 4). Interestingly, simvastatin and pravastatin "per se" induced an increase in GS activity.

Serum biochemical parameters

In order to evaluate the effect of ICV-STZ on serum glucose levels, and the effect of statins on the serum lipidic profile we carried out biochemical assays. STZ injection did not alter the serum glucose levels, triglycerides and total cholesterol levels. Moreover, statin administration had no effect on the lipid profile. We also measured serum transaminases to evaluate possible hepatic damage, induced by statins. No effect was observed (Table 1).

Discussion

The STZ model of dementia has been widely used, and the injection of this drug causes increased oxidative stress and alterations in glucose metabolism, accompanied by progressive deficits in cognition and behavior (Lannert and Hoyer 1998; Terwel et al. 1995). Our data showed a reduction in GSH content and GS activity with no alterations in glutamate uptake in animals that received the ICV-STZ injection, in agreement with previous results from our group (Rodrigues et al. 2009).

A variety of compounds have shown neuroprotective activities, including resveratrol (Sharma and Gupta 2002), aminoguanidine (Rodrigues et al. 2009) and tacrine and donepezil (Saxena et al. 2008), in the dementia model induced

by ICV injection of STZ. Many studies have shown that statins, a class of drugs that act by the inhibition of cholesterol synthesis, can exert a neuroprotective role in the central nervous system (Sharma et al. 2008; Vaughan and Delanty 1999). In our study, we observed that simvastatin and pravastatin were able to reverse some effects of STZ, without reducing the plasma cholesterol levels in STZ or control rats.

Although these statins have discrete structural differences, they exhibit different activities in diverse parameters analyzed *in vitro* and *in vivo* (Sierra et al. 2010). Simvastatin is more lipophilic and potentially crosses the blood-brain-barrier more easily than pravastatin (Saheki et al. 1994; Sierra et al. 2010). However, pravastatin inhibits HMG-CoA redutase more effectively in neural cultures (Sierra et al. 2010) and brain tissue (Kirsch et al. 2003). Importantly, despite these differences, both statins used in this study exhibited beneficial effects on cognitive and neurochemical parameters.

Despite the fact that high levels of plasma cholesterol are possibly involved in the pathogenesis of AD (Canevari and Clark 2007), and the presence of cholesterol in amyloid plaques in the APP transgenic AD model in mice (Mori et al. 2001). However, no changes have been observed in total brain cholesterol in APP transgenic mice (Kurata et al. 2011). Moreover, there are no reports regarding the brain content of cholesterol in the STZ model of AD.

We did not find any changes in peripheral cholesterol in ICV-STZ treated rats, that received, or not, statins. In agreement with this finding, atorvastatin (30 mg/kg) and pitavastatin (3 mg/kg) improved the cognitive deficits in APP transgenic mice, but were not able to modify serum levels of cholesterol (Kurata et al. 2011). It is important to mention that even when an elevated dose of

simvastatin was administered to guinea pigs (250 mg/Kg), no changes were observed in the brain content of cholesterol (Fassbender et al. 2001). It is believed that statins could act via inhibition of isoprenoid synthesis, and that isoprenoids are important for the posttranslational modifications of a variety of proteins, including G proteins (Kuipers and van den Elsen 2007; Rikitake and Liao 2005). Moreover, brain cholesterol content is not dependent upon peripheral synthesis and, in AD, the release of cholesterol is altered in neurons (Kandiah and Feldman 2009). It has also been proposed that statins stabilize the cholesterol in membrane micro domains, preventing the cleavage of APP into βA toxic isoforms (Kirsch et al. 2003).

There is evidence that dementia is accompanied by oxidative stress and damage to the brain tissue (Butterfield et al. 2010; Markesbery and Carney 1999). The exact mechanism of the action of STZ in not well understood, but it is well known that it causes central damage and neuronal death by induction of oxidative stress (Rodrigues et al. 2009; Sharma and Gupta 2001; Sharma and Gupta 2002). The protective effect of statins in oxidative stress is well known in cardiovascular diseases (Nagay Hernandez et al. 2008), and some recent studies demonstrate the protective effect of statins in the brains of mice exposed to beta-amyloid peptide. Previous studies also report a reduction in oxidative stress in mice (Barone et al. 2011; Piermartiri et al. 2010). In present work, ICV-STZ injection reduced the total content of GSH in hippocampal slices, according to previous reports (Rodrigues et al. 2009),and both statins were capable of reversing this effect, in agreement with previous studies that demonstrated the antioxidant potential of statins (Bandoh et al. 2000; Piermartiri et al. 2010; Vaughan and Delanty 1999).

There is some evidence that the metabolism of glutamate is altered in AD, and neurons and astrocytes present lower levels of GS (Robinson 2001). The dementia model induced by ICV injection of βA causes a reduction in glutamate uptake in hippocampal slices (Piermartiri et al. 2010); however, in our model, STZ injection did not alter the hippocampal glutamate uptake, in agreement with previous results (Rodrigues et al. 2009). Interestingly, simvastatin presented an effect *per se*, increasing the glutamate uptake in the hippocampus. Previous reports have demonstrated the effect of statins against glutamate excitotoxicity in cortical neurons (Bosel et al. 2005; Zacco et al. 2003). Atorvastatin reversed the reduction in glutamate transporter expression in a dementia model with βA, probably mediated by PI3K and AKT pathways (Piermartiri et al. 2010). More recently, it has been suggested that simvastatin, but not pravastatin, is an effective neuroprotectant in a mouse model of neurodegeneration induced by kainate (Ramirez et al. 2011). It is possible that the effect of simvatatin (but not pravastatin) on glutamate uptake that we observed in this study could contribute to this protection against excitotoxicity.

It is important to mention that we used a low dose of simvastatin (5 mg/Kg), when compared to the maximum dose approved for human use (80 mg/day) (Fassbender et al. 2001) or to the amount administered in studies aiming for neuroprotection (Li et al. 2006; Ramirez et al. 2011). However, low doses of statins also showed protection in cognitive parameters in transgenic (Kurata et al. 2011) and STZ (Sharma et al. 2008) models of dementia. Moreover, in the STZ model of dementia, as occurs in AD, we observed a decrease in glutamine synthetase activity, and both statins prevented this effect. In fact, both statins exhibited an effect “per se” on this enzyme.

Glutamatergic neurotransmission, glutamate metabolism and antioxidant defenses (particularly mediated by GSH) are strongly coupled. Our data suggest that simvastatin and pravastatin can interfere in these parameters. Together with previous reports our data support the neuroprotective role of statins in AD, based on changes in biochemical and cognitive parameters in the STZ model of dementia. In summary, our results confirm the cognitive deficit observed in ICV-STZ models of dementia and that this deficit was accompanied by changes in glutamate metabolic parameters. Simvastatin (and pravastatin) exhibited a neuroprotective effect on these parameters. These results support the idea that these drugs could be effective in the prevention of alterations observed in the STZ dementia model, and may contribute to reduce cognitive impairment and brain damage in AD patients.

Acknowledgements

This work was supported by the Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq), Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES), FINEP/ Rede IBN 01.06.0842-00 and INCT-National Institute of Science and Technology for Excitotoxicity and Neuroprotection.

References

Adamson P, Greenwood J (2003) How do statins control neuroinflammation? Inflamm Res 52:399-403.

Bandoh T, Mitani H, Niihashi M, Kusumi Y, Kimura M, Ishikawa J, Totsuka T, Sakurai I, Hayashi S (2000) Fluvastatin suppresses atherosclerotic progression, mediated through its inhibitory effect on endothelial dysfunction, lipid peroxidation, and macrophage deposition. J Cardiovasc Pharmacol 35:136-144.

Barone E, Cenini G, Di Domenico F, Martin S, Sultana R, Mancuso C, Murphy MP, Head E, Butterfield DA (2011) Long-term high-dose atorvastatin decreases brain oxidative and nitrosative stress in a preclinical model of Alzheimer disease: A novel mechanism of action. Pharmacol Res 63:172-180.

Bosel J, Gandor F, Harms C, Synowitz M, Harms U, Djoufack PC, Megow D, Dirnagl U, Hortnagl H, Fink KB, Endres M (2005) Neuroprotective effects of atorvastatin against glutamate-induced excitotoxicity in primary cortical neurones. J Neurochem 92:1386-1398.

Browne RW, Armstrong D (1998) Reduced glutathione and glutathione disulfide. Methods Mol Biol 108:347-352.

Butterfield DA, Galvan V, Lange MB, Tang H, Sowell RA, Spilman P, Fombonne J, Gorostiza O, Zhang J, Sultana R, Bredesen DE (2010) In vivo oxidative stress in brain of Alzheimer disease transgenic mice: Requirement for methionine 35 in amyloid beta-peptide of APP. Free Radic Biol Med 48:136-144.

Candore G, Bulati M, Caruso C, Castiglia L, Colonna-Romano G, Bona DD, Duro G, Lio D, Matranga D, Pellicano M, Risso C, Scapagnini G, Vasto S (2010) Inflammation, Cytokines, Immune Response, Apolipoprotein E, Cholesterol, and Oxidative Stress in Alzheimer Disease: Therapeutic Implications. Rejuvenation Research 13:301-313.

Canevari L, Clark JB (2007) Alzheimer's disease and cholesterol: the fat connection. Neurochem Res 32:739-750.

Danbolt NC (2001) Glutamate uptake. Prog Neurobiol 65:1-105.

dos Santos AQ, Nardin P, Funchal C, de Almeida LM, Jacques-Silva MC, Wofchuk ST, Goncalves CA, Gottfried C (2006) Resveratrol increases glutamate uptake and glutamine synthetase activity in C6 glioma cells. Arch Biochem Biophys 453:161-167.

Dringen R, Hirrlinger J (2003) Glutathione pathways in the brain. Biol Chem 384:505-516.

Fassbender K, Simons M, Bergmann C, Stroick M, Lutjohann D, Keller P, Runz H, Kuhl S, Bertsch T, von Bergmann K, Hennerici M, Beyreuther K, Hartmann T (2001) Simvastatin strongly reduces levels of Alzheimer's disease beta -amyloid peptides Abeta 42 and Abeta 40 in vitro and in vivo. Proc Natl Acad Sci U S A 98:5856-5861.

Gottfried C, Tramontina F, Goncalves D, Goncalves CA, Moriguchi E, Dias RD, Wofchuk ST, Souza DO (2002) Glutamate uptake in cultured astrocytes depends on age: a study about the effect of guanosine and the sensitivity to oxidative stress induced by H(2)O(2). Mech Ageing Dev 123:1333-1340.

Grunblatt E, Salkovic-Petrisic M, Osmanovic J, Riederer P, Hoyer S (2007) Brain insulin system dysfunction in streptozotocin intracerebroventricularly treated rats generates hyperphosphorylated tau protein. J Neurochem 101:757-770.

Hoglund K, Blennow K (2007) Effect of HMG-CoA reductase inhibitors on beta-amyloid peptide levels: implications for Alzheimer's disease. CNS Drugs 21:449-462.

Jick H, Zornberg GL, Jick SS, Seshadri S, Drachman DA (2000) Statins and the risk of dementia. Lancet 356:1627-1631.

Kandiah N, Feldman HH (2009) Therapeutic potential of statins in Alzheimer's disease. J Neurol Sci 283:230-234.

Kirsch C, Eckert GP, Mueller WE (2003) Statin effects on cholesterol micro-domains in brain plasma membranes. Biochem Pharmacol 65:843-856.

Kuipers HF, van den Elsen PJ (2007) Immunomodulation by statins: inhibition of cholesterol vs. isoprenoid biosynthesis. Biomed Pharmacother 61:400-407.

Kurata T, Miyazaki K, Kozuki M, Panin VL, Morimoto N, Ohta Y, Nagai M, Ikeda Y, Matsuura T, Abe K (2011) Atorvastatin and pitavastatin improve cognitive function and reduce senile plaque and phosphorylated tau in aged APP mice. Brain Res 1371:161-170.

Lannert H, Hoyer S (1998) Intracerebroventricular administration of streptozotocin causes long-term diminutions in learning and memory abilities and in cerebral energy metabolism in adult rats. Behav Neurosci 112:1199-1208.

Li B, Mahmood A, Lu D, Wu H, Xiong Y, Qu C, Chopp M (2009) Simvastatin attenuates microglial cells and astrocyte activation and decreases interleukin-1beta level after traumatic brain injury. Neurosurgery 65:179-185; discussion 185-176.

Li L, Cao D, Kim H, Lester R, Fukuchi K (2006) Simvastatin enhances learning and memory independent of amyloid load in mice. Ann Neurol 60:729-739.

Liao JK, Laufs U (2005) Pleiotropic effects of statins. Annu Rev Pharmacol Toxicol 45:89-118.

Lowry OH, Rosebrough NJ, Farr AL, Randall RJ (1951) Protein measurement with the Folin phenol reagent. J Biol Chem 193:265-275.

M. Weinstock SS (2004) Rat models of dementia based on reductions in regional glucose metabolism, cerebral blood flow and cytochrome oxidase activity. Journal of Nerural Transmission.

Maccioni RB, Munoz JP, Barbeito L (2001) The molecular bases of Alzheimer's disease and other neurodegenerative disorders. Arch Med Res 32:367-381.

Markesbery WR, Carney JM (1999) Oxidative alterations in Alzheimer's disease. Brain Pathol 9:133-146.

Mori T, Paris D, Town T, Rojiani AM, Sparks DL, Delledonne A, Crawford F, Abdullah LI, Humphrey JA, Dickson DW, Mullan MJ (2001) Cholesterol accumulates in senile plaques of Alzheimer disease patients and in transgenic APP(SW) mice. J Neuropathol Exp Neurol 60:778-785.

Morris R (1984) Developments of a water-maze procedure for studying spatial learning in the rat. J Neurosci Methods 11:47-60.

Mrak RE, Griffinbc WS (2001) The role of activated astrocytes and of the neurotrophic cytokine S100B in the pathogenesis of Alzheimer's disease. Neurobiol Aging 22:915-922.

Nagay Hernandez S, Flores Molina JJ, Ilarraza Lomeli H, Martinez Sanchez C, del Valle Mondragon L, Tenorio Lopez FA, Pastelin Hernandez G (2008) [Influence of rosuvastatin in endothelial function and oxidative stress, in patients with acute coronary syndrome]. Arch Cardiol Mex 78:379-383.

Pasquier F, Boulogne A, Leys D, Fontaine P (2006) Diabetes mellitus and dementia. Diabetes Metab 32:403-414.

Paxinos (1997) The Rat Nervous System. Academic Press.

Piermartiri TC, Figueiredo CP, Rial D, Duarte FS, Bezerra SC, Mancini G, de Bem AF, Prediger RD, Tasca CI (2010) Atorvastatin prevents hippocampal cell death, neuroinflammation and oxidative stress following amyloid-beta(1-40) administration in mice: evidence for dissociation between cognitive deficits and neuronal damage. Exp Neurol 226:274-284.

Ramirez C, Tercero I, Pineda A, Burgos JS (2011) Simvastatin is the statin that most efficiently protects against kainate-induced excitotoxicity and memory impairment. J Alzheimers Dis 24:161-174.

Rikitake Y, Liao JK (2005) Rho GTPases, statins, and nitric oxide. Circ Res 97:1232-1235.

Robinson SR (2001) Changes in the cellular distribution of glutamine synthetase in Alzheimer's disease. J Neurosci Res 66:972-980.

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

Saheki A, Terasaki T, Tamai I, Tsuji A (1994) In vivo and in vitro blood-brain barrier transport of 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase inhibitors. Pharm Res 11:305-311.

Saxena G, Singh SP, Agrawal R, Nath C (2008) Effect of donepezil and tacrine on oxidative stress in intracerebral streptozotocin-induced model of dementia in mice. Eur J Pharmacol 581:283-289.

Schonbeck U, Libby P (2004) Inflammation, immunity, and HMG-CoA reductase inhibitors: statins as antiinflammatory agents? Circulation 109:II18-26.

Sharma B, Singh N, Singh M (2008) Modulation of celecoxib- and streptozotocin-induced experimental dementia of Alzheimer's disease by pitavastatin and donepezil. J Psychopharmacol 22:162-171.

Sharma M, Gupta YK (2001) Intracerebroventricular injection of streptozotocin in rats produces both oxidative stress in the brain and cognitive impairment. Life Sci 68:1021-1029.

Sharma M, Gupta YK (2002) Chronic treatment with trans resveratrol prevents intracerebroventricular streptozotocin induced cognitive impairment and oxidative stress in rats. Life Sci 71:2489-2498.

Sierra S, Ramos MC, Molina P, Esteo C, Vazquez JA, Burgos JS (2010) Statins as neuroprotectants: a comparative in vitro study of lipophilicity, blood-brain-barrier penetration, lowering of brain cholesterol, and decrease of neuron cell death. J Alzheimers Dis 23:307-318.

Silva MC, Rocha J, Pires CS, Ribeiro LC, Brolese G, Leite MC, Almeida LM, Tramontina F, Ziegler DR, Goncalves CA (2005) Transitory gliosis in the CA3 hippocampal region in rats fed on a ketogenic diet. Nutr Neurosci 8:259-264.

Silvestrelli G, Lanari A, Parnetti L, Tomassoni D, Amenta F (2006) Treatment of Alzheimer's disease: from pharmacology to a better understanding of disease pathophysiology. Mech Ageing Dev 127:148-157.

Stefani M, Liguri G (2009) Cholesterol in Alzheimer's disease: unresolved questions. Curr Alzheimer Res 6:15-29.

Terwel D, Prickaerts J, Meng F, Jolles J (1995) Brain enzyme activities after intracerebroventricular injection of streptozotocin in rats receiving acetyl-L-carnitine. Eur J Pharmacol 287:65-71.

Thomazi AP, Godinho GF, Rodrigues JM, Schwalm FD, Frizzo ME, Moriguchi E, Souza DO, Wofchuk ST (2004) Ontogenetic profile of glutamate uptake in brain structures slices from rats: sensitivity to guanosine. Mech Ageing Dev 125:475-481.

Vaughan CJ, Delanty N (1999) Neuroprotective properties of statins in cerebral ischemia and stroke. Stroke 30:1969-1973.

Weinstock M, Shoham S (2004) Rat models of dementia based on reductions in regional glucose metabolism, cerebral blood flow and cytochrome oxidase activity. J Neural Transm 111:347-366.

Wolozin B, Wang SW, Li NC, Lee A, Lee TA, Kazis LE (2007) Simvastatin is associated with a reduced incidence of dementia and Parkinson's disease. BMC Med 5:20.

Yoshida M (2003) Potential role of statins in inflammation and atherosclerosis. J Atheroscler Thromb 10:140-144.

Zacco A, Togo J, Spence K, Ellis A, Lloyd D, Furlong S, Piser T (2003) 3-hydroxy-3-methylglutaryl coenzyme A reductase inhibitors protect cortical neurons from excitotoxicity. J Neurosci 23:11104-11111.

Zipp F, Waiczies S, Aktas O, Neuhaus O, Hemmer B, Schraven B, Nitsch R, Hartung HP (2007) Impact of HMG-CoA reductase inhibition on brain pathology. Trends Pharmacol Sci 28:342-349.

Figure Legends

Figure 1. *Cognitive performance of rats submitted to ICV-STZ injection* and oral simvastatin or pravastatin administration*, evaluated by the water maze.* (**A**) Performance in the reference memory protocol, based on escape latency. Each line represents the mean ± standard error. * Significant differences were detected from day 1 onwards, when compared to the control group (N = 8, repeated measures analysis of variance, $p < 0.05$); (**B**) Memory in the probe trial of reference memory, based on escape latency. Values are mean ± standard error. * Significantly different from all other groups (N = 8, two-way ANOVA followed by Tukey's test, $p < 0.05$); (**C**) Number of crossings over the platform position. Values are mean ± standard error. * Significantly different from all other groups (N = 8, two-way ANOVA followed by Tukey's test, $p < 0.05$). (**D**) Memory in the probe trial of reference memory, as measured by time spent (in s) in the target and opposite quadrant. Values are mean ± standard error. * Time spent in target quadrant by STZ group was significantly different from all other groups (N = 8, two-way ANOVA followed by Tukey's test, $p < 0.05$).

Figure 2. *Glutamate uptake in the hippocampus of rats submitted to the ICV-STZ model of dementia.* Adult rats were submitted to ICV injection of STZ and oral simvastatin or pravastatin administration. Four weeks later, hippocampi were dissected out and the glutamate uptake assay performed in hippocampal slices. Values are mean ± standard error. * Significant difference between simvastatin and sham group (N = 6-7, two-way ANOVA followed by Tukey's test, $p < 0.05$).

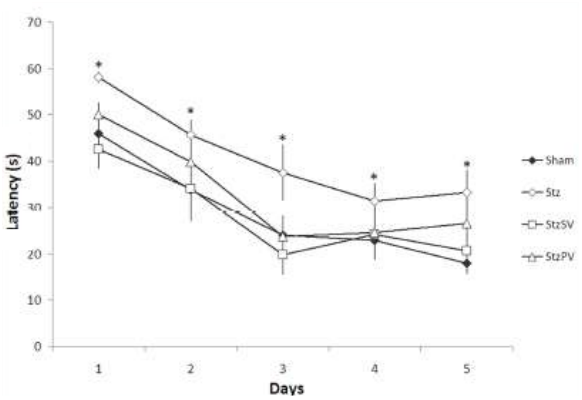
Figure 3. *Glutathione content in the hippocampus of rats submitted to ICV-STZ injection.* Adult rats were submitted to ICV injection of STZ and simvastatin or pravastatin oral administration. Four weeks later, hippocampi were dissected out and homogenized for measurement of glutathione content. Values are means ± standard error. * Significantly different from the sham groups (with or without statins). N = 7, two-way ANOVA followed by Tukey's test, $p < 0.05$).

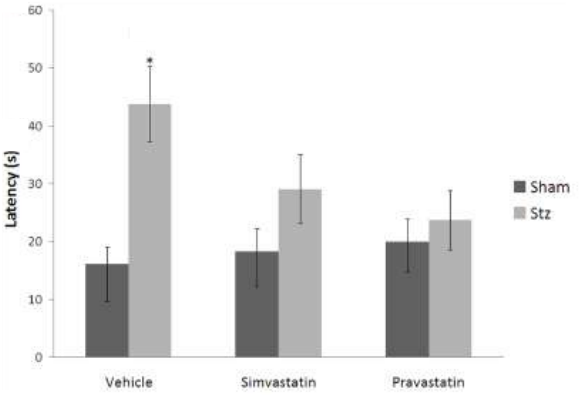
Figure 4. *Glutamine Synthetase activity in the hippocampus of rats submitted to ICV-STZ injection.* Adult rats were submitted to ICV injection of STZ and simvastatin or pravastatin oral administration. Four weeks later, hippocampi were dissected out and homogenized for measurement the glutamine synthetase activity. Values are mean ± standard error. [a] Significantly different from all other groups, [b] Significantly different from the sham and STZ group, [c] Significantly different from the STZ group (N = 7, two-way ANOVA followed by Tukey's test, $p < 0.05$).

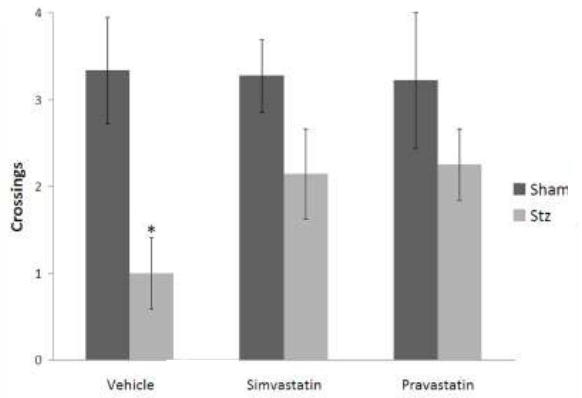
Table 1. *Serum biochemical parameters of rats submitted to ICV-STZ injection* and oral simvastatin or pravastatin administration.

TCholesterol, total serum cholesterol; SGPT, serum glutamic pyruvic transaminase; SGOT, serum glutamic oxalacetic transaminase. Data are expressed as means ± standard error (N = 7, two-way ANOVA).

Figure 1


Latency (s)
Days
Sham
Stz
StzSV
StzPV


Latency (s)
Vehicle
Simvastatin
Pravastatin
Sham
Stz


Crossings
Vehicle
Simvastatin
Pravastatin
Sham
Stz

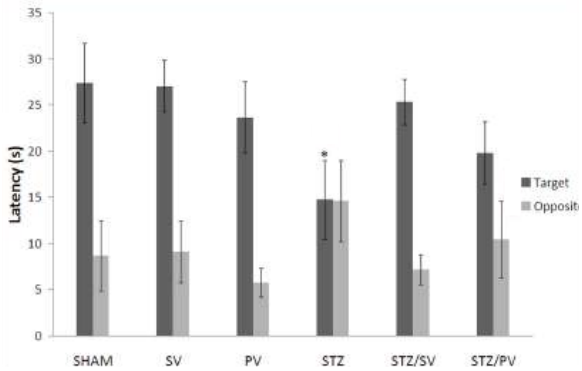

Latency (s)
SHAM
SV
PV
STZ
STZ/SV
STZ/PV
Target

Figure 2

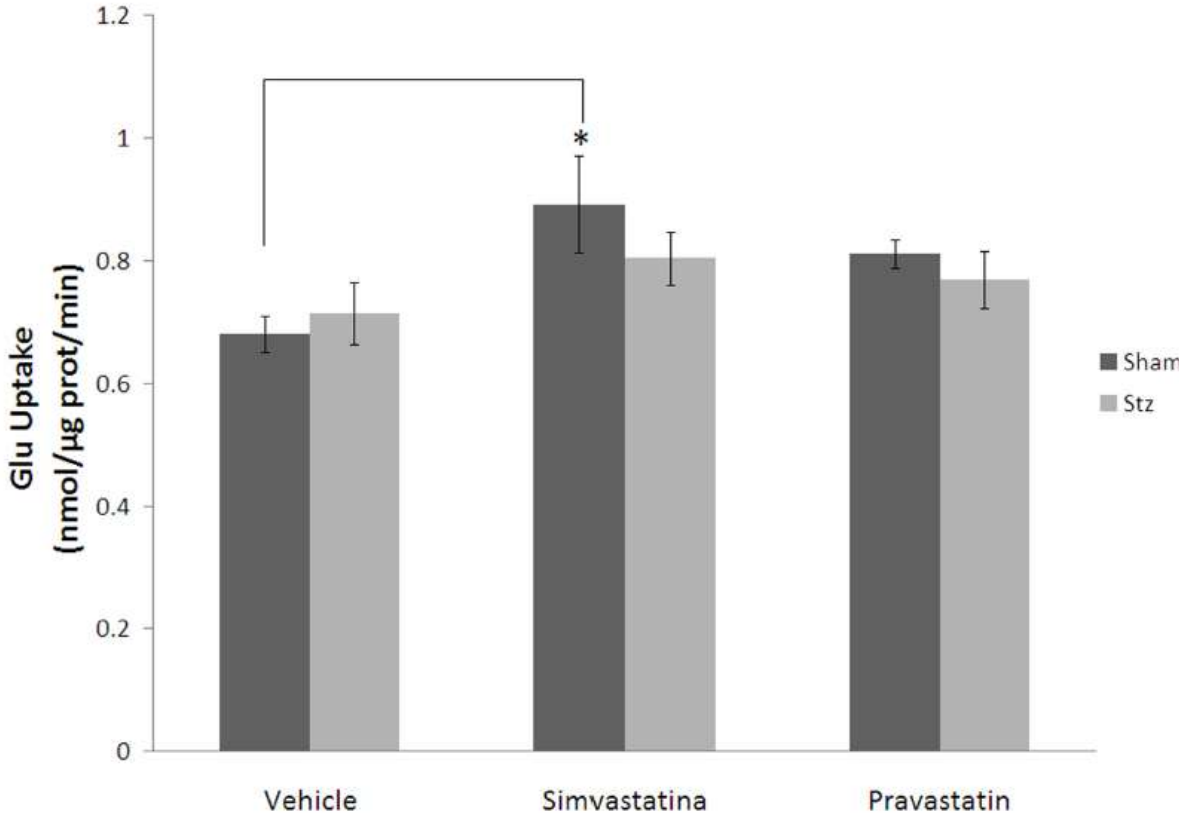

1.2
1
0.8
0.6
0.4
0.2
0
Glu Uptake
(nmol/µg prot/min)
*
Vehicle
Simvastatina
Pravastatin
Sham
Stz

Figure 3

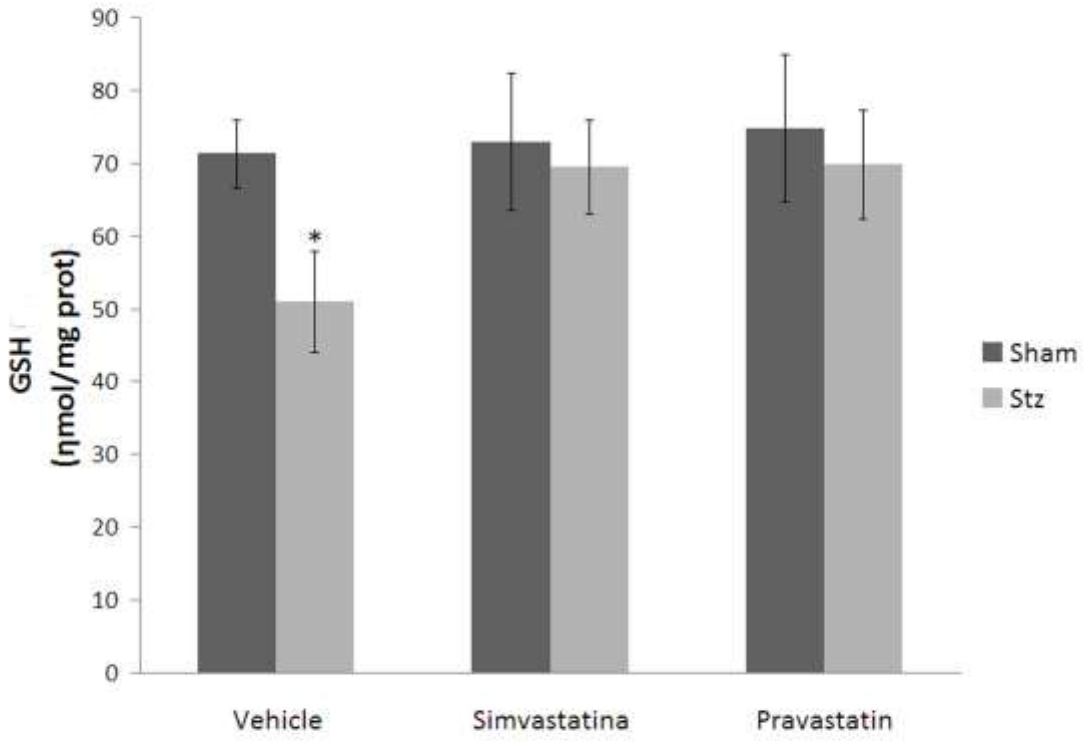

GSH
(ηmol/mg prot)
90
80
70
60
50
40
30
20
10
0
*
Vehicle
Simvastatina
Pravastatin
Sham
Stz

Figure 4

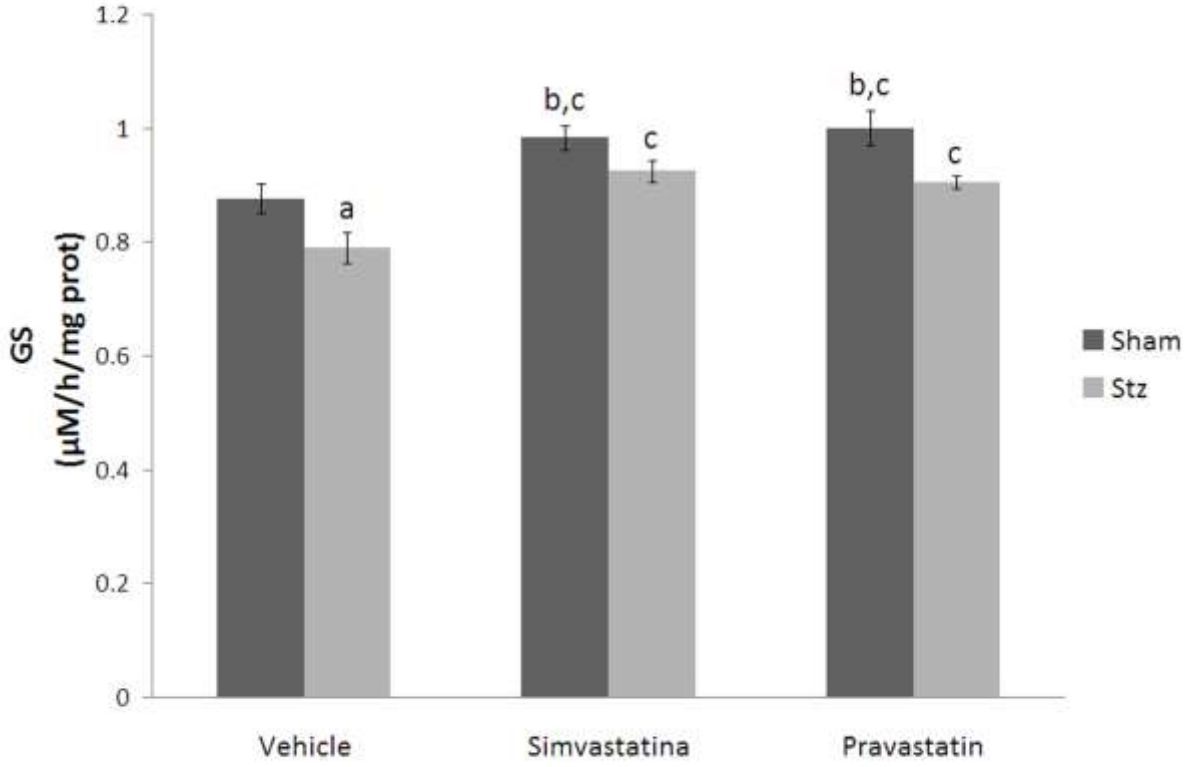

GS
(µM/h/mg prot)
1.2
1
0.8
0.6
0.4
0.2
0
a
b,c
c
b,c
c
Vehicle
Simvastatina
Pravastatin
Sham
Stz

Tabela 1

Table 1. Serum biochemical parameters of rats submited to ICV-STZ injection and statin treatment.

Serum measurements						
Treatments	Sham Group			STZ Group		
	Vehicle	Simvastatin	Pravastatin	Vehicle	Simvastatin	Pravastatin
Glucose (mg/dl)	170.1±27.0	174.0±23.7	193.5±19.0	166.0±29.1	160.0±20.1	176.0±20.0
TCholesterol(mg/dl)	84.0±10.2	88.1±10.1	78.1±9.3	90.5±20.2	87.5±12.5	89.5±12.5
Triglicerides (mg/dl)	125.3±12.1	122.0±15.0	114.5±16.4	117.3±24.3	121.2±14.4	118.0±13.4
SGOT (UI)	50.1±22.5	42.5±13.5	56.0±23.0	40.1±17.8	37.2±16.3	45.1±20.5
SGPT(UI)	20.8±5.7	20.3±4.5	21.1±6.0	23.0±7.8	20.1±4.3	21.1±3.9

CAPÍTULO III

Statins improve glucose uptake and acetyl cholinesterase activities in streptozotocin-induced model of dementia in rats.

Artigo a ser submetido para o periódico Journal of Alzheimer's Disease

Statins improve glucose uptake and acetyl cholinesterase activities in streptozotocin-induced model of dementia in rats.

Ana Carolina Tramontina[1*], Krista Minéia Wartchow[1], Regina Biasibeti[1], André Quincozes-Santos[1], Larissa Bobermin[1], Paula Lunardi[1], Ana Paula Costa[1], Caren Bernardi[2], Lucas Tortorelli[1], Carlos-Alberto Gonçalves[1].

1 – Programa de Pós-Graduação em Bioquímica, 2 – Programa de Pós-Graduação em Neurociências, Instituto de Ciências Básicas da Saúde, Universidade Federal do Rio Grande do Sul, Porto Alegre, Brazil.

* Corresponding author: Ana Carolina Tramontina

Depto Bioquímica, ICBS, UFRGS

Ramiro Barcelos, 2600-anexo

Porto Alegre, RS, Brazil

90035-003

Fax: 55-51-3308 5535

E-mail: tramontina_ana@yahoo.com.br

Abstract

Alzheimer's disease is the most common form of dementia, and its incidence has increased greatly in recent decades, which is directly associated with the increase in life expectancy. The exact cause of Alzheimer's remains elusive, however some risk factors are associated with the development of the disease. There are strong correlations between Alzheimer's disease and diabetes mellitus, and clinical studies reveal that diabetic patients , with type II diabetes, has two to three fold increased risk for development Alzheimer's. Both diseases share some pathological characteristics, like insulin metabolism disturbance, as well as reduction in glucose utilization. The aim of this study was to evaluate glucose uptake and acetyl cholinesterase activity in hippocampal tissue of rats exposed to intracerebroventricular streptozotocin injection, as well as the neuroprotective effect of two statins (simvastatin and pravastatin). We also evaluated cognitive deficit, astrocyte markers (GFAP and S100B) and parameters of oxidative stress. Our results confirm the spatial cognitive deficit and oxidative stress in hippocampal tissue in streptozotocin rats, as well as the decrease of glucose uptake, the cholinergic deficit (based on increased acetyl cholinesterase activity) observed in Alzheimer's disease, and reduced levels of liquor S100B, also observed in other models of dementia. All these alterations could be assumed as early alterations in Alzheimer's disease, since beta-amyloid plaques appear later in streptozotocin model of dementia. Simvastatin and pravastatin prevented theses alterations reinforcing the potential neuroprotective role of statins.

Introduction

Alzheimer's disease (AD) is the most common form of dementia, and its incidence has increased greatly in recent decades, which is directly associated with and increase in life expectancy [1]. AD patients present progressive memory deficits and cognitive impairment, and histologically, AD is characterized by two main hallmarks: the neuritic plaques and neurofibrillary tangles (NTFs) [2]. Neuritic plaques are extracellular deposits of β-amyloid peptide (βA), and NTFs are intraneuronal aggregates of microtubule-associated protein tau [3]. Associated with this alterations, also occurs synaptic modifications, accompanied by neuronal death [4].

Beyond the neuronal damage in AD, astrocytes are also implicated in the pathogenesis of the disease. Activated astrocytes are found in association with neuritic plaques, and the activation levels are correlated with the extend of amyloid deposits [5]. Reactive astrocytes found in AD brain overexpress S100B protein and GFAP [6], and the degree of astrogliosis is directly correlated with cognitive decline [7].

The exact cause of AD remains elusive, however some risk factors are associated with the development of the disease. There are strong correlations between AD and diabetes mellitus, and clinical studies reveal that patients who suffer from diabetes mellitus type II has two to three fold increased risk for development AD [8, 9]. Both diseases share some pathological changes, like insulin metabolism disturbance, as well as modifications in glucose utilization [10].

Insulin is involved in the modulation of various brain functions, such as learning, memory and synaptic plasticity [11]. Dysfunction in insulin and insulin receptor (IR) could be involved in the pathogenesis of AD, leading to a metabolic impairment and modifications in the APP processing [12]. In addition, the phosphorylation of tau protein, which leads to NFTs generation, could be affected by some molecules of the insulin signaling cascade [13]. Some studies also show a reduction in IR and insulin signaling molecules in postmortem analysis brains from AD patients, which may indicate that insulin signaling is reduced is the AD brain [14, 15].

Besides the alterations in insulin signaling, another common alterations among DA and diabetes mellitus, is the disturbance in glucose utilization, which lead some authors to define AD as Type III diabetes [16]. Reduction in cerebral glucose utilization occurs in many brain areas in the AD patients [17], causing metabolic alterations. Considering that the metabolism of glucose in brain is the main source of ATP and acetyl-CoA [18], metabolite of great importance in acetylcholine synthesis, the disturbance in glucose metabolism could lead to a reduction in acetylcholine synthesis. The cholinergic deficit is well described in AD [19], and it has been correlated to increased cognitive decline [20].

Another well established parameter who increase sporadic AD risk is the ε4 variant for ApoE [21], the major apolipoprotein in brain [22]. Clinical studies with patients lacking or expressing the Apo ε4 allele showed that Apo ε4 is associated with increased levels of Aβ deposits [23, 24], however the mechanism by which Apo ε4 causes this increment is not well known. Some studies suggest that Apo ε4 can increase the Aβ deposits, promote the aggregation [25] and increase the peptide stability, probably by decreasing its

clearance in the brain [3]. A growing amount of evidence has linking cholesterol metabolism and AD, and some studies suggests that increased levels of serum cholesterol could be associated with an increased risk of AD [26, 27]. Taking into account the possible participation of cholesterol in the development of AD, statins has been suggested to play an important role in protection against dementia [28, 29].

Statins are cholesterol-lowering agents, and acts in the key enzyme in cholesterol synthesis, 3-hydroxy-3-methylglutaryl coenzyme A reductase (HMGCoA reductase) [30]. Beyond the effect in cholesterol synthesis, statins also cause a reduction in isoprenoid intermediates, which are generated in cholesterol synthesis pathway [31]. These cholesterol-independent effects are known as pleiotropic effects, and includes anti-inflammatory [32] and immunomodulatory activities [33]. Moreover, our group also show antioxidant effects of statins in a dementia model [34]. The effects of statins in cardiovascular disease are well established [35], however the possible neuroprotective role of this drugs are still in controversial and need better clarification.

Several non-transgenic animal models for AD are employed to mimic some of the neurochemical and behavioral characteristics presented in sporadic AD, however the intracerebroventricular (icv) streptozotocin (STZ) model demonstrates metabolic changes very similar to those that occur in sporadic AD, including alterations in cerebral insulin and glucose metabolism [18, 36]. STZ model of dementia also cause a reduced brain antioxidant capacity [37], and increase in oxidative stress [38], alterations that also occur in AD, and could be implicated in the pathogenesis of the disease [39].

The aim of this study was to evaluate glucose uptake and acetyl cholinesteraseactivity in hippocampal tissue of rats exposed to icv-STZ, as well as the neuroprotective effect of two statins (simvastatin and pravastatin). We also evaluated cognitive deficit, astrocyte markers (GFAP and S100B) and parameters of oxidative stress.

Material and Methods

Chemicals

Streptozotocin, simvastatin, pravastatin, albumin, o-phenylenediamine, dichlorofluorescein diacetate (DCF-DA), acetylthiocholine iodide, 5,5-ditio-bis-(2-nitrobenzóico) and monoclonal anti- S100B antibody were purchased from Sigma. Anti-S100 antibody conjugated with peroxidase and anti-GFAP antibody were purchased from Dako. Anti-rabbit peroxidase-conjugated anti-IgG was purchased from Amersham. [2,3-^{3}H]deoxi-D- glucose was purchased from Perkin Elmer. Protein Carbonyls determination kit (OxyBlotTM Protein Oxidation Detection Kit (S7150)) was obtained from Millipore. All other reagents were purchased from local suppliers.

Animals

Forty-two male Wistar rats (90 days old, weighing 250–320 g) were obtained from our breeding colony (at the Department of Biochemistry, Universidade Federal do Rio Grande do Sul), and were maintained under controlled light and environmental conditions (12 hour light/12 hour dark cycle at a constant temperature of 22 ± 1◦C) with free access to food and water. All animal experiments were carried out in accordance with the National Institute of Health Guide for the Care and Use of Laboratory Animals (NIH Publications No. 80-23) revised 1996, and following the regulations of the local animal house authorities.

Rats were divided into 6 groups: sham (N = 7), sham-simvastatin (N = 7), sham-pravastatin (N=7), STZ (N = 7), STZ-simvastatin (N=7) and STZ-pravastatin (N = 7). After behavioral tasks, rats were anaesthetized, as subsequently described, for CSF puncture and brain slice preparation.

Experimental protocol

Rats were divided into 6 groups of 7 rats: sham, sham-simvastatin, sham-pravastatin, STZ, STZ-simvastatin, and STZ-pravastatin. (a) Sham group: the animals received ICV injection of STZ vehicle (HBSS). On day two after the surgery and every two days they received the statin vehicle, p.o.; (b) Sham-simvastatin group: the animals received an ICV injection of STZ vehicle (HBSS, detailed further). On day two after the surgery, and every two days, they received simvastatin (5mg/kg) p.o.; (c) Sham-pravastatin group: Sham-simvastatin group: the animals received an ICV injection of the STZ vehicle (HBSS). On day two after the surgery and every two days they received pravastatin (5mg/kg) p.o.; (d) STZ group: the animals received an ICV injection of STZ (3mg/kg). On day two after the surgery and each two days they received the statins vehicle, p.o.; (e) STZSimvastatin group: the animals received an ICV injection of STZ (3mg/kg). On day two after the surgery and every two days they received simvastatin (5mg/kg) p.o.; and (f) STZ-pravastatin group: the animals received an ICV injection of STZ (3mg/kg). On day two after the surgery and every two days they received pravastatin (5mg/kg) p.o.

Surgical procedure

Streptozotocin was ICV infused based on previous work [40]. Briefly, on the day of the surgery animals were anesthetized with ketamine/xylazine (75 and 10 mg/Kg, respectively, i.p.) and placed in a stereotaxic apparatus. A midline sagittal incision was made in the scalp. Burr holes were drilled in the skull on

both the sides over the lateral ventricles. The lateral ventricles were accessed using the following coordinates [41]: 0.9 mm posterior to bregma; 1.5 mm lateral to sagittal suture; 3.6mmbeneath the surface of brain. Rats received a single bilateral infusion of 5 µL STZ (3 mg/Kg) or vehicle (Hank's balanced salt solution – HBSS – containing in mM: 137 NaCl; 0.63 Na2HPO4; 4.17 NaHCO3; 5.36 KCl; 0.44 KH2PO4; 1.26 CaCl2; 0.41 MgSO4; 0.49 MgCl2 and 10 glucose, in pH 7.4) using a 5 µL Hamilton syringe. After the surgical procedure, rats were placed on a heating pad to maintain body temperature at 37.5 ± 0.5◦C and were kept there until recovery from anesthesia. The animals were submitted to behavioral tasks and biochemical analysis 4 weeks after SZT-injection.

Cognitive evaluation

Three weeks after surgery, rats were submitted to training in the Morris water maze [42, 43].The apparatus consisted of a circular pool (180 cm diameter, 60 cm high) filled with water (depth 30 cm; 24± 1◦C), placed in a room with consistently located spatial cues. An escape platform (10 cm diameter) was placed in the middle of one of the quadrants, 1.5 cm below the water surface, equidistant from the sidewall and the middle of the pool. The platform provided the only escape from the water and was located in the same quadrant every trial. Four different starting positions were equally spaced around the perimeter of the pool. On each training day, all four start positions were used once in a random sequence, i.e., four training trials per day. A trial began by placing the animal in the water facing the wall of the pool at one of the starting points. If the animal failed to escape within 60 s it was gently conducted to the platform by the experimenter. The rat was allowed to stay there for 20 s. The inter-trial interval was 10 min. After each trial, the rats were dried, and returned to their

cages at the end of the session. Animals were trained for 5 days. 24 h after the last training session, the rats were submitted to a test session (four weeks after surgery). Before this session, the submerged platform was removed. The retention test consisted of placing the animals in the water for 1 min. The number of crossings over the original position of the platform and time spent in the target quadrant compared to the opposite quadrant were measured.

Obtaining CSF, hippocampal and cortical samples

Animals were anesthetized as described above and then positioned in a stereotaxic holder and CSF was obtained by cisterna magna puncture using an insulin syringe (27 gauge × 1/2" length). CSF was frozen (−20◦C) until further analysis [44].The animals were killed by decapitation, and the brains were removed and placed in cold saline medium with the following composition (in mM): 120 NaCl; 2 KCl; 1 CaCl2; 1 MgSO4; 25 HEPES; 1 KH2PO4 and 10 glucose, adjusted to pH 7.4 and previously aerated with O2. The hippocampi and cortex were dissected, and cut into transverse slices of 0.3 mm using a Mcllwain tissue for, and the slicer were homogeneized for TBARS, DCF, NO, S100B, GFAP, acetyl cholinesterase activity and Proteins Carbonyls measurement. For glucose uptake, slices were transferred immediately to 24-well culture plates, each well containing 0.3 mL of HBSS for measuring glucose uptake.

Obtaining HIppocampal Slices for Glucose Uptake *in vitro*

Briefly the animals were killed by decapitation, the brains were removed and placed in cold saline medium with the following composition (in mM): 120 NaCl; 2 KCl; 1 CaCl2; 1 MgSO4; 25 HEPES; 1 KH2PO4 and 10 glucose, adjusted to pH 7.4 and previously aerated with O2. The hippocampi were dissected and

transverse slices of 0.3 mm were obtained using a McIlwain Tissue Chopper. Slices were then transferred immediately into 24-well culture plates, each well containing 0.3 ml of physiological medium and only one slice. The medium was changed every 15 min with fresh saline medium at room temperature for equilibration (maintained at 25ºC). Following a 120-min equilibration period, slices were incubated in a medium containing STZ in different concentrations, as indicated in results, for 1 hour. After the STZ incubation, the media was replaced with HBSS and glucose uptake was started.

Glucose Uptake

Glucose uptake was performed in hipocampal slices. Briefly, slices were transferred to 24-well plates and incubated for 30 min at 37°C in a Hank's balanced salt solution (HBSS) containing (in mM): 137 NaCl, 5.36 KCl, 1.26 $CaCl_2$, 0.41 $MgSO_4$, 0.49 $MgCl_2$, 0.63 $Na_2HPO_4.7H_2O$, 0.44 KH_2PO_4, 4.17 $NaHCO_3$ and 5.6 glucose, adjusted to pH 7.4. The assay was started by addition of 0.1 µCi/ml [2,3-^{3}H]deoxi-D- glucose. Incubation was stopped after 30 min by removal of the medium and rinsing the cells twice with ice-cold HBSS. The slices were then lysed in a solution containing 0.5 M NaOH. Radioactivity was measured in a scintillation counter. Non-specific uptake was determined by using 25uM cytochalasin B. Final glucose uptake was obtained by subtracting the non-specific uptake of the total one to obtain the specific uptake.

Acetylcholinesterase Activity Determination

HIppocampal and cortex slices homogenates were centrifugated at 10000g for 10 min at 4°C; supernatants used as acetylcholinesterase sources were keeped into aliquots and stored at - 20°C. Acetylcholinesterase activity was measured by the principle of the Ellman method (Ellman et. al., 1961)[1]. Enzyme samples

in 20mM phosphate buffer, pH 7.4 were incubated 150 s with 0.8mM acetylthiocholine iodide in the presence of 10 mM 5,5-ditio-bis-(2-nitrobenzóico) (DTNB), for color development. Production of the yellow anion of 5-thio-2-nitrobenzoic acid was measured with a SPECTRAmax 190, 96-well plate reader, at 415 nm.

Quantification of S100B and GFAP

S100B content in the hippocampus, cortex and CSF was measured by ELISA [45] 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 (SH-B1). Polyclonal anti-S100Bwas 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. The standard S100B curve ranged from 0.025 to 2.5 ng/mL. ELISA for GFAP [46] was carried out by coating the microtiter plate with 100 µL samples containing 30 µg of protein for 48 h at 4◦C. Incubation with a rabbit polyclonal anti-GFAP for 2 h was followed by incubation with a secondary antibody conjugated with peroxidase for 1h, at room temperature; the standard GFAP curve ranged from 0.1 to 10 ng/mL.

Evaluation of intracellular ROS production

Intracellular ROS production was detected using the nonfluorescent cell permeating compound, 2'–7'-dichlorofluorescein diacetate (DCF-DA). DCF-DA is hydrolyzed by intracellular esterases and then oxidized by ROS to a fluorescent compound 2'–7'-dichlorofluorescein (DCF). Hipocampal and cortical slices homogenates were treated with DCF-DA (10 µM) for 30 min at 37°C and rinsed with DMEM without serum. Following DCF-DA exposure, the cells were

rinsed and then scraped into PBS with 0.2% Triton X-100. The fluorescence was measured in a plate reader (Spectra Max GEMINI XPS, Molecular Devices, USA) with excitation at 485 nm and emission at 520 nm [47].

Nitric oxide (NO) production

Nitric oxide was determined by measurement of nitrite (a stable oxidation product of NO), based on the Griess reaction. The Griess reagent was prepared by mixing equal volumes of 1% sulfanilamide in 0.5 N HCl and 0.1% N-(1-naphthyl) ethylenediamine in deionized water. The assay was performed as described [48], with modifications. Briefly, the Griess reagent and the tissue homogenate were added in microplates and maintained under reduced light at room temperature during 15 min. Samples were analyzed at 550 nm on a microplate spectrophotometer. Controls and blanks were run simultaneously. Nitrite concentrations were calculated using a standard curve prepared with sodium nitrite (0–50 µM).

Protein carbonyl assay (OxyblotTM analyses)

Protein carbonyls were assayed by Western blot analysis in hippocampal slices, according to the manufacturer's instructions (OxyBlotTM Protein Oxidation Detection Kit (S7150); Millipore Corporation, USA). In brief, 15 µg protein from hippocampal slices obtained in phosphate-buffered saline (PBS) were reacted with 2,4 dinitrophenylhydrazine and Western blotted using a primary antibody specific to dinitrophenylhydrazone-derivatized residues (OxyBlotTM) and a secondary antibody (OxyBlotTM). Protein carbonyls were visualized by phosphoimaging or revealed using enhanced chemioluminescence and quantified by densitometry. The optical density (O.D.) of the bands was quantified using Scion ImageTM (Frederick, MD, USA).

Lipid Peroxidation Assay

As an index of lipid peroxidation, the formation of thiobarbituric acid reactive species (TBARS) was monitored during an acidheating reaction, which is widely adopted for measurement of lipid redox state, as previously described [49]. The samples (slices homogenates) were mixed with 0.6mL of 10% trichloroacetic acid and 0.5mL of 0.67% thiobarbituric acid, and then heated in a boiling water bath for 25 min. Thiobarbituric acid reactive species were determined by the absorbance in a spetrophotometer at 532 nm. Result were expressed as malondialdeide (MDA)/mg protein.

Protein determination

Protein content was measured by Lowry's method using bovine serum albumin as standard [50].

Statistical analysis

Parametric data from the experiments are presented as means±standard error and statistically evaluated by two-way analysis of variance, followed by the Tukey's test, assuming $p < 0.05$. The escape latency parameter in the water maze task was evaluated by repeated measures analysis of variance, assuming $p < 0.05$.

Results

1. Statins prevents the cognitive deficit induced by STZ.

The Morris water maze was used to evaluate reference memory in the six groups: sham, sham-simvastatin, sham-pravastatin, STZ, STZ-simvastatin and STZ-pravastatin. In the training sessions, from day 3 onwards, there was a

significant increase of STZ group in the average time to find the platform (escape latency) when compared sham group (Fig. 1A) (F (3, 23) = 2,606, *p<0,05*). In the trial session, STZ rats spent more time to find platform (F (3,26)= 3,836, p<0,05) when compared to other groups (Fig. 1B). The number of crossings over the platform location was lower in the STZ group, compared to sham (Fig.1C), (F (3,26)= 4,256, p<0,05) and STZ group also spent less time in the target quadrant (Fig.1D), (F(3,26)=2,464, p<0,05). The performance of STZ/simvastatin and STZ/pravastatin was not different from the sham group, based on the average time to find the platform, time spent in the target and opposite quadrant. Notice that simvastatin and pravastatin "per se" had no effect on comportamental task.

2. Decrease in glucose uptake is prevented by statins

Hippocampal slices of rats exposed to ICV-STZ exhibited a reduced glucose uptake activity (Fig 2 A) ($F_{(5,36)} = 2.837$, $p < 0.05$). Interestingly, simvastatin and pravastatin treated rats were able to prevent this decrease induced by ICV-STZ administration. No statins affected the glucose uptake *per se.* Moreover, no effect of STZ on glucose uptake was observed when this compound as directly administered to hippocampal slices, at concentration from 0.02 to 2 mM (Fig 2B)($F_{(3,23)} = .111$, $p > 0.05$).

3. Increase of acetyl cholinesterase activity is attenuated by statins

ICV-STZ administration caused an increase in hippocampal AchE activity about 30% ($F_{(5,34)} = 35.622$, $p<0.05$). However, in ICV-STZ rats that received

simvastin or pravastatin the increase in hippocampal AchE activity was significantly lower than sham or ICV-STZ groups . STZ-SV group presented a 65% reduction in AchE activity when compared to the sham group, and STZ-PV, a 70% reduction (Fig 3)($F_{(5,34)}$ = 35.622, p=0.000).

4. Increase of reactive oxygen and nitrogen species in ICV-STZ are reduced by statins

ICV-STZ induces an increment in intracellular production of ROS in hippocampal slices ($F_{(5,40)}$ = 5.473, $p < 0.05$) measured by DCF assay (Fig 4A). Simvastin or pravastatin administration was able to prevent this effect. Statins by themselves did not alter ROS production. Moreover, NO production, measured by nitrite content was increased in hippocampus in ICV-STZ rats (Fig 4B). Both statins were able to prevent this effect, and did not show any *per se* effect ($F_{(5,38)}$ = 2.457, $p < 0.05$).

5. Oxidative damage is attenuated by statins

In ICV-STZ rats exhibited an increased lipid peroxidation measured by TBARS, in hippocampus ($F_{(5,39)}$ = 7.433, $p<0.05$) (Fig 5A). Simvastin or pravastatin administration partially prevent the effect of ICV-STZ administration, and no present effect *per se*. In cerebral cortex, neither STZ nor statins presents any effects in lipid damage (data not shown). In order to evaluate protein oxidative damage induced by ICV-STZ administration, we evaluated the content of protein carbonyls in the hippocampus (Fig 5B). STZ did not change the content

of protein carbonyls when compared to the sham group, and statins *per se* did not caused any effect on this parameter($F_{(5,30)} = 0.570$, $p>0.05$).

6. Cerebrospinal fluid S100B is decreased in ICV-STZ rats and normalized by statin administration

ICV-STZ administration did not altered the content of astroglial markers in hippocampus: GFAP (Fig 6A) or S100B (Fig 6B) measured by ELISA. Simvastatin or pravastatin ($F_{(5,43)} = 1.775$, $p = 0.333$) administration *per se* did not alter the content of these proteins in hippocampus. Moreover, no changes were observed in cerebral cortex concerning to these two astroglial markers (data not shown). However, CSF S100B content was reduced in ICS-STZ rats (Fig 6C) ($F_{(5,35)}= 2.537$, $p<0.05$) and, interestingly, simvastatin and pravastatin administration was able to prevent this effect. Both statins *per se* did not alter the CSF S100B content.

Discussion

Streptozotocin model has been widely used to mimic the effects of AD in the brain. In fact, the intracerebroventricular injection of this drug cause cognitive damage [34, 38, 40], accompanied by alterations in glucose metabolism[36], changes in enzymes of insulin pathway [10, 51], and reduced brain antioxidant capacity [37], alterations which also occurs in AD brain.

Glucose is indubitably the main energetic substrate for CNS, as well as the substrate for neurotransmitter synthesis (e.g. glutamate and acetylcholine) and brain antioxidant defense (via synthesis of NADPH) and lipid synthesis. The

most abundant isoforms of glucose transporters in CNS are GLUT-1 (endothelium, astrocytes and oligodendrocytes) and GLUT-3 (specifically in neurons). A decrease in glucose uptake has been demonstrated to precede neuropsychological impairment in AD [52, 53]. Based on this, some authors [10] have suggested that glucose metabolism impairment is causally associated to the neurodegenerative aspects of this disease and that insulin resistance observed in AD [54] is underlying the glucose metabolism alteration[55].

ICV-STZ administration in rats caused a decrease of glucose uptake in specific brain regions measured by ^{14}C-deoxyglucose autoradiography [36] and in brain slices by glucose consumption in the incubation medium [56].

We observed glucose uptake impairment in hippocampal slices in this model of dementia induced by ICV-STZ administration, and simvastatin and pravastatin administration were able to prevent this effect. The effect caused by STZ on glucose uptake was not observed *in* vitro, when we incubated hippocampal slices with different STZ concentrations. This suggests non-acute changes on glucose uptake induced by STZ. The mechanism of STZ is still unknown as well as if its effects on CNS depend on STZ uptake through GLUT-2 as occurs in beta cells in pancreas [51].

There are a few studies which demonstrate benefic effects of statins in diabetes mellitus [57, 58], however none of them has evaluated the effect of statins in glucose utilization. To our knowledge, is the first time that this effect is reported and particularly on hippocampal tissue.

Degeneration of cholinergic system is an important component in the pathogenesis of AD [59]. Loss of cholinergic neuron is a hallmark of the disease, and occurs especially is areas related to learning and memory[60], and

cholinergic deficit is associated with the degree of cognitive impairment[20]. In this sense, drugs with anticholinesterase activity have been used in AD patients [59].

In ICV-STZ model, an increase in AChE is described, and anticholinesterase drugs are used to prevent this alteration, and they also prevent the cognitive deficit [61]. Interestingly, both statins used here were able to prevent the increase in AChE activity, as well as the cognitive deficit. Some works investigated the effect of statins on AChE activity. Administration of simvastatin in normal rats decrease the AChE activity [62], however in STZ model of dementia, pitavastatin administration did not revert the effects in AChE [61]. Cibickova *et al.*2009 demonstrate that the effects of statins in AChE activity is directly associated with their lipophilicity, being more liposoluble statins that most affect AChE activity [62]. We used two statins with different lipophilicity. However, no differences among simvastatin and pravastatin were observed in their ability to affect hippocampal AChE activity of ICV-STZ treated rats.

We also observed in STZ dementia model the presence of oxidative stress, alteration that is well described in this model [37, 40, 63]. We observed an increase in ROS generation hippocampus (and cerebral cortex – data not show), accompanied by an increase in NO production in hippocampus. Besides the increase in reactive species generation, we previously described that STZ injection cause reduction in glutathione content, the most important antioxidant in brain [34]. Simvastatin and pravastatin administration prevented the over production of reactive species.

The antioxidant effect of statins is well described in the literature, and is suggested that this activity is independent of reduction in cholesterol levels [30].

Statins acts in the key enzyme in cholesterol synthesis, and beyond cholesterol reduction, these drugs reduce synthesis of mevalonate, which are important for the isoprenoid synthesis. These compounds are essential for isoprenylation, a post-translational modification that affects the activity of several proteins [31]. For example, adhesion of monocytes in inflammatory response involves a isoprenoid-dependent generation of ROS, and that this effect is inhibited by simvastatin [64]. Therefore, a mechanism involving a decrease of isoprenoid synthesis could be underlying the antioxidant effect simvastatin and pravastatin in hippocampal tissue of ICV-STZ rats. However, we did not measure the content of isoprenoids or even the cholesterol content in the hippocampus of these animals.

Astrocytes have been implicated in the AD pathogenesis, and in order to evaluate the role of these cells in STZ induced dementia model, we measured the content of two important glial markers: GFAP and S100B in hippocampus. We did not find changes in GFAP or S100B contents. However, we observed a decrease in CSF S100B levels of ICV-STZ infused rats, and both statins were able to prevent this effect. This alteration reinforces the glial involvement and confirms our previous observations in this model of dementia [63]. Interestingly, in other dementia models, like okadaic acid injection [65] and chronic hipoperfusion, also was observed a decrease of CSF S100B. Many studies have supported the concept that elevated S100B expression has pathological relevance for degeneration of the central nervous system in AD [66]. Although the CSF S100B content is not necessarily increased in AD subjects [67]. Moreover, serum S100B decrease has been described in AD patients [68].

In summary, our results confirm the spatial cognitive deficit and oxidative stress in hippocampal tissue in ICV- STZ rats, as well as the decrease of glucose uptake, the cholinergic deficit (based on increased AChE activity) observed in AD, and reduced levels of CSF S100B, also observed in other models of dementia. All these alterations could be assumed as early alterations in AD, since beta-amyloid plaques appear later in STZ model of dementia [69]. Simvastatin and pravastatin prevented theses alterations reinforcing the potential neuroprotective role of statins.

Figure Legends

Fig.1. *Cognitive performance of rats submitted to ICV-STZ injection evaluated in the water maze.* (**A**) Performance in the reference memory protocol, based on escape latency. Each line represents the mean ± standard error. * Significant differences were detected from day 3 onwards when compared to all other groups (N = 7, repeated measures analysis of variance, $p < 0.05$); (**B**) Memory in the probe trial of reference memory, based on escape latency. Values are mean ± standard error .* Significantly different from all other groups (N = 7, two-way ANOVA followed by Tukey's test, $p < 0.05$); (**C**) Number of crossings over the platform position. Values are mean ± standard error. * Significantly different from all other groups (N = 7, two-way ANOVA followed by Tukey's test, $p < 0.05$). (**D**) Memory in the probe trial of reference memory, as measured by time spent (in s) in the target quadrant. Values are mean ± standard error. *Significantly different from the control group (N = 7, two-way ANOVA followed by Tukey's test, $p < 0.05$).

Fig 2. *Glucose uptake in hippocampus of rats submitted to ICV-STZ injection and hippocampal slices treated with STZ.* (**A**) Adult rats were submitted to ICV injection of STZ. Four weeks later, hippocampi were dissected out and performed the glucose uptake assay in hippocampal slices. Values are mean ± standard error. * Significantly different from all other groups (N = 6-7, two-way ANOVA followed by Tukey's test, $p < 0.05$). (**B**) Hippocampal slices were treated for 1h with different STZ concentrations (0,2 ηM - 2μM) and after were

performed the glucose uptake. Values are mean ± standard error. N = 6, two-way ANOVA followed by Tukey's test, $p < 0.05$).

Fig 3. *Acetylcholinesterase activity in hippocampus of rats submited to ICV-STZ injection.* Adult rats were submitted to ICV injection of STZ. Four weeks later, hippocampi and cortex were dissected out and performed the acetylcholinesterase activity assay. Values are mean ± standard error. [a] Significantly different from the sham group, with and without statins, [b] Significantly different from the sham group with and without statins, and from the STZ group (N = 5-6, two-way ANOVA followed by Tukey's test, $p < 0.05$).

Fig 4. *Oxidative and nitrosative species in hippocampus of rats submitted to ICV-STZ injection.* Adult rats were submitted to ICV injection of STZ. Four weeks later, hippocampi and cortex were dissected out and the ROS content was evaluated by DCF-DA assay and NO levels were evaluated by nitrite content measured by Griess reaction. Values were measured in a fluorescence microplate reader (excitation 485 nm and emission 520 nm) and sham group without statins were assumed as being 100%. Values are mean ± standard error (**A**) ROS content in hippocampus. .* Significantly different from all other groups (N = 6-7, two-way ANOVA followed by Tukey's test, $p < 0.05$). (**B**) NO content in hippocampus. * Significantly different from all other groups (N = 6-7, two-way ANOVA followed by Tukey's test, $p < 0.05$).

Fig 5. *Oxidative damage in hippocampus of rats submitted to ICV-STZ injection.* Adult rats were submitted to ICV injection of STZ. Four weeks later, hippocampi were dissected out and submitted to lipid damage evaluation, by detection of thiobarbituric acid reactive species (TBARS) and to protein carbonyls measurement (Oxyblot™). Sham group without statins were assumed as being

100%. Values are mean ± standard error. N = 4, two-way ANOVA followed by Tukey's test, $p < 0.05$. (**A**) Hippocampal content of malondialdeide (MDA).[a] Significantly difference from sham groups, with and without statins, [b] Significantly difference from STZ group, without statins (N = 6-7, two-way ANOVA followed by Tukey's test, $p < 0.05$). (**B**) Hippocampal protein carbonyls content. Sham group was assumed to be100%. (N = 5, two-way ANOVA followed by Tukey's test, $p < 0.05$).

Fig 6. *GFAP and S100B content in hippocampus, and S100B CSF content of rats submitted to ICV-STZ injection.* Adult rats were submitted to ICV injection of STZ. Four weeks later, CSF was collected and hippocampi were dissected out to measurement of S100B and GFAP content by ELISA. Values are mean ± standard error (**A**) Hippocampal GFAP content (N = 7-8, two-way ANOVA followed by Tukey's test, $p < 0.05$). (**B**) Hippocampus content of S100B (N = 10, two-way ANOVA followed by Tukey's test, $p < 0.05$). (**C**) CSF content of S100B. * Significantly different from all other groups (N = 5-6, two-way ANOVA followed by Tukey's test, $p < 0.05$).

References

[1] Candore G, Bulati M, Caruso C, Castiglia L, Colonna-Romano G, Di Bona D, Duro G, Lio D, Matranga D, Pellicano M, Rizzo C, Scapagnini G, Vasto S (2010) Inflammation, cytokines, immune response, apolipoprotein E, cholesterol, and oxidative stress in Alzheimer disease: therapeutic implications. *Rejuvenation Res* **13**, 301-313.

[2] Maccioni RB, Munoz JP, Barbeito L (2001) The molecular bases of Alzheimer's disease and other neurodegenerative disorders. *Arch Med Res* **32**, 367-381.

[3] Selkoe DJ (2001) Alzheimer's disease: genes, proteins, and therapy. *Physiol Rev* **81**, 741-766.

[4] Arendt T (2009) Synaptic degeneration in Alzheimer's disease. *Acta Neuropathol* **118**, 167-179.

[5] Mrak RE, Griffinbc WS (2001) The role of activated astrocytes and of the neurotrophic cytokine S100B in the pathogenesis of Alzheimer's disease. *Neurobiol Aging* **22**, 915-922.

[6] Nagele RG, D'Andrea MR, Lee H, Venkataraman V, Wang HY (2003) Astrocytes accumulate A beta 42 and give rise to astrocytic amyloid plaques in Alzheimer disease brains. *Brain Res* **971**, 197-209.

[7] Simpson JE, Ince PG, Lace G, Forster G, Shaw PJ, Matthews F, Savva G, Brayne C, Wharton SB (2010) Astrocyte phenotype in relation to Alzheimer-type pathology in the ageing brain. *Neurobiol Aging* **31**, 578-590.

[8] Kodl CT, Seaquist ER (2008) Cognitive dysfunction and diabetes mellitus. *Endocr Rev* **29**, 494-511.

[9] Ott A, Stolk RP, van Harskamp F, Pols HA, Hofman A, Breteler MM (1999) Diabetes mellitus and the risk of dementia: The Rotterdam Study. *Neurology* **53**, 1937-1942.

[10] Hoyer S (2004) Glucose metabolism and insulin receptor signal transduction in Alzheimer disease. *Eur J Pharmacol* **490**, 115-125.

[11] Zhao WQ, Townsend M (2009) Insulin resistance and amyloidogenesis as common molecular foundation for type 2 diabetes and Alzheimer's disease. *Biochim Biophys Acta* **1792**, 482-496.

[12] Takeda S, Sato N, Rakugi H, Morishita R (2011) Molecular mechanisms linking diabetes mellitus and Alzheimer disease: beta-amyloid peptide, insulin signaling, and neuronal function. *Mol Biosyst* **7**, 1822-1827.

[13] Iqbal K, Grundke-Iqbal I (2008) Alzheimer neurofibrillary degeneration: significance, etiopathogenesis, therapeutics and prevention. *J Cell Mol Med* **12**, 38-55.

[14] Freude S, Hettich MM, Schumann C, Stohr O, Koch L, Kohler C, Udelhoven M, Leeser U, Muller M, Kubota N, Kadowaki T, Krone W, Schroder H, Bruning JC, Schubert M (2009) Neuronal IGF-1 resistance reduces Abeta accumulation and protects against premature death in a model of Alzheimer's disease. *FASEB J* **23**, 3315-3324.

[15] Rivera EJ, Goldin A, Fulmer N, Tavares R, Wands JR, de la Monte SM (2005) Insulin and insulin-like growth factor expression and function deteriorate with progression of Alzheimer's disease: link to brain reductions in acetylcholine. *J Alzheimers Dis* **8**, 247-268.

[16] Steen E, Terry BM, Rivera EJ, Cannon JL, Neely TR, Tavares R, Xu XJ, Wands JR, de la Monte SM (2005) Impaired insulin and insulin-like growth factor expression and signaling mechanisms in Alzheimer's disease--is this type 3 diabetes? *J Alzheimers Dis* **7**, 63-80.

[17] Baker LD, Cross DJ, Minoshima S, Belongia D, Watson GS, Craft S (2010) Insulin resistance and Alzheimer-like reductions in regional cerebral glucose metabolism for cognitively normal adults with prediabetes or early type 2 diabetes. *Arch Neurol* **68**, 51-57.

[18] Grunblatt E, Salkovic-Petrisic M, Osmanovic J, Riederer P, Hoyer S (2007) Brain insulin system dysfunction in streptozotocin intracerebroventricularly treated rats generates hyperphosphorylated tau protein. *J Neurochem* **101**, 757-770.

[19] Kuhl DE, Minoshima S, Frey KA, Foster NL, Kilbourn MR, Koeppe RA (2000) Limited donepezil inhibition of acetylcholinesterase measured with positron emission tomography in living Alzheimer cerebral cortex. *Ann Neurol* **48**, 391-395.

[20] Bohnen NI, Kaufer DI, Hendrickson R, Ivanco LS, Lopresti B, Davis JG, Constantine G, Mathis CA, Moore RY, DeKosky ST (2005) Cognitive correlates of alterations in acetylcholinesterase in Alzheimer's disease. *Neurosci Lett* **380**, 127-132.

[21] Corder EH, Saunders AM, Strittmatter WJ, Schmechel DE, Gaskell PC, Small GW, Roses AD, Haines JL, Pericak-Vance MA (1993) Gene dose of apolipoprotein E type 4 allele and the risk of Alzheimer's disease in late onset families. *Science* **261**, 921-923.

[22] Stefani M, Liguri G (2009) Cholesterol in Alzheimer's disease: unresolved questions. *Curr Alzheimer Res* **6**, 15-29.

[23] Gearing M, Mori H, Mirra SS (1996) Abeta-peptide length and apolipoprotein E genotype in Alzheimer's disease. *Ann Neurol* **39**, 395-399.

[24] Schmechel DE, Saunders AM, Strittmatter WJ, Crain BJ, Hulette CM, Joo SH, Pericak-Vance MA, Goldgaber D, Roses AD (1993) Increased amyloid beta-peptide deposition in cerebral cortex as a consequence of apolipoprotein E genotype in late-onset Alzheimer disease. *Proc Natl Acad Sci U S A* **90**, 9649-9653.

[25] Vasto S, Mocchegiani E, Candore G, Listi F, Colonna-Romano G, Lio D, Malavolta M, Giacconi R, Cipriano C, Caruso C (2006) Inflammation, genes and zinc in ageing and age-related diseases. *Biogerontology* **7**, 315-327.

[26] Matsuzaki T, Sasaki K, Hata J, Hirakawa Y, Fujimi K, Ninomiya T, Suzuki SO, Kanba S, Kiyohara Y, Iwaki T (2011) Association of Alzheimer disease pathology with abnormal lipid metabolism: the Hisayama Study. *Neurology* **77**, 1068-1075.

[27] Presecki P, Muck-Seler D, Mimica N, Pivac N, Mustapic M, Stipcevic T, Smalc VF (2011) Serum lipid levels in patients with Alzheimer's disease. *Coll Antropol* **35 Suppl 1**, 115-120.

[28] Wolozin B, Wang SW, Li NC, Lee A, Lee TA, Kazis LE (2007) Simvastatin is associated with a reduced incidence of dementia and Parkinson's disease. *BMC Med* **5**, 20.

[29] Hoglund K, Blennow K (2007) Effect of HMG-CoA reductase inhibitors on beta-amyloid peptide levels: implications for Alzheimer's disease. *CNS Drugs* **21**, 449-462.

[30] Rosendo AB, Dal-Pizzol F, Fiegenbaum M, Almeida S (2007) [Pharmacogenetics and anti-inflammatory effect of HMG-CoA reductase inhibitors]. *Arq Bras Endocrinol Metabol* **51**, 520-525.

[31] Liao JK, Laufs U (2005) Pleiotropic effects of statins. *Annu Rev Pharmacol Toxicol* **45**, 89-118.

[32] Yoshida M (2003) Potential role of statins in inflammation and atherosclerosis. *J Atheroscler Thromb* **10**, 140-144.

[33] Kuipers HF, van den Elsen PJ (2007) Immunomodulation by statins: inhibition of cholesterol vs. isoprenoid biosynthesis. *Biomed Pharmacother* **61**, 400-407.

[34] Tramontina AC, Wartchow KM, Rodrigues L, Biasibetti R, Quincozes-Santos A, Bobermin L, Tramontina F, Goncalves CA (2011) The neuroprotective effect of two statins: simvastatin and pravastatin on a streptozotocin-induced model of Alzheimer's disease in rats. *J Neural Transm* **118**, 1641-1649.
[35] Schonbeck U, Libby P (2004) Inflammation, immunity, and HMG-CoA reductase inhibitors: statins as antiinflammatory agents? *Circulation* **109**, II18-26.
[36] Duelli R, Schrock H, Kuschinsky W, Hoyer S (1994) Intracerebroventricular injection of streptozotocin induces discrete local changes in cerebral glucose utilization in rats. *Int J Dev Neurosci* **12**, 737-743.
[37] Tahirovic I, Sofic E, Sapcanin A, Gavrankapetanovic I, Bach-Rojecky L, Salkovic-Petrisic M, Lackovic Z, Hoyer S, Riederer P (2007) Reduced brain antioxidant capacity in rat models of betacytotoxic-induced experimental sporadic Alzheimer's disease and diabetes mellitus. *Neurochem Res* **32**, 1709-1717.
[38] Rodrigues L, Biasibetti R, Swarowsky A, Leite MC, Quincozes-Santos A, Quilfeldt JA, Achaval M, Goncalves CA (2009) Hippocampal alterations in rats submitted to streptozotocin-induced dementia model are prevented by aminoguanidine. *J Alzheimers Dis* **17**, 193-202.
[39] Bonda DJ, Wang X, Perry G, Nunomura A, Tabaton M, Zhu X, Smith MA (2010) Oxidative stress in Alzheimer disease: a possibility for prevention. *Neuropharmacology* **59**, 290-294.
[40] Sharma M, Gupta YK (2001) Intracerebroventricular injection of streptozotocin in rats produces both oxidative stress in the brain and cognitive impairment. *Life Sci* **68**, 1021-1029.
[41] Paxinos (1997) The Rat Nervous System. *Academic Press*.
[42] Morris R (1984) Developments of a water-maze procedure for studying spatial learning in the rat. *J Neurosci Methods* **11**, 47-60.
[43] Silva MC, Rocha J, Pires CS, Ribeiro LC, Brolese G, Leite MC, Almeida LM, Tramontina F, Ziegler DR, Goncalves CA (2005) Transitory gliosis in the CA3 hippocampal region in rats fed on a ketogenic diet. *Nutr Neurosci* **8**, 259-264.
[44] Vicente E, Tramontina F, Leite MC, Nardin P, Silva M, Karkow AR, Adolf R, Lucion AB, Netto CA, Gottfried C, Goncalves CA (2007) S100B levels in the cerebrospinal fluid of rats are sex and anaesthetic dependent. *Clin Exp Pharmacol Physiol* **34**, 1126-1130.
[45] 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.
[46] Tramontina F, Leite MC, Cereser K, de Souza DF, Tramontina AC, Nardin P, Andreazza AC, Gottfried C, Kapczinski F, Goncalves CA (2007) Immunoassay for glial fibrillary acidic protein: antigen recognition is affected by its phosphorylation state. *J Neurosci Methods* **162**, 282-286.
[47] de Almeida LM, Pineiro CC, Leite MC, Brolese G, Tramontina F, Feoli AM, Gottfried C, Goncalves CA (2007) Resveratrol increases glutamate uptake, glutathione content, and S100B secretion in cortical astrocyte cultures. *Cell Mol Neurobiol* **27**, 661-668.
[48] Hevel JM, Marletta MA (1994) Nitric-oxide synthase assays. *Methods Enzymol* **233**, 250-258.
[49] Draper HH, Hadley M (1990) Malondialdehyde determination as index of lipid peroxidation. *Methods Enzymol* **186**, 421-431.
[50] Lowry OH, Rosebrough NJ, Farr AL, Randall RJ (1951) Protein measurement with the Folin phenol reagent. *J Biol Chem* **193**, 265-275.
[51] Salkovic-Petrisic M, Tribl F, Schmidt M, Hoyer S, Riederer P (2006) Alzheimer-like changes in protein kinase B and glycogen synthase kinase-3 in rat frontal

cortex and hippocampus after damage to the insulin signalling pathway. *J Neurochem* **96**, 1005-1015.

[52] Azari NP, Pettigrew KD, Schapiro MB, Haxby JV, Grady CL, Pietrini P, Salerno JA, Heston LL, Rapoport SI, Horwitz B (1993) Early detection of Alzheimer's disease: a statistical approach using positron emission tomographic data. *J Cereb Blood Flow Metab* **13**, 438-447.

[53] Silverman DH, Small GW, Chang CY, Lu CS, Kung De Aburto MA, Chen W, Czernin J, Rapoport SI, Pietrini P, Alexander GE, Schapiro MB, Jagust WJ, Hoffman JM, Welsh-Bohmer KA, Alavi A, Clark CM, Salmon E, de Leon MJ, Mielke R, Cummings JL, Kowell AP, Gambhir SS, Hoh CK, Phelps ME (2001) Positron emission tomography in evaluation of dementia: Regional brain metabolism and long-term outcome. *JAMA* **286**, 2120-2127.

[54] Frolich L, Blum-Degen D, Bernstein HG, Engelsberger S, Humrich J, Laufer S, Muschner D, Thalheimer A, Turk A, Hoyer S, Zochling R, Boissl KW, Jellinger K, Riederer P (1998) Brain insulin and insulin receptors in aging and sporadic Alzheimer's disease. *J Neural Transm* **105**, 423-438.

[55] Cardoso S, Correia S, Santos RX, Carvalho C, Santos MS, Oliveira CR, Perry G, Smith MA, Zhu X, Moreira PI (2009) Insulin is a two-edged knife on the brain. *J Alzheimers Dis* **18**, 483-507.

[56] Pathan AR, Viswanad B, Sonkusare SK, Ramarao P (2006) Chronic administration of pioglitazone attenuates intracerebroventricular streptozotocin induced-memory impairment in rats. *Life Sci* **79**, 2209-2216.

[57] Olafsdottir E, Aspelund T, Sigurdsson G, Thorsson B, Eiriksdottir G, Harris TB, Launer LJ, Benediktsson R, Gudnason V (2011) Effects of statin medication on mortality risk associated with type 2 diabetes in older persons: the population-based AGES-Reykjavik Study. *BMJ Open* **1**, e000132.

[58] Riad A, Du J, Stiehl S, Westermann D, Mohr Z, Sobirey M, Doehner W, Adams V, Pauschinger M, Schultheiss HP, Tschope C (2007) Low-dose treatment with atorvastatin leads to anti-oxidative and anti-inflammatory effects in diabetes mellitus. *Eur J Pharmacol* **569**, 204-211.

[59] Sabbagh M, Cummings J (2011) Progressive cholinergic decline in Alzheimer's Disease: consideration for treatment with donepezil 23 mg in patients with moderate to severe symptomatology. *BMC Neurol* **11**, 21.

[60] Lahiri DK, Rogers JT, Greig NH, Sambamurti K (2004) Rationale for the development of cholinesterase inhibitors as anti-Alzheimer agents. *Curr Pharm Des* **10**, 3111-3119.

[61] Sharma B, Singh N, Singh M (2008) Modulation of celecoxib- and streptozotocin-induced experimental dementia of Alzheimer's disease by pitavastatin and donepezil. *J Psychopharmacol* **22**, 162-171.

[62] Cibickova L, Palicka V, Cibicek N, Cermakova E, Micuda S, Bartosova L, Jun D (2007) Differential effects of statins and alendronate on cholinesterases in serum and brain of rats. *Physiol Res* **56**, 765-770.

[63] Rodrigues L, Dutra MF, Ilha J, Biasibetti R, Quincozes-Santos A, Leite MC, Marcuzzo S, Achaval M, Goncalves CA (2010) Treadmill training restores spatial cognitive deficits and neurochemical alterations in the hippocampus of rats submitted to an intracerebroventricular administration of streptozotocin. *J Neural Transm* **117**, 1295-1305.

[64] Park SY, Lee JS, Ko YJ, Kim AR, Choi MK, Kwak MK, Choi HG, Yong CS, Kim JA (2008) Inhibitory effect of simvastatin on the TNF-alpha- and angiotensin II-induced monocyte adhesion to endothelial cells is mediated through the suppression of geranylgeranyl isoprenoid-dependent ROS generation. *Arch Pharm Res* **31**, 195-204.

[65] Costa AP, Tramontina AC, Biasibetti R, Batassini C, Lopes MW, Wartchow KM, Bernardi C, Tortorelli LS, Leal RB, Goncalves CA (2010) Neuroglial alterations

in rats submitted to the okadaic acid-induced model of dementia. *Behav Brain Res*.

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

[67] Peskind ER, Griffin WS, Akama KT, Raskind MA, Van Eldik LJ (2001) Cerebrospinal fluid S100B is elevated in the earlier stages of Alzheimer's disease. *Neurochem Int* **39**, 409-413.

[68] Chaves ML, Camozzato AL, Ferreira ED, Piazenski I, Kochhann R, Dall'Igna O, Mazzini GS, Souza DO, Portela LV (2010) Serum levels of S100B and NSE proteins in Alzheimer's disease patients. *J Neuroinflammation* **7**, 6.

[69] Salkovic-Petrisic M, Osmanovic-Barilar J, Bruckner MK, Hoyer S, Arendt T, Riederer P (2011) Cerebral amyloid angiopathy in streptozotocin rat model of sporadic Alzheimer's disease: a long-term follow up study. *J Neural Transm* **118**, 765-772.

Figure 1

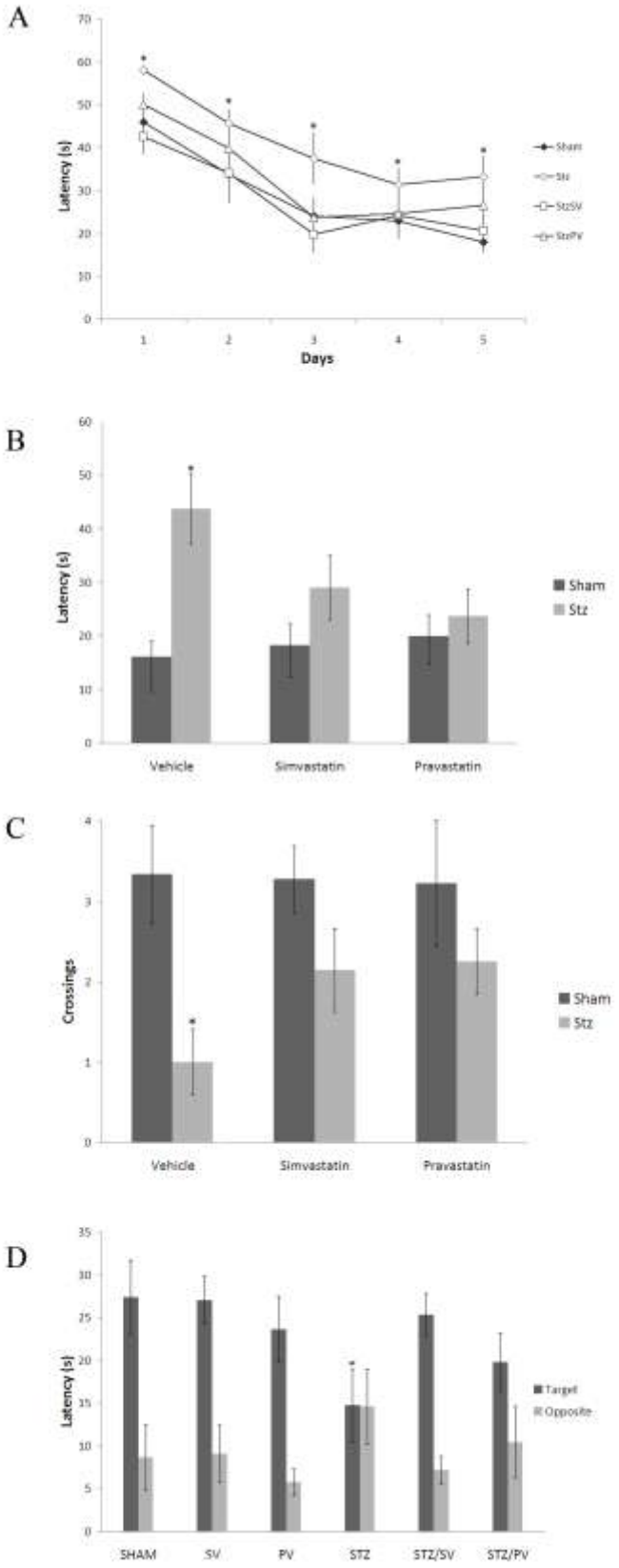

A
Latency (s)
Days
Sham
Stz
StzSV
StzPV
B
Latency (s)
Vehicle
Simvastatin
Pravastatin
Sham
Stz
C
Crossings
Vehicle
Simvastatin
Pravastatin
Sham
Stz
D
Latency (s)
SHAM
SV
PV
STZ
STZ/SV
STZ/PV
Target
Opposite

Figure 2

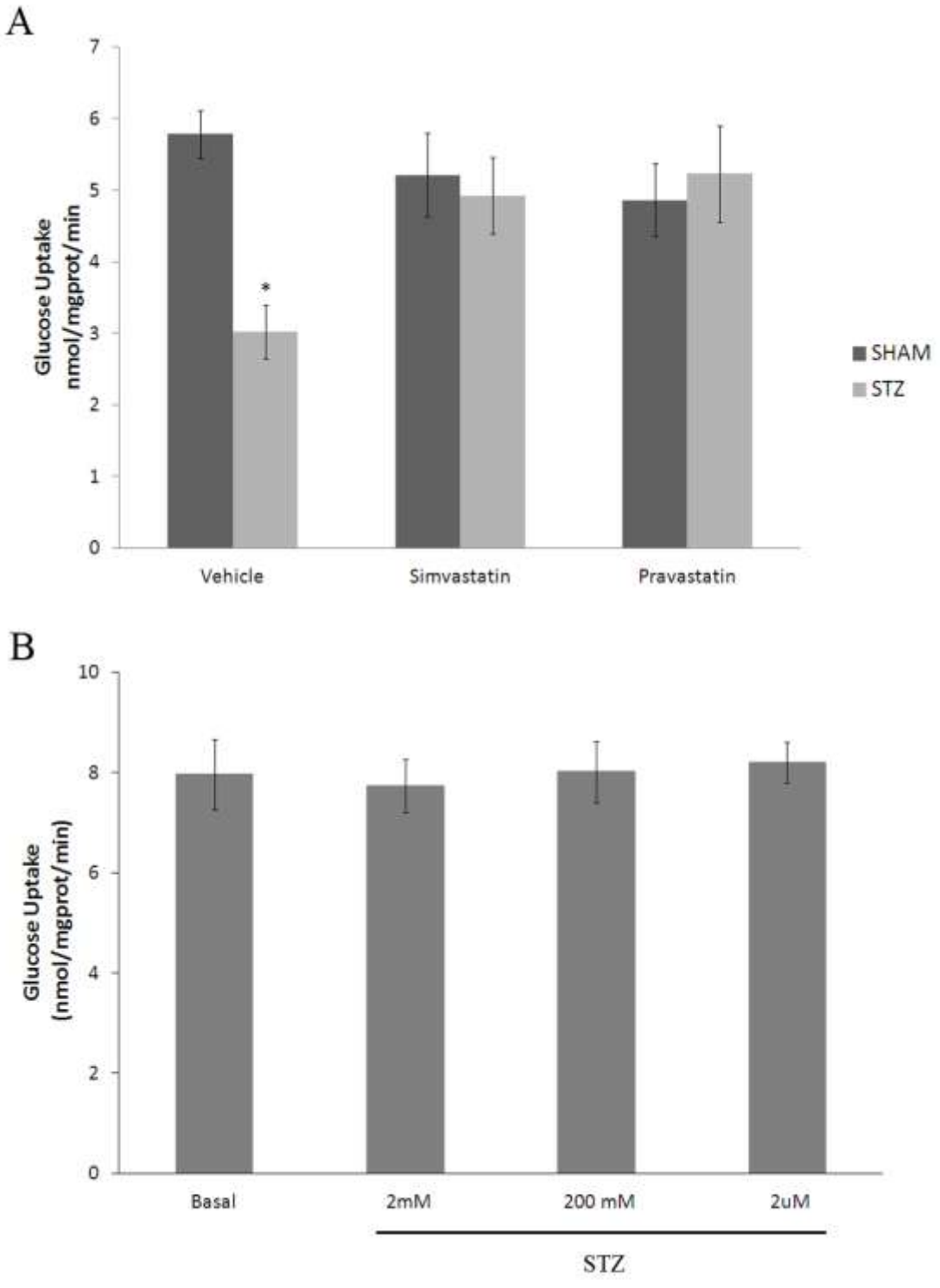

A
Glucose Uptake
nmol/mgprot/min
7
6
5
4
3
2
1
0
*
Vehicle
Simvastatin
Pravastatin
SHAM
STZ
B
Glucose Uptake
(nmol/mgprot/min)
10
8
6
4
2
0
Basal
2mM
200 mM
2uM
STZ

Figure 3

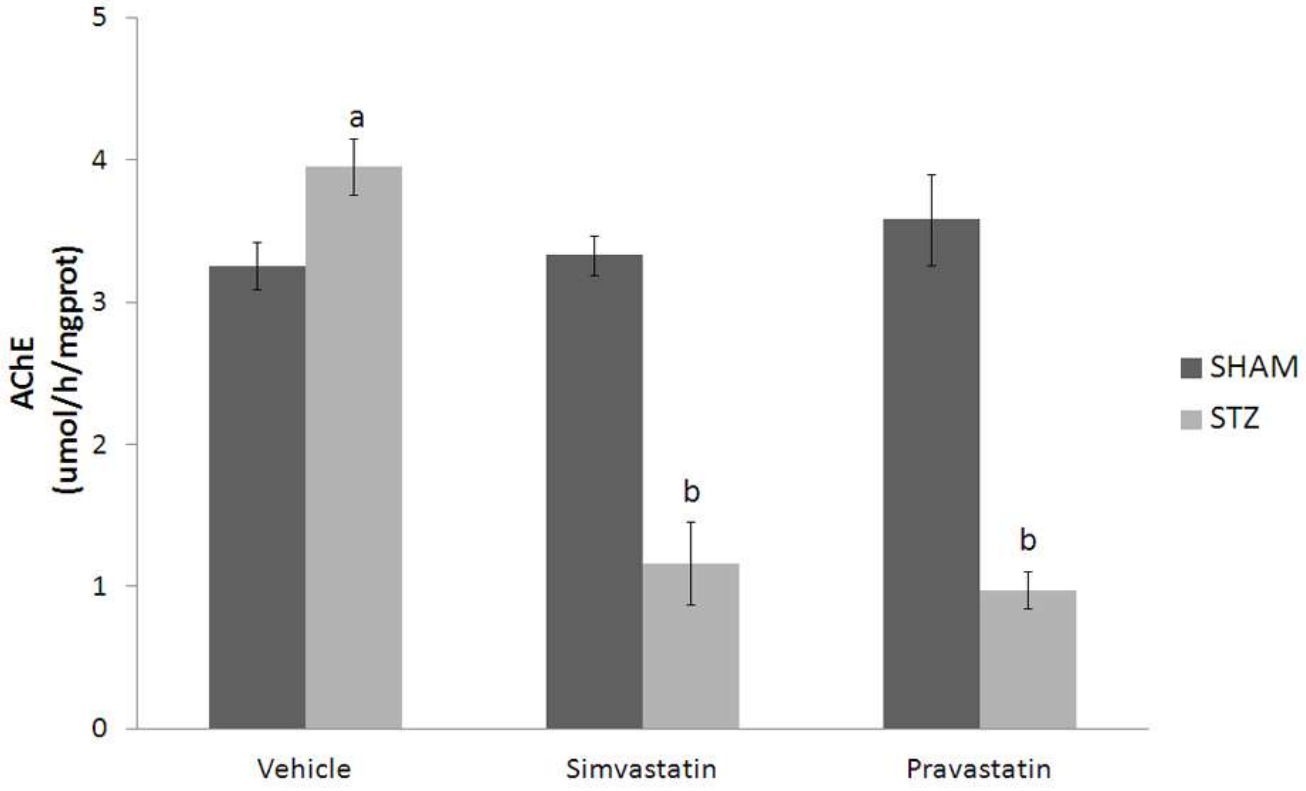

AChE
(umol/h/mgprot)
5
4
3
2
1
0
a
b
b
Vehicle
Simvastatin
Pravastatin
SHAM
STZ

Figure 4

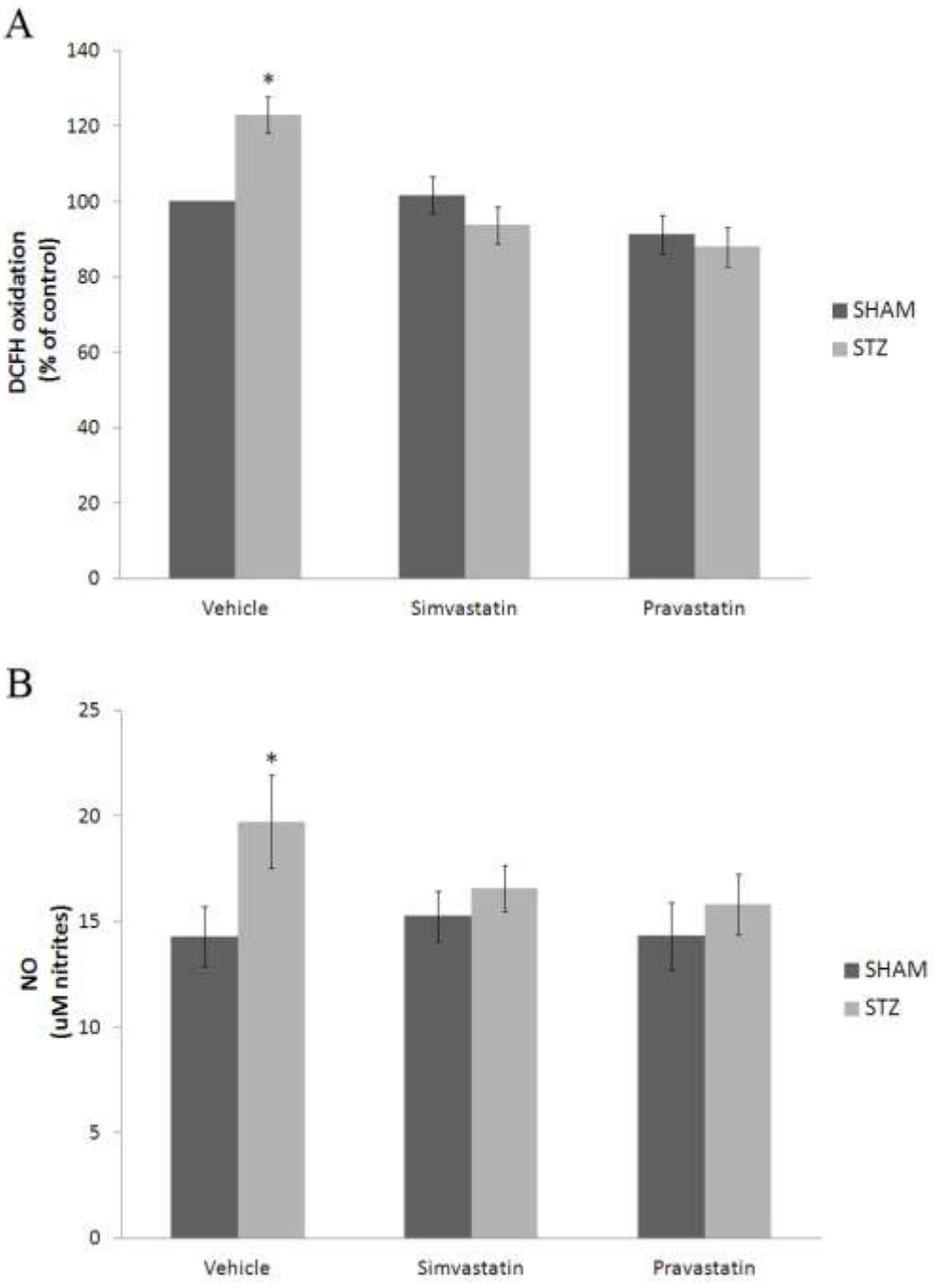

A
140
120
100
80
60
40
20
0
DCFH oxidation
(% of control)
*
Vehicle
Simvastatin
Pravastatin
SHAM
STZ
B
25
20
15
10
5
0
NO
(uM nitrites)
*
Vehicle
Simvastatin
Pravastatin
SHAM
STZ

Figure 5

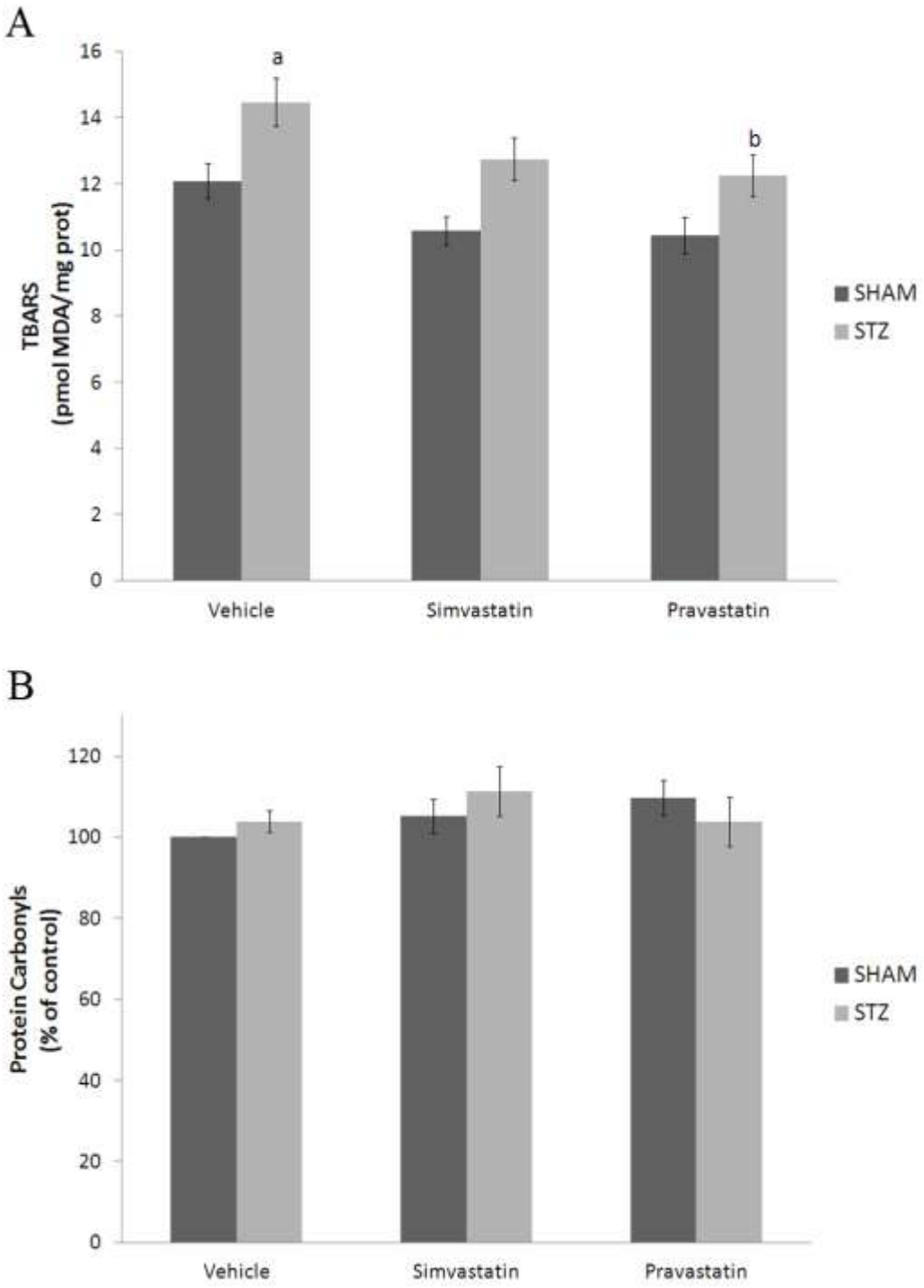
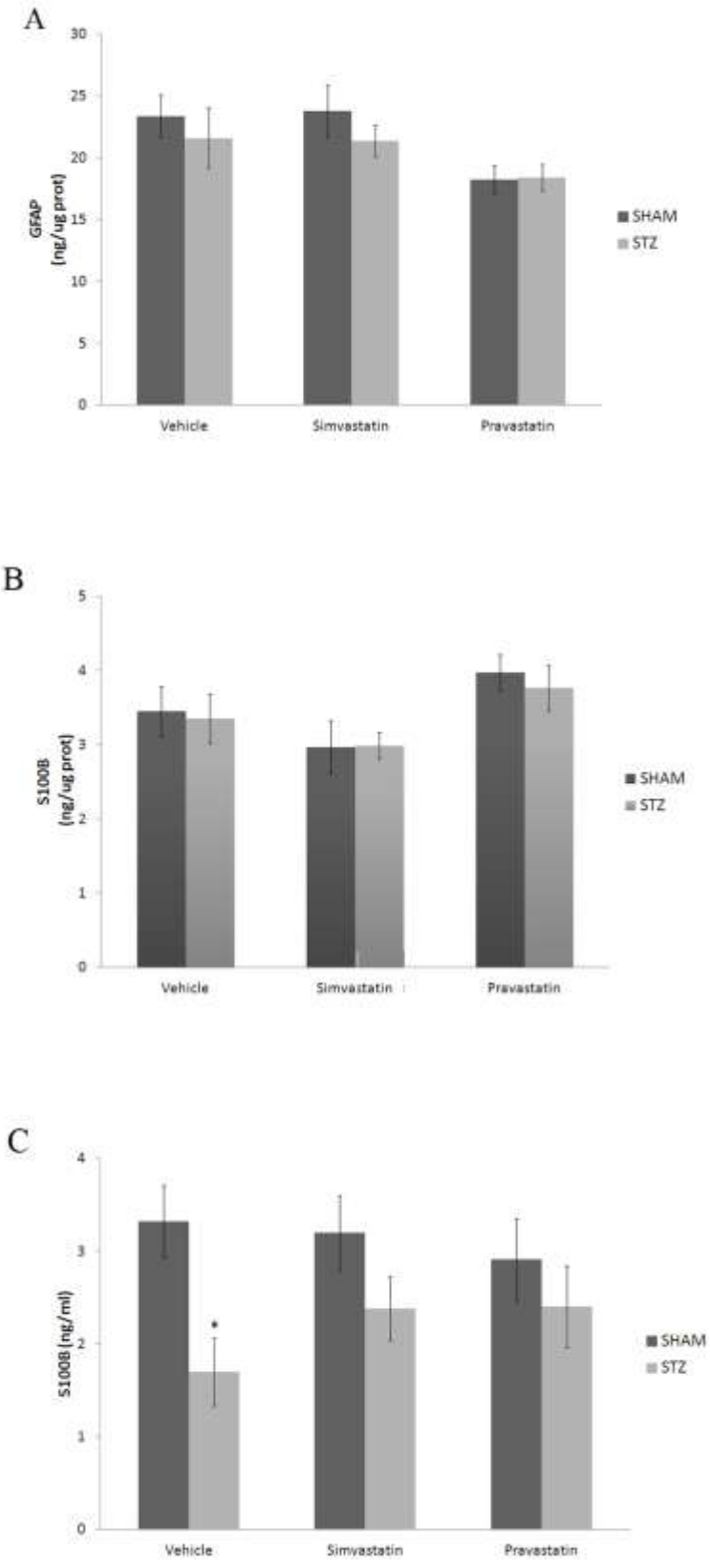

A
TBARS (pmol MDA/mg prot)
16
14
12
10
8
6
4
2
0
a
b
Vehicle
Simvastatin
Pravastatin
SHAM
STZ
B
Protein Carbonyls (% of control)
120
100
80
60
40
20
0
Vehicle
Simvastatin
Pravastatin
SHAM
STZ

Figure 6


A
GFAP (ng/ug prot)
30
25
20
15
10
5
0
Vehicle
Simvastatin
Pravastatin
SHAM
STZ
B
S100B (ng/ug prot)
5
4
3
2
1
0
Vehicle
Simvastatin
Pravastatin
SHAM
STZ
C
S100B (ng/ml)
4
3
2
1
0
*
Vehicle
Simvastatin
Pravastatin
SHAM
STZ

PARTE III

Discussão

1. Resultados *in vitro*

O DM é uma desordem metabólica frequentemente associada a diversas complicações, tanto periféricas quanto centrais [41]. No SNC ocorrem inúmeras alterações, incluindo alterações de neurotransmissores, bem como alterações eletrofisiológicas, mudanças estruturais e prejuízo na memória e cognição [163].

Uma das primeiras alterações que ocorrem em resposta a altas concentrações de glicose é o desenvolvimento de estresse oxidativo[164], e no SNC as altas concentrações de glicose podem levar à neurotoxicidade. De acordo com estudos prévios, a incubação das células de glioma C6 com altas concentrações de glicose causou um aumento na produção de ERO e nitrogênio, bem como a redução dos níveis do antioxidante GSH.

Um dos possíveis mecanismos pelo qual a glicose pode levar ao desenvolvimento de estresse oxidativo é o aumento da atividade da via do poliol [165]. Nessa via, a glicose é reduzida a sorbitol e frutose, ocorrendo consumo de NADPH, que é essencial para a regeneração da GSH. Ocorrendo redução dos níveis de NADPH, a regeneração da GSH fica prejudicada, e os baixos níveis de GSH na célula podem ser refletidos no desenvolvimento de estresse oxidativo [164].

Altas concentrações de glicose também levam à geração de superóxido através da interrupção da cadeia transportadora de elétrons no complexo III [166]. A combinação de superóxido com óxido nítrico (NO) pode levar à formação de peroxinitrito, que atua diretamente sobre o DNA[164]. De fato, nossos resultados estão de acordo com esses dados, visto que as altas

concentrações de glicose causaram aumento da geração de ERO e NO, e o desenvolvimento do estresse oxidativo levou a um aumento muito grande na lesão do DNA, quando comparadas com células mantidas em condições normais.

A fim de avaliar os efeitos da produção de NO, avaliamos além do dano ao DNA, a atividade da enzima GS. Essa enzima está presente em astrócitos, e é responsável pela conversão do glutamato captado em glutamina, que será utilizada pelos neurônios para a renovação desse neurotransmissor [65]. Sua atividade é modulada negativamente pelo NO [167], o que vem de acordo com os resultados apresentados, visto que a atividade da GS foi reduzida nas células expostas a altas concentrações de glicose.

Alguns estudos correlacionam o desenvolvimento do estresse oxidativo com alterações no metabolismo do glutamato [168, 169], as quais são descritas no DM [170], porém os efeitos das altas concentrações de glicose na captação deste neurotransmissor ainda não são totalmente elucidados. Elevações dos níveis extracelulares de glutamato têm sido descritas em pacientes com DM [171], e receptores e transportadores de glutamato têm sido estudados em pacientes e modelos da doença.

Estudos indicam que os transportadores de glutamato são regulados pelo estado redox, possuindo maior atividade no estado reduzido [169], sendo o estresse oxidativo um fator que atua reduzindo a captação desse neurotransmissor.

Inesperadamente, a incubação de células de glioma C6 com altas concentrações de glicose causou um aumento na captação de glutamato, mesmo na presença de estresse oxidativo, efeito não observado em culturas

de astrócitos cultivadas nas mesmas condições [172]. Recentemente, aumento da captação de glutamato tem sido descrita em células gliais de retina de ratos diabéticos [173], porém resultados opostos já foram descritos, onde a captação reduziu em preparados de vesículas gliais de ratos diabéticos [75].

Astrócitos cultivados expostos a altas concentrações de glicose não tem a captação de glutamato alterada [172], ao contrário das células C6, porém essa diferença pode ser devida aos transportadores de glutamato expressos nos dois tipos celulares. Os astrócitos expressam principalmente os transportadores GLAST/EAAT1 e GLT1/EAAT2, enquanto as células C6 também apresentam o transportador EAAT3, o mesmo presente em neurônios [174].

Tendo em vista os diferentes perfis de captação de glutamato entre os astrócitos e células C6 expostas à alta concentração de glicose, e os diferentes perfis de expressão de transportadores de glutamato, acreditamos que o efeito modulador do estresse oxidativo sobre os mesmos possa ocorrer de acordo com o tipo de transportador.

Para avaliar se o efeito das altas concentrações de glicose sobre a captação de glutamato foi devido a alterações na expressão do transportador ou ao aumento da afinidade do mesmo, realizamos a avaliação da expressão de EAAT3 por western blotting, e não observamos alteração na expressão dos mesmos. Vários estudos sobre a expressão de transportadores de glutamato em modelos animais de DM têm demonstrado que não ocorre a alteração na expressão dos mesmos, em astrócitos de retina [173] e em hipocampo, cerebelo e córtex [75].

Tem sido demonstrado que a neurotoxicidade da glicose pode ser mediada não só pelos mecanismos já comentados, mas também pela formação de AGEs, que atuam contribuindo para o aumento do estresse oxidativo em condições de alta concentração de glicose [165]. Para avaliar se o efeito das altas concentrações de glicose sobre a captação de glutamato é devido ao estresse oxidativo ou à formação de AGEs, utilizamos a NAC, que possui efeito antioxidante, e a AG, que possui efeito anti-glicação.

A AG reduz a formação de AGEs por interagir com os produtos de Amadori, intermediários da via de formação dos AGEs, e estudos já demonstraram efeitos benéficos deste composto em ratos diabéticos transgênicos [175]. A NAC possui efeito antioxidante bem descrito, e estudos têm demonstrado efeitos benéficos deste composto em modelos animais de DA [102] e DM [176].

A AG e a NAC não foram capazes de reverter o efeito das altas concentrações de glicose sobre a captação de glutamato. Entretanto, a AG foi preveniu o aumento da captação de glutamato, enquanto a NAC não apresentou efeito preventivo. Avaliando esses resultados, acreditamos que a modulação da captação de glutamato causada pelas altas concentrações de glicose em células de glioma C6 é decorrente de efeitos de glicação, e não de oxidação.

Estudos prévios do nosso grupo demonstraram que as altas concentrações de glicose aumentam não apenas a captação de glutamato em células de glioma C6, mas também a secreção de S100B [172]. Novamente utilizamos NAC e AG para avaliar os efeitos da oxidação e glicação sobre a secreção de S100B, porém nenhum dos compostos foi capaz de prevenir ou

reverter os efeitos das altas concentrações de glicose sobre a secreção da proteína.

Tramontina *et al.* demonstrou que a captação de glutamato e secreção da proteína S100B estão interligados de alguma forma. A utilização de antioxidantes como as epicatequinas e o resveratrol estimulam tanto a secreção de S100B como a captação de glutamato [177, 178]. Também já foi demonstrado pelo nosso grupo que a adição de S100B causa um aumento na captação de glutamato em culturas de astrócitos hipocampais [179], resultado esse que repetimos em células C6, que possuem expressão diferenciada de transportadores de glutamato, quando comparadas com astrócitos. É importante ressaltar que o efeito da S100B sobre a secreção de glutamato, tanto em cultura de astrócitos quanto em células C6, é independente das altas concentrações de glicose.

Dessa forma, avaliando os resultados que foram discutidos, observamos o desenvolvimento de estresse oxidativo em células C6 cultivas com altas concentrações de glicose, causando dano ao DNA. Esses achados vêm de encontro com dados presentes na literatura, que demonstram o envolvimento do estresse oxidativo nas complicações do DM[164]. Surpreendentemente, a captação de glutamato foi aumentada com os altos níveis de glicose, e esse efeito foi prevenido pela administração de AG. A alteração de parâmetros gliais na presença de altas concentrações de glicose reforça que a glia pode estar envolvida na patogênese do DM.

2. Resultados *in vivo*

A DA é uma desordem neurodegenerativa progressiva, e possui características histopatológicas e bioquímicas conhecidas, embora sua causa ainda seja desconhecida [40]. Os pacientes com DA apresentam problemas na cognição, com progressivas perdas de memória e alterações de comportamento [6]. Na DA, além da presença de depósitos amilóides e da proteína tau fosforilada, ocorre o desenvolvimento de estresse oxidativo, alterações no metabolismo energético, alterações de neurotransmissores e uma extensa perda neuronal [40].

A DA tem sido extensamente associada a alterações no metabolismo lipídico, sendo a hipercolesterolemia um dos fatores que poderiam levar ao aumento do risco de desenvolvimento da doença [130], bem como a presença do alelo ε4 para a ApoE [34]. O envolvimento do colesterol na patogênese da DA ainda é controverso, porém inúmeros estudos apontam os efeitos deletérios do colesterol. Grupos de pesquisa têm demonstrado o acúmulo de colesterol nas placas neuríticas, tanto em cérebros de pacientes com DA como em modelos animais da doença [22]. Aumento de produtos de metabolização do colesterol, como o 24-hidroxi-colesterol, que é derivado quase que exclusivamente do colesterol cerebral [18], estão aumentados em plasma de pacientes com DA moderada[180], e a marcação de colesterol em cérebro de pacientes com DA está aumentada em neurônios presentes na periferia dos emaranhados neurofibrilares[181].

Devido à possibilidade de envolvimento do colesterol na patogênese da DA, inúmeros grupos de pesquisa têm avaliado o efeito de drogas redutoras de colesterol, principalmente as estatinas na redução da incidência de DA[35] ou

em modelos animais da doença [100, 155]. Jick *et al* [35] demonstrou que a administração de estatinas em pacientes com mais de 50 anos está diretamente relaciona à redução da incidência de DA, independente da presença ou ausência de hipercolesterolemia.

A infusão ICV de STZ é um modelo que tem sido extensamente utilizado para mimetizar as alterações que ocorrem na DA [78, 113]. Vários autores têm demonstrado que a injeção de STZ causa déficit cognitivo, de duas a oito semanas após a cirurgia, tanto em avaliação por labirinto aquático de Morris [78] [123], como na esquiva inibitória [113].

De acordo com estudos prévios, demonstramos que ratos expostos à injeção ICV de STZ apresentam um acentuado déficit cognitivo, que foi avaliado em labirinto aquático de Morris. Além da presença do déficit cognitivo no modelo, observamos que tanto a PV quanto a SV foram capazes de prevenir o dano causado por STZ, e não apresentaram efeito "*per* se". É importante ressaltar, que os efeitos das estatinas na cognição e nos demais parâmetros estudados, foram independentes da redução do colesterol plasmático. Estudos têm demonstrado os efeitos das estatinas sobre o déficit cognitivo que ocorre em pacientes com DA e em modelos animais de demência, porém os resultados ainda são controversos.

Em modelo de demência induzido em ratos por administração de βA, a administração de atorvastatina não foi capaz de reverter o dano cognitivo [100], enquanto a administração de fluvastatina, em animais submetidos ao mesmo modelo de demência foi capaz de prevenir os danos na cognição [99]. A administração de pitavastatina também foi capaz de reverter o dano cognitivo, dessa vez em modelo por injeção ICV de STZ em ratos [155]. Um estudo de

2010 demonstrou que a administração de atorvastatina a pacientes com sintomas moderados de DA causou uma melhora nos resultados em testes de avaliação cognitiva [182].

Um estudo publicado em 2006 por Li *et al.* [183], demonstrou que a SV, além de reverter o déficit cognitivo encontrado em ratos transgênicos com maior expressão de APP, causou uma melhora na cognição em ratos não transgênicos, efeito esse que não observamos no nosso trabalho.

Sabe-se que alterações de mecanismos de neurotransmissão podem estar envolvidas na patogênese da DA, podendo levar às alterações na cognição [100]. O déficit colinérgico já é bem descrito na DA [184], porém a neurotransmissão glutamatérgica também está alterada.

O glutamato, principal neurotransmissor excitatório do SNC, tem envolvimento em processos de aprendizado e memória [100], e seu metabolismo inclui a participação ativa dos astrócitos, através da captação de glutamato [65]. Na DA, dois estágios de alterações glutamatérgicas são descritos, ocorrendo hipo ou hiperatividade dependendo do estágio da doença [185].

A fim de avaliar um possível envolvimento da excitotoxicidade glutamatérgica e dos astrócitos no modelo de DA induzido por STZ, avaliamos a captação de glutamato e o efeito das estatinas em fatias hipocampais. De acordo com trabalho prévio publicado por nosso grupo [78], observamos neste estudo que a STZ não causou alteração na captação de glutamato, entretanto a SV apresentou efeito "*per se"*, levando a um aumento na captação de glutamato.

Outros modelos de demência já demonstraram alterações no metabolismo glutamatérgico, com redução da captação de glutamato em fatias de hipocampo de ratos submetidos à administração de βA, com reversão pela adição de atorvastatina [100], em modelo de destruição do núcleo basal magnocelular [106], e no modelo de hipoperfusão cerebral crônica [186].

Ao nosso conhecimento, é a primeira vez que se descreve o efeito da SV sobre a captação de glutamato em fatias hipocampais, porém outros grupos de pesquisa já demonstraram que essa estatina possui a capacidade de interferir no metabolismo glutamatérgico.

Em modelo de demência induzido pela administração de βA, a atorvastatina foi capaz de reverter a redução da expressão dos transportadores de glutamato GLAST e GLT-1 [100]. Estudo utilizando modelo de doença de Parkinson demonstrou que após administração de 6-hidroxidopamina ocorre uma redução dos receptores do tipo NMDA de glutamato, enquanto o tratamento com SV é capaz de reverter esse efeito [187]. Em estudo com ratos sadios, a SV causou um aumento nos receptores NMDA [188], e em modelo de excitotoxicidade por administração de kainato, um agonista glutamatérgico, a SV foi a estatina mais eficiente na prevenção dos danos [189], porém o mecanismo pelo qual a SV exerce seus efeitos sobre o metabolismo glutamatérgico ainda é desconhecido.

Apesar de não ter ocorrido alteração na captação de glutamato, a STZ induziu uma redução na atividade da GS, a enzima responsável pela conversão de glutamato em glutamina nos astrócitos, de acordo com estudos prévios do nosso grupo [78]. Tanto a SV quanto a PV preveniram esse efeito, e além do

efeito de prevenção, as estatinas apresentaram efeito nos animais não tratados com STZ, causando um aumento na atividade da GS.

A atividade da enzima GS é diretamente modulada por NO. Segundo Kosenko *et al.* [167], a atividade da enzima é diminuída pelo NO, via nitração ou nitrosilação. As estatinas são agentes que sabidamente causam um aumento da atividade da óxido nítrico sintase endotelial (eNOS) [190], o que levaria a um aumento da NO, porém estudos demonstram que as estatinas possuem um papel dual na modulação da NOS.

A produção de NO no sistema nervoso central em resposta à inflamação ocorre em astrócitos, a partir da ativação da iNOS, o que levaria ao aumento excessivo de NO, desenvolvendo estresse por espécies reativas de nitrogênio. Trabalhos demonstram que as estatinas, além de causarem aumento na ativação da eNOS, são capazes de reduzir a atividade da iNOS, o que seria decorrente da redução da liberação de citocinas pró-inflamatórias, como o fator de necrose tumoral-α (TNFα) [191].

Uma vez possuindo esse efeito, explica-se facilmente a ação das estatinas sobre a GS, sendo que a redução da liberação de NO pela SV e PV afeta diretamente a atividade da GS. Efeito semelhante já foi demonstrado pelo nosso grupo, onde a AG, que também atua modulando o NO, causou uma reversão na redução de GS causada por STZ, além de aumentar a atividade da mesma nos animais não submetidos ao modelo de demência [78].

Além do desenvolvimento de alterações cognitivas, a injeção ICV de STZ também leva ao desenvolvimento de estresse oxidativo [113, 135], alterações no metabolismo energético, com redução da utilização da glicose [112] e alterações nas vias de sinalização da insulina [123, 147].

Nossos resultados demonstram claramente o efeito da STZ sobre a geração de radicais livres e redução da capacidade antioxidante. A STZ causou uma redução na quantidade de GSH em hipocampo, e ambas as estatinas utilizadas preveniram esse efeito. O efeito das estatinas sobre a GSH já foi demonstrado em modelo pré-clínico de DA em cachorros, e o aumento no conteúdo de GSH por atorvastatina foi diretamente correlacionado ao aumento nos níveis da heme oxigenase-1 (HO-1) [192]. A HO-1 e a γ-glutamilcisteina sintetase, enzima chave da síntese de GSH, tem sua expressão mediada por Nrf2, e já foram demonstrados efeitos estimulatórios das estatinas sobre esse fator [193].

Além da redução da GSH, outra evidência da ocorrência de estresse oxidativo no modelo de demência induzido por STZ é o aumento na produção de ERO em hipocampo e córtex, avaliado pela técnica de DCF-DA. Mais uma vez as estatinas utilizadas demonstraram efeito benéfico, prevenindo o aumento de ERO. Em córtex, a PV foi capaz de prevenir totalmente o efeito da STZ, enquanto a SV preveniu parcialmente. Em hipocampo, ambas as estatinas preveniram totalmente o efeito da STZ. Novamente, é importante lembrar que o efeito das estatinas foi independente da redução dos níveis plasmáticos de colesterol.

Os efeitos antioxidantes das estatinas são bem descritos na literatura, e acredita-se que eles estejam relacionados aos efeitos pleiotrópicos das estatinas, ou seja, efeitos independentes da redução do colesterol. As estatinas atuam sobre a enzima chave da síntese de colesterol, a HMG-CoA redutase, reduzindo não só os níveis de colesterol, mas também os níveis de mevalonato. Tendo reduzida a produção desse intermediário, também ocorre

uma redução da síntese de isoprenóides, moléculas responsáveis por modificações pós-traducionais de proteínas [87] .

A redução da formação de isoprenóides afeta diretamente as GTPases, proteínas que possuem inúmeras funções celulares. Uma subfamília de GTPases que é diretamente afetada pela redução da isoprenilação é a das Rho GTPases, que desempenham importantes papéis na sinalização inflamatória [89]. Um membro dessa subfamília, a Rac, é um fator essencial para a ativação da enzima NADPH oxigenase, responsável pela produção de ERO [194]. Com a redução da isoprenilação da Rac pelas estatinas, ocorre uma redução da sua função [89], o que poderia levar à redução da atividade da NADPH oxidase, sendo esse um dos possíveis mecanismos pelos quais as estatinas reduzem a produção de ERO.

Além do aumento da produção de ERO, a STZ também causou um aumento na produção de NO em hipocampo, avaliada através do conteúdo de nitritos. Novamente as estatinas foram capazes de prevenir o aumento na geração de NO, não apresentando efeito *"per se"*. Não ocorreu alteração na produção de NO em córtex.

O aumento na produção de NO pela administração de STZ já foi descrito por trabalhos prévios do nosso grupo de pesquisa [78], e esse aumento de NO pode estar diretamente relacionado ao desenvolvimento de estresse oxidativo. A ação das estatinas sobre o NO já foi descrita anteriormente, sendo que essas drogas atuam aumentando a eNOS, porém causando uma redução na expressão da iNOS, o que estaria levando à prevenção do efeito da STZ.

A administração de estatinas também está relacionada com o aumento da expressão da enzima HO-1 [192], e a atuação dessa enzima gera

metabólitos como o monóxido de carbono e ferro, que poderiam estar atuando na redução da expressão da iNOS, contribuindo ainda mais para a redução da produção de NO [195].

O aumento da produção de ERO e NO, juntamente com a redução da GSH levou ao aumento da lipoperoxidação nos animais tratados com STZ, avaliada pelo conteúdo de malondialdeído, em córtex e hipocampo. Nesse parâmetro, apenas a PV apresentou efeito protetor, prevenindo o efeito da STZ tanto em córtex quanto em hipocampo. Estudos prévios demonstraram o efeito da pitavastatina na redução de peroxidação lipídica em modelo de demência induzido por STZ [155], e da atorvastatina em modelo de demência induzido pela administração de βA [100].

Além da peroxidação lipídica, avaliamos também o conteúdo de proteínas carboniladas, porém para nossa surpresa a STZ não foi capaz de causar dano a proteínas. No modelo de demência induzido por ácido ocadáico, o dano a proteínas pode ser observado pelo aumento no conteúdo de carbonilação [196], e em modelo de demência em cachorros também ocorre aumento do estresse oxidativo e dano à proteínas, sendo que a administração de atorvastatina foi capaz de reverter o dano[98]. Até onde sabemos, não existem trabalhos publicados demonstrando efeito da STZ sobre a carbonilação protéica.

Alterações metabólicas ocorrem desde os estágios iniciais da DA, e incluem redução da utilização de glicose no cérebro e alteração das vias de sinalização da insulina, podendo ocorrer resistência à ação dessa molécula. Essas alterações são semelhantes àquelas que ocorrem em pacientes com DM, o que levou grupos de pesquisa a denominar a DA como o “Diabetes tipo

III”[197]. Essa hipótese foi investigada através da avaliação *post-mortem* de cérebro de paciente com DA. De la Monte *et al.* demonstrou que a DA avançada está diretamente associada à redução dos níveis de insulina, fator de crescimento semelhante à insulina (IGF), adicionado ao fato de o metabolismo energético estar seriamente alterado na DA [40].

A injeção ICV de STZ tem sido descrita como um modelo que mimetiza bem os achados do Diabetes tipo III, e alterações nas vias de sinalização da insulina, com redução dos RI e de IGF já foram descritas no modelo [123], bem como a redução da atividade de enzimas da via glicolítica [111].

Um dos achados mais interessantes do nosso trabalho, foi a redução da captação de glicose causada pela administração de STZ, e o efeito de prevenção que as estatinas apresentaram. A redução da utilização de glicose em animais submetidos ao modelo de demência por STZ já foi extensamente descrita [111, 112], porém acreditamos ser a primeira vez que é demonstrada redução da captação de glicose em fatias hipocampais. O efeito da SV e da PV na prevenção desse efeito também é muito interessante. Alguns trabalhos têm demonstrado efeitos benéficos das estatinas em pacientes com DM [153] e em modelo animal da doença[154], porém nenhum trabalho demonstrou o efeito das estatinas sobre a captação de glicose, ou sobre os níveis de glicose séricos.

A utilização de glicose através das reações da via glicolítica é a principal fonte de ATP e acetil-CoA no SNC [123], e com sua captação diminuída, esses compostos ficam reduzidos, o que poderia prejudicar a síntese de acetilcolina. O déficit colinérgico é bem descrito na DA, e tem sido extensamente relacionado ao grau do déficit cognitivo [125]. Além da redução dos compostos

energéticos, as altas concentrações de glicose circulantes podem levar à produção de AGEs, que por sua vez atuam aumentando ainda mais a produção de ERO, contribuindo para o estresse oxidativo desenvolvido no modelo.

No modelo de demência utilizado, observamos que a STZ causa um aumento na atividade da AChE, enzima responsável pela degradação da acetilcolina. Essa alteração já foi demonstrada por outros grupos de pesquisa [155]. Observamos também que a administração de SV e PV foi capaz de prevenir a alteração causada por STZ, causando uma redução da atividade da AChE em torno de 60 a 70%.

Não sabemos exatamente como as estatinas atuam modulando a atividade dessa enzima, mas estudos prévios demonstram a redução da atividade da enzima em córtex de animais sadios tratados com SV [156]. Neste estudo, porém, apenas a estatina capaz de cruzar a BHE foi efetiva, enquanto no nosso trabalho, ambas as estatinas exerceram efeito sobre a atividade da enzima. Outros estudos, entretanto, apresentam efeitos contrários, onde a pitavastina não foi capaz de reverter o aumento de AChE causado pela administração ICV de STZ [155].

A capacidade que a PV e SV apresentaram de prevenir os danos cognitivos causados pela STZ pode estar diretamente associada à redução da atividade da AChE, aumentando assim a disponibilidade de acetilcolina, neurotransmissor importante nos processos de aprendizado e memória.

Além da extensa morte de neurônios colinérgicos que ocorre na DA, alterações astrocíticas também estão presentes, e podem contribuir para a patogênese da doença [70]. A presença de astrócitos reativos na periferia das placas amilóides já foi descrita, e esses astrócitos apresentam uma expressão

aumentada da proteína S100B, indicando que essa citocina poderia estar envolvida no desenvolvimento das placas [68, 69].Os astrócitos reativos também apresentam alterações estruturais, com aumento da marcação para a GFAP [70].

A fim de avaliar a participação astrocítica nas alterações que ocorrem no modelo de demência por injeção ICV de STZ, avaliados o imunoconteúdo de S100B e GFAP no córtex e hipocampo dos animais, bem como o conteúdo de S100B no líquor. Ao contrário do que nosso grupo de pesquisa já havia demonstrado [78], não observamos alterações no conteúdo de S100B em córtex e hipocampo, porém houve uma redução no conteúdo de S100B no líquor dos animais, com prevenção pelas estatinas. É importante ressaltar que a alterações na secreção da proteína S100B nem sempre estão relacionadas a alterações na sua expressão [198].

Levando-se em conta que a S100B extracelular funciona como um fator neurotrófico, promovendo aumento da sobrevivência neuronal, neuritogênese e até mesmo o aumento da função sináptica[57], e os achados deletérios encontrados neste modelo de doença de Alzheimer esporádica, podemos inferir que possivelmente a atividade neuronal está comprometida. Também é interessante enfatizar o fato de houve alteração numa importante proteína astrocítica, a S100B, sem ter havido alteração no tradicional marcador de astrogliose, a proteína de citoesqueleto GFAP. Tal achado também salienta o fato de que a resposta astrocítica em eventos de insulto pode ser complexa e ampla, devendo ser analisada por diferentes parâmetros.

Sobre as estatinas, é importante comentar novamente, que todos os efeitos foram independentes da redução dos níveis de colesterol plasmático,

porém o efeito sobre o colesterol cerebral não foi avaliado. Alguns estudos demonstram que as estatinas diminuem o conteúdo de colesterol cerebral [199], porém outros demonstram que essas drogas não têm a capacidade de atuar sobre a síntese do colesterol cerebral [183], e que seu efeito sobre a cognição e demais parâmetros avaliados é independente da alteração do colesterol, tanto central quanto periférico.

Outra dúvida que é levantada por muitos grupos de pesquisa, é se as estatinas possuem efeitos diferentes de acordo com sua capacidade de atravessar a BHE. No nosso trabalho observamos que tanto a SV, que é mais lipofílica e atravessa a BHE, quanto a PV, que é mais hidrofílica, possuíram efeitos semelhantes, sendo que em alguns parâmetros, como a redução do dano oxidativo a lipídeos, a PV apresentou um melhor efeito. Entretanto, alguns estudos sugerem que apenas as estatinas lipofílicas apresentam melhores resultados na prevenção dos danos causados na DA e em modelos animais da doença [199].

Perspectivas

1) Avaliar o conteúdo de colesterol cerebral no modelo de demência por injeção ICV de STZ;

2) Avaliar a atividade da enzima iNOS ;

3) Avaliar conteúdo de nitrotirosina no modelo de demência por injeção ICV de STZ;

4) Avaliar o conteúdo de RAGE no modelo de demência por injeção ICV de STZ;

5) Quantificar AGEs no modelo de demência por injeção ICV de STZ;

6) Avaliar o possível papel protetor das estatinas em células C6 cultivadas em altas concentrações de glicose.

Referências Bibliográficas

1. Trahan MA, Kahng S, Fisher AB, Hausman NL: **Behavior-Analytic Research on Dementia in Older Adults.** *J Appl Behav Anal* 2011, **44:**687-691.
2. Ritchie K, Lovestone S: **The dementias.** *Lancet* 2002, **360:**1759-1766.
3. Stefani M, Liguri G: **Cholesterol in Alzheimer's disease: unresolved questions.** *Curr Alzheimer Res* 2009, **6:**15-29.
4. Langa KM, Larson EB, Wallace RB, Fendrick AM, Foster NL, Kabeto MU, Weir DR, Willis RJ, Herzog AR: **Out-of-pocket health care expenditures among older Americans with dementia.** *Alzheimer Dis Assoc Disord* 2004, **18:**90-98.
5. Haan MN, Wallace R: **Can dementia be prevented? Brain aging in a population-based context.** *Annu Rev Public Health* 2004, **25:**1-24.
6. Candore G, Bulati M, Caruso C, Castiglia L, Colonna-Romano G, Di Bona D, Duro G, Lio D, Matranga D, Pellicano M, et al: **Inflammation, cytokines, immune response, apolipoprotein E, cholesterol, and oxidative stress in Alzheimer disease: therapeutic implications.** *Rejuvenation Res* 2010, **13:**301-313.
7. Duyckaerts C, Delatour B, Potier MC: **Classification and basic pathology of Alzheimer disease.** *Acta Neuropathol* 2009, **118:**5-36.
8. Selkoe DJ: **Alzheimer's disease: genes, proteins, and therapy.** *Physiol Rev* 2001, **81:**741-766.
9. Vasto S, Candore G, Listi F, Balistreri CR, Colonna-Romano G, Malavolta M, Lio D, Nuzzo D, Mocchegiani E, Di Bona D, Caruso C: **Inflammation, genes and zinc in Alzheimer's disease.** *Brain Res Rev* 2008, **58:**96-105.
10. Whitehouse PJ: **The cholinergic deficit in Alzheimer's disease.** *J Clin Psychiatry* 1998, **59 Suppl 13:**19-22.
11. Sabbagh M, Cummings J: **Progressive cholinergic decline in Alzheimer's Disease: consideration for treatment with donepezil 23 mg in patients with moderate to severe symptomatology.** *BMC Neurol* 2011, **11:**21.
12. Lahiri DK, Rogers JT, Greig NH, Sambamurti K: **Rationale for the development of cholinesterase inhibitors as anti-Alzheimer agents.** *Curr Pharm Des* 2004, **10:**3111-3119.
13. Selkoe DJ: **Alzheimer's disease is a synaptic failure.** *Science* 2002, **298:**789-791.
14. Mattson MP, Chan SL: **Neuronal and glial calcium signaling in Alzheimer's disease.** *Cell Calcium* 2003, **34:**385-397.
15. Wang X, Su B, Zheng L, Perry G, Smith MA, Zhu X: **The role of abnormal mitochondrial dynamics in the pathogenesis of Alzheimer's disease.** *J Neurochem* 2009, **109 Suppl 1:**153-159.
16. Kang DE, Saitoh T, Chen X, Xia Y, Masliah E, Hansen LA, Thomas RG, Thal LJ, Katzman R: **Genetic association of the low-density lipoprotein receptor-related protein gene (LRP), an apolipoprotein E receptor, with late-onset Alzheimer's disease.** *Neurology* 1997, **49:**56-61.
17. Eckert GP, Wood WG, Muller WE: **Statins: drugs for Alzheimer's disease?** *J Neural Transm* 2005, **112:**1057-1071.
18. Canevari L, Clark JB: **Alzheimer's disease and cholesterol: the fat connection.** *Neurochem Res* 2007, **32:**739-750.
19. Puglielli L, Tanzi RE, Kovacs DM: **Alzheimer's disease: the cholesterol connection.** *Nat Neurosci* 2003, **6:**345-351.
20. Koudinov AR, Koudinova NV: **Cholesterol homeostasis failure as a unifying cause of synaptic degeneration.** *J Neurol Sci* 2005, **229-230:**233-240.
21. Sjogren M, Mielke M, Gustafson D, Zandi P, Skoog I: **Cholesterol and Alzheimer's disease--is there a relation?** *Mech Ageing Dev* 2006, **127:**138-147.
22. Mori T, Paris D, Town T, Rojiani AM, Sparks DL, Delledonne A, Crawford F, Abdullah LI, Humphrey JA, Dickson DW, Mullan MJ: **Cholesterol accumulates in senile plaques of Alzheimer disease patients and in transgenic APP(SW) mice.** *J Neuropathol Exp Neurol* 2001, **60:**778-785.

23. Ledesma MD, Dotti CG: **Amyloid excess in Alzheimer's disease: what is cholesterol to be blamed for?** *FEBS Lett* 2006, **580:**5525-5532.
24. Pike LJ: **Lipid rafts: bringing order to chaos.** *J Lipid Res* 2003, **44:**655-667.
25. Turley SD, Burns DK, Rosenfeld CR, Dietschy JM: **Brain does not utilize low density lipoprotein-cholesterol during fetal and neonatal development in the sheep.** *J Lipid Res* 1996, **37:**1953-1961.
26. Pfrieger FW: **Cholesterol homeostasis and function in neurons of the central nervous system.** *Cell Mol Life Sci* 2003, **60:**1158-1171.
27. Beach TG, Wilson JR, Sue LI, Newell A, Poston M, Cisneros R, Pandya Y, Esh C, Connor DJ, Sabbagh M, et al: **Circle of Willis atherosclerosis: association with Alzheimer's disease, neuritic plaques and neurofibrillary tangles.** *Acta Neuropathol* 2007, **113:**13-21.
28. Ledesma MD, Dotti CG: **The conflicting role of brain cholesterol in Alzheimer's disease: lessons from the brain plasminogen system.** *Biochem Soc Symp* 2005**:**129-138.
29. Chen TY, Liu PH, Ruan CT, Chiu L, Kung FL: **The intracellular domain of amyloid precursor protein interacts with flotillin-1, a lipid raft protein.** *Biochem Biophys Res Commun* 2006, **342:**266-272.
30. Kojro E, Gimpl G, Lammich S, Marz W, Fahrenholz F: **Low cholesterol stimulates the nonamyloidogenic pathway by its effect on the alpha -secretase ADAM 10.** *Proc Natl Acad Sci U S A* 2001, **98:**5815-5820.
31. Wolozin B: **A fluid connection: cholesterol and Abeta.** *Proc Natl Acad Sci U S A* 2001, **98:**5371-5373.
32. Kivipelto M, Solomon A: **Cholesterol as a risk factor for Alzheimer's disease - epidemiological evidence.** *Acta Neurol Scand Suppl* 2006, **185:**50-57.
33. de Chaves EP, Narayanaswami V: **Apolipoprotein E and cholesterol in aging and disease in the brain.** *Future Lipidol* 2008, **3:**505-530.
34. Dufouil C, Richard F, Fievet N, Dartigues JF, Ritchie K, Tzourio C, Amouyel P, Alperovitch A: **APOE genotype, cholesterol level, lipid-lowering treatment, and dementia: the Three-City Study.** *Neurology* 2005, **64:**1531-1538.
35. Jick H, Zornberg GL, Jick SS, Seshadri S, Drachman DA: **Statins and the risk of dementia.** *Lancet* 2000, **356:**1627-1631.
36. Hajjar I, Schumpert J, Hirth V, Wieland D, Eleazer GP: **The impact of the use of statins on the prevalence of dementia and the progression of cognitive impairment.** *J Gerontol A Biol Sci Med Sci* 2002, **57:**M414-418.
37. Wolozin B, Wang SW, Li NC, Lee A, Lee TA, Kazis LE: **Simvastatin is associated with a reduced incidence of dementia and Parkinson's disease.** *BMC Med* 2007, **5:**20.
38. Siegel GJ, Chauhan NB, Feinstein DL, Li G, Larson EB, Breitner JC, Montine TJ: **Statin therapy is associated with reduced neuropathologic changes of Alzheimer disease.** *Neurology* 2008, **71:**383; author reply 383.
39. McCall AL: **Cerebral glucose metabolism in diabetes mellitus.** *Eur J Pharmacol* 2004, **490:**147-158.
40. de la Monte SM, Wands JR: **Alzheimer's disease is type 3 diabetes-evidence reviewed.** *J Diabetes Sci Technol* 2008, **2:**1101-1113.
41. Biessels GJ, van der Heide LP, Kamal A, Bleys RL, Gispen WH: **Ageing and diabetes: implications for brain function.** *Eur J Pharmacol* 2002, **441:**1-14.
42. Sima AA: **Encephalopathies: the emerging diabetic complications.** *Acta Diabetol* 2010, **47:**279-293.
43. Kodl CT, Seaquist ER: **Cognitive dysfunction and diabetes mellitus.** *Endocr Rev* 2008, **29:**494-511.
44. Haan MN: **Therapy Insight: type 2 diabetes mellitus and the risk of late-onset Alzheimer's disease.** *Nat Clin Pract Neurol* 2006, **2:**159-166.
45. Ott A, Stolk RP, van Harskamp F, Pols HA, Hofman A, Breteler MM: **Diabetes mellitus and the risk of dementia: The Rotterdam Study.** *Neurology* 1999, **53:**1937-1942.
46. Zhao WQ, Townsend M: **Insulin resistance and amyloidogenesis as common molecular foundation for type 2 diabetes and Alzheimer's disease.** *Biochim Biophys Acta* 2009, **1792:**482-496.

47. Kalousova M, Zima T, Tesar V, Dusilova-Sulkova S, Skrha J: **Advanced glycoxidation end products in chronic diseases-clinical chemistry and genetic background.** *Mutat Res* 2005, **579:**37-46.
48. Ramasamy R, Vannucci SJ, Yan SS, Herold K, Yan SF, Schmidt AM: **Advanced glycation end products and RAGE: a common thread in aging, diabetes, neurodegeneration, and inflammation.** *Glycobiology* 2005, **15:**16R-28R.
49. Yan SD, Chen X, Fu J, Chen M, Zhu H, Roher A, Slattery T, Zhao L, Nagashima M, Morser J, et al: **RAGE and amyloid-beta peptide neurotoxicity in Alzheimer's disease.** *Nature* 1996, **382:**685-691.
50. Stern D, Yan SD, Yan SF, Schmidt AM: **Receptor for advanced glycation endproducts: a multiligand receptor magnifying cell stress in diverse pathologic settings.** *Adv Drug Deliv Rev* 2002, **54:**1615-1625.
51. Gasparini L, Gouras GK, Wang R, Gross RS, Beal MF, Greengard P, Xu H: **Stimulation of beta-amyloid precursor protein trafficking by insulin reduces intraneuronal beta-amyloid and requires mitogen-activated protein kinase signaling.** *J Neurosci* 2001, **21:**2561-2570.
52. Phiel CJ, Wilson CA, Lee VM, Klein PS: **GSK-3alpha regulates production of Alzheimer's disease amyloid-beta peptides.** *Nature* 2003, **423:**435-439.
53. Qiu WQ, Folstein MF: **Insulin, insulin-degrading enzyme and amyloid-beta peptide in Alzheimer's disease: review and hypothesis.** *Neurobiol Aging* 2006, **27:**190-198.
54. Iqbal K, Grundke-Iqbal I: **Alzheimer neurofibrillary degeneration: significance, etiopathogenesis, therapeutics and prevention.** *J Cell Mol Med* 2008, **12:**38-55.
55. Takeda S, Sato N, Rakugi H, Morishita R: **Molecular mechanisms linking diabetes mellitus and Alzheimer disease: beta-amyloid peptide, insulin signaling, and neuronal function.** *Mol Biosyst* 2011, **7:**1822-1827.
56. Perea G, Araque A: **Glial calcium signaling and neuron-glia communication.** *Cell Calcium* 2005, **38:**375-382.
57. 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.
58. Perea G, Navarrete M, Araque A: **Tripartite synapses: astrocytes process and control synaptic information.** *Trends Neurosci* 2009, **32:**421-431.
59. Pellerin L, Magistretti PJ: **Glutamate uptake into astrocytes stimulates aerobic glycolysis: a mechanism coupling neuronal activity to glucose utilization.** *Proc Natl Acad Sci U S A* 1994, **91:**10625-10629.
60. Eng LF, Ghirnikar RS, Lee YL: **Glial fibrillary acidic protein: GFAP-thirty-one years (1969-2000).** *Neurochem Res* 2000, **25:**1439-1451.
61. Donato R: **Intracellular and extracellular roles of S100 proteins.** *Microsc Res Tech* 2003, **60:**540-551.
62. Goncalves CA, Leite MC, Nardin P: **Biological and methodological features of the measurement of S100B, a putative marker of brain injury.** *Clin Biochem* 2008, **41:**755-763.
63. Donato R, Sorci G, Riuzzi F, Arcuri C, Bianchi R, Brozzi F, Tubaro C, Giambanco I: **S100B's double life: intracellular regulator and extracellular signal.** *Biochim Biophys Acta* 2009, **1793:**1008-1022.
64. Verkhratsky A, Olabarria M, Noristani HN, Yeh CY, Rodriguez JJ: **Astrocytes in Alzheimer's disease.** *Neurotherapeutics* 2010, **7:**399-412.
65. Danbolt NC: **Glutamate uptake.** *Prog Neurobiol* 2001, **65:**1-105.
66. Hirrlinger J, Dringen R: **The cytosolic redox state of astrocytes: Maintenance, regulation and functional implications for metabolite trafficking.** *Brain Res Rev* 2010, **63:**177-188.
67. Heneka MT, Rodriguez JJ, Verkhratsky A: **Neuroglia in neurodegeneration.** *Brain Res Rev* 2010, **63:**189-211.
68. Mrak RE, Griffin WS: **Glia and their cytokines in progression of neurodegeneration.** *Neurobiol Aging* 2005, **26:**349-354.
69. Van Eldik LJ, Griffin WS: **S100 beta expression in Alzheimer's disease: relation to neuropathology in brain regions.** *Biochim Biophys Acta* 1994, **1223:**398-403.
70. Mrak RE, Griffinbc WS: **The role of activated astrocytes and of the neurotrophic cytokine S100B in the pathogenesis of Alzheimer's disease.** *Neurobiol Aging* 2001, **22:**915-922.

71. Pike CJ, Cummings BJ, Cotman CW: **Early association of reactive astrocytes with senile plaques in Alzheimer's disease.** *Exp Neurol* 1995, **132:**172-179.
72. Pekny M, Pekna M: **Astrocyte intermediate filaments in CNS pathologies and regeneration.** *J Pathol* 2004, **204:**428-437.
73. Liberto CM, Albrecht PJ, Herx LM, Yong VW, Levison SW: **Pro-regenerative properties of cytokine-activated astrocytes.** *J Neurochem* 2004, **89:**1092-1100.
74. Hovsepyan MR, Haas MJ, Boyajyan AS, Guevorkyan AA, Mamikonyan AA, Myers SE, Mooradian AD: **Astrocytic and neuronal biochemical markers in the sera of subjects with diabetes mellitus.** *Neurosci Lett* 2004, **369:**224-227.
75. Coleman ES, Dennis JC, Braden TD, Judd RL, Posner P: **Insulin treatment prevents diabetes-induced alterations in astrocyte glutamate uptake and GFAP content in rats at 4 and 8 weeks of diabetes duration.** *Brain Res* 2010, **1306:**131-141.
76. Revsin Y, Saravia F, Roig P, Lima A, de Kloet ER, Homo-Delarche F, De Nicola AF: **Neuronal and astroglial alterations in the hippocampus of a mouse model for type 1 diabetes.** *Brain Res* 2005, **1038:**22-31.
77. Silvestrelli G, Lanari A, Parnetti L, Tomassoni D, Amenta F: **Treatment of Alzheimer's disease: from pharmacology to a better understanding of disease pathophysiology.** *Mech Ageing Dev* 2006, **127:**148-157.
78. Rodrigues L, Biasibetti R, Swarowsky A, Leite MC, Quincozes-Santos A, Quilfeldt JA, Achaval M, Goncalves CA: **Hippocampal alterations in rats submitted to streptozotocin-induced dementia model are prevented by aminoguanidine.** *J Alzheimers Dis* 2009, **17:**193-202.
79. van der Most PJ, Dolga AM, Nijholt IM, Luiten PG, Eisel UL: **Statins: mechanisms of neuroprotection.** *Prog Neurobiol* 2009, **88:**64-75.
80. Sultana R, Robinson RA, Di Domenico F, Abdul HM, Clair DK, Markesbery WR, Cai J, Pierce WM, Butterfield DA: **Proteomic identification of specifically carbonylated brain proteins in APP(NLh)/APP(NLh)xPS-1(P264L)/PS-1(P264L) human double mutant knock-in mice model of Alzheimer disease as a function of age.** *J Proteomics* 2011, **74:**2430-2440.
81. Hiramatsu M: **A role for guanidino compounds in the brain.** *Mol Cell Biochem* 2003, **244:**57-62.
82. Ahmed N, Ahmed U, Thornalley PJ, Hager K, Fleischer G, Munch G: **Protein glycation, oxidation and nitration adduct residues and free adducts of cerebrospinal fluid in Alzheimer's disease and link to cognitive impairment.** *J Neurochem* 2005, **92:**255-263.
83. Giardino I, Fard AK, Hatchell DL, Brownlee M: **Aminoguanidine inhibits reactive oxygen species formation, lipid peroxidation, and oxidant-induced apoptosis.** *Diabetes* 1998, **47:**1114-1120.
84. Stadler K, Jenei V, Somogyi A, Jakus J: **Beneficial effects of aminoguanidine on the cardiovascular system of diabetic rats.** *Diabetes Metab Res Rev* 2005, **21:**189-196.
85. Bolton WK, Cattran DC, Williams ME, Adler SG, Appel GB, Cartwright K, Foiles PG, Freedman BI, Raskin P, Ratner RE, et al: **Randomized trial of an inhibitor of formation of advanced glycation end products in diabetic nephropathy.** *Am J Nephrol* 2004, **24:**32-40.
86. Cash D, Beech JS, Rayne RC, Bath PM, Meldrum BS, Williams SC: **Neuroprotective effect of aminoguanidine on transient focal ischaemia in the rat brain.** *Brain Res* 2001, **905:**91-103.
87. Rosendo AB, Dal-Pizzol F, Fiegenbaum M, Almeida S: **[Pharmacogenetics and anti-inflammatory effect of HMG-CoA reductase inhibitors].** *Arq Bras Endocrinol Metabol* 2007, **51:**520-525.
88. Liao JK, Laufs U: **Pleiotropic effects of statins.** *Annu Rev Pharmacol Toxicol* 2005, **45:**89-118.
89. Cordle A, Koenigsknecht-Talboo J, Wilkinson B, Limpert A, Landreth G: **Mechanisms of statin-mediated inhibition of small G-protein function.** *J Biol Chem* 2005, **280:**34202-34209.
90. Yoshida M: **Potential role of statins in inflammation and atherosclerosis.** *J Atheroscler Thromb* 2003, **10:**140-144.
91. Greenwood J, Steinman L, Zamvil SS: **Statin therapy and autoimmune disease: from protein prenylation to immunomodulation.** *Nat Rev Immunol* 2006, **6:**358-370.

92. Miida T, Takahashi A, Ikeuchi T: **Prevention of stroke and dementia by statin therapy: experimental and clinical evidence of their pleiotropic effects.** *Pharmacol Ther* 2007, **113:**378-393.
93. Fassbender K, Simons M, Bergmann C, Stroick M, Lutjohann D, Keller P, Runz H, Kuhl S, Bertsch T, von Bergmann K, et al: **Simvastatin strongly reduces levels of Alzheimer's disease beta -amyloid peptides Abeta 42 and Abeta 40 in vitro and in vivo.** *Proc Natl Acad Sci U S A* 2001, **98:**5856-5861.
94. Kurata T, Miyazaki K, Kozuki M, Panin VL, Morimoto N, Ohta Y, Nagai M, Ikeda Y, Matsuura T, Abe K: **Atorvastatin and pitavastatin improve cognitive function and reduce senile plaque and phosphorylated tau in aged APP mice.** *Brain Res* 2011, **1371:**161-170.
95. Friedhoff LT, Cullen EI, Geoghagen NS, Buxbaum JD: **Treatment with controlled-release lovastatin decreases serum concentrations of human beta-amyloid (A beta) peptide.** *Int J Neuropsychopharmacol* 2001, **4:**127-130.
96. Simons M, Schwarzler F, Lutjohann D, von Bergmann K, Beyreuther K, Dichgans J, Wormstall H, Hartmann T, Schulz JB: **Treatment with simvastatin in normocholesterolemic patients with Alzheimer's disease: A 26-week randomized, placebo-controlled, double-blind trial.** *Ann Neurol* 2002, **52:**346-350.
97. Kuipers HF, van den Elsen PJ: **Immunomodulation by statins: inhibition of cholesterol vs. isoprenoid biosynthesis.** *Biomed Pharmacother* 2007, **61:**400-407.
98. Barone E, Cenini G, Di Domenico F, Martin S, Sultana R, Mancuso C, Murphy MP, Head E, Butterfield DA: **Long-term high-dose atorvastatin decreases brain oxidative and nitrosative stress in a preclinical model of Alzheimer disease: a novel mechanism of action.** *Pharmacol Res* 2011, **63:**172-180.
99. Kurinami H, Sato N, Shinohara M, Takeuchi D, Takeda S, Shimamura M, Ogihara T, Morishita R: **Prevention of amyloid beta-induced memory impairment by fluvastatin, associated with the decrease in amyloid beta accumulation and oxidative stress in amyloid beta injection mouse model.** *Int J Mol Med* 2008, **21:**531-537.
100. Piermartiri TC, Figueiredo CP, Rial D, Duarte FS, Bezerra SC, Mancini G, de Bem AF, Prediger RD, Tasca CI: **Atorvastatin prevents hippocampal cell death, neuroinflammation and oxidative stress following amyloid-beta(1-40) administration in mice: evidence for dissociation between cognitive deficits and neuronal damage.** *Exp Neurol* 2010, **226:**274-284.
101. Guasti L, Marino F, Cosentino M, Maio RC, Rasini E, Ferrari M, Castiglioni L, Klersy C, Gaudio G, Grandi AM, et al: **Prolonged statin-associated reduction in neutrophil reactive oxygen species and angiotensin II type 1 receptor expression: 1-year follow-up.** *Eur Heart J* 2008, **29:**1118-1126.
102. Robinson RA, Joshi G, Huang Q, Sultana R, Baker AS, Cai J, Pierce W, St Clair DK, Markesbery WR, Butterfield DA: **Proteomic analysis of brain proteins in APP/PS-1 human double mutant knock-in mice with increasing amyloid beta-peptide deposition: Insights into the effects of in vivo treatment with N-acetylcysteine as a potential therapeutic intervention in mild cognitive impairment and Alzheimer's disease.** *Proteomics* 2011, **11:**4243-4256.
103. Pocernich CB, La Fontaine M, Butterfield DA: **In-vivo glutathione elevation protects against hydroxyl free radical-induced protein oxidation in rat brain.** *Neurochem Int* 2000, **36:**185-191.
104. Farr SA, Poon HF, Dogrukol-Ak D, Drake J, Banks WA, Eyerman E, Butterfield DA, Morley JE: **The antioxidants alpha-lipoic acid and N-acetylcysteine reverse memory impairment and brain oxidative stress in aged SAMP8 mice.** *J Neurochem* 2003, **84:**1173-1183.
105. Lopes JP, Oliveira CR, Agostinho P: **Neurodegeneration in an Abeta-induced model of Alzheimer's disease: the role of Cdk5.** *Aging Cell* 2010, **9:**64-77.
106. Swarowsky A, Rodrigues L, Biasibetti R, Leite MC, de Oliveira LF, de Almeida LM, Gottfried C, Quillfeldt JA, Achaval M, Goncalves CA: **Glial alterations in the hippocampus of rats submitted to ibotenic-induced lesion of the nucleus basalis magnocellularis.** *Behav Brain Res* 2008, **190:**206-211.

107. Baydas G, Reiter RJ, Yasar A, Tuzcu M, Akdemir I, Nedzvetskii VS: **Melatonin reduces glial reactivity in the hippocampus, cortex, and cerebellum of streptozotocin-induced diabetic rats.** *Free Radic Biol Med* 2003, **35:**797-804.
108. Hosokawa M, Dolci W, Thorens B: **Differential sensitivity of GLUT1- and GLUT2-expressing beta cells to streptozotocin.** *Biochem Biophys Res Commun* 2001, **289:**1114-1117.
109. Weinstock M, Shoham S: **Rat models of dementia based on reductions in regional glucose metabolism, cerebral blood flow and cytochrome oxidase activity.** *J Neural Transm* 2004, **111:**347-366.
110. Ishrat T, Khan MB, Hoda MN, Yousuf S, Ahmad M, Ansari MA, Ahmad AS, Islam F: **Coenzyme Q10 modulates cognitive impairment against intracerebroventricular injection of streptozotocin in rats.** *Behav Brain Res* 2006, **171:**9-16.
111. Plaschke K, Hoyer S: **Action of the diabetogenic drug streptozotocin on glycolytic and glycogenolytic metabolism in adult rat brain cortex and hippocampus.** *Int J Dev Neurosci* 1993, **11:**477-483.
112. Duelli R, Schrock H, Kuschinsky W, Hoyer S: **Intracerebroventricular injection of streptozotocin induces discrete local changes in cerebral glucose utilization in rats.** *Int J Dev Neurosci* 1994, **12:**737-743.
113. Sharma M, Gupta YK: **Intracerebroventricular injection of streptozotocin in rats produces both oxidative stress in the brain and cognitive impairment.** *Life Sci* 2001, **68:**1021-1029.
114. Maccioni RB, Munoz JP, Barbeito L: **The molecular bases of Alzheimer's disease and other neurodegenerative disorders.** *Arch Med Res* 2001, **32:**367-381.
115. Arendt T: **Synaptic degeneration in Alzheimer's disease.** *Acta Neuropathol* 2009, **118:**167-179.
116. Nagele RG, D'Andrea MR, Lee H, Venkataraman V, Wang HY: **Astrocytes accumulate A beta 42 and give rise to astrocytic amyloid plaques in Alzheimer disease brains.** *Brain Res* 2003, **971:**197-209.
117. Simpson JE, Ince PG, Lace G, Forster G, Shaw PJ, Matthews F, Savva G, Brayne C, Wharton SB: **Astrocyte phenotype in relation to Alzheimer-type pathology in the ageing brain.** *Neurobiol Aging* 2010, **31:**578-590.
118. Hoyer S: **Glucose metabolism and insulin receptor signal transduction in Alzheimer disease.** *Eur J Pharmacol* 2004, **490:**115-125.
119. Freude S, Hettich MM, Schumann C, Stohr O, Koch L, Kohler C, Udelhoven M, Leeser U, Muller M, Kubota N, et al: **Neuronal IGF-1 resistance reduces Abeta accumulation and protects against premature death in a model of Alzheimer's disease.** *FASEB J* 2009, **23:**3315-3324.
120. Rivera EJ, Goldin A, Fulmer N, Tavares R, Wands JR, de la Monte SM: **Insulin and insulin-like growth factor expression and function deteriorate with progression of Alzheimer's disease: link to brain reductions in acetylcholine.** *J Alzheimers Dis* 2005, **8:**247-268.
121. Steen E, Terry BM, Rivera EJ, Cannon JL, Neely TR, Tavares R, Xu XJ, Wands JR, de la Monte SM: **Impaired insulin and insulin-like growth factor expression and signaling mechanisms in Alzheimer's disease--is this type 3 diabetes?** *J Alzheimers Dis* 2005, **7:**63-80.
122. Baker LD, Cross DJ, Minoshima S, Belongia D, Watson GS, Craft S: **Insulin resistance and Alzheimer-like reductions in regional cerebral glucose metabolism for cognitively normal adults with prediabetes or early type 2 diabetes.** *Arch Neurol* 2010, **68:**51-57.
123. Grunblatt E, Salkovic-Petrisic M, Osmanovic J, Riederer P, Hoyer S: **Brain insulin system dysfunction in streptozotocin intracerebroventricularly treated rats generates hyperphosphorylated tau protein.** *J Neurochem* 2007, **101:**757-770.
124. Kuhl DE, Minoshima S, Frey KA, Foster NL, Kilbourn MR, Koeppe RA: **Limited donepezil inhibition of acetylcholinesterase measured with positron emission tomography in living Alzheimer cerebral cortex.** *Ann Neurol* 2000, **48:**391-395.
125. Bohnen NI, Kaufer DI, Hendrickson R, Ivanco LS, Lopresti B, Davis JG, Constantine G, Mathis CA, Moore RY, DeKosky ST: **Cognitive correlates of alterations in acetylcholinesterase in Alzheimer's disease.** *Neurosci Lett* 2005, **380:**127-132.

126. Corder EH, Saunders AM, Strittmatter WJ, Schmechel DE, Gaskell PC, Small GW, Roses AD, Haines JL, Pericak-Vance MA: **Gene dose of apolipoprotein E type 4 allele and the risk of Alzheimer's disease in late onset families.** *Science* 1993, **261:**921-923.
127. Gearing M, Mori H, Mirra SS: **Abeta-peptide length and apolipoprotein E genotype in Alzheimer's disease.** *Ann Neurol* 1996, **39:**395-399.
128. Schmechel DE, Saunders AM, Strittmatter WJ, Crain BJ, Hulette CM, Joo SH, Pericak-Vance MA, Goldgaber D, Roses AD: **Increased amyloid beta-peptide deposition in cerebral cortex as a consequence of apolipoprotein E genotype in late-onset Alzheimer disease.** *Proc Natl Acad Sci U S A* 1993, **90:**9649-9653.
129. Vasto S, Mocchegiani E, Candore G, Listi F, Colonna-Romano G, Lio D, Malavolta M, Giacconi R, Cipriano C, Caruso C: **Inflammation, genes and zinc in ageing and age-related diseases.** *Biogerontology* 2006, **7:**315-327.
130. Matsuzaki T, Sasaki K, Hata J, Hirakawa Y, Fujimi K, Ninomiya T, Suzuki SO, Kanba S, Kiyohara Y, Iwaki T: **Association of Alzheimer disease pathology with abnormal lipid metabolism: the Hisayama Study.** *Neurology* 2011, **77:**1068-1075.
131. Presecki P, Muck-Seler D, Mimica N, Pivac N, Mustapic M, Stipcevic T, Smalc VF: **Serum lipid levels in patients with Alzheimer's disease.** *Coll Antropol* 2011, **35 Suppl 1:**115-120.
132. Hoglund K, Blennow K: **Effect of HMG-CoA reductase inhibitors on beta-amyloid peptide levels: implications for Alzheimer's disease.** *CNS Drugs* 2007, **21:**449-462.
133. Tramontina AC, Wartchow KM, Rodrigues L, Biasibetti R, Quincozes-Santos A, Bobermin L, Tramontina F, Goncalves CA: **The neuroprotective effect of two statins: simvastatin and pravastatin on a streptozotocin-induced model of Alzheimer's disease in rats.** *J Neural Transm* 2011, **118:**1641-1649.
134. Schonbeck U, Libby P: **Inflammation, immunity, and HMG-CoA reductase inhibitors: statins as antiinflammatory agents?** *Circulation* 2004, **109:**II18-26.
135. Tahirovic I, Sofic E, Sapcanin A, Gavrankapetanovic I, Bach-Rojecky L, Salkovic-Petrisic M, Lackovic Z, Hoyer S, Riederer P: **Reduced brain antioxidant capacity in rat models of betacytotoxic-induced experimental sporadic Alzheimer's disease and diabetes mellitus.** *Neurochem Res* 2007, **32:**1709-1717.
136. Bonda DJ, Wang X, Perry G, Nunomura A, Tabaton M, Zhu X, Smith MA: **Oxidative stress in Alzheimer disease: a possibility for prevention.** *Neuropharmacology* 2010, **59:**290-294.
137. Paxinos: **The Rat Nervous System.** *Academic Press* 1997.
138. Morris R: **Developments of a water-maze procedure for studying spatial learning in the rat.** *J Neurosci Methods* 1984, **11:**47-60.
139. Silva MC, Rocha J, Pires CS, Ribeiro LC, Brolese G, Leite MC, Almeida LM, Tramontina F, Ziegler DR, Goncalves CA: **Transitory gliosis in the CA3 hippocampal region in rats fed on a ketogenic diet.** *Nutr Neurosci* 2005, **8:**259-264.
140. Vicente E, Tramontina F, Leite MC, Nardin P, Silva M, Karkow AR, Adolf R, Lucion AB, Netto CA, Gottfried C, Goncalves CA: **S100B levels in the cerebrospinal fluid of rats are sex and anaesthetic dependent.** *Clin Exp Pharmacol Physiol* 2007, **34:**1126-1130.
141. Leite MC, Galland F, Brolese G, Guerra MC, Bortolotto JW, Freitas R, Almeida LM, Gottfried C, Goncalves CA: **A simple, sensitive and widely applicable ELISA for S100B: Methodological features of the measurement of this glial protein.** *J Neurosci Methods* 2008, **169:**93-99.
142. Tramontina F, Leite MC, Cereser K, de Souza DF, Tramontina AC, Nardin P, Andreazza AC, Gottfried C, Kapczinski F, Goncalves CA: **Immunoassay for glial fibrillary acidic protein: antigen recognition is affected by its phosphorylation state.** *J Neurosci Methods* 2007, **162:**282-286.
143. de Almeida LM, Pineiro CC, Leite MC, Brolese G, Tramontina F, Feoli AM, Gottfried C, Goncalves CA: **Resveratrol increases glutamate uptake, glutathione content, and S100B secretion in cortical astrocyte cultures.** *Cell Mol Neurobiol* 2007, **27:**661-668.
144. Hevel JM, Marletta MA: **Nitric-oxide synthase assays.** *Methods Enzymol* 1994, **233:**250-258.
145. Draper HH, Hadley M: **Malondialdehyde determination as index of lipid peroxidation.** *Methods Enzymol* 1990, **186:**421-431.

146. Lowry OH, Rosebrough NJ, Farr AL, Randall RJ: **Protein measurement with the Folin phenol reagent.** *J Biol Chem* 1951, **193:**265-275.
147. Salkovic-Petrisic M, Tribl F, Schmidt M, Hoyer S, Riederer P: **Alzheimer-like changes in protein kinase B and glycogen synthase kinase-3 in rat frontal cortex and hippocampus after damage to the insulin signalling pathway.** *J Neurochem* 2006, **96:**1005-1015.
148. Azari NP, Pettigrew KD, Schapiro MB, Haxby JV, Grady CL, Pietrini P, Salerno JA, Heston LL, Rapoport SI, Horwitz B: **Early detection of Alzheimer's disease: a statistical approach using positron emission tomographic data.** *J Cereb Blood Flow Metab* 1993, **13:**438-447.
149. Silverman DH, Small GW, Chang CY, Lu CS, Kung De Aburto MA, Chen W, Czernin J, Rapoport SI, Pietrini P, Alexander GE, et al: **Positron emission tomography in evaluation of dementia: Regional brain metabolism and long-term outcome.** *JAMA* 2001, **286:**2120-2127.
150. Frolich L, Blum-Degen D, Bernstein HG, Engelsberger S, Humrich J, Laufer S, Muschner D, Thalheimer A, Turk A, Hoyer S, et al: **Brain insulin and insulin receptors in aging and sporadic Alzheimer's disease.** *J Neural Transm* 1998, **105:**423-438.
151. Cardoso S, Correia S, Santos RX, Carvalho C, Santos MS, Oliveira CR, Perry G, Smith MA, Zhu X, Moreira PI: **Insulin is a two-edged knife on the brain.** *J Alzheimers Dis* 2009, **18:**483-507.
152. Pathan AR, Viswanad B, Sonkusare SK, Ramarao P: **Chronic administration of pioglitazone attenuates intracerebroventricular streptozotocin induced-memory impairment in rats.** *Life Sci* 2006, **79:**2209-2216.
153. Olafsdottir E, Aspelund T, Sigurdsson G, Thorsson B, Eiriksdottir G, Harris TB, Launer LJ, Benediktsson R, Gudnason V: **Effects of statin medication on mortality risk associated with type 2 diabetes in older persons: the population-based AGES-Reykjavik Study.** *BMJ Open* 2011, **1:**e000132.
154. Riad A, Du J, Stiehl S, Westermann D, Mohr Z, Sobirey M, Doehner W, Adams V, Pauschinger M, Schultheiss HP, Tschope C: **Low-dose treatment with atorvastatin leads to anti-oxidative and anti-inflammatory effects in diabetes mellitus.** *Eur J Pharmacol* 2007, **569:**204-211.
155. Sharma B, Singh N, Singh M: **Modulation of celecoxib- and streptozotocin-induced experimental dementia of Alzheimer's disease by pitavastatin and donepezil.** *J Psychopharmacol* 2008, **22:**162-171.
156. Cibickova L, Palicka V, Cibicek N, Cermakova E, Micuda S, Bartosova L, Jun D: **Differential effects of statins and alendronate on cholinesterases in serum and brain of rats.** *Physiol Res* 2007, **56:**765-770.
157. Rodrigues L, Dutra MF, Ilha J, Biasibetti R, Quincozes-Santos A, Leite MC, Marcuzzo S, Achaval M, Goncalves CA: **Treadmill training restores spatial cognitive deficits and neurochemical alterations in the hippocampus of rats submitted to an intracerebroventricular administration of streptozotocin.** *J Neural Transm* 2010, **117:**1295-1305.
158. Park SY, Lee JS, Ko YJ, Kim AR, Choi MK, Kwak MK, Choi HG, Yong CS, Kim JA: **Inhibitory effect of simvastatin on the TNF-alpha- and angiotensin II-induced monocyte adhesion to endothelial cells is mediated through the suppression of geranylgeranyl isoprenoid-dependent ROS generation.** *Arch Pharm Res* 2008, **31:**195-204.
159. Costa AP, Tramontina AC, Biasibetti R, Batassini C, Lopes MW, Wartchow KM, Bernardi C, Tortorelli LS, Leal RB, Goncalves CA: **Neuroglial alterations in rats submitted to the okadaic acid-induced model of dementia.** *Behav Brain Res* 2010.
160. 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:**409-413.
161. Chaves ML, Camozzato AL, Ferreira ED, Piazenski I, Kochhann R, Dall'Igna O, Mazzini GS, Souza DO, Portela LV: **Serum levels of S100B and NSE proteins in Alzheimer's disease patients.** *J Neuroinflammation* 2010, **7:**6.

162. Salkovic-Petrisic M, Osmanovic-Barilar J, Bruckner MK, Hoyer S, Arendt T, Riederer P: **Cerebral amyloid angiopathy in streptozotocin rat model of sporadic Alzheimer's disease: a long-term follow up study.** *J Neural Transm* 2011, **118:**765-772.
163. Gardoni F, Kamal A, Bellone C, Biessels GJ, Ramakers GM, Cattabeni F, Gispent WH, Di Luca M: **Effects of streptozotocin-diabetes on the hippocampal NMDA receptor complex in rats.** *J Neurochem* 2002, **80:**438-447.
164. Allen DA, Yaqoob MM, Harwood SM: **Mechanisms of high glucose-induced apoptosis and its relationship to diabetic complications.** *J Nutr Biochem* 2005, **16:**705-713.
165. Tomlinson DR, Gardiner NJ: **Glucose neurotoxicity.** *Nat Rev Neurosci* 2008, **9:**36-45.
166. Brownlee M: **Biochemistry and molecular cell biology of diabetic complications.** *Nature* 2001, **414:**813-820.
167. Kosenko E, Llansola M, Montoliu C, Monfort P, Rodrigo R, Hernandez-Viadel M, Erceg S, Sanchez-Perez AM, Felipo V: **Glutamine synthetase activity and glutamine content in brain: modulation by NMDA receptors and nitric oxide.** *Neurochem Int* 2003, **43:**493-499.
168. Gottfried C, Tramontina F, Goncalves D, Goncalves CA, Moriguchi E, Dias RD, Wofchuk ST, Souza DO: **Glutamate uptake in cultured astrocytes depends on age: a study about the effect of guanosine and the sensitivity to oxidative stress induced by H(2)O(2).** *Mech Ageing Dev* 2002, **123:**1333-1340.
169. Miralles VJ, Martinez-Lopez I, Zaragoza R, Borras E, Garcia C, Pallardo FV, Vina JR: **Na+ dependent glutamate transporters (EAAT1, EAAT2, and EAAT3) in primary astrocyte cultures: effect of oxidative stress.** *Brain Res* 2001, **922:**21-29.
170. Valastro B, Cossette J, Lavoie N, Gagnon S, Trudeau F, Massicotte G: **Up-regulation of glutamate receptors is associated with LTP defects in the early stages of diabetes mellitus.** *Diabetologia* 2002, **45:**642-650.
171. Hansson E, Muyderman H, Leonova J, Allansson L, Sinclair J, Blomstrand F, Thorlin T, Nilsson M, Ronnback L: **Astroglia and glutamate in physiology and pathology: aspects on glutamate transport, glutamate-induced cell swelling and gap-junction communication.** *Neurochem Int* 2000, **37:**317-329.
172. Nardin P, Tramontina F, Leite MC, Tramontina AC, Quincozes-Santos A, de Almeida LM, Battastini AM, Gottfried C, Goncalves CA: **S100B content and secretion decrease in astrocytes cultured in high-glucose medium.** *Neurochem Int* 2007, **50:**774-782.
173. Ward MM, Jobling AI, Kalloniatis M, Fletcher EL: **Glutamate uptake in retinal glial cells during diabetes.** *Diabetologia* 2005, **48:**351-360.
174. Palos TP, Ramachandran B, Boado R, Howard BD: **Rat C6 and human astrocytic tumor cells express a neuronal type of glutamate transporter.** *Brain Res Mol Brain Res* 1996, **37:**297-303.
175. Wilkinson-Berka JL, Kelly DJ, Koerner SM, Jaworski K, Davis B, Thallas V, Cooper ME: **ALT-946 and aminoguanidine, inhibitors of advanced glycation, improve severe nephropathy in the diabetic transgenic (mREN-2)27 rat.** *Diabetes* 2002, **51:**3283-3289.
176. Kamboj SS, Chopra K, Sandhir R: **Neuroprotective effect of N-acetylcysteine in the development of diabetic encephalopathy in streptozotocin-induced diabetes.** *Metab Brain Dis* 2008, **23:**427-443.
177. dos Santos AQ, Nardin P, Funchal C, de Almeida LM, Jacques-Silva MC, Wofchuk ST, Goncalves CA, Gottfried C: **Resveratrol increases glutamate uptake and glutamine synthetase activity in C6 glioma cells.** *Arch Biochem Biophys* 2006, **453:**161-167.
178. Abib RT, Quincozes-Santos A, Nardin P, Wofchuk ST, Perry ML, Goncalves CA, Gottfried C: **Epicatechin gallate increases glutamate uptake and S100B secretion in C6 cell lineage.** *Mol Cell Biochem* 2008, **310:**153-158.
179. Tramontina F, Tramontina AC, Souza DF, Leite MC, Gottfried C, Souza DO, Wofchuk ST, Goncalves CA: **Glutamate uptake is stimulated by extracellular S100B in hippocampal astrocytes.** *Cell Mol Neurobiol* 2006, **26:**81-86.
180. Papassotiropoulos A, Lutjohann D, Bagli M, Locatelli S, Jessen F, Rao ML, Maier W, Bjorkhem I, von Bergmann K, Heun R: **Plasma 24S-hydroxycholesterol: a peripheral indicator of neuronal degeneration and potential state marker for Alzheimer's disease.** *Neuroreport* 2000, **11:**1959-1962.

181. Distl R, Meske V, Ohm TG: **Tangle-bearing neurons contain more free cholesterol than adjacent tangle-free neurons.** *Acta Neuropathol* 2001, **101:**547-554.
182. Willey JZ, Elkind MS: **3-Hydroxy-3-methylglutaryl-coenzyme A reductase inhibitors in the treatment of central nervous system diseases.** *Arch Neurol* 2010, **67:**1062-1067.
183. Li L, Cao D, Kim H, Lester R, Fukuchi K: **Simvastatin enhances learning and memory independent of amyloid load in mice.** *Ann Neurol* 2006, **60:**729-739.
184. Grutzendler J, Morris JC: **Cholinesterase inhibitors for Alzheimer's disease.** *Drugs* 2001, **61:**41-52.
185. Schaeffer EL, Gattaz WF: **Cholinergic and glutamatergic alterations beginning at the early stages of Alzheimer disease: participation of the phospholipase A2 enzyme.** *Psychopharmacology (Berl)* 2008, **198:**1-27.
186. Vicente E, Degerone D, Bohn L, Scornavaca F, Pimentel A, Leite MC, Swarowsky A, Rodrigues L, Nardin P, de Almeida LM, et al: **Astroglial and cognitive effects of chronic cerebral hypoperfusion in the rat.** *Brain Res* 2009, **1251:**204-212.
187. Yan J, Xu Y, Zhu C, Zhang L, Wu A, Yang Y, Xiong Z, Deng C, Huang XF, Yenari MA, et al: **Simvastatin prevents dopaminergic neurodegeneration in experimental parkinsonian models: the association with anti-inflammatory responses.** *PLoS One* 2011, **6:**e20945.
188. Wang Q, Zengin A, Deng C, Li Y, Newell KA, Yang GY, Lu Y, Wilder-Smith EP, Zhao H, Huang XF: **High dose of simvastatin induces hyperlocomotive and anxiolytic-like activities: The association with the up-regulation of NMDA receptor binding in the rat brain.** *Exp Neurol* 2009, **216:**132-138.
189. Ramirez C, Tercero I, Pineda A, Burgos JS: **Simvastatin is the statin that most efficiently protects against kainate-induced excitotoxicity and memory impairment.** *J Alzheimers Dis* 2011, **24:**161-174.
190. Rikitake Y, Liao JK: **Rho GTPases, statins, and nitric oxide.** *Circ Res* 2005, **97:**1232-1235.
191. Cucchiara B, Kasner SE: **Use of statins in CNS disorders.** *J Neurol Sci* 2001, **187:**81-89.
192. Butterfield DA, Barone E, Di Domenico F, Cenini G, Sultana R, Murphy MP, Mancuso C, Head E: **Atorvastatin treatment in a dog preclinical model of Alzheimer's disease leads to up-regulation of haem oxygenase-1 and is associated with reduced oxidative stress in brain.** *Int J Neuropsychopharmacol* 2011**:**1-7.
193. Hsieh CH, Rau CS, Hsieh MW, Chen YC, Jeng SF, Lu TH, Chen SS: **Simvastatin-induced heme oxygenase-1 increases apoptosis of Neuro 2A cells in response to glucose deprivation.** *Toxicol Sci* 2008, **101:**112-121.
194. Lambeth JD: **NOX enzymes and the biology of reactive oxygen.** *Nat Rev Immunol* 2004, **4:**181-189.
195. Syapin PJ: **Regulation of haeme oxygenase-1 for treatment of neuroinflammation and brain disorders.** *Br J Pharmacol* 2008, **155:**623-640.
196. Costa AP, Tramontina AC, Biasibetti R, Batassini C, Lopes MW, Wartchow KM, Bernardi C, Tortorelli LS, Leal RB, Goncalves CA: **Neuroglial alterations in rats submitted to the okadaic acid-induced model of dementia.** *Behav Brain Res* 2011.
197. Kroner Z: **The relationship between Alzheimer's disease and diabetes: Type 3 diabetes?** *Altern Med Rev* 2009, **14:**373-379.
198. Tramontina F, Conte S, Goncalves D, Gottfried C, Portela LV, Vinade L, Salbego C, Goncalves CA: **Developmental changes in S100B content in brain tissue, cerebrospinal fluid, and astrocyte cultures of rats.** *Cell Mol Neurobiol* 2002, **22:**373-378.
199. Cibickova L, Hyspler R, Micuda S, Cibicek N, Zivna H, Jun D, Ticha A, Brcakova E, Palicka V: **The influence of simvastatin, atorvastatin and high-cholesterol diet on acetylcholinesterase activity, amyloid beta and cholesterol synthesis in rat brain.** *Steroids* 2009, **74:**13-19.