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Efeitos do metilglioxal em parâmetros comportamentais e neuroquímicos *in vitro* e *ex vivo*

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“A verdadeira ciência ensina sobretudo a duvidar e a ser ignorante”

Miguel Unamuno

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RESUMO

O diabetes *mellitus* é uma doença metabólica caracterizada por níveis elevados de glicose no jejum e está associada com a perda da função cognitiva e um maior risco de desenvolvimento de doenças neurodegenerativas, tais como a Doença de Alzheimer (DA). As complicações do diabetes estão relacionadas com a hiperglicemia, níveis elevados de compostos reativos, como o metilglioxal (MG) e a formação de produtos finais de glicação avançada (do inglês *Avanced Glycation End Products* - AGEs). Os AGEs e o MG - um aldeído reativo envolvido no estresse dicarbonil e na formação de AGEs – encontram-se elevados em pacientes com diabetes e DA e têm sido sugeridos como mediadores do declínio cognitivo observado nessas patologias. Estudos indicaram que os astrócitos possuem o sistema glioxalase mais efetivo que os neurônios e, portanto, podem proteger os neurônios do estresse dicarbonil. Entretanto, as reações de glicação estão associadas com o prejuízo no funcionamento dos astrócitos, que, desta forma, comprometem a atividade neuronal. Tendo em vista que as reações de glicação estão exacerbadas no diabetes e em doenças neurodegenerativas sugere-se que nestas situações o dano neuronal seja ainda maior. Este trabalho teve como objetivo avaliar a suscetibilidade ao dano induzido pelo MG em culturas de astrócitos e de glioma C6 e examinar o efeito da alta concentração de glicose, do MG e da carboxietil-lisina (CEL), um AGE derivado da reação do MG com a lisina, em parâmetros oxidativos, metabólicos e específicos de astrócitos em fatias de hipocampo *in vitro*. Além disso, verificar se a administração intracerebroventricular (ICV) de MG causa alterações comportamentais, relacionadas com declínio cognitivo e ansiedade, e alterações bioquímicas em hipocampo, córtex cerebral e líquido cefalorraquidiano (LCR) *ex vivo*. Em culturas de células observou-se uma alta eficiência do sistema glioxalase em astrócitos em comparação com células C6. O conteúdo de glutationa diminuiu a partir de 1 hora após a exposição ao MG apenas em C6. A captação de glutamato reduziu em astrócitos e aumentou em C6, sendo que este efeito estava relacionado com reações de glicação, mas não com a expressão de transportadores de glutamato analisados. A formação de espécies reativas, a secreção de S100B e o conteúdo da proteína glial fibrilar ácida (GFAP) não foram modificados pelo tratamento com MG nas células estudadas. Em fatias de hipocampo (*in vitro*) verificou-se que a glicose, o MG e a CEL não alteraram a formação de espécies reativas, a captação de glicose e a atividade da glutamina sintetase. No entanto, a captação de glutamato e a secreção de S100B diminuiram após o tratamento com MG e CEL. Estas mudanças não foram mediadas pela ativação de RAGE e por reações de glicação. A partir do estudo *in vivo* verificou-se que o MG não causou deficiência nos processos de aprendizagem e memória investigados pelas tarefas de habituação, labirinto em Y e reconhecimento de objetos e não alterou a atividade locomotora dos animais. Contudo, o aumento agudo dos níveis exógenos de MG diminuiu o comportamento do tipo ansiedade, observado através do teste de campo aberto. Em relação às análises *ex vivo*, o MG induziu alterações persistentes relacionados com a atividade da glioxalase 1, conteúdo de AGEs e captação de glutamato no hipocampo. A formação de espécies reativas, o conteúdo de glutationa, o conteúdo tecidual de GFAP e S100B e a atividade da glutamina sintetase não foram alterados com a administração ICV de MG. É possível concluir que a exposição aguda, *in vitro*, de fatias de hipocampo ao MG e CEL, mas não à glicose, foi capaz de induzir efeitos semelhantes em fatias de hipocampo, sugerindo que a alta concentração de glicose é tóxica principalmente por elevar a concentração de compostos glicantes, como o MG, e gerar ligações cruzadas com proteínas. O MG foi capaz de induzir prejuízo na captação de glutamato em culturas de astrócitos e fatias de hipocampo, indicando que o MG gera alterações na homestase cerebral através do comprometimento da remoção do glutamato da fenda sináptica, deste modo, contribuindo com as alterações neurológicas relacionadas com o diabetes *mellitus*. Em suma, as concentrações exógenas de MG e o tempo de exposição a elevadas concentrações deste composto determinam as diferentes características que podem ser observadas em pacientes diabéticos.

ABSTRACT

Diabetes *mellitus* is a metabolic disease characterized by high fasting-glucose levels and is associated with loss of cognitive function and a higher risk of developing neurodegenerative diseases, such as Alzheimer's disease (AD). Diabetic complications have been associated with hyperglycemia, high levels of reactive compounds, such as methylglyoxal (MG) and advanced glycation end products (AGEs) formation. AGEs and MG - a reactive aldehyde, involved in dicarbonyl stress and AGEs formation - are elevated in diabetes and AD and have been suggested as mediators of cognitive decline observed in these pathologies. Astrocytes are impaired to glycation processes, although, they have an improved glyoxalase system compared to neurons that protect them from dicarbonyl stress, suggesting that damage to neuronal functions could be increased under diabetes and neurodegenerative diseases. This study aimed to evaluate the susceptibility of astrocytes and C6 glioma cells cultures to MG damage and to examine the effect of high glucose, MG and carboxyethyllysine (CEL), a MG-derived AGE of lysine, on oxidative, metabolic and astrocyte-specific parameters in hippocampal slices *in vitro*. Furthermore, verify if intracerebroventricular (ICV) administration of MG cause behavioral changes, related to cognitive decline and anxiety, and biochemical alterations in the hippocampus, cerebral cortex and cerebrospinal fluid *ex vivo.* Since our cultures studies we observed a high efficiency of the glyoxalase system in astrocytes compared to C6 cells. The content of glutathione decreased from 1 hour after MG exposure only in C6 cells. Glutamate uptake was decreased in astrocytes and increased in C6 cells and this effect is related to glycation processes, but not to glutamate transporters expression. The reactive species formation, S100B secretion and glial fibrillary acidic protein (GFAP) content were not modified by MG treatment in either culture studied. With hippocampal slices inbucation (*in vitro*) it was found that glucose, MG and CEL did not alter reactive species formation, glucose uptake or glutamine synthetase activity. However, glutamate uptake and S100B secretion were decreased after MG and CEL exposure. RAGE activation and glycation reactions did not mediate these changes. From *in vivo* research it was found that MG did not cause impairment in learning-memory processes investigated by habituation, Y-maze and object recognition tasks and did not alter locomotion behavior of animals. However, the acute increase of MG exogenous levels reduced anxiety-related behavior evaluated in the open field test. *Ex vivo* findings support that MG induced persistent alterations related to glyoxalase 1 activity, AGEs content and glutamate uptake in hippocampus. The reactive species formation, glutathione content, GFAP and S100B content, as well as glutamine synthetase activity were not altered by MG ICV administration. It is possible conclude that acute MG and CEL exposure, but not glucose, were able to induce similar effects on hippocampal slices *in vitro*, suggesting that conditions of high glucose concentrations are primarily toxic by elevate the rates of these glycation compounds, as MG, and generate protein cross-links. MG-induced astrocyte glutamate uptake impairment was seen in astrocyte cultures and hippocampal slices, indicating that MG produces changes in brain homeostasis by the impairment of glutamate removal of the synaptic cleft, thus contributing to neurological changes related to diabetes *mellitus*. The exogenous concentrations of MG and the time of exposure to high concentrations of this compound determine the different features that can be seen in diabetic patients.

LISTA DE ABREVIATURAS

ADN: Ácido desoxirribonucleico

AGEs: Produtos Finais de Glicação Avançada (do inglês *Avanced Glycation End Products*)

AMPA: Alfa-amino-3-hidroxi-metil-5-4-isoxazolpropiônico

CEL: Carboxietil-lisina

DA: Doença de Alzheimer

DM 1: Diabetes *mellitus* tipo 1

DM 2: Diabetes *mellitus* tipo 2

GABA: Ácido γ-aminobutírico

GFAP: Proteína Glial Fibrilar Ácida

GLAST: Transportador de glutamato/aspartato (do inglês *Glutamate Aspartate Transporter)*

GLO1: Glioxalase 1

GLO2: Glioxalase 2

GLT-1: Transportador glial de glutamato 1 (do inglês, *Glial Glutamate Transporter 1*)

GSH: Glutationa reduzida

ICV: Intracerebroventricular

LCR: Líquido cefalorraquidiano

MG: Metilglioxal

MG-H1: Hidroimidazolona derivada do metilglioxal

NMDA: N-metil-D-aspartato

RAGE: Receptor para AGEs

INTRODUÇÃO

1 Diabetes

O diabetes *mellitus* (DM) é uma doença metabólica crônica, caracterizada por hiperglicemia e por alterações no metabolismo de lipídios, carboidratos e proteínas (McNeill, 2000). Existem dois tipos de diabetes *mellitus.* O diabetes *mellitus* tipo 1 (DM 1), que na maioria dos casos é caracterizado pela destruição autoimune das células β do pâncreas. O diabetes *mellitus* tipo 2 (DM 2) que é caracterizado pela disfunção das células β pancreáticas, envolvendo deficiência na produção de insulina e/ou resistência à insulina (Biessels et al., 2002). Existem outros tipos de diabetes, um dos que merece destaque devido a sua prevalência e impacto social é o diabetes gestacional, que é caracterizado por hiperglicemia no período gestacional, desenvolvida geralmente em torno da vigésima quarta semana de gravidez (http://www.diabetes.org, página eletrônica da *American Diabetes Association,* 2015).

Em longo prazo, o diabetes gera complicações micro e macrovasculares, dentre as quais a retinopatia, nefropatia e neuropatia são complicações microvasculares frequentemente observadas (Brownlee, 2001; Gross et al., 2005), sendo uma das principais causas de cegueira, doença renal terminal e uma variedade de neuropatias debilitantes. Além disso, o diabetes está associado com a progressão de doenças macrovasculares ateroscleróticas por afetar artérias que suprem o coração, o cérebro e os membros inferiores. Como resultado, os pacientes com diabetes têm um risco

aumentado de infarto do miocárdio, acidente vascular cerebral e amputação de membros (Bloomgarden, 2003; Brownlee, 2001).

Em 2014 estimou-se que, no mundo, 387 milhões de pessoas possuíam diabetes e que em 2035 este número irá aumentar para 592 milhões. Na América do Sul e Central o número de pessoas com diabetes irá aumentar em torno de 60% até 2035. No Brasil, em 2014, aproximadamente 12 milhões de pessoas entre 20 e 79 anos possuíam diabetes, o que representava prevalência nacional de 8,7%. Os casos não diagnosticados estavam em torno de 3 milhões (http://www.idf.org/diabetesatlas/update-2014, página eletrônica da *International Diabetes Federation*, 2015).

1.1 Diabetes, cognição e demência

Há anos tem sido reconhecido que o diabetes pode afetar a cognição (Miles e Root, 1922). Estudos com diabéticos (DM 1 ou 2) corroboram com essa evidência, pois mostram que esses pacientes apresentam declínio cognitivo (Strachan et al., 1997; Stewart e Liolitsa, 1999; Brands et al., 2005; Cukierman et al., 2005), principalmente relacionado a processos que envolvem memória e aprendizagem (Ryan, 1988). Além disso, foi proposto que o diabetes aumenta o risco de desenvolvimento de demência, e especialmente demência do tipo Alzheimer (Biessels et al., 2006; Kopf e Frolich, 2009), particularmente durante o envelhecimento (Biessels et al., 2002). O DM 2 está associado com o aumento de 1,5 a 2,5 vezes no risco de demência (Strachan et al., 2011).

O termo “encefalopatia diabética” tem sido utilizado para caracterizar a associação entre diabetes, declínio cognitivo moderado e mudanças estruturais

e neurofisiológicas que ocorrem no encéfalo (Biessels et al., 2002). Além disso, a relação entre diabetes e demência continua sendo extensivamente estudada e, em especial, a relação entre o DM 2 e a doença de Alzheimer (DA). Observou-se que a resistência à insulina é uma alteração presente nestas patologias e, em virtude disso, a DA tem sido denominada como “diabetes tipo 3” ou “DM 2 específico do cérebro” (Sebastião et al., 2014).

Foi proposto que a hiperglicemia crônica pode afetar o encéfalo, sendo citada como um dos fatores que está diretamente relacionado com o declínio cognitivo observado nos pacientes diabéticos (Strachan et al., 1997). A associação entre elevados níveis de glicose e desenvolvimento de demência é reforçada por estudos que observaram que os níveis elevados de glicose são um fator de risco para demência mesmo entre pessoas não diabéticas (Crane et al., 2013) e que a prevalência de diabetes (glicemia de jejum maior ou igual a 126 mg/dL) e intolerância à glicose (glicemia de jejum 110 a 125 mg/dL) é maior em indivíduos com DA (81%) do que em indivíduos que não apresentam este tipo de demência (Janson et al., 2004).

Diversos fatores além da hiperglicemia têm sido estudados e sugeridos como mediadores envolvidos no diabetes e na demência, dentre eles está o acúmulo de produtos finais de glicação avançada (AGEs, do inglês *Advanced Glycation End Products*) (Strachan et al., 2011; Yan e Song, 2013; Bornstein et al., 2014). Contudo, os mecanismos pelos quais estes mediadores interferem na cognição, podendo gerar ou agravar o quadro de demência, não estão bem esclarecidos. Sabendo-se que a prevalência de DM 2 continua a aumentar e a população continua a envelhecer, é imperativo estender este conhecimento

para melhor entender como DM, comprometimento cognitivo, idade e DA interagem.

2 Glicação

Os efeitos tóxicos das altas concentrações de glicose são mediados, pelo menos em parte, por reações de glicação. Nestas reações, a glicose modifica irreversivelmente macromoléculas, tais como proteínas estruturais e funcionais de meia vida longa, lipídios e ácidos nucleicos (Brownlee, 2001).

As reações de glicação ocorrem entre açúcares redutores livres, como a glicose, e grupos amino livres de diversas moléculas, que formam inicialmente compostos instáveis, conhecidos como bases de Schiff. As bases de Schiff dão origem a cetoaminas estáveis, conhecidas como produtos de Amadori, que, através de reações de desidratação e oxidação, podem ser degradados e gerar compostos dicarbonil (glioxal, metilglioxal (MG) e 3-deoxiglicosona). Estes três compostos são muito mais reativos do que os açúcares a partir dos quais eles são derivados, portanto, eles agem como propagadores das reações de glicação. Através da reação destes propagadores com grupos amino livres ocorre a formação dos AGEs, que são compostos irreversíveis e insolúveis. Esta série de reações é conhecida como Reação de Maillard ou reações não enzimáticas da glicação (Lapolla et al., 2005b).

2.1 Sistema glioxalase

Existem mecanismos de defesa a fim de evitar os danos causados pela glicação e a formação de AGEs, que não envolvem a homeostase da glicose plasmática, mas sim a detoxificação dos propagadores dicarbonil. O sistema

glioxalase é considerado o principal mecanismo de defesa contra a toxicidade do MG e está presente no citosol de todos os tecidos de organismos eucariontes e procariontes (Vander Jagt e Hunsaker, 2003). Este sistema é composto por duas enzimas, a glioxalase 1 (GLO1) e a glioxalase 2 (GLO2) e é limitado pela disponibilidade de glutationa reduzida (GSH), que juntamente com o MG forma o substrato da GLO1. Os produtos da reação da GLO2 incluem GSH e D-lactato (Figura 1) (Thornalley, 1993).

A atividade da enzima GLO1 previne o acúmulo de MG e de glioxal, além de diminuir as reações de glicação mediadas por estes compostos dicarbonil (Shinohara et al., 1998). A manipulação do sistema glioxalase, especificamente da GLO1 por ser a enzima limitante deste sistema, através da superexpressão ou inibição desta enzima, tem sido utilizada em diversos estudos *in vitro* e *in vivo* com o objetivo de verificar os efeitos de alterações nas concentrações de MG e dos AGEs derivados deste composto (Thornalley et al., 1996; Kuhla et al., 2006; Nigro et al., 2014).

Outra via de detoxificação do MG, porém de menor relevância quando comparada com o sistema glioxalase, é a aldose redutase, entretanto sua distribuição nos tecidos humanos é restrita. Esta enzima realiza a redução de MG em lactaldeído na presença de GSH e em acetol na ausência de GSH e, após, lactaldeído e acetol são convertidos a 1,2-propanodiol (Figura 1) (Vander Jagt e Hunsaker, 2003).

2.2 Metilglioxal

O MG é um composto dicarbonil que pode ser originado de fonte endógenas e exógenas. A formação endógena de MG ocorre principalmente a

partir de intermediários da via glicolítica (as trioses-fosfato - diidroxiacetona fosfato e o gliceraldeído-3 fosfato) (Figura 1), através da eliminação espontânea (não-enzimática) do grupo fosfato ou pela ação da triose fosfato isomerase (Pompliano et al., 1990; Richard, 1993). Esta via de formação de MG é responsável pela alta taxa de produção relativa deste composto (0,05 a 0,1% do fluxo glicolítico) (Rabbani e Thornalley, 2015). É importante considerar que o MG pode ser gerado a partir de outras vias metabólicas onde trioses-fosfatos são intermediários, como a gliconeogênese e a gliceroneogênese (Rabbani e Thornalley, 2015).

O metabolismo de corpos cetônicos, através da ação das enzimas citocromo P450 e mieloperoxidase, e o catabolismo do aminoácido treonina, pela amino oxidase, produzem MG no espaço intracelular (Figura 1) (Thornalley, 1993). O MG pode ser formado também, de forma não-enzimática, pela lenta degradação da glicose e dos produtos de Amadori através da reação de Maillard (Thornalley et al., 1999) e pela oxidação do acetol e a peroxidação lipídica (Nemet et al., 2006; Vander Jagt e Hunsaker, 2003).

As fontes exógenas de MG incluem diversos alimentos, bebidas, fumaça do cigarro e a atmosfera urbana. As fontes do MG em alimentos e bebidas são os açúcares, os produtos da reação de Maillard, lipídios e microorganismos formados durante o processamento industrial, cozimento e armazenamento prolongado (Nemet et al., 2006). Fatores como a temperatura e o tempo de preparação dos alimentos influenciam na maior formação de MG (Martins et al., 2003), além da presença de monossacarídeos e a composição lipídica (Nemet et al., 2006).

O MG presente nos alimentos é completamente ou parcialmente metabolizado e/ou reage com as proteínas antes da absorção no trato gastrointestinal, portanto, parece não exercer influência direta sobre a sua concentração *in vivo* (Degen et al., 2013). Já os AGEs presentes nos alimentos e bebidas exercem grande influência nas concentrações plasmáticas destes compostos e causam alterações metabólicas como o aumento da inflamação, estresse oxidativo e resistência à insulina em roedores e em humanos (Sandu et al., 2005; Uribarri et al., 2005). Estratégias dietoterápicas para redução da ingestão de AGEs tem sido propostas a fim de reduzir o impacto negativo do consumo de AGEs no desenvolvimento ou agravamento de doenças cardiovasculares, diabetes e DA (Uribarri et al., 2010; Uribarri et al., 2011; West et al., 2014). A pirralina é um AGE formado exclusivamente em temperaturas elevadas e, portanto, é um marcador da exposição de AGEs a partir de alimentos (Rabbani e Thornalley, 2015).

2.3 AGEs derivados do MG

A glicação ocorre em condições fisiológicas e em algumas condições patológicas ela é exacerbada. A implicação das reações de glicação está relacionada com o dano a proteínas celulares e extracelulares em sistemas fisiológicos, acometendo entre 0,1 a 0,2% dos resíduos de arginina e lisina (Thornalley, 2002; Thornalley et al., 2003), devido à suscetibilidade desses aminoácidos à glicação. A carboxietil-lisina (CEL) (Figura 1) e a hidroimidazolona derivada do MG (MG-H1) são AGEs derivados da reação do MG com lisina e arginina, respectivamente (Thornalley, 2005; Thornalley e Rabbani, 2011).

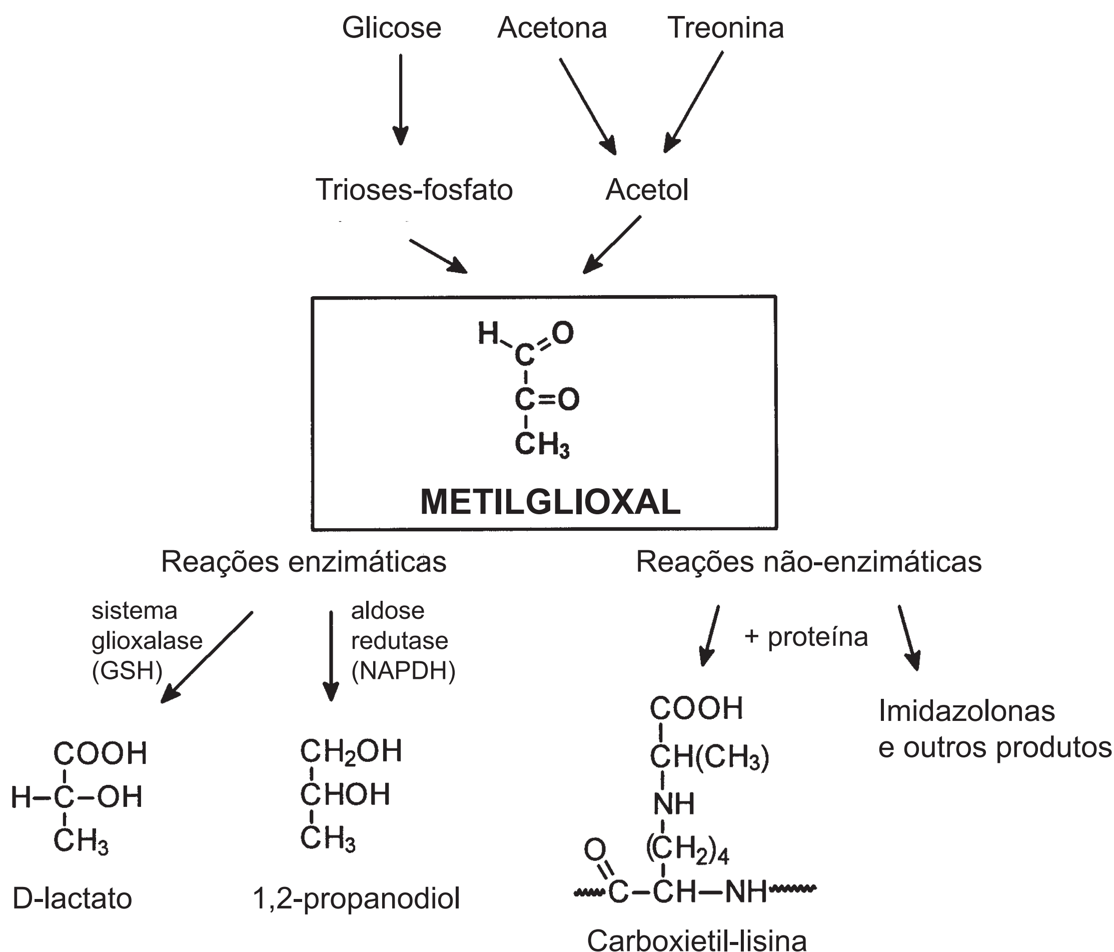

Figura 1. Fontes e reações do MG. Adaptado de Ahmed et al., 1997.

A CEL é um AGE formado a partir da glicação de lisina pelo MG, podendo ser gerada também a partir de trioses-fosfato e outros açúcares (Ahmed et al., 1997). Elevadas concentrações de CEL foram encontradas no plasma e urina de pacientes com diabetes e no líquido cefalorraquidiano (LCR) de indivíduos com DA (Ahmed et al., 1997; Ahmed et al., 2005; Barzilay et al., 2013; Hanssen et al., 2013; Beisswenger et al., 2013; Beisswenger et al., 2014). Já a MG-H1 é um importante AGE formado a partir da glicação de arginina pelo MG, que corresponde a cerca de 90% dos adutos (Rabbani e Thornalley, 2012b). Quantitativamente, este é o principal AGE encontrado no plasma, LCR, urina, retina, nervos e glomérulos renais em modelos

experimentais ou clínicos de diabetes (Thornalley et al., 2003; Karachalias et al., 2003; Karachalias et al., 2010; Ahmed et al., 2004; Ahmed et al., 2005; Ahmed et al., 2006).

O fato de terem sido encontrados elevados níveis de CEL e MG-H1 no LCR de pacientes com DA (Ahmed et al., 2005) reforça a hipótese de que os AGEs e seus precursores estão envolvidos na fisiopatologia desta doença. Além disso, foi sugerido que três AGEs, dentre eles CEL e MG-H1, podem ser indicadores precoces da progressão de importantes lesões na nefropatia diabética, inclusive melhores preditores da rápida progressão do que a hemoglobina glicada (Beisswenger et al., 2013).

2.4 Níveis de MG em condições fisiológicas e patológicas

Alguns autores propuseram que as concentrações fisiológicas de MG (intra e extracelulares) estão entre 0,1 – 2,0 μM (Ohmori et al., 1987). Outros inferiram que a concentração de MG no plasma humano é de aproximadamente 0,1 μM (Beisswenger et al., 1999; Nicolay et al., 2006) e que a concentração de MG intracelular é geralmente de 1,0 a 4,0 μM (Phillips et al., 1993; Dobler et al., 2006; Nicolay et al., 2006).

A elevada produção de MG ocorre em condições de hiperglicemia (Lee et al., 2009) e a partir do metabolismo de corpo cetônicos, que tem maior relevância em situações de cetose e cetoacidose (Rabbani e Thornalley, 2015). Dependendo do controle glicêmico, o fluxo de formação de MG pode aumentar de 2 a 4 vezes em pacientes diabéticos, assim como a formação de AGEs derivados do MG (Rabbani e Thornalley, 2014b). No DM 1 as concentrações de MG estão elevadas de 5 a 6 vezes e no DM 2 de 2 a 3 vezes (Thornalley,

1993). Por consequência, os AGEs derivados do MG estão elevados nos fluidos corporais em modelo experimental e clínico de diabetes (Ahmed et al., 2005; Karachalias et al., 2010). Em pacientes com DA a média das concentrações de MG encontradas no LCR foi de 22 μM, que são concentrações maiores do que as encontradas em pacientes sem esta doença (Kuhla et al., 2005).

O MG é um composto capaz de permear a membrana plasmática das células por difusão passiva na forma não hidratada, portanto, a desidratação do MG é um passo limitante que influencia diretamente na meia-vida deste composto (Rabbani e Thornalley, 2014a). Foi proposto que a meia-vida do MG é de aproximadamente 4 minutos (Rabbani e Thornalley, 2014a), contudo, quando considerado o metabolismo do MG pelo sistema glioxalase a meia-vida seria de cerca de 10 minutos (estimada a partir de taxas de formação de D-lactato *in situ* nas células). A maior parte do MG livre (> 95%) está ligada à proteínas de forma reversível. O tempo médio de ligação com proteínas de forma irreversível no plasma é de aproximadamente 3,6 horas (Rabbani e Thornalley, 2015).

Foi proposto que os níveis de MG no plasma são aproximadamente 50.000 vezes menores do que os de glicose, considerando o sistema glioxalase atuante. Portanto, pode-se estabelecer que em condições pós-prandiais ou em diabetes descompensado, quando os níveis de glicose plasmática estão em torno de 10 mM (Lapolla et al., 2005a; Khan et al., 2006; Lund et al., 2008; Barzilay et al., 2013), as concentrações de MG estarão em torno de 0,2 μM (50.000 vezes menor). Contudo, o MG apresenta reatividade para glicação muito maior que a glicose (10.000 a 50.000 vezes maior) (Thornalley, 2005).

Assim sendo, a formação de adutos de proteínas através da glicação mediada pelo MG *in vivo* ocorre em um fluxo muito semelhante ao da glicação mediada pela glicose (Rabbani e Thornalley, 2012b).

As concentrações de MG e tempos de tratamento utilizados diferem entre os estudos publicados, tanto *in vitro* quanto *in vivo*. Culturas de neurônios foram incubadas com 100 μM de MG por 24 horas (Di Loreto et al., 2004; Di Loreto et al., 2008), culturas de astrócitos - 400 a 800 μM por 24 horas (Chu et al., 2014), fatias de córtex cerebral - 400 μM por 1 hora (Schmidt et al., 2010) e fatias de córtex e de hipocampo foram tratadas com 100 μM a 5000 μM de MG durante 1 hora (Heimfarth et al., 2013). Já em estudos de eletrofisiologia foram utilizadas culturas de neurônios de hipocampo e cerebelo incubadas com 0,01 a 100 μM de MG (Distler et al., 2012) ou utilizadas fatias de cérebro de camundongo, que foram tratadas com 10 μM de MG (Hambsch et al., 2010). Em estudos *in vivo*, quando foi realizada admininistração oral de MG utilizou-se concentrações de 600 mg/kg de peso corporal durante 4 semanas em ratos (Lee et al., 2012). Em estudos com administração intraperitoneal as doses utilizadas estão na faixa de 50 a 300 mg/kg de peso corporal em camundongos (Distler et al., 2012; Distler et al., 2013) ou 50 a 75 mg/kg de peso corporal em camungondos ou ratos (Nigro et al., 2014). Existe um estudo publicado, até o momento, que utilizou administração intracerebroventricular (ICV) de MG, no qual a concentração de 467 mM de MG foi injetada por 6 dias consecutivos em camundongos (Hambsch et al., 2010).

No entanto, a tarefa de mimetizar os efeitos do MG através do uso de concentrações fisiológicas e, até mesmo patológicas, é um desafio especialmente devido ao tempo de incubação possível para cada modelo

utilizado e à manutenção dos níveis deste composto. É importante ressaltar que o aumento da exposição do proteoma ao MG através de níveis extremamente elevados (suprafisiológicos) de MG exógeno - como em concentrações milimolares, que podem ocorrer apenas em situações de toxicidade aguda (Jerzykowski et al., 1975; Kang et al., 1996) – eleva a propensão a modificar e prejudicar a função de muitas proteínas que não são suscetíveis a esse efeito sob condições fisiológicas, mesmo em situações patológicas (Rabbani e Thornalley, 2012b).

2.5 Estresse dicarbonil

A expressão “estresse dicarbonil” é frequentemente utilizada na literatura científica e é caracterizada pelo acúmulo anormal de metabólitos dicarbonil que leva a um aumento da taxa de reação de compostos dicarbonil com proteínas, nucleotídeos e fosfolipídios e a formação de adutos – AGEs (Rabbani e Thornalley, 2015). A causa do estresse dicarbonil se deve ao aumento da formação e/ou redução do metabolismo de compostos dicarbonil, e exposição a dicarbonil exógenos (Rabbani e Thornalley, 2015). Alguns exemplos de situações em que ocorre estresse dicarbonil incluem o diabetes, onde foi observado aumento da concentração plasmática e tecidual de MG (McLellan et al., 1994), a insuficiência renal pelo aumento da concentração de MG, glioxal, 3-deoxiglicosona (Rabbani e Thornalley, 2012a) e o envelhecimento do cristalino verificado pelo aumento da modificação de proteínas causada pelo MG (Ahmed et al., 1997; Ahmed et al., 2003).

A extensão da glicação de proteínas por compostos dicarbonil é baixa, geralmente de 1 a 5%, mas pode aumentar no envelhecimento e em situações

patológicas, contribuindo para a disfunção de células e tecidos. As proteínas modificadas por MG e glioxal são reconhecidas como mal enoveladas e direcionados para o proteassoma a fim de que ocorra proteólise (Rabbani e Thornalley, 2015).

Quando o estresse dicarbonil está instalado ocorre dano exacerbado ao proteoma e ao genoma, que leva ao acúmulo de AGEs, à glicação de proteínas, do ADN e de moduladores de transcrição. Essas alterações são capazes de gerar apoptose, mudanças na transcrição gênica, ligações cruzadas de proteínas, inflamação, dano tecidual (Ramasamy et al., 2006) (Figura 2), quebras na cadeia do ADN e mutagênese (Thornalley et al., 2010).

Os AGEs podem causar ligação cruzada com proteínas e fosfolipídios presentes em membranas basais e estruturais, desse modo, modificando estruturas celulares essenciais que atuam na permeabilidade e motilidade celular (Ramasamy et al., 2005). Além disso, eles podem interagir com diferentes receptores celulares, como o receptor para AGEs (RAGE) (Figura 2). Através da ligação com o RAGE, os AGEs regulam positivamente a produção de uma série de moléculas inflamatórias e radicais livres, que provocam lesão aos tecidos, o que contribui para o desenvolvimento de doenças cardiovasculares, neurodegenerativas e diabetes (Thornalley, 1998; Lapolla et al., 2005b).

O uso de bloqueadores da produção de AGEs ou moléculas que estimulem a degradação deles ou de seus precursores pode representar um passo importante na prevenção e/ou tratamento de patologias onde o estresse dicarbonil está presente (Ramasamy et al., 2005; Rabbani e Thornalley, 2015). Neste sentido, estudos mostraram que a metformina - um composto

guanidínico, que tem ação hipoglicemiante e é utilizado no tratamento do diabetes - possui ação anti-glicação, pois se liga e inativa o MG, reduzindo a formação de AGEs derivados dele (Beisswenger e Ruggiero-Lopez, 2003; Beisswenger, 2014). Já a suplementação de tiamina mostrou ser benéfica como estratégica anti-glicação em estudos clínicos, atuando na redução da formação de MG (Rabbani et al., 2009; Alaei Shahmiri et al., 2013).

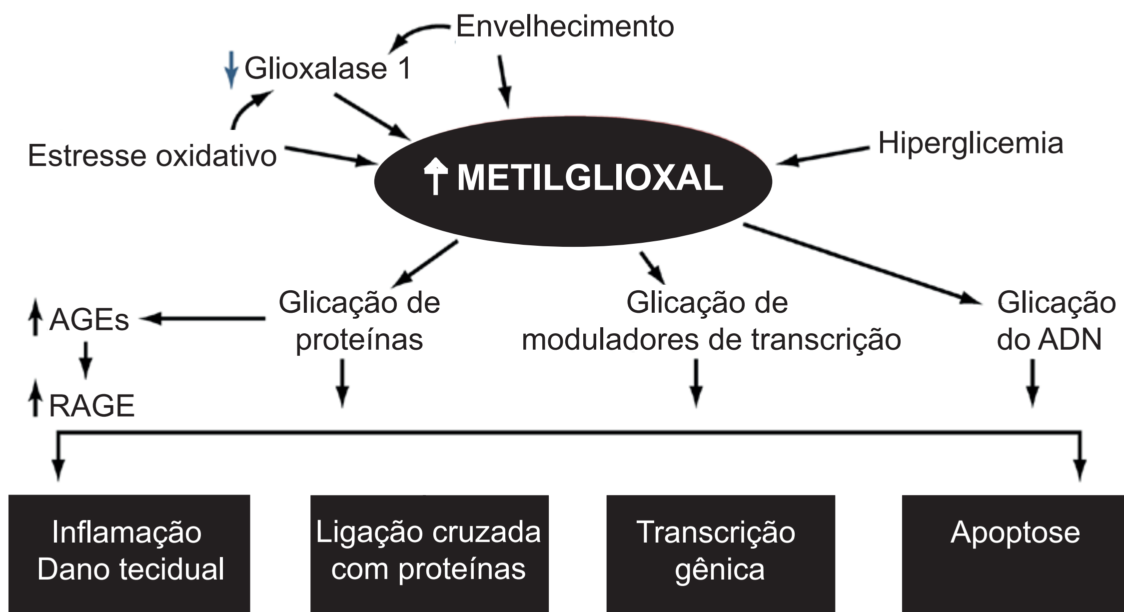

Figura 2. Alterações causadas pelo MG no proteoma e no genoma. Adaptado de Ramasamy et al., 2006.

Foram descritos outros receptores de AGEs e, embora o estudo desses receptores ainda seja escasso, evidências sugerem que eles possuem papel fisiológico diferente e, possivelmente oposto, do RAGE (Vlassara e Striker, 2011). Ou seja, quando ocorre a ligação e ativação desses receptores a resposta inflamatória e a formação de radicais livres é reduzida. Assim, o estudo desses receptores pode auxiliar na compreensão e desenvolvimento de

estratégias terapêuticas que evitem ou limitem o estresse dicarbonil (Thornalley, 1998; Vlassara, 2001; Cai et al., 2014; Ott et al., 2014).

3 Glicação e doenças neurodegenerativas

Nos últimos anos tem-se aprofundado a investigação e a discussão da importância da regulação dos níveis de MG para a homeostase do Sistema Nervoso Central (Williams et al., 2011; Hambsch, 2011). Estudos experimentais e clínicos indicam a associação do diabetes com a DA e sugerem que a hiperglicemia, o MG e os AGEs podem ser mediadores desse dano (Krautwald e Münch, 2010; West et al., 2014). Os níveis séricos de MG foram associados com rápido declínio cognitivo em indivíduos idosos (Beeri et al., 2011) e elevadas concentrações plasmáticas de AGEs, oriundas da ingestão de alimentos com AGEs, foram relacionadas com prejuízo cognitivo envolvendo memória em idosos não dementes e em camundongos (Cai et al., 2014; West et al., 2014). Como os indivíduos diabéticos possuem elevados níveis de AGEs circulantes, esse achado reforça a hipótese dos altos níveis de declínio cognitivo e DA em indivíduos com diabetes (West et al., 2014).

Além disso, foi demonstrada diminuição da atividade do sistema glioxalase no envelhecimento e nos estágios intermediário e tardio da DA e proposto que essa redução levaria ao aumento da glicação e do dano aos tecidos (Thornalley, 2003; Kuhla et al., 2007), possivelmente, através do aumento da concentração de MG. Curiosamente, na fase inicial da DA ocorre regulação positiva da GLO1, provavelmente de maneira compensatória, para evitar o aumento de MG (Kuhla et al., 2005; Kuhla et al., 2007). Como

consequência, previne-se o aumento de AGEs que tem sido demonstrado com a superexpressão da GLO1 (Thornalley, 2003).

Foi proposto que a glicação acelera os processos de polimerização do β-amilóide e da hiperfosforilação da tau, que são marcadores histopatológicos da DA e que essas alterações levariam à formação facilitada das placas senis e emaranhados neurofibrilares, respectivamente (Li et al., 2012; Li et al., 2013). Este achado corrobora com estudos que verificaram que os AGEs se acumulam intra e extracelularmente no cérebro durante o envelhecimento e na DA (Jono et al., 2002; Münch et al., 2003; Grillo e Colombatto, 2008). Nos pacientes com DA foi verificada a co-localização dos AGEs com as placas senis e os emaranhados neurofibrilares (Smith et al., 1994; Kroner, 2009) e um aumento de AGEs de aproximadamente 3 vezes nas placas senis comparado com controles pareados por idade (Vitek et al., 1994).

Em modelo experimental de diabetes observou-se que os níveis de MG estão elevados e relacionados com o prejuízo de memória e aprendizagem (Brouwers et al., 2011; Huang et al., 2012). A superexpressão da GLO1 diminuiu significativamente (81%) os níveis de glioxal e de MG bem como de AGEs (aproximadamente 50%) no plasma de ratos diabéticos (Brouwers et al., 2011). Outro estudo mostrou que o aumento da expressão e da atividade da GLO1 foi capaz de diminuir os níveis de AGEs e de RAGE no hipocampo e melhorar o dano cognitivo induzido pelo modelo de diabetes em ratos (Liu et al., 2013). Estes achados indicam que a regulação dos níveis de MG é essencial para evitar o dano cognitivo que ocorre no diabetes.

Um importante papel fisiológico que foi atribuído ao MG é sua ação agonista ao receptor ácido γ-aminobutírico ($GABA_A$) (Dislter et al., 2012). Os

autores propuseram a associação deste efeito agonista com o comportamento do tipo ansiedade e o controle de convulsões em modelo animal. Estudos recentes demonstraram que a administração de MG reduziu o comportamento do tipo ansiedade e que o aumento da expressão e atividade da GLO1 elevou o comportamento associado à ansiedade através da redução dos níveis de MG (Hovatta et al., 2005; Hambsch et al., 2010; Distler et al., 2012). Uma pesquisa sugere que a inibição da GLO1 atenua as convulsões em modelo animal de epilepsia pelo aumento dos níveis de MG e, consequente maior ativação do receptor $GABA_A$ (Distler et al., 2013). Doses mais elevadas de MG produziram depressão locomotora, ataxia e hipotermia em camundongos, alterações que também podem estar relacionadas com a ativação do receptor $GABA_A$ (Distler et al., 2012).

A deficiência experimental de α-sinucleína aumentou o conteúdo de dicarbonil e a expressão de GLO1 no tronco cerebral, mesencéfalo e córtex, sugerindo que a α-sinucleína pode prevenir o estresse dicarbonil e esta função pode ser prejudicada na doença de Parkinson (Kurz et al., 2011). Em relação a esquizofrenia, observou-se que a deficiência clínica rara de GLO1 estava associada a um risco elevado de esquizofrenia grave (Arai et al., 2010).

3.1 Glicação e células do Sistema Nervoso Central

Os astrócitos, assim chamados por sua forma estrelar, são as células mais abundantes pertencentes a glia, que durante muito tempo foi considerada somente como um suporte estrutural para os neurônios (Kimelberg e Norenberg, 1989). Dentre as inúmeras funções exercidas pelos astrócitos pode-se citar: a formação e manutenção da barreira hemato-encefálica (Abbott

et al., 2006), a produção de fatores tróficos (Eriksen e Druse, 2001), o suporte energético para os neurônios (Pellerin, 2005), a regulação dos níveis de glutamato na sinapse (Bernardinelli et al., 2004), a síntese e liberação de GSH (Dringen et al., 2000; Shih et al., 2006) e a capacidade de funcionar como células imunopotentes (Dong e Benveniste, 2001). Os astrócitos participam também da sinalização celular, fazendo parte das sinapses, formando uma unidade multifuncional juntamente com os neurônios (Fellin e Carmignoto, 2004).

Em condições fisiológicas e patológicas, os astrócitos desempenham funções essenciais para a sobrevivência dos neurônios (Nedergaard et al., 2003). Estudos sugerem que a diminuição das funções astrocíticas está associada a doenças neurodegenerativas (Fuller et al., 2010), como a DA, e que os AGEs se acumulam em neurônios e astrócitos, bem como em placas senis (Luth et al., 2005).

Diversos estudos têm demonstrado o efeito de compostos dicarbonil sobre funções do Sistema Nervoso Central em culturas de células primárias e linhagens tumorais. Os neurônios foram as células mais investigadas, porém, nos últimos anos, tem se voltado a atenção para o papel dos astrócitos na glicação. Evidências indicam que os astrócitos possuem um eficiente sistema glioxalase comparado com os neurônios e isso se caracterizaria como uma forma de cooperação entre esses tipos celulares, retratada como um mecanismo de neuroproteção contra o estresse dicarbonil (Bélanger et al., 2011b; Allaman et al., 2015). Também em linhagens tumorais foi demonstrado que as células gliais e os neurônios respondem de forma diferente frente à exposição ao MG. Em células de glioblastoma humano (ADF) foi observado

aumento da atividade da GLO1 e GLO2 através do tratamento com MG, todavia, em linhagem de células de neuroblastoma humano (SH-SY5Y) a atividade da GLO1 reduziu com a exposição ao MG (Amicarelli et al., 2003).

As células de glioma C6 têm sido utilizadas como modelo para o estudo de parâmetros gliais, permitindo o melhor entendimento do funcionamento e mecanismo de resposta da glia frente à diferentes insultos (Hansen et al., 2012; de Souza et al., 2013). O glioma C6 constitui-se numa linhagem neural, apresentando características de astrócitos (Parker et al., 1980) a partir da centésima passagem de cultivo (de Souza et al., 2009). A expressão das proteínas gliais S100B e proteína glial fibrilar ácida (GFAP) confirma o seu caráter astrocitário (Benda et al., 1971).

OBJETIVOS

Objetivo geral

Investigar os efeitos do MG em parâmetros comportamentais e neuroquímicos *in vitro* e *ex vivo*.

Objetivos específicos

1. Avaliar a toxicidade do MG frente a exposição de curto (1 hora) e longo (24 horas) prazo em culturas de astrócitos e de glioma C6;
2. Examinar algumas diferenças metabólicas e constitucionais existentes entre as culturas de astrócitos e glioma C6 que permitam elucidar a suscetibilidade ao dano causado pelo MG;
3. Examinar os efeitos de elevadas concentrações de glicose, de MG e CEL, sobre parâmetros oxidativos, metabólicos e específicos de ativação e função astrocítica em fatias de hipocampo;
4. Investigar alguns dos mecanismos que podem mediar os efeitos de elevadas concentrações de glicose, de MG e CEL, em fatias de hipocampo;
5. Verificar se a administração ICV de MG causa alterações comportamentais, relacionadas com declínio cognitivo e ansiedade, e alterações bioquímicas *ex vivo* em hipocampo, córtex cerebral e LCR de ratos Wistar.

CAPÍTULO 1

Glutamate uptake activity and glutathione levels diminish following methylglyoxal exposure in glial cells: differences of the glyoxalase system

Submetido ao periódico Glia

Glutamate uptake activity and glutathione levels diminish following methylglyoxal exposure in glial cells: differences of the glyoxalase system

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Glutamate uptake activity and glutathione levels diminish following methylglyoxal exposure in glial cells: differences of the glyoxalase system

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Running title: Glyoxalase and glutamate in glial cells

This article has 2 tables and 6 figures.

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Main Points:

- High efficiency of the glyoxalase system in astrocytes representing an important mechanism for avoiding dicarbonyl stress
- MG-induced astrocyte glutamate uptake activity occurs without changes in MG detoxification by the glyoxalase system

ABSTRACT

In diabetes and neurodegenerative diseases, astrocytes are impaired to glycation processes, although, they have an improved glyoxalase system compared to neurons that protect them from dicarbonyl stress, suggesting that damage to neuronal functions could be increased under these pathological conditions. It is important to establish the effects of methylglyoxal (MG), a reactive aldehyde, involved in dicarbonyl stress and advanced glycation endproducts (AGEs) formation, on astrocyte-related parameters to elucidate the alterations that may impair brain homeostasis. Herein, we evaluate the susceptibility of astrocytes and C6 glioma cells cultures (an astrocyte-like cell line) to MG damage upon short and long-term exposure to MG. Interestingly, we observed a high efficiency of the glyoxalase system in astrocytes, compared to C6 cells representing an important mechanism for avoiding dicarbonyl stress and AGEs formation. Moreover, in C6 cells, glutathione levels are decreased from 1 h after MG exposure, despite the fact that reactive species formation is not altered. Glutamate uptake was decreased in astrocytes and increased in C6 cells and this effect is related to glycation processes, but not to glutamate transporters expression. S100B secretion and glial fibrillary acidic protein (GFAP) content were not modified by MG treatment in either culture studied. In conclusion, impairment in MG-induced astrocyte glutamate uptake activity occurs without changes in MG detoxification by the glyoxalase system. The increased levels of glutamate can cause excitotoxicity in neurons and contributes to neurological changes related to diabetes. Data obtained from C6 cultures indicate that non-astrocyte glutamate transporters may demonstrate altered activity following MG exposure.

KEY WORDS: Advanced glycation endproducts; Astrocytes; C6 glioma cells; Dicarbonyl stress

INTRODUCTION

Astrocytes outnumber neurons in the human brain and are essential to the survival of neurons in physiological and pathological conditions (Nedergaard et al., 2003). Data indicate that the impairment of astrocyte functions is linked to neurodegenerative diseases (Fuller et al., 2010), such as Alzheimer's disease, where advanced glycation endproducts (AGEs) accumulate in neurons and astroglia, as well as in neuritic amyloid plaques (Luth et al., 2005). The key role of astrocytes in brain includes defense against oxidative stress, synthesis and release of glutathione (GSH), glutamate uptake, modulation of synaptic activity and synapse formation and remodeling (Volterra and Meldolesi, 2005; Bélanger et al., 2011a).

Physiological and pathological increases in glucose, as occur in diabetes, can cause the elevation of the concentration of methylglyoxal (MG), a reactive aldehyde, involved in dicarbonyl stress and AGEs formation. MG is generated from the glycolytic pathway, by triosephosphate deviation (about 0.1 a 1%) and also by glycine, threonine, aminoacetone and acetone metabolism (Phillips and Thornalley, 1993; Amicarelli et al., 2003). In this context, the glyoxalase system is important to detoxify MG and, as a consequence, to maintain MG concentration under control, avoiding AGEs formation (Thornalley, 1993). AGEs are generated as a result of glycation reactions by MG with amino acids, proteins, nucleic acids or lipids (Di Loreto et al., 2008).

Despite knowledge that astrocytes have a higher glyoxalase system, compared to the neurons, which protects them from dicarbonyl stress (Bélanger et al., 2011b; Allaman et al., 2015), it has been demonstrated that astrocytes are damaged by glycation processes in diabetes and neurodegenerative diseases (Luth et al., 2005; Shaikh and Nicholson, 2008), suggesting that the damage to neuronal functions could be aggravated under these pathological conditions. It is important to establish the effects of MG on astrocyte functions and on astrocyte-related parameters to elucidate the alterations that occur in this cell type and that may impair brain homeostasis. Herein, we evaluate the susceptibility of primary astrocytes cultures from Wistar rats to MG damage following short (1 h) and long-term (24 h) exposure. We also used C6 glioma cells in this study; metabolic differences in the metabolism of glucose and glutamate have previously been described for C6 cells and astrocytes (Nardin et al., 2007; Tramontina et al., 2012), and this study aims to improve our understanding of MG-induced damage in glial cells.

MATERIALS AND METHODS

Materials

Methylglyoxal (MG), propidium iodide, [3(4,5-dimethylthi-azol-2-yl)-2,5-diphenyl tetrazolium bromide] (MTT), neutral red, carbenoxolone disodium salt (CBX), 2′,7′-dichlorofluorescein diacetate (DCF-DA), standard glutathione, Triton X-100, o-phthaldialdehyde, S100B protein, anti-S100B (SH-B1), L-glutamate, 4-(2-hydroxyethyl) piperazine-L-ethanesulfonic acid (HEPES), o-phenylenediamine (OPD), meta-phosphoric acid, N-methyl-D-glucamine, aminoguanidine hemisulfate salt (AG) and cell culture materials were purchased from Sigma (Saint Louis, MO, USA). Dulbecco's Modified Eagle Medium (DMEM) and Dulbecco's Phosphate-Buffered Saline (DPBS) were purchased from Gibco BRL (Carlsbad, CA, USA). Fetal bovine serum was obtained from Cultilab (Campinas, SP, Brazil) and L-[2,3-^{3}H] glutamate was purchased from Amersham International (United Kingdom). Polyclonal anti-S100B and polyclonal anti-GFAP were purchased from DAKO (São Paulo, SP, Brazil), anti-rabbit peroxidase linked antibody was purchased from GE Healthcare (Little Chalfont, United Kingdom), polyclonal anti-EAAT1 (GLAST) and anti-EAAT2 (GLT-1) were purchased from Abcam (Cambridge, MA, USA) and polyclonal anti-GLO1 was purchased from Santa Cruz Biotechnology, Inc. (Dallas, Texas, USA). Alexa fluor 568 was purchased from Life Technologies (Eugene, OR, USA). Rhodamine phalloidin was purchased from Molecular Probes® (Eugene, OR, USA). All other chemicals were purchased from local commercial suppliers.

Primary astrocyte culture

Primary astrocyte cultures from Wistar rats were prepared as previously described (Gottfried et al., 2003). Procedures were in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals and were approved by the local authorities. Briefly, the cerebral cortex of newborn Wistar rats (1–2 days old) were removed and mechanically dissociated in Ca^{2+}- and Mg^{2+}- free Dulbecco's Phosphate-Buffered Saline (DPBS), pH 7.2, containing (in mM): 137.93 NaCl, 2.66 KCl, 8.09 Na_2HPO_4, 1.47 KH_2PO_4 and 5.55 glucose. The cortex was cleaned of meninges and mechanically dissociated by sequential passage through a Pasteur pipette. After centrifugation at 1,400 rpm for 5 min, the pellet was resuspended in DMEM (pH 7.6)

supplemented with 8.39 mM HEPES, 23.8 mM $NaHCO_3$, 0.1% amphotericin B, 0.032% garamycin and 10% fetal bovine serum. Approximately 300,000 cells were seeded in each well in 24-well plates and maintained in DMEM containing 10% fetal bovine serum in 5% CO_2/95% air at 37°C, then allowed to grow to confluence and used at 21 days *in vitro*. The medium was replaced by DMEM with 1% fetal bovine serum in the absence or presence of MG at 1 or 24 h. CBX (0.1 mM) was added 15 min before MG exposure and afterwards cells were co-incubated with MG at 24 h. Co-incubation of MG and AG (400 µM) at 24 h was also performed.

C6 glioma cell culture

The C6 glioma cell line was obtained from the American Type Culture Collection (Rockville, Maryland, USA). Late passage cells, harvested after at least 100 passages, are an astrocyte-like cell line, were seeded in 24-well plates at densities of 10.000 cells/well and cultured in DMEM (pH 7.6) supplemented with 5% fetal bovine serum, 2.5 mg/mL Fungizone and 100 U/L gentamicin in 5% CO_2/95% air at 37°C. After the cells had reached confluence, the culture medium was replaced by DMEM without serum in the absence or presence of MG at 1 or 24 h (De Souza et al., 2009). Co-incubation of MG and AG (400 µM) at 24 h also was performed.

Propidium iodide uptake assay

Cells were treated with MG concomitantly with 7.5 µM propidium iodide (PI) and incubated for 1 h or 24 h. Cells were viewed with a Nikon inverted microscope with a TE-FM Epi-Fluorescence accessory; images were transferred to a computer with a digital camera. Optical density was determined with the Optiquant version 02.00 software (Packard Instrument Company) and positive cell counts for PI were measured (Leite et al., 2009).

MTT reduction assay

Cells were treated with 0.5 mg/mL MTT for 30 min in 5 % CO2/95 % air at 37°C. The medium was then removed and MTT crystals were dissolved in DMSO. Absorbance values were measured at 560 and 650 nm. The reduction of MTT was calculated by the following formula: [(abs 560 nm) − (abs 650 nm)] (Hansen et al., 1989). Results were expressed as percentages of the control.

Neutral Red incorporation assay

Cells were treated with 50 μg/mL neutral red (NR) for 30 min in 5% CO_2/95% air at 37°C. Afterwards, the cells were rinsed twice with phosphate-buffered saline (PBS) for 5 min each time. The NR dye taken up by viable cells was then extracted with 500 μL of acetic acid/ethanol/water (1/50/49, v/v). Absorbance values were measured at 560 nm (Leite et al., 2009).

Evaluation of intracellular reactive species production

Intracellular reactive species production was detected via the nonfluorescent cell permeating compound, DCF-DA, which is hydrolyzed by intracellular esterases and then oxidized by reactive species to a fluorescent compound, 2′,7′-dichlorofluorescein (DCFH). Cells were treated with DCF-DA (10 μM) for 30 min at 37°C. 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. All procedures were performed in the dark, and blanks containing only DCF-DA were processed for measurement of autofluorescence (Quincozes-Santos et al., 2010). Values were obtained as fluorescence units/mg protein and were expressed as percentages of the control.

Glutathione (GSH) content assay

Intracellular GSH levels (nmol/mg protein) were measured as previously described (Browne and Armstrong, 1998). This assay detects only the reduced glutathione content. Cell homogenates were diluted in ten 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-phthaldialdehyde (1 mg/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 formed using standard GSH solutions (0–500 μM). GSH concentration was expressed as the percentage of the control.

Evaluation of glyoxalase system

Primarily, the astrocytes or C6 cultures were lysed and homogenized in sodium phosphate buffer, pH 7.4. Cell homogenates were centrifuged at 13,000 rpm for 15 min at 4°C and the supernatant was used for enzymatic activities and protein content

measurements. Glyoxalase I activity was then determined as previously described (Mannervik et al., 1981) with some modifications. The assay was carried out in 96-well microplates using a microplate spectrophotometer (UV Star - Greiner). The reaction mixture (200 µL/well) contained 50 mM sodium-phosphate buffer pH 7.2, 2 mM MG and 1 mM GSH (pre-incubated for 30 min at room temperature). Protein from the sample (10–20 µg per well) was added to the buffer. The formation of S-(D)-lactoylglutathione was linear and monitored at 240 nm for 15 min at 25°C. A unit of glyoxalase I activity is defined as the amount of enzyme that catalyzes the formation of 1 µmol of S-(D)-lactoylglutathione per minute. Specific activity is expressed in milliunits per milligram of protein.

D-lactate, the final product of the glyoxalase system, was assessed using a commercial kit obtained from the Cayman Chemical Company (Ann Arbor, MI, USA). The assay of intracelular D-lactate was performed according to the supplier's protocol.

Glutamate uptake assay

Glutamate uptake was performed as previously described (Gottfried et al., 2002) with some modifications. Cells were briefly 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 $Na_2HPO_4 \cdot 7H_2O$, 0.44 KH_2PO_4, 4.17 $NaHCO_3$ and 5.6 glucose, adjusted to pH 7.2. The assay was initiated 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 7 min for the astrocyte cultures and after 10 min for the C6 cultures by removing the medium and rinsing the cells twice with ice-cold HBSS. Cells were then lysed in a 0.5 M NaOH solution. 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 the specific uptake. Radioactivity was measured in a scintillation counter. Results were calculated as nmol/mg protein/min and were expressed as percentage of the control.

Western blot analysis

After 24 h of MG addition, astrocytes or C6 cultures were processed for electrophoresis/western blotting by directly homogenized in electrophoresis sample buffer at pH 6.8 (containing 62.5 mM Tris-HCl, 2% (w/v) SDS, 5% (w/v) β-mercaptoethanol, 10% (v/v) glycerol, 0.002% (w/v) bromophenol blue) and boiled for 2

min. Protein samples (20 μg per lane), prepared for electrophoresis as described above, were analyzed by 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to nitrocellulose membranes using a semidry blotting apparatus (1.2 mA/cm2; 1 h) (Karl et al., 2000). The membranes were blocked overnight with 5% bovine BSA in Tris-buffered saline (TBS) (10 mM Tris, 150 mM NaCl, pH 7.5) and then incubated for 3 h with an anti-EAAT-1 or anti-EAAT-2 antibody (diluted 1:1000, 1:200, respectively, in TBS containing Tween-20 and 2% BSA), or overnight with an anti-GLO1 antibody (diluted 1:200 in TBS containing Tween-20 and 2% BSA). Next, membranes were incubated for 2 h at room temperature with horseradish peroxidase (HRP)-conjugated anti-mouse secondary antibody. The chemiluminescent reactions were developed using luminol as the substrate (ECL Western Blotting Analysis System, GE Healthcare) and registered on radiographic film. The immunocontent of EAAT-1, EAAT-2 and GLO1 was determined for optical density. The bands were quantified using Scion Image® software.

S100B measurement

S100B was measured by an enzyme-linked immunosorbent assay, as previously described (Leite et al., 2008). Briefly, 50 μL of sample (previously diluted when necessary) plus 50 μL of Tris buffer were incubated for 2 h on a microtiter plate previously coated overnight with monoclonal anti-S100B (SH-B1) antibody. Polyclonal anti-S100 antibody was incubated for 30 min, and then peroxidase-conjugated anti-rabbit antibody was added for an additional 30 min. The microtiter plate was rinsed three times with a wash solution between each step of the technique. A colorimetric reaction with o-phenylenediamine was measured at 492 nm. The standard S100B curve ranged from 0.002 to 1 ng/mL. Data were calculated as ng/mL and were expressed as percentages of the control.

Glial Fibrillary Acidic Protein (GFAP) content

Enzyme-linked immunosorbent assay for GFAP was carried out, as previously described (Tramontina et al., 2007), by coating the microtiter plate with 100 μL samples, overnight at 4°C. Incubation with a polyclonal anti-GFAP from rabbit for 1 h was followed by incubation with a secondary antibody conjugated with peroxidase for 1 h, at room temperature. A colorimetric reaction with o-phenylenediamine was measured at 492 nm. The standard human GFAP (from Calbiochem) curve ranged from 0.1 to 5.0

ng/mL. Data were calculated as ng/μg total protein and were expressed as percentages of the control.

Immunocytochemistry

After 24 h of MG addition, cells were fixed for 20 min with 4% paraformaldehyde in phosphate buffer (PBS), containing (in mM): 51.33 NaCl, 19.13 $NaH_2PO_4.H_2O$, 81.01 Na_2HPO_4. Cells were then rinsed with PBS and permeabilized for 10 min in PBS containing 0.2% Triton X-100. Fixed cells were then blocked for 60 min with PBS containing 5% bovine serum albumin and incubated overnight with anti-GFAP (1:1000 in astrocytes; 1:250 in C6). Following incubation with primary antibody, the cultures were washed once in PBS containing 0.2% Triton X-100 and, subsequently, 3 times in PBS. Finally, cells were incubated for 2 h with Alexa 568 (goat anti-rabbit IgG; red fluorescence, 1:1000). Others cells were incubated for 20 min with rhodamine phalloidin (2.5 units/mL) after the blocked period (Pinto et al., 2000). Cells were viewed with a Nikon inverted microscope and images taken with a digital camera were transferred to a computer (Sound Vision Inc., Wayland, MA, USA).

Protein determination

Protein content was measured by Lowry's method using bovine serum albumin as standard (Peterson, 1977).

Statistical analysis

Data are reported as the mean ± standard error and analyzed statistically by Student's t-test, or by one-way or two-way analysis of variance (ANOVA). Statistically significant one-way ANOVA were followed by a post hoc Dunnett's test. Data were considered significant when $p<0.05$. All analyses were carried out using the SPSS software package or Prism 5.0 (GraphPad).

RESULTS

Cytotoxicity induced by MG in astrocytes is blocked by CBX

We evaluated the effect of MG (100, 400 and 1000 μM; 24 h) on astrocyte and C6 glioma cell viability by propidium iodide (PI) uptake, MTT reduction and NR incorporation assays. Only the highest concentration of MG tested induced cytotoxicity

in astrocytes and C6 glioma cells (Fig. 1) (Hansen et al., 2012). Interestingly, this effect of MG (1000 μM) on PI incorporation was inhibited by CBX, a gap junction inhibitor, and only in astrocytes cultures (Fig. 1). MTT reduction (Fig. 2) and NR incorporation (data not shown) was not altered by MG exposure for 24 h in either of the cell cultures. As 1000 μM MG had a cytotoxic effect on glial cells, we chose an intermediate concentration of MG (400 μM) for further experiments. Moreover, this is a concentration range of MG classically used in different cellular models to study glycation processes (Amicarelli et al., 2003; Kawaguchi et al., 2005; Di Loreto et al., 2008; Bélanger et al., 2011b; Chu et al., 2014).

Differences in the glyoxalase system of astrocytes and C6 glioma cells

To elucidate whether the glyoxalase system modulates the effects of MG on the cell cultures studied, we measured glyoxalase 1 expression and activity in cells, as this is the first enzyme involved in this system. We also determined D-lactate, which is the final product of the glyoxalase 2 reaction. Interestingly, the relative expression and activity of glyoxalase 1 are significantly higher in astrocytes than in C6 cells (3.3 and 0.7-fold, respectively) (Fig. 3A, B). Moreover, astrocytes present a 1.5-fold increase in glyoxalase 1 expression/activity ratio, compared to C6 glioma cells (Fig. 3C). The treatment did not alter the expression or activity of this enzyme (Fig. 3A, B), nor was the content of D-lactate modified by MG exposure at 24 h in either cellular cultures tested (data not shown).

MG exposure decreases GSH levels in C6 cells, but does not alter DCFH oxidation in either cultures studied

In order to establish whether MG could induce modifications in redox state and in glutathione (GSH), an important cellular defense enzyme that protects against oxidative and dicarbonyl stress, we measured DCFH oxidation and GSH content at 1 and 24 h of incubation. Reactive species formation was not altered by MG exposure; however, the GSH levels decreased in C6 cells at 1 and 24 h (Table 1).

Glutamate uptake was altered differently in astrocytes and C6 glioma cells following MG treatment

We performed glutamate uptake assays to establish whether MG could impair glutamate uptake in glial cells, constituting a mechanism that may explain its toxicity.

Curiously, glutamate uptake was altered differently in astrocytes and C6 cells at 24 h; where MG treatment decreased and increased glutamate uptake in astrocytes and C6 cells, respectively (Table 2). To investigate whether glycation processes mediated this MG-induced effect, we used aminoguanidine (AG), a well-known anti-glycation compound. In astrocyte cultures, the factor MG significantly reduced glutamate uptake and, while the factor AG significantly increased glutamate uptake. However, no statistical significance was found when analyzing the interaction between MG and AG (Fig. 4A). In C6 glioma cells, MG significantly increased glutamate uptake, but the factor AG did not alter this parameter. As in astrocytes cultures, no interaction between the MG and AG factors was observed (Fig. 4B). To clarify the interpretation of these results, particularly as to whether co-incubation of AG with MG could reverse the MG-induced effect, we also performed one-way ANOVA followed by Dunnett's post hoc test. This statistical analysis revealed that AG was able to the reverse MG-induced decrease in glutamate uptake in astrocytes cultures (ANOVA - $p=0.004$, Dunnett's test - Control X MG $p=0.015$ and Control X MG + AG $p=0.963$) and the increase caused by MG in C6 cells (ANOVA - $p=0.029$, Dunnett's test - Control X MG $p=0.014$ and Control X MG + AG $p=0.125$).

MG treatment does not alter glutamate transporters expression

To determine whether these differences in glutamate uptake were related to glutamate transporter expression, we performed Western blot analysis. Interestingly, MG treatment at 24 h did not change GLAST and GLT-1 expression in either cell cultures studied. The expression of these glutamate transporters was significantly higher in astrocytes than in C6 cells (Fig. 5).

S100B secretion and GFAP content were not modified by MG in astrocytes or C6 glioma cells

To clarify the effect of MG on astrocyte-related parameters, we analyzed S100B secretion and GFAP content in the cells. Interestingly, MG exposure did not alter S100B secretion nor the GFAP content, as demonstrated by enzyme-linked immunosorbent assay (Table 2) and immunocytochemistry (Fig. 6). To further investigate the effects of MG exposure on cytoskeletal organization, we examined the actin stress fiber. We observed that the organization of actin filaments was slightly

altered upon MG treatment in astrocytes with decrease of stress fiber, but not in C6 cells (Fig. 6).

DISCUSSION

We herein evaluated the short (1 h) and long-term (24 h) effect of MG on astrocyte cell cultures and on C6 lineage cells (an astrocyte-like cell model). We found that 1000 μM MG induces cytotoxicity in astrocytes and C6 glioma cells at 24 h. The specific fluorescence induced by MG (1000 μM), observed in the PI assay indicates that gap junctions may mediate this effect. Indeed, CBX, a gap junction inhibiter reverses the cytotoxicity induced by MG. C6 cells did not demonstrate a similar pattern of fluorescence to that of the astrocytes of the PI assay, in accordance with the fact that C6 cultures only contain low levels of gap junction channels, when compared with astrocytes (Leite et al., 2009). These data indicate that gap junctions have a pivotal role in MG-induced damage in astrocytes.

Studies in animal models of diabetes and *in vitro* experiments that determine the effects of high glucose levels on astrocytes cultures (Gandhi et al., 2010; Ball et al., 2011), as well as AGE-BSA treatment of aortic endothelial cells (Wang et al., 2011), demonstrate that these compounds reduce gap junction communications. The interpretation of these findings is in apparent conflict with our findings, possibly indicating a pathological effect or a response to limit the extension of the lesion and increase the chances of cell survival. Moreover, cytotoxicity induced by MG in astrocytes cultures was observed to be related to cellular confluence and the duration of treatments. Astrocyte cultures at high confluence were more resistant to alterations caused by MG (data not shown).

Unexpectedly, reactive species formation was not related to MG-induced changes in either cell cultures examined at 24 h. However, in several studies performed *in vitro* with neuron-containing preparations, MG was able to increase reactive species levels (Kikuchi et al., 1999; Amicarelli et al., 2003; Di Loreto et al., 2004; Heimfarth et al., 2013). In astrocytes, which have higher levels of GSH, compared to C6 cells (Guerra et al., 2011; de Souza et al., 2013), no change on DCFH oxidation was expected; however, in C6 cells we observed a rapid and persistent decrease in GSH levels upon MG treatment. It has been previously demonstrated that incorporation of glycine in proteins is significantly decreased within 1 h and 3 h of MG exposure in C6

cells (Hansen et al., 2012). As glycine is an amino acid that is involved in GSH synthesis (Bélanger et al., 2011a) this explains, at least in part, the reduction in GSH at 1 h of MG treatment in C6 cultures.

Data indicate that astrocytes are very resistant to high concentrations of MG (Chu et al., 2014), compared to neurons (Bélanger et al., 2011b). In fact, we observed that glyoxalase 1 expression and activity is higher in astrocytes than in C6 glioma cells; however MG did not induce alterations in the glyoxalase system in either of the cell cultures (two-way ANOVA). This important difference in the glyoxalase system is accompanied by GSH levels regarding astrocytes and C6 cells and may explain the distinct susceptibility of MG-induced changes regarding these glial cells.

With regard to glutamate uptake, we observed an impairment in uptake upon MG treatment in astrocytes, but not in C6 cells, where an increase in glutamate uptake was verified. The exposure of astrocytes to another glycant agent, glyoxal, indicates the formation of a GLT-1 CML adduct and a reduction in glutamate uptake activity (Kawaguchi et al., 2005). Moreover, data from our laboratory show that C6 cells cultured in high levels of glucose have an increased glutamate uptake (Tramontina et al., 2012).

Co-incubation with AG reversed the MG-induced change in glutamate uptake in astrocytes and C6 glioma cells cultures, indicating that these alterations are associated with glycation processes within 24 h of exposure. However, these alterations in glutamate transporter activity are not related to glutamate transporter expression since MG exposure did not modify GLAST and GLT-1 expression. Accordingly, astrocyte glutamate transporters were not affected in an animal model of diabetes (Coleman et al., 2004), where high levels of MG may be found due to hyperglycemia.

It is important to mention that astrocytes and C6 cell cultures exhibit differences in glutamate transporters. Astrocytes express and demonstrate functional activity mainly of GLAST and GLT-1, although they can also express EAAT3 (Kim et al., 2011). On the other hand, C6 cells express GLAST and GLT-1 (Baber and Haghighat, 2010) and have expression and activity of EAAT3, a characteristic neuronal glutamate transporter (Bianchi et al., 2006; Tramontina et al., 2012). We found that glutamate uptake in primary astrocytes cultures was higher than that verified in C6 cells (data not shown); furthermore, glutamate transporter expression (GLAST and GLT-1) was also higher.

As verified, MG treatment alters the activity of glutamate transporters in a distinct manner that is linked to glycation reactions. The increase in glutamate uptake

observed upon MG exposure in C6 cells may be related to the fact that excess of extracellular glutamate indirectly contributes to the proliferation of malignant gliomas, and this may be impaired by MG (Vanhoutte and Hermans, 2008). Moreover, a reduced expression of glial glutamate transporters is a common feature of several glioma cell lines (Ye et al., 1999).

S100B secretion and GFAP content performed in astrocytes and C6 lineage cells reveal that these parameters are not affected by MG at a concentration of 400 μM. However, under the conditions that neuron-astrocyte communications are maintained, S100B secretion may respond differently, since the regulation of extracellular levels of S100B functions as a signal, exerting neurotrophic or neurotoxic effects on concentration-dependent neurons (Donato et al., 2009). The streptozotocin (STZ)-induced model of diabetes is associated with a significant decrease in GFAP protein levels in distinct brain regions (Coleman et al., 2004). Nevertheless, GFAP levels were increased in the retinal Müller glia of the STZ-model of diabetes and overexpression of glyoxalase 1 reduced this diabetes-mediated response (Berner et al., 2012). A significant increase in GFAP expression was also observed in astrocytes upon incubation with 700 μM MG (Chu et al., 2014). These data suggest that MG can induce changes in GFAP expression, but that these alterations occur in a time-dependent manner and may be related to MG concentration.

In conclusion, MG-induced impairment in astrocyte glutamate uptake activity occurs without changes in MG detoxification by the glyoxalase system. Increased levels of glutamate can cause excitotoxicity in neurons and contribute to neurological changes related to diabetes. Moreover, data obtained from C6 cultures indicate that non-astrocyte glutamate transporters may have their activity altered due to MG exposure and confirm that astrocyte-related parameters, S100B secretion and GFAP levels, were not modified by MG during 24 h in cell cultures. Finally, the high efficiency of the glyoxalase system in astrocyte cultures is an important characteristic to be included as one of the functions of astrocytes in order to avoid carbonyl stress, and, consequently, AGEs formation.

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FIGURE LEGENDS

Fig. 1. MG (1000 μM) induced cytotoxicity in astrocyte cultures, and reversal by CBX. Representative fields of astrocytes and C6 cultures treated with 1000 μM MG for 24 h in the presence of PI. CBX was added 15 min before MG exposure and cells were then co-incubated with MG at 24 h. Phase contrast and nuclear fluorescent staining is shown under control conditions, MG exposure at 1000 μM and MG 1000 μM + CBX 0.1 mM. All images are representative fields from at least three experiments, carried out in triplicate. Scale bar = 50 μm.

Fig. 2. Cellular viability was not altered by different concentrations of MG in astrocytes and C6 cells. MTT reduction assay was performed to assess cellular viability after 24 h of MG exposure. Each value represents mean (± standard error) of eight independent experiments performed in triplicate from astrocytes and three independent experiments performed in triplicate from C6 cells, assuming the control value as 100%. Data from each cell culture were analyzed by one-way ANOVA followed by the Dunnett's test, assuming $p<0.05$.

Fig. 3. Glyoxalase 1 expression and activity are significantly higher in primary astrocytes than C6 cells. Rat primary astrocyte cultures and C6 glioma cells were exposed to 400 μM MG at 24 h. Glyoxalase 1 expression (A), glyoxalase 1 activity (B) and glyoxalase 1 expression/activity ratio (C). In A and C, the control value of C6 cells is assumed as 1.0. Each value represents mean (± standard error) of three independent experiments performed in duplicate from Western blot analysis and six independent experiments performed in triplicate from glyoxalase 1 activity assays in both cell cultures. Data were analyzed by two-way ANOVA with regard to cellular type and treatments, assuming $p<0.05$ (A, B). Statistical significance is indicated in the graph in bold type.

Fig. 4. MG alters glutamate uptake in astrocytes and C6 cultures. Rat primary astrocyte cultures (A) and C6 glioma cells (B) were exposed to 400 μM MG and 400 μM AG at 24 h. Each value represents mean (± standard error) of ten independent experiments performed in triplicate from astrocytes and six independent experiments performed in triplicate from C6 cells, assuming the control value as 100%. Data from

each cell culture were analyzed by two-way ANOVA with regard to the presence or not of MG and presence or not of AG, assuming $p<0.05$. Statistical significance is indicated in the graph in bold type.

Fig. 5. GLAST and GLT-1 expression is significantly higher in primary astrocytes than in C6 cells. Rat primary astrocyte cultures and C6 glioma cells were exposed to 400 μM MG for 24 h. GLAST (A) and GLT-1 expression (B), assuming the control value of C6 cells as 1.0. Each value represents mean (± standard error) of four independent experiments performed in duplicate. Data were analyzed by two-way ANOVA with regard to cell type and treatments, assuming $p<0.05$. Statistical significance is indicated in the graph in bold.

Fig. 6. Immunocontent of GFAP was not modified in astrocytes and C6 glioma cells, however a slight actin cytoskeletal reorganization was induced in astrocytes following MG exposure. Immunocytochemistry for GFAP and for actin of astrocytes and C6 in control and 400 μM MG-treated cultures (24 h) are shown. All images are representative fields from at least three experiments, carried out in triplicate. Scale bar = 50 μm.

Table 1. GSH content decreased with MG treatment at 1 and 24 h in C6 cells[1]

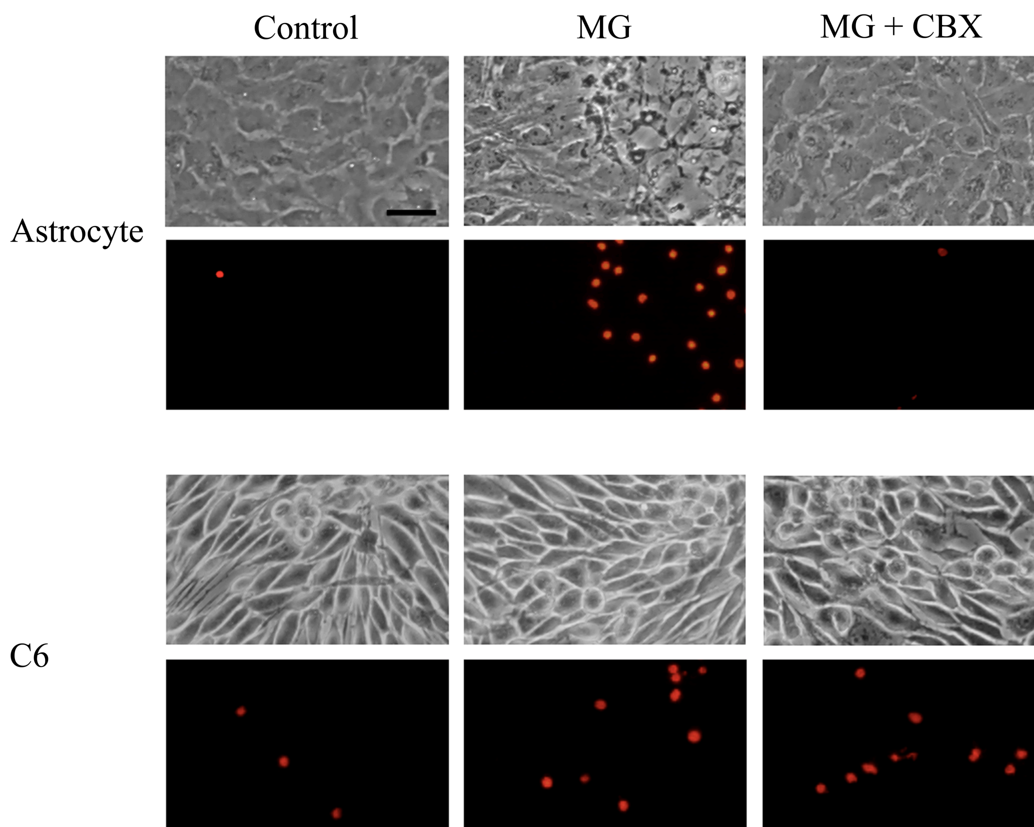
		Astrocytes		C6 glioma cells	
Parameter analyzed	**Treatment**	**1 h**	**24 h**	**1 h**	**24 h**
DCFH oxidation	Control	100,0 ± 22,3	100,0 ± 17,5	100,0 ± 7,9	100,0 ± 28,5
	MG	105,6 ± 7,8	93,8 ± 4,1	115,3 ± 1,5	125,0 ± 2,0
Glutathione	Control	100,0 ± 3,4	100,0 ± 1,8	100,0 ± 6,5	100,0 ± 7,2
(GSH)	MG	99,2 ± 2,2	101,6 ± 2,4	73,7 ± 3,5*	65,1 ± 3,9*

[1] Rat primary astrocyte cultures and C6 glioma cells were exposed to 400 µM MG for 1 or 24 h (the latter also represented in graphs in Fig. 3). DCFH oxidation and GSH content are shown. Each value represents mean (± standard error) of five independent experiments performed in triplicate, assuming control value as 100% for each type of cell culture. Data were analyzed by Student's t-test, assuming $p<0.05$. *Significantly different from control.

Table 2. Glutamate uptake was altered differently by MG in astrocytes and C6 glioma cells[2]

		Astrocytes		C6 glioma cells	
Parameter analyzed	**Treatment**	**1 h**	**24 h**	**1 h**	**24 h**
Glutamate uptake	Control	100,0 ± 4,8	100,0 ± 6,6	100,0 ± 27,6	100,0 ± 14,9
	MG	92,6 ± 3,5	72,9 ± 4,0*	93,3 ± 3,0	147,1 ± 8,4*
S100B secretion	Control	100,0 ± 19,4	100,0 ± 16,9	100,0 ± 9,6	100,0 ± 15,4
	MG	95,7 ± 13,1	106,5 ± 6,2	104,7 ± 3,7	104,9 ± 9,5
GFAP content	Control	100,0 ± 20,7	100,0 ± 22,8	100,0 ± 24,8	100,0 ± 16,0
	MG	116,5 ± 33,5	94,8 ± 12,8	78,8 ± 6,8	85,5 ± 6,3

[2] Rat primary astrocyte cultures and C6 glioma cells were exposed to 400 µM MG for 1 or 24 h. Glutamate uptake, S100B secretion and GFAP content are shown. Each value represents mean (± standard error) of eight independent experiments performed in triplicate from astrocytes and five independent experiments performed in triplicate from C6 cells, assuming control value as 100% for each type of cell culture. Data were analyzed by Student's t-test, assuming $p<0.05$. *Significantly different from control.



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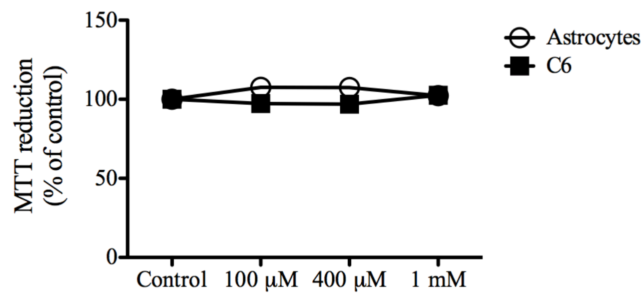

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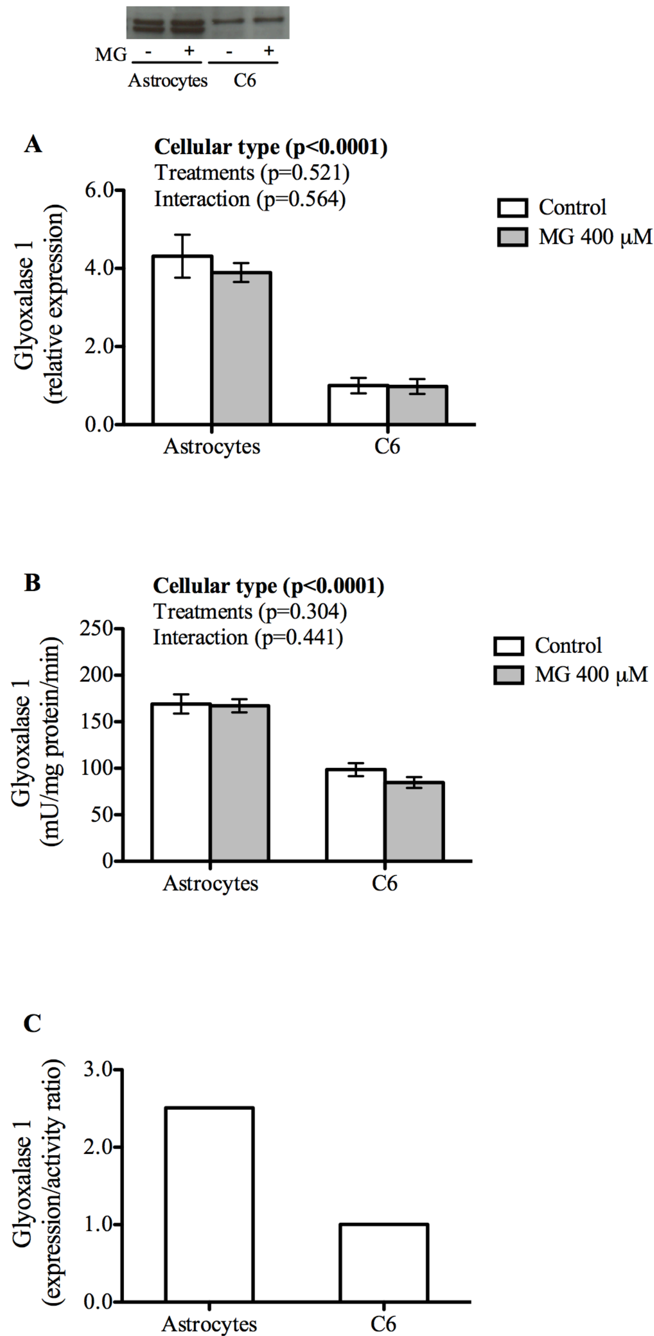

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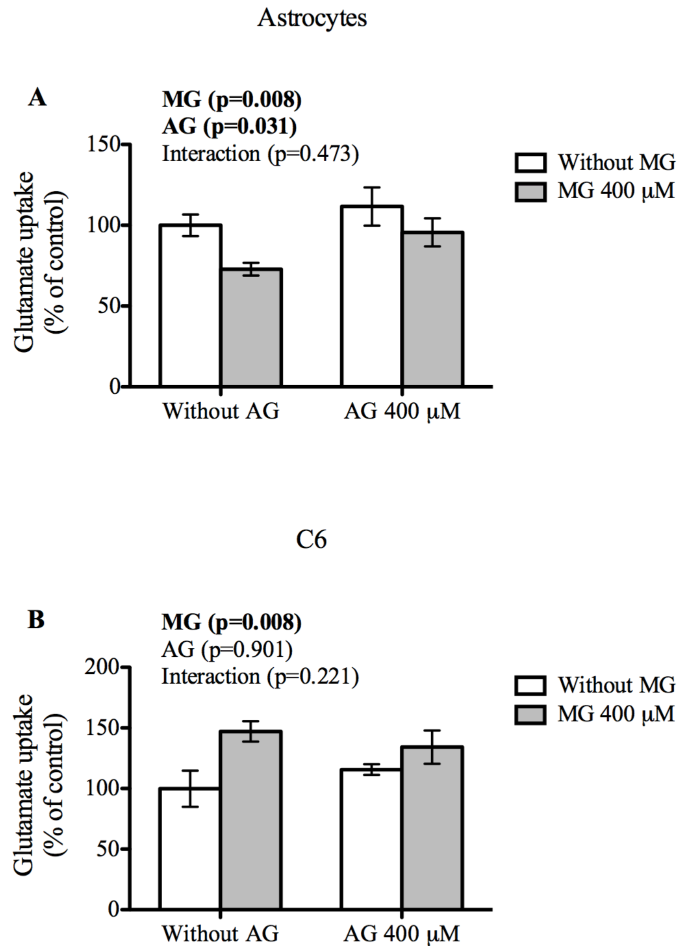

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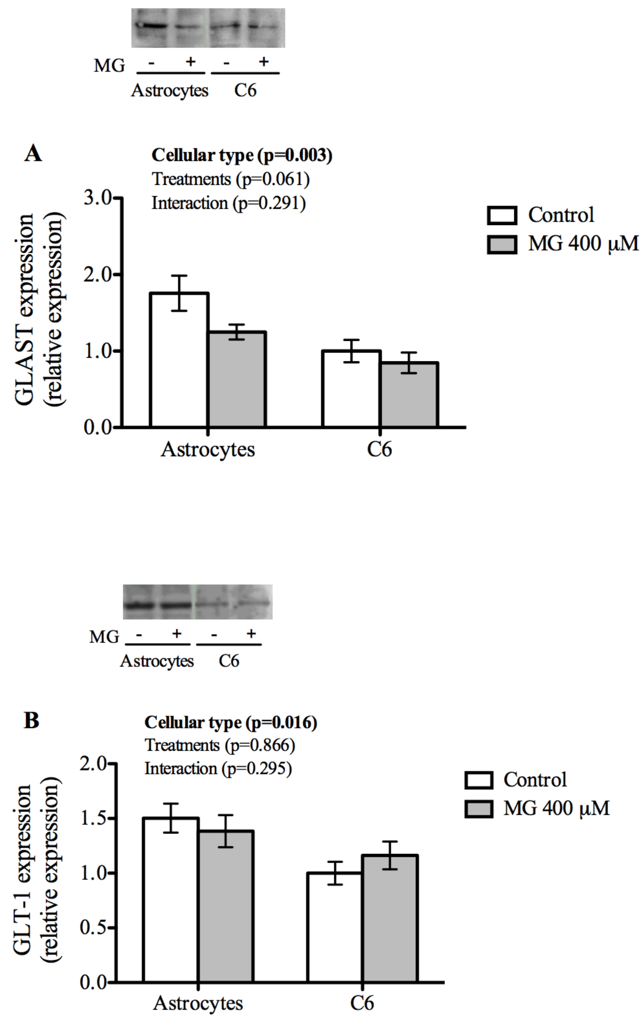

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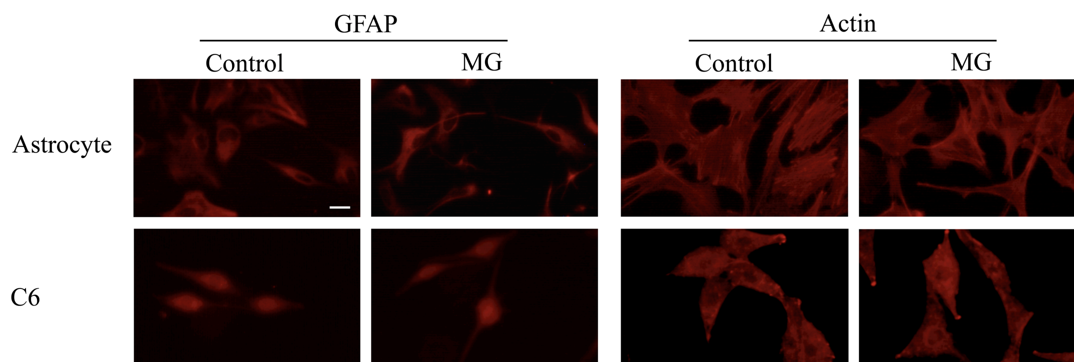

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CAPÍTULO 2

Methylglyoxal and carboxyethyllysine reduce glutamate uptake and S100B secretion in the hippocampus independently of RAGE activation

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ORIGINAL ARTICLE

Methylglyoxal and carboxyethyllysine reduce glutamate uptake and S100B secretion in the hippocampus independently of RAGE activation

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Abstract Diabetes is a metabolic disease characterized by high fasting-glucose levels. Diabetic complications have been associated with hyperglycemia and high levels of reactive compounds, such as methylglyoxal (MG) and advanced glycation endproducts (AGEs) formation derived from glucose. Diabetic patients have a higher risk of developing neurodegenerative diseases, such as Alzheimer's disease or Parkinson's disease. Herein, we examined the effect of high glucose, MG and carboxyethyllysine (CEL), a MG-derived AGE of lysine, on oxidative, metabolic and astrocyte-specific parameters in acute hippocampal slices, and investigated some of the mechanisms that could mediate these effects. Glucose, MG and CEL did not alter reactive oxygen species (ROS) formation, glucose uptake or glutamine synthetase activity. However, glutamate uptake and S100B secretion were decreased after MG and CEL exposure. RAGE activation and glycation reactions, examined by aminoguanidine and L-lysine co-incubation, did not mediate these changes. Acute MG and CEL exposure, but not glucose, were able to induce similar effects on hippocampal slices, suggesting that conditions of high glucose concentrations are primarily toxic by elevating the rates of these glycation compounds, such as MG, and by generation of protein cross-links. Alterations in the secretion of S100B and the glutamatergic activity mediated by MG and AGEs can contribute to the brain dysfunction observed in diabetic patients.

Keywords Diabetes · Hyperglycemia · Methylglyoxal · Advanced glycation endproducts · Glutamate uptake · S100B secretion

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Introduction

Diabetes is a metabolic disease characterized by high fasting-glucose levels. Diabetic complications have been associated with hyperglycemia and high levels of reactive compounds, such as methylglyoxal (MG) and advanced glycation endproducts (AGEs) formation (Brownlee 2001). MG is an endogenous dicarbonyl compound that is physiologically and predominantly produced by the glycolytic pathway from triose phosphate degradation, non-enzymatic and/or enzymatic elimination of phosphate from dihydroxyacetone phosphate and glyceraldehyde-3-phosphate. MG can also be formed by the catabolism of threonine and acetone (Thornalley 1993), which have a greater relevance in diabetic ketosis. High production of MG occurs in hyperglycemic conditions (Lee et al. 2009). In diabetic patients, the flux of formation of MG is increased 2- to 4-fold, depending on glycemic control, and the formation of MG-derived AGEs is increased similarly (Rabbani and Thornalley 2014). Major level of MG can be found in the plasma of diabetic patients with uncontrolled glycemia,

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since the ketogenic bodies are increased progressively and may contribute to MG elevation.

Glucose and MG can form different AGEs by participating in glycation reactions with proteins, lipids or nucleic acids. Arginine and lysine amino acids are more susceptible to the glycation process and their modification by reactive compounds may be damaging as these amino acids are common in functional domains of proteins (Thornalley 2005; Thornalley and Rabbani 2011). Carboxyethyllysine (CEL) and methylglyoxal hydroimidazolone (MGHI) are MG-derived AGEs of lysine and arginine, respectively. A high concentration of MG has been found in body fluids and in the tissues of diabetic patients and animal models of diabetes (Phillips et al. 1993; Lapolla et al. 2003). In type 1 and type 2 diabetes, MG concentrations are elevated 5- to 6-fold and 2- to 3-fold, respectively (Thornalley 1993) and MG-derived AGEs are also elevated in body fluids of experimental and clinical diabetes patients (Ahmed et al. 2005; Karachalias et al. 2010) (Table 1).

Diabetic patients have a higher risk of developing neurodegenerative diseases, such as Alzheimer's disease or Parkinson's disease (Strachan et al. 2011; Xu et al. 2011). Formation of AGEs has been found in neurofibrillary tangles of Alzheimer disease (Wong et al. 2001) and in Lewy bodies of Parkinson disease (Lo et al. 1994), suggesting that these protein deposits have been exposed to MG or other AGE precursors.

Despite the existing data regarding the concentrations and involvement of glucose metabolism, MG and AGE molecules in diabetes and neurodegenerative diseases, little is known of the effect of these compounds on brain cell functions, particularly in brain slices where neuroglial circuitry is partially preserved (Thomazi et al. 2004; Buyukuysal 2005; Sajikumar and Navakkode 2005). Clarification of these mechanisms is important to establish the possible alterations in each one of these compounds and to design therapeutic approaches with targets of clinical relevance. In this study, we examine the effects of high glucose, MG and CEL on oxidative, metabolic and astrocyte-specific parameters in acute hippocampal slices and explore some of the mechanisms that could be involved in these effects (Scheme 1).

Materials and methods

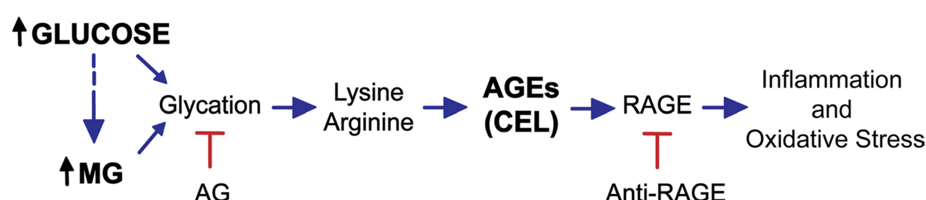
Materials

Methylglyoxal (MG), DCF-DA, standard GSH, o-phthaldialdehyde, anti-S100B (SH-B1), L-glutamate, 4-(2-hydroxyethyl) piperazine-L-ethanesulfonic acid (HEPES), *o*-phenylenediamine (OPD), meta-phosphoric acid, [3(4,5-dimethylthi-azol-2-yl)-2,5-diphenyl tetrazolium bromide] (MTT), γ-glutamylhydroxamate acid,

Table 1 Blood levels of glucose, MG and CEL from clinical studies

	Glucose	MG	CEL
DM type 1	≅219 mg/dL[a, b, c]	50–250 nM[d] 86–1044 pmol/g blood[e]	58–77 nM[f,g,h]
DM type 2	≅145 mg/dL[i, j, k]	189 nM[l] 55–2366 pmol/g blood[e] 29 μg/mL[i]	0.12 μmol/mol amino acid modified[j] 49 (39–64) nmol/L (free AGEs) or 31 (22–39) nmol/mmol lysine[k]

[a] Hamilton et al. (2003), [b] Khan et al. (2006), [c] Lund et al. (2008), [d] Beisswenger et al. (2005), [e] Thornalley (1993), [f] Beisswenger et al. (2013), [g] Beisswenger et al. (2014), [h] Ahmed et al. (2005), [i] Lapolla et al. (2005), [j] Barzilay et al. (2014), [k] Hanssen et al. (2013), [l] Beisswenger et al. (1999)



Scheme 1 AGEs formation from glucose and MG and strategies to block these reactions. Physiologically, the glycolytic flux causes MG formation and this process is increased in pathological conditions, such as diabetes, during hyperglycemia. Glucose and MG are able to glycate different compounds, such as nucleic acids, lipids and proteins (amino acid residues, predominantly arginine and lysine), which could generate AGEs. CEL is an AGE produced by glycation reactions of glucose or MG with lysine residues. AGEs bind RAGE and cause inflammation and oxidative stress. Strategies to prevent or block the glycation reactions or block the binding of RAGE are important to elucidate the mechanism of action of glucose, MG and AGEs

N-methyl-D-glucamine, aminoguanidine hemisulfate salt (AG) and L-lysine were purchased from Sigma (Saint Louis, MO, USA). Epsilon-*N*-carboxyethyl-L-lysine (mixture of two diastereoisomers) was purchased from PolyPeptide Group (San Diego, CA, USA), L-[2,3-^{3}H] glutamate was purchased from Amersham International (United Kingdom) and deoxy-D-glucose, 2-[3H(G)] (10 Ci/mmol) was purchased from American Radiolabeled Chemicals, Inc. (Saint Louis, MO, USA). Polyclonal anti-S100B and anti-rabbit peroxidase-linked antibodies were purchased from DAKO (São Paulo, Brazil) and GE, respectively (Little Chalfont, United Kingdom) and polyclonal anti-RAGE (N16) was purchase from Santa Cruz Biotechnology, Inc. (Dallas, Texas, USA). All other chemicals were purchased from local commercial suppliers.

Animals

Forty male 60-day-old Wistar rats were obtained from our breeding colony (Department of Biochemistry, UFRGS, Porto Alegre, Brazil). The animals were maintained under controlled light and environmental conditions (12 h light/12 h dark cycle at a constant temperature of 22 ± 1 °C) and had free access to commercial chow and water. This study was performed in accordance with Institutional Guidelines for the Care and Use of Animals and the Institutional Animal Care and Use Committee (IACUC) approved the procedures conducted in this work (approval number 28035).

Preparation and incubation of hippocampal slices

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 $CaCl_2$; 1 $MgSO_4$; 25 HEPES; 1 KH_2PO_4 and 10 glucose, adjusted to pH 7.4. 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 replaced every 15 min with fresh saline medium at room temperature. Following a 120-min equilibration period, the medium was removed and replaced with basal or specific treatments (glucose, MG and CEL) for 60 min at 30 °C on a warm plate (Nardin et al. 2009).

To perform the experiments with AG and anti-RAGE (1:50), the hippocampal slices followed a 30-min equilibration period where medium was replaced every 15 min with fresh saline at room temperature. Slices were pre-incubated with AG or anti-RAGE for 30 min before adding AG or anti-RAGE with or without other specific treatments for each experiment for 60 min. In the experiments with L-lysine, the hippocampal slices underwent a 60-min equilibration period where the medium was replaced every 15 min with fresh saline at room temperature. After 60 min, the medium was removed and slices were incubated with L-lysine and/or with other specific treatments for a further 60 min.

MTT reduction assay

A slice viability assay was performed using the colorimetric MTT method (Hansen et al. 1989). Slices were incubated with 0.5 mg/mL MTT at 30 °C for 30 min. The formazan product generated during the incubation was solubilized in dimethyl sulfoxide and measured at 560 and 630 nm. Results were expressed as percentages of the control.

Cell integrity assay

Lactate dehydrogenase (LDH) activity in the incubation medium was determined by a colorimetric commercial kit (from Doles, Brazil), according to the manufacturer's instructions. Results were expressed as percentages of the control.

Evaluation of intracellular reactive oxygen species (ROS) production

Intracellular ROS production was detected via the non-fluorescent 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 (DCFH). Slices were treated with DCF-DA (10 μM) for 30 min at 37 °C. Following DCF-DA exposure, the slices were lysed in PBS with 0.2 % Triton X-100. Fluorescence was measured on a plate reader (Spectra Max Gemini XPS, Molecular Devices, USA) with excitation at 485 nm and emission at 520 nm. All procedures were performed in the dark, and blanks containing only DCF-DA were processed for measurement of autofluorescence (Quincozes-Santos et al. 2010, 2014). Values were obtained as fluorescence units/mg protein and were expressed as percentages of the control.

Glutathione (GSH) content assay

Intracellular GSH levels (nmol/mg protein) were measured as previously described (Browne and Armstrong 1998). This assay detects only the reduced glutathione content. Slices were diluted in ten 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*-phthaldialdehyde (1 mg/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 employed using standard GSH solutions (0–500 μM). GSH concentrations were expressed as percentages of the control.

Glucose uptake assay

Glucose uptake was measured as previously described (Pellerin and Magistretti 1994), with modifications. Hippocampal slices of all treatments were rinsed thrice with Hank's balanced salt solution (HBSS) to ensure that the excess of glucose was removed, especially following high glucose treatment. Subsequently, slices were incubated at 35 °C in HBSS containing (in mM): 137 NaCl, 5.36 KCl, 1.26 $CaCl_2$, 0.41 $MgSO_4$, 0.49 $MgCl_2$, 0.63 $Na_2HPO_4 \cdot 7H_2O$, 0.44 KH_2PO_4, 4.17 $NaHCO_3$ and 5.6 glucose, adjusted to pH 7.2. The assay was initiated by the addition of 0.1 μCi/well deoxy-D-glucose, 2-[3H(G)]. The incubation was stopped after 30 min by removing the medium and rinsing the slices twice with ice-cold HBSS. The slices were then lysed in a solution containing 0.5 M NaOH. Glucose uptake was calculated by subtracting the non-specific uptake, obtained by the glucose transporter inhibitor, cytochalasin B (10 μM), from the total uptake in order to obtain the specific uptake. Radioactivity was measured using a scintillation counter. Results were calculated as nmol/mg protein/min and were expressed as percentages of the control.

Glutamate uptake assay

Glutamate uptake was performed as previously described (Thomazi et al. 2004). Slices were incubated at 37 °C in HBSS (described above). The assay was started by the addition of 0.1 mM L-glutamate and 0.66 μCi/mL L-[2,3-^{3}H] glutamate. Incubation was stopped after 5 min by removing the medium and rinsing the slices twice with ice-cold HBSS. The slices were then lysed in a 0.5 M NaOH solution. 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 the specific uptake. Radioactivity was measured in a scintillation counter. Results were calculated as nmol/mg protein/min and were expressed as percentages of the control.

Glutamine synthetase (GS) activity

The enzymatic activity of glutamine synthetase was determined using the procedures described previously (Minet et al. 1997) with modifications. Briefly, slices were homogenized in 50 mM imidazole. Homogenates were incubated with (mM): 50 imidazole, 50 hydroxylamine, 100 L-glutamine, 25 sodium arsenate dibasic heptahydrate, 0.2 ADP, 2 manganese chloride, pH 6.2 for 15 min at 37 °C. The reactions were terminated by the addition of 0.2 ml of 0.37 M $FeCl_3$, 50 mM trichloroacetic acid, and 100 mM HCl. After centrifugation, the absorbance of the supernatant was measured at 530 nm and compared to the absorbance generated by standard quantities of γ-glutamylhydroxamate acid treated with ferric chloride reagent. GS activity was expressed as μmol/h/mg protein and was expressed as a percentage of the control.

S100B measurement

S100B was measured by an enzyme-linked immunosorbent assay, as previously described (Leite et al. 2008). Briefly, 50 μL of sample and 50 μL of Tris buffer were incubated for 2 h on a microtiter plate previously coated with monoclonal anti-S100B (SH-B1) antibody. Polyclonal anti-S100 antibody was incubated for 30 min, and then peroxidase-conjugated anti-rabbit antibody was added for an additional 30 min. A colorimetric reaction with *o*-phenylenediamine was measured at 492 nm. The standard S100B curve ranged from 0.002 to 1 ng/mL. Data were calculated as ng/mL and were expressed as percentages of the control.

Protein determination

Protein content was measured by Lowry's method using bovine serum albumin as standard (Peterson 1977).

Statistical analysis

Data are reported as the mean ± standard error and analyzed statistically by one-way analysis of variance (ANOVA) followed by Dunnett's test or Duncan's test. Differences were considered to be significant when $p < 0.05$. All analyses were performed using the SPSS software package 20.0 or Prism 5.0 (GraphPad).

Results

Effect of glucose, MG and CEL on hippocampal slice viability and cell integrity

We initially evaluated the viability and cell integrity of hippocampal slices incubated for 60 min with three different concentrations of glucose (10 mM (control), 25 and 50 mM), MG (0.05, 0.5 and 1 mM) and CEL (0.5, 1 and 2.5 mM). Cell viability and integrity were measured by MTT reduction assay and extracellular LDH activity, respectively. Any of the concentrations tested altered these parameters when compared to the control (Fig. 1). It

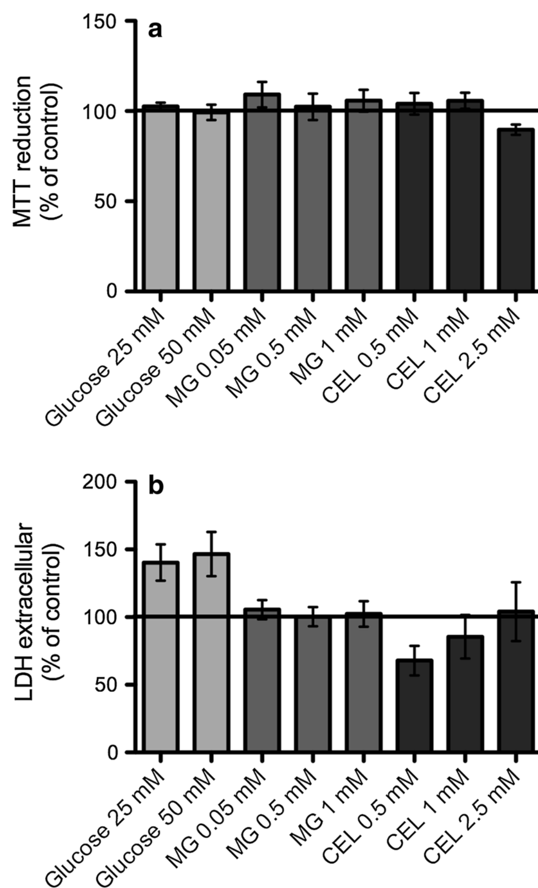


Fig. 1 Viability and cell integrity were not altered by different concentrations of treatments—glucose, MG and CEL. Hippocampal slices were treated for 60 min, after a 120-min equilibration period. **a** MTT reduction by hippocampal slices and **b** LDH activity in the medium. Each value represents mean (±standard error) of $n = 8$ (performed in triplicate), assuming a control value of 100 %. Data were analyzed by one-way ANOVA followed by Dunnett's test, assuming $p < 0.05$

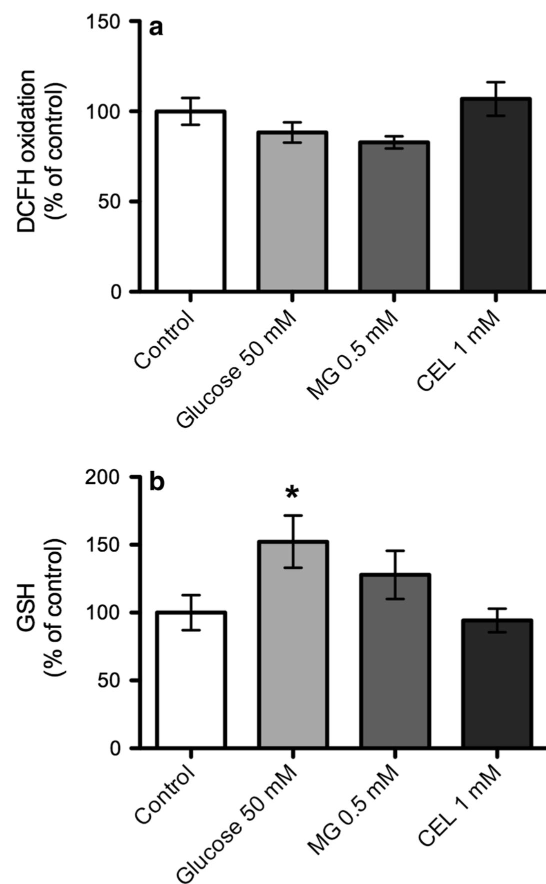


Fig. 2 GSH content was increased with high glucose treatment. Hippocampal slices were treated for 60 min, after a 120-min equilibration period. **a** DCFH oxidation and **b** GSH content. Each value represents mean (±standard error) of $n = 12$ (performed in triplicate), assuming the control value as 100 %. Data were analyzed by one-way ANOVA followed by Duncan's test, assuming $p < 0.05$. *Significantly different from control, mannitol (data not shown) and CEL

is known that α-oxoaldehydes are up to 20,000-fold more reactive than glucose in glycation processes (Thornalley 2005), as such we chose the highest concentration of glucose (50 mM) and the intermediate concentration of MG (0.5 mM) and CEL (1 mM) to perform subsequent analyses. As the effects of glucose could be due to its effects on osmolarity, we used mannitol at the same concentration as that of glucose (50 mM), as a control in cases in which high glucose was able to induce alterations in the parameters analyzed.

High glucose, MG and CEL do not change ROS formation in hippocampal slices

We hypothesized that glucose, MG and CEL mediate toxicity on hippocampal slices through their oxidative effects, leading to the formation of ROS and/or reducing cellular defenses, such as GSH. This tripeptide participates in the Central Nervous System's defense against oxidant agents and carbonyl stress via the glyoxalase system. Data from the DCFH oxidation assay demonstrated that ROS formation in acute hippocampal slices was not changed by these compounds. However, the GSH content increased only with high glucose incubation (Fig. 2) and this augment was not related to osmolarity, as mannitol did not cause the same effect [mannitol = 89.42 ± 12.79 % (mean ± standard error); $n = 12$; Duncan's test $p > 0.05$].

Effect of glycation agents and CEL on glucose and glutamate uptake

Although significant differences were not observed directly in the redox state (based on reduced GSH content and DCFH oxidation assay) of hippocampal slices incubated with MG or CEL, we also determined whether glucose

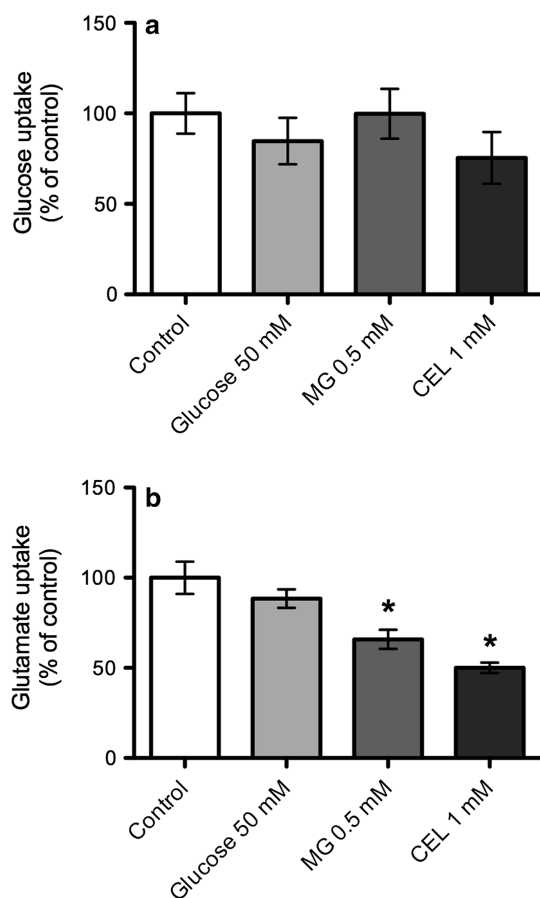


Fig. 3 MG and CEL treatments induced a decrease in glutamate uptake. Hippocampal slices were treated for 60 min, after a 120-min equilibration period. **a** Glucose uptake and **b** glutamate uptake. Each value represents mean (±standard error) of $n = 9$ (performed in triplicate), assuming a control value of 100 %. Data were analyzed by one-way ANOVA followed by Duncan's test, assuming $p < 0.05$. *Significantly different from control and glucose

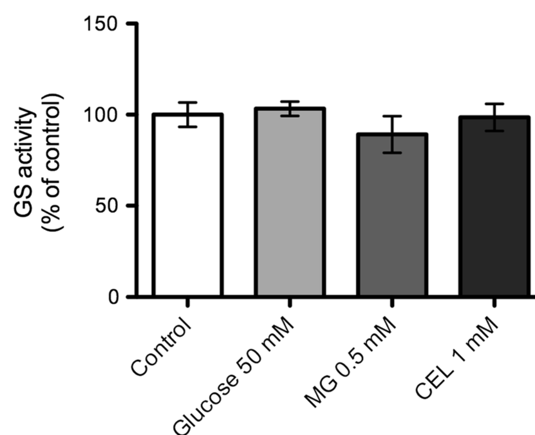


Fig. 4 GS activity was not altered by different treatments. Hippocampal slices were treated for 60 min after a 120-min equilibration period. Each value represents mean (±standard error) of $n = 8$ (performed in triplicate), assuming a control value of 100 %. Data were analyzed by one-way ANOVA followed by the Duncan's test, assuming $p < 0.05$

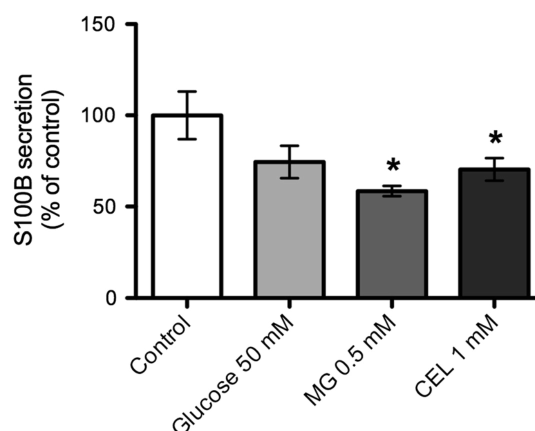


Fig. 5 S100B secretion is decreased by MG and CEL treatments. Hippocampal slices were treated for 60 min, after a 120-min equilibration period. Each value represents mean (±standard error) of $n = 20$ (performed in triplicate), assuming a control value of 100 %. Data were analyzed by one-way ANOVA followed by Duncan's test, assuming $p < 0.05$. *Significantly different from control

or glutamate uptake were altered by these compounds, as NADPH (generated by glucose metabolism via pentose pathway) and glutamate are necessary for the recycling and synthesis of GSH, respectively. Surprisingly, glucose uptake was not altered; however, the glutamate uptake decreased significantly following MG and CEL treatments, but not following glucose incubation (Fig. 3).

High glucose, MG and CEL do not affect GS activity in the hippocampus

Once taken up by astrocytes, glutamate is converted into glutamine by GS, where glutamine is the source for neuronal glutamate synthesis. To determine whether the reduction in glutamate uptake affects GS activity, an enzyme expressed in astrocytes but absent in neurons, we measured the GS activity in hippocampal slices. The compounds investigated did not alter the GS activity in the hippocampal slices (Fig. 4).

S100B secretion by the hippocampus is reduced following MG and CEL incubation

We measured the S100B secretion in the extracellular medium of hippocampal slices treated with high glucose, MG and CEL. This protein is synthesized and secreted by astrocytes and has been related to brain injury. Moreover, data from our laboratory demonstrate that high levels

of extracellular glutamate are able to induce alterations in S100B secretion. The secretion of S100B was reduced by incubation with MG (0.5 mM) and CEL (1 mM). The decrease in S100B secretion induced by high glucose incubation was apparent, but not statistically significant (Fig. 5).

MG-induced reductions in glutamate uptake and S100B secretion are not prevented by AG

In order to clarify whether MG induced alterations in glutamate uptake and S100B secretion in hippocampal slices via glycation processes, we performed these experiments with an anti-glycation compound, AG. Curiously, incubation with AG, at the concentrations tested, did not prevent the effect of MG on either of the parameters analyzed (Fig. 6). To exclude the possibility that CEL mediated the decreases in these same parameters via glycation, we also repeated these experiments with CEL and AG. Similarly, AG failed to prevent the effect of CEL on either of the parameters analyzed (data not shown).

The CEL-induced decrease in glutamate uptake and S100B secretion are not mediated by RAGE

To determine whether the decrease in glutamate uptake and S100B secretion, induced by CEL, are mediated through RAGE, we performed experiments with an anti-RAGE antibody (1:50). The CEL-induced alterations in acute hippocampal slices were not mediated by RAGE (Fig. 7).

The effects of MG and CEL on glutamate uptake and S100B secretion are not prevented by L-lysine

In order to investigate the mechanism of MG toxicity, hippocampal slices were co-incubated with MG (0.5 mM) and L-lysine (1 mM), a competitive substrate for MG protein glycation. L-lysine was unable to prevent the effect of MG on glutamate uptake and S100B secretion (Fig. 8). Slices were also incubated with CEL (1 mM) and L-lysine; similarly, L-lysine did not prevent the toxicity of CEL on glutamate uptake and S100B secretion (Fig. 8). In contrast, the toxic effect of CEL on glutamate uptake was statistically higher in the presence of L-lysine.

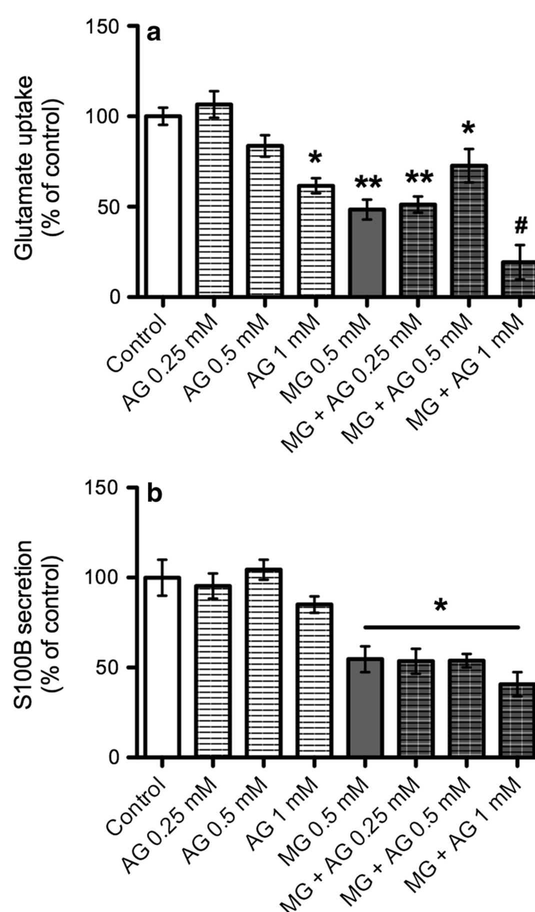


Fig. 6 Effect of MG on glutamate uptake and S100B secretion was not prevented by the anti-glycating compound, AG. Hippocampal slices were treated for 60 min after a 30-min equilibration period, preceded by pre-incubation with different concentrations of AG for 30 min. Each value represents mean (±standard error) of $n = 12$ (performed in triplicate), assuming a control value of 100 %. Data were analyzed by one-way ANOVA followed by Duncan's test, assuming $p < 0.05$. **a** Glutamate uptake—*significantly different from control and AG 0.25 mM. **Significantly different from control and AG (0.25 and 0.5 mM). #Significantly different from all groups. **b** S100B secretion—*significantly different from control and AG (0.25, 0.5 and 1 mM)

Discussion

We, herein, evaluated the effects of the acute exposure (short-treatment) of hippocampal slices to high concentrations of glucose, MG and CEL. Glucose and MG are glycating agents, while CEL is the final product of these reactions; as such these experiments were carried out to determine whether these compounds exert similar effects on the hippocampus (Scheme 1). Firstly, it is important to mention that the concentration of glucose, MG and CEL in diabetic patients is below the level that we used in this study; however, we chose these concentrations based on the acute treatment employed (60 min). We did not observe any toxic effects (leading to alterations in cell viability and integrity) of these compounds at these concentrations.

The use of acute hippocampal slices allows us to understand the effects of these molecules when the neuroglial circuitry is partially preserved and to clarify the partnership between astrocytes and neurons, which play important roles in the mechanisms that underlie brain lesions (Thomazi

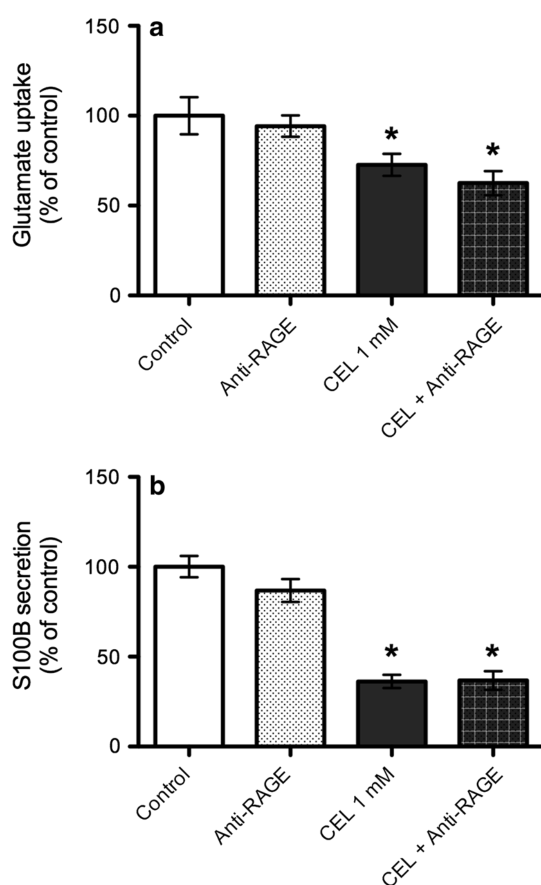


Fig. 7 The decreases in glutamate uptake and S100B secretion, induced by CEL, are not mediated by RAGE. Hippocampal slices were treated for 60 min, after a 30-min equilibration period, preceded by pre-incubated with anti-RAGE (1:50) for 30 min. Each value represents mean (±standard error) of $n = 9$ (performed in triplicate), assuming a control value of 100 %. Data were analyzed by one-way ANOVA, followed by Duncan's test, assuming $p < 0.05$. **a** Glutamate uptake and **b** S100B secretion—*significantly different from control and anti-RAGE

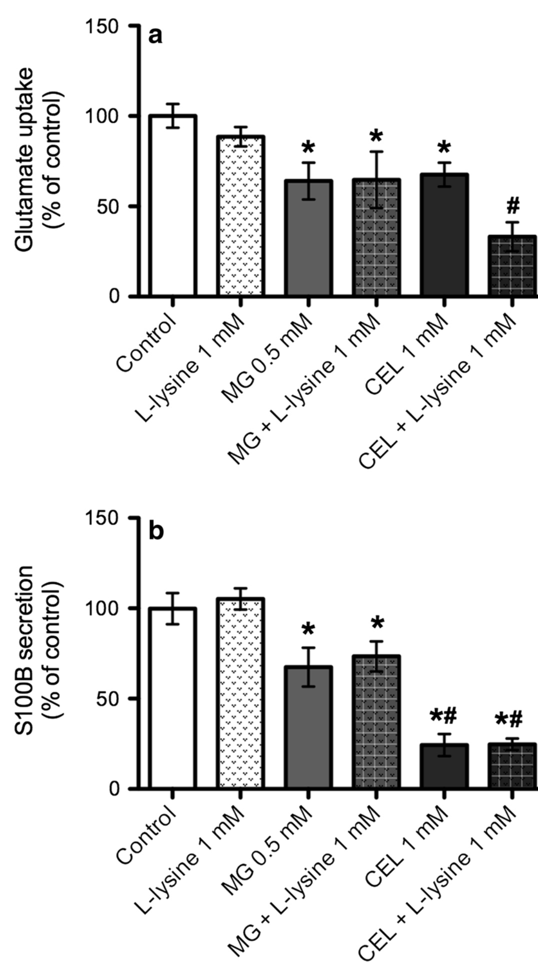


Fig. 8 The effects of MG and of CEL on glutamate uptake and S100B secretion were not prevented by L-lysine. Hippocampal slices were treated for 60 min, after a 60-min equilibration period. Each value represents mean (±standard error) of $n = 9$ (performed in triplicate), assuming a control value of 100 %. Data were analyzed by one-way ANOVA, followed by Duncan's test, assuming $p < 0.05$. **a** Glutamate uptake—*significantly different from control. #Significantly different from all groups. **b** S100B secretion—*significantly different from control and L-lysine (1 mM). #Significantly different from MG and MG + L-lysine (1 mM)

et al. 2004; Volterra and Meldolesi 2005; Nardin et al. 2009; Quincozes-Santos et al. 2014; Takano et al. 2014). Furthermore, studies that have evaluated the methylglyoxal-induced changes in cultures of neurons or neuroblastoma have shown that these cells have a high susceptibility to damage caused by MG (De Arriba et al. 2006; Di Loreto et al. 2008). In contrast, astrocytes, which play a key role in neuronal cell survival and brain homeostasis, are more resistant to MG-induced impairment, in part because they present higher expression and activity of the glyoxalase system compared to neurons (Bélanger et al. 2011).

In the present study, we observed that acute exposure to glucose, MG and CEL did not cause changes in ROS production, glucose uptake or glutamine synthetase activity. However, an unexpected increase in GSH levels was observed when hippocampal slices were exposed to high glucose levels. Previous study has reported that astrocytes cultured in high-glucose medium demonstrate a reduction in GSH levels (Nardin et al. 2007), suggesting that the increased levels of GSH observed in hippocampal slices may be transient.

Extracellular S100B has been used as a marker of glial activation in several conditions of brain injury (Steiner et al. 2011), and in vitro studies indicate that S100B has a trophic (at nM levels) or apoptotic (at µM levels) effect on neurons (Donato et al. 2009). The basal extracellular levels found in our preparations are compatible with the neurotrophic activity of this protein (Nardin et al. 2009). Therefore, we suggest that high levels of MG and CEL affect astrocyte activity, reducing extracellular levels of S100B

and that this may, subsequently, affect neuronal activity and survival.

We found that glutamate uptake decreased following MG and CEL exposure, demonstrating the impairment of astrocytic glutamatergic transporter activity. Unchanged levels of astrocyte glutamate transporters studied in STZ-induced diabetic rats have been reported (Coleman et al. 2004), while others have related the involvement of NMDA (*N*-methyl-D-aspartate) receptor activation following MG treatment (De Arriba et al. 2006), indicating that alterations in glutamatergic transmission and excitotoxicity observed in diabetic rats could involve changes in the affinity and expression of glutamate receptors (Gardoni et al. 2002). Our findings suggest that the impairment of glutamate uptake, which in turn could increase glutamate levels in the synaptic cleft, generates glutamatergic dysfunction. Therefore, these results could be indicative of alterations in diabetic patients, since MG and CEL levels are increased in these individuals and are involved in the physiopathology of this disease (Brownlee 2001).

The ability of MG and CEL to decrease glutamate uptake is clear; however, the mechanism by which this occurs requires further comprehension. Glutamate transporters are sensitive to oxidative stress (Trotti et al. 1998) and susceptible to glycation (Kawaguchi et al. 2005); however, we did not find any evidence of the induction of oxidative stress by MG or CEL under our experimental conditions that could explain this decrease. The activity of GS, an astrocyte-specific enzyme responsible for the conversion of glutamate to glutamine (Hertz and Zielke 2004; Albrecht et al. 2007), is sensitive to oxidative stress (Quincozes-Santos et al. 2014), but was also not affected by these compounds. Moreover, we investigated the toxicity of these compounds by incubating them with AG (an anti-glycation compound) or L-lysine (a competitive substrate for glycation). Neither AG nor L-lysine prevented the decrease in glutamate that was induced by MG or CEL. Therefore, we can rule out oxidative stress and protein glycation as mediators of the acute toxicity of these compounds on glutamate uptake activity, but other possible mechanisms require further investigation. In addition, anti-RAGE was not able to prevent the effect of CEL on glutamate uptake activity, suggesting that RAGE is not involved.

Presumably, glucose requires more time to elicit similar effects to those observed following MG and CEL incubation. This difference could be due to the fact that glucose is less reactive than MG and also because the amount of MG and CEL generated during glucose exposure for the incubation time used is low. This finding may partially explain the increase in GSH levels, which is required to avoid oxidative and carbonyl stress. Astrocytes cultured in high glucose medium have also been reported to demonstrate a decrease in S100B secretion, but with no associated change in glutamate uptake (Nardin et al. 2007).

The degree of glycation of AGEs is known to be related to cytotoxicity (Bigl et al. 2008). The AGE used in this study, CEL, is a glycation-free adduct, that is not bound to protein, and was able to induce the same changes in hippocampal slices as MG. As such, whether protein-linked CEL (glycation adduct residues) would induce different or more deleterious effects, compared to the alterations reported in this study, remain unknown.

Experiments performed with RAGE did not prevent the CEL-induced reduction in glutamate uptake and S100B secretion; similarly L-lysine was unable to prevent these alterations. These findings suggest that CEL can exert intracellular effects, as cell activation due to AGE has been suggested to occur via non-receptor pathways or via free radical generation (Vlassara 2001). Although other AGE receptors have been described in neuronal and glial cells (Vlassara 2001; Ott et al. 2014), these are still poorly studied.

The possibility that increased glutamate levels cause a reduction in S100B secretion from astrocytes (Tramontina et al. 2006) remains. This hypothesis is strengthened by the fact that incubation with AG and L-lysine, used to prevent the glycation reactions, did not prevent the MG-induced decreases in glutamate uptake and S100B secretion. Nevertheless, it is possible that distinct mechanisms are involved in the effects of MG and CEL exposure on hippocampal slices.

Conclusion

This study provides important data to show that acute MG and CEL exposure, but not glucose, induces similar effects in hippocampal slices, suggesting that conditions of high-glucose concentrations are primarily toxic due to the elevation of the glycation rates of compounds, such as MG, and the generation of protein cross-links. A reduction in the secretion of S100B and alterations in glutamatergic activity may be mechanisms involved in the toxicity of these glucose-derived products in vivo and may contribute to the brain dysfunction observed in diabetic patients.

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Compliance with ethical standards

Ethical approval All applicable international, national, and/or institutional guidelines for the care and use of animals were followed, and all procedures performed in this study were in accordance with the ethical standards of the institution at which the studies were conducted (Institutional Animal Care and Use Committee (IACUC), approval number 28035).

Conflict of interest The authors declare that they have no conflicts of interest.

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CAPÍTULO 3

Could methylglyoxal mediate behavioral and biochemical alterations in diabetic rats?

Submetido ao periódico Neuroscience

Could methylglyoxal mediate behavioral and biochemical alterations in diabetic rats?

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Abstract

Diabetes is associated with loss of cognitive function and increased risk for Alzheimer's disease (AD). Advanced glycation end products (AGEs) are elevated in diabetes and AD and have been suggested to act as mediators of the cognitive decline observed in these pathologies. Methylglyoxal (MG) is an extremely reactive carbonyl compound that propagates glycation reactions and is, therefore, able to generate AGEs. Herein, we evaluated persistent behavioral and biochemical parameters linked to explore the hypothesis that increased exogenous levels of MG, induced by intracerebroventricular (ICV) infusion, could mediate cognitive impairment in Wistar rats and could be used as an animal model of dementia. We found that acute high MG levels induced alterations related to anxiety behavior, diminishing the anxiety parameters evaluated in the open field test. In contrast, MG did not alter locomotion behavior and was not able to cause impairment in learning-memory processes investigated by habituation in the open field, Y-maze and object recognition tasks. Our biochemical findings support the hypothesis that MG induces persistent alterations in the hippocampus, but not in the cortex, related to GLO1 activity, AGEs content and glutamate uptake. GFAP and S100B content, as well as S100B secretion (astroglial-related parameters of brain injury), were not altered by MG ICV administration. Taken together, our data suggest that MG interferes directly in brain function and that the time and the levels of exogenous MG determine the different features that can be seen in diabetic patients.

Keywords: Advanced glycation end products; anxiety; dementia; diabetes; glyoxalase 1; methylglyoxal.

Introduction

It is well recognized that diabetes can affect cognition (Miles and Root, 1922; Biessels et al., 2006). Studies with diabetic patients indicate that they present a cognitive decline that particularly affects learning and memory, behavioral flexibility and mental speed (Strachan et al., 1997; Ryan, 1988; Stewart and Liolitsa, 1999; Brands et al., 2005; Cukierman et al., 2005). Consistent data suggest that diabetes is related to a high risk of developing Alzheimer's disease (AD) (Biessels et al., 2006; Kopf and Frolich, 2009; Lourenco et al., 2015). In this context, some molecules have been studied and suggested as mediators of the association between diabetes and brain damage, such as elevated levels of glucose and accumulation of advanced glycation end products (AGEs) (Krautwald and Münch, 2010; Yang and Song, 2013; Bornstein et al., 2014).

Several findings support the hypothesis that glycation is involved in the pathophysiology of diabetes and AD. Studies have found elevated concentrations of AGEs and one of its main precursors, methylglyoxal (MG), in the plasma of diabetes patients and in the cerebrospinal fluid (CSF) of patients with AD (Ahmed et al., 2005; Kuhla et al., 2005; Beisswenger et al., 2013; Beisswenger, 2014). Moreover, it has been proposed that the polymerization of β-amyloid and tau hyperphosphorylation are processes exacerbated by glycation (Li et al., 2012; Li et al., 2013). Clinical reports indicate that AGEs are co-located with senile plaques and neurofibrillary tangles (Smith et al., 1994; Kroner, 2009) and that the senile plaques of AD patients contain about three times more AGEs than observed in age-matched controls (Vitek et al., 1994). In addition, elevated levels of MG have been described in experimental studies using the streptozotocin-induced diabetes model (Brouwers et al., 2011), in association with cognitive impairments in memory and learning (Huang et al., 2012). Studies investigating the chronic oral or intraperitoneal administration of MG have found induced endothelial insulin resistance and suggested the use of this protocol as an animal model of diabetes (Lee et al., 2012; Nigro et al., 2014). Furthermore, clinical findings indicate that higher serum levels of MG are associated with a faster rate of cognitive decline in elderly individuals

(Beeri et al., 2011).

Studies that have looked at the glyoxalase pathway, which is a major defense against MG toxicity (Thornalley et al., 1996), have strengthened the idea that an appropriate regulation of MG levels is important to avoid cognitive impairment (Thornalley, 2006; Hambsch, 2011). It has been shown that increases in glyoxalase 1 (GLO1) expression and activity significantly decrease MG and AGEs levels in plasma (Brouwers et al., 2011) and diminish AGEs levels in the hippocampus, as well as reversing the cognitive decline in an animal model of diabetes (Liu et al., 2013). Moreover, other behavioral alterations have been related to MG levels, but these findings are still in discussion (Thornalley, 2006; Hambsch et al., 2010). Reports have demonstrated that MG administration reduces anxiety-like behavior and that GLO1 increases anxiety by reducing levels of MG (Hovatta et al., 2005; Hambsch et al., 2010; Distler et al., 2012). Recent findings have also identified the role of MG levels in epilepsy and suggested that the inhibition of GLO1 attenuates seizures (Distler et al., 2013). Nevertheless, it has been proposed, through clinical studies, that aberrant expression of GLO1 is linked to major depression, panic disorders and schizophrenia (Hambsch, 2011) and that higher doses of MG can cause locomotor depression, ataxia and hypothermia in mice (Distler et al., 2013).

We hypothesized that elevated MG concentrations, induced by intracerebroventricular (ICV) infusion, lead to cognitive decline and changes in anxiety-related behavior in Wistar rats. This study investigated behavioral and biochemical alterations induced by different procedures of ICV MG administration.

Experimental Procedures

Materials

Methylglyoxal (MG), 2′,7′-dichlorofluorescein diacetate (DCF-DA), standard glutathione (GSH), Triton X-100, o-phthaldialdehyde, S100B protein, anti-S100B (SH-B1), L-glutamate, o-phenylenediamine (OPD), meta-phosphoric acid, γ-glutamylhydroxamate acid and N-methyl-D-glucamine were purchased from Sigma (Saint Louis, MO, USA). L-[2,3-^{3}H] glutamate was

purchased from Amersham International (United Kingdom). Polyclonal anti-S100B and polyclonal anti-GFAP were purchased from DAKO (São Paulo, SP, Brazil), anti-rabbit peroxidase linked antibody was purchased from GE Healthcare (Little Chalfont, United Kingdom). Anti-AGE antibody (6D12) was purchased from Cosmo Bio (Tokyo, Japan). All other chemicals were purchased from local commercial suppliers.

Animals

Fifty-eight male Wistar rats (90-days old) were obtained from our breeding colony (Department of Biochemistry, UFRGS, Porto Alegre, Brazil). The animals were maintained under controlled light and environmental conditions (12 h light/12 h dark cycle at a constant temperature of 22±1°C) and had free access to commercial chow 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 4 groups: SHAM (N = 16), MG 1 day (N = 10), MG 3 days (N = 16) and MG 6 days (N = 16). After behavioral tasks, rats were anaesthetized, as subsequently described, for CSF puncture and posterior hippocampal and frontal cortex slice preparation, aiming to evaluate reactive species levels, glutathione contents (GSH), GLO1 and glutamine synthetase (GS) activities, glutamate uptake, S100B secretion, AGEs, S100B and glial fibrillary acidic protein (GFAP) content.

Surgical procedure and treatments

MG was ICV infused based on a previous study (Hambsch et al., 2010). Animals were anesthetized with ketamine (80 mg/kg) and xylazine (10 mg/kg) intraperitoneally. The head of the animal was fixed in a stereotaxic instrument, and the skin covering the skull was cut with a 3-cm-long rostro-caudal incision along the midline. After exposure of the skull bone surface, a 27-gauge 9-mm guide cannula was unilaterally placed at 0.9 mm posterior to the bregma, 1.5 mm right from the midline and 1.0 mm above the right lateral brain ventricle. The cannula was

implanted 3.0 mm ventral to the superior surface of the skull through a 2-mm hole made in the cranial bone. The positioning of the cannula was fixed with dental acrylic cement, and a screw used for the fixation of the dental acrylic helmet to the bone was used as ground (Torres et al., 2010). The appropriate stereotactic location of the cannula had been previously verified by Ponceau-stain injection. After the surgical procedure, rats were placed on a heating pad to maintain body temperature at 37°C and were kept there until recovery from anesthesia. After 5 days of recovery, a total volume of 5 μL of MG or vehicle (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 $Na_2HPO_4 \cdot 7H_2O$, 0.44 KH_2PO_4, 4.17 $NaHCO_3$ and 5.6 glucose, adjusted to pH 7.2) was injected once a day into the capillary tubules, using a Hamilton syringe.

All animals treated with ICV MG received the same infused final concentration (3 μmol/μL), but the distribution of this total concentration was different among these 3 groups: MG 1 day – one injection; MG 3 days – three injections on alternate days (1 μmol/μL per day); MG 6 days - six injections on 6 subsequent days (0.5 μmol/μL per day). The SHAM group received six injections of vehicle on 6 subsequent days. According to these groups, the single or last ICV MG injection was performed on the same day (see Fig. 1). The animals were submitted to behavioral tasks at 3 weeks after MG injection. The tasks were performed following the following sequence: open field (OF) test, object-recognition (OR) task and Y-maze, always with a 24-h interval between each behavioral test. Twelve days after the Y-maze test the rats were submitted to biochemical analysis (approximately 6 weeks after MG injection) (see Fig. 1).

Behavioral tests

Locomotion, anxiety-related behavior and habituation

The motor activity, the anxiety-related behavior and the habituation of rats were evaluated in the OF test. The OF was made of wood covered with impermeable wood, had a black floor measuring 60 cm^2, and was surrounded by 60-cm-high walls. This test consists of a large arena containing an aversive central area and represents a widely used paradigm for the evaluation of

both locomotor activity and anxiety-like behavior (Prut and Belzung, 2003). In the habituation task, the rats were allowed to explore the OF for 10 min on two consecutive days. The distance traveled was registered on the first day as an index of general activity (Rodgers, 1997; Pierozan et al., 2014). Number of entries and distance traveled in peripheral squares (adjacent to the walls - peripheral locomotion), number of entries and distance traveled in central squares (away from the walls - central locomotion) and percentage of central locomotion in relation to the total locomotion (peripheral locomotion plus central locomotion) were registered on the first day as an index of anxiety-like behavior. The rats were individually placed in the center of the OF, and behavioral parameters were recorded and subsequently elaborated with an automated activity-monitoring system (Any-maze; Stoelting, Wood Dale, IL, USA).

OR task

The OR task was conducted in the OF, as previously described (Ennaceur and Delacour, 1988 adapted by Pamplona et al., 2009). It consisted of three distinct phases: habituation, sample, and discrimination. In the habituation phase, the rats were allowed to explore the OF for 10 min on two consecutive days. In the sample phase, two identical objects (C1 and C2; cubes) were placed in opposite corners of the OF, 20 cm distant from the walls and ~ 60 cm apart from each other, and the rats were allowed to explore them for 5 min. After the end of the sample phase, the rats were removed from the OF and kept in the home cage. After a delay period of 30 min, in the discrimination phase, an identical copy of the familiar object (C3) and a novel T-shaped object (T) were placed in the locations previously occupied by C1 and C2, and the rats were allowed to explore the objects for 5 min. All of the objects were constructed with plastic LEGO blocks. The locations of the objects were counterbalanced in each session. The time spent by the rats exploring each object was monitored with a video system placed in an adjacent room. Exploration of an object was defined as directing the nose to the object at a distance of ≤ 2 cm and/or touching it with the nose. Analyses were performed on the following measures: the total time spent exploring the two objects in the sample phase (C1 + C2) and the discrimination index, which is defined by the difference in exploration time between the novel and the familiar

objects, divided by the total time spent exploring these two objects in the discrimination phase [(T - C3)/(T + C3)].

Y-maze task

The spatial recognition memory of animals was investigated using a Y-maze paradigm. The apparatus consisted of three arms (50 x 10 x 20 cm^3 and 120° apart) made of black plastic, placed in a room with visual cues on the walls. Y-maze testing consisted of two trials separated by an interval of one hour (Dellu et al., 1997; Pandolfo et al., 2013). In the first trial, the animal was placed at the end of one arm and allowed access to that arm and another arm for 5 min. The third arm (the novel arm) was blocked by a guillotine door. The rat was then removed from the maze and returned to its home cage. For the second trial, the rat was placed back into the start arm of the maze and given free access to all three arms for 5 min. The number of entries and the time spent in each arm were recorded. The percentage of entries and time spent in the novel arm was compared to random exploration of the three arms of the maze (i.e., 33%).

All behavioral experiments were conducted in a sound-attenuated room under low-intensity light (12 lx) and were monitored by an experimenter who was unaware of the drug treatments. All apparatuses were cleaned with a 10% ethanol solution and then dried with a paper towel after each trial.

Biochemical analysis

Obtaining CSF and hippocampal and frontal cortex samples

Animals were anesthetized 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 samples were frozen (−20°C) until further analysis (Rodrigues et al., 2009; Guerra et al., 2011). The animals were killed by decapitation, the brains were removed and placed in cold sodium phosphate buffer with the following composition (in mM): 51.33 NaCl, 19.13 $NaH_2PO_4.H_2O$, 81.01

Na_2HPO_4, pH 7.4. The hippocampi and frontal cortex were dissected and transverse slices of 0.3 mm were obtained using a McIlwain Tissue Chopper (Nardin et al., 2009). Slices were then frozen (−80°C) or transferred immediately to 24-well culture plates for measuring glutamate uptake, as subsequently described.

Evaluation of intracellular reactive species production

Intracellular reactive species production was detected via the nonfluorescent cell-permeating compound, 2′,7′-dichlorofluorescein diacetate (DCF-DA). DCF-DA is hydrolyzed by intracellular esterases and then oxidized by reactive species to a fluorescent compound, 2′,7′-dichlorofluorescein (DCFH). Slices were treated with DCF-DA (10 μM) for 30 min at 37°C. Following DCF-DA exposure, the slices were lysed into PBS with 0.2% Triton X-100. Fluorescence was measured on a plate reader (Spectra Max Gemini XPS, Molecular Devices, USA) with excitation at 485 nm and emission at 520 nm. All procedures were performed in the dark, and blanks containing only DCF-DA were processed for measurement of autofluorescence (Quincozes-Santos et al., 2010). Values were obtained as fluorescence units/mg protein and were expressed as percentages of the control.

GSH content assay

Intracellular GSH levels were measured as previously described (Browne and Armstrong, 1998). This assay detects only the reduced glutathione content. Slices were homogenized in sodium phosphate buffer (0.1 M, pH 8.0) containing 5 mM EDTA and protein was precipitated with 1.7% meta-phosphoric acid. Supernatant was assayed with o-phthaldialdehyde (1 mg/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 employed using standard GSH solutions (0–500 μM). GSH concentrations were calculated as nmol/mg protein and were expressed as percentages of the control.

GLO1 activity assay

Slices were lysed and homogenized in sodium phosphate buffer, pH 7.4. Subsequently, slices were centrifuged at 13,000 rpm for 15 min at 4°C and the supernatant was used for enzymatic activity and protein content measurements. GLO1 activity was assayed according to Mannervik et al., 1981 with some modifications. The assay was carried out in 96-well microplates using a microplate spectrophotometer (UV Star - Greiner). The reaction mixture (200 μL/well) contained 50 mM sodium-phosphate buffer pH 7.2, 2 mM methylglyoxal (MG) and 1 mM GSH (pre-incubated for 30 min at room temp). Protein from the sample (10– 20 μg per well) was added to the buffer. The formation of S-(D)-lactoylglutathione was linear and monitored at 240 nm for 15 min at 30°C. A unit of GLO1 activity is defined as the amount of enzyme that catalyzes the formation of 1 μmol of S-(D)-lactoylglutathione per minute. Specific activity was calculated in milliunits per milligram of protein (mU/mg protein) and was expressed as percentages of the control.

AGEs content

AGEs were measured by an enzyme-linked immunosorbent assay, as previously described (Ikeda et al., 1996), with some modifications. The wells of a microtiter plate were coated overnight with 0.1 μg protein in 0.1 mL of 50 mM carbonate bicarbonate buffer (pH 9.6). The wells were washed three times with washing buffer (PBS containing 0.05% Tween 20) and then incubated for 3 h with 2% albumin from chicken egg white to block nonspecific binding. Subsequently, wells were washed again with washing buffer and incubated with 100 μL anti-AGE (6D12) for 1 h. After three washes, wells were incubated with 100 μL peroxidase-conjugated secondary antibody for 60 min. The reactivity of peroxidase was determined by incubation with o-phenylenediamine dihydrochloride (OPD) for 30 min. The reaction was stopped by the addition of 50 μL sulfuric acid (3 M). Absorbance measurements were taken at 492 nm. Results were calculated and expressed as percentages of the control.

GFAP content

Enzyme-linked immunosorbent assay for GFAP was carried out, as previously described

(Tramontina et al., 2007), by coating the microtiter plate with 100 μL samples for 24 h at 4°C. Incubation with a rabbit polyclonal anti-GFAP for 1 h was followed by incubation with a secondary antibody conjugated with peroxidase for 1 h, at room temperature. A colorimetric reaction with o-phenylenediamine was measured at 492 nm. The standard human GFAP (from Calbiochem) curve ranged from 0.1 to 5.0 ng/mL. Data were calculated as ng/μg total protein and were expressed as percentages of the control.

S100B measurement

S100B was measured by an enzyme-linked immunosorbent assay, as previously described (Leite et al., 2008). Briefly, 50 μL of sample (previously diluted when necessary) plus 50 μL of Tris buffer were incubated for 2 h on a microtiter plate previously coated overnight with monoclonal anti-S100B (SH-B1) antibody. For measurement of S100B content in the hippocampus and frontal cortex slices, polyclonal anti-S100B and peroxidase-conjugated anti-rabbit antibodies were incubated at the same time for 60 min at 37°C. The microtiter plate was rinsed three times with a wash solution between each step of the technique. A colorimetric reaction with o-phenylenediamine was measured at 492 nm. The standard S100B curve ranged from 0.010 to 10 ng/mL. For CSF samples, polyclonal anti-S100 antibody was incubated for 30 min, and then peroxidase-conjugated anti-rabbit antibody was added for an additional 30 min, at 37°C. The standard S100B curve ranged from 0.002 to 1 ng/mL. Data were calculated as ng/μg protein and were expressed as percentages of the control.

Glutamate uptake assay

Glutamate uptake was performed as previously described (Thomazi et al., 2004). Slices were incubated at 37°C in HBSS, as described previously. The assay was started by the addition of 0.1 mM L-glutamate and 0.66 μCi/mL L-[2,3-^{3}H] glutamate. Incubation was stopped after 5 min by removing the medium and rinsing the slices twice with ice-cold HBSS. The slices were then lysed in a 0.5 M NaOH solution. 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 the specific uptake. Radioactivity was measured in a scintillation counter. Results were calculated as nmol/mg protein/min and were expressed as percentages of the control.

GS activity

The enzymatic activity of glutamine synthetase was determined using the procedures described previously (Minet et al., 1997) with modifications. Briefly, slices were homogenized in 50 mM imidazole. Homogenates were incubated with (mM): 50 imidazole, 50 hydroxylamine, 100 L-glutamine, 25 sodium arsenate dibasic heptahydrate, 0.2 ADP, 2 manganese chloride, pH 6.2 for 15 min at 37°C. The reactions were terminated by the addition of 0.2 ml of 0.37 M $FeCl_3$, 50 mM trichloroacetic acid, and 100 mM HCl. After centrifugation, the absorbance of the supernatant was measured at 530 nm and compared to the absorbance generated by standard quantities of γ-glutamylhydroxamate acid treated with ferric chloride reagent. Glutamine synthetase activity was expressed as μmol/mg protein/h and was expressed as a percentage of the control.

Protein determination

Protein content was measured by Lowry's method using bovine serum albumin as standard (Peterson, 1977).

Statistical analysis

Data are reported as means ± standard errors and analyzed statistically by one-way or two-way analysis of variance (ANOVA), followed by Duncan's or Bonferroni's test, when the *F*-test was significant. Differences were considered to be significant when $p<0.05$. All analyses were performed using the SPSS software package 20.0 or Prism 5.0 (GraphPad).

Results

Behavioral effects

We first tested the effect of the ICV administration of MG on the performance of adult Wistar rats in the OF test. Locomotion activity (Fig. 2B) and anxiety-like behavior (Fig. 2C, D) were evaluated on the first day after treatment. Habituation in the OF task, measured as the distance traveled, was measured on day 1 and 2 (Fig. 2A). Locomotor activity was not altered by MG injection, as compared to the SHAM group ($F_{(3,47)}$=6.5, p=0.0009) (Fig. 2B); however, anxiety-like behavior was significantly reduced when MG was administrated in single dose (MG 1 day), as demonstrated by the increase in the central locomotion (Fig. 2C,D) shown as a ratio of distance traveled ($F_{(3,45)}$=18.3, p<0.0001) and the percentage of visits ($F_{(3,44)}$=6.8, p=0.0007). To evaluate the habituation of rats, two-way ANOVA revealed that the factor "Days in the OF" and the factor "Treatments" were statistically significant, where no interaction between these factors was observed. Taken together, results indicate that the habituation of all groups occurred in the OF task on two consecutive days (24 h of interval). Bonferroni's *post hoc* analysis showed that MG, only when administered on alternative days (MG 3 days), significantly affected behavior in the OF when compared to the SHAM group on day 2 (Fig. 2A).

Subsequently, we assessed the effect of MG administration on the sample phase (Fig. 3A) and discrimination phase (Fig. 3B) in the OR task. Results for the sample phase revealed that MG administration for 6 consecutive days increased the time spent exploring the objects, when compared to SHAM and MG 1 day (Fig. 3A) ($F_{(3,42)}$=3.0, p=0.0382). However, the results for the discrimination phase revealed, curiously, no significant difference in discrimination score between groups (Fig. 3B) ($F_{(3,45)}$=2.1, p=0.1086), indicating no short-term memory impairment in MG-treated rats in this specific OR task.

To further investigate the effects of MG injection on short-term spatial memory the rats were subjected to a Y-maze test one day after the OR task. There were no significant differences between the groups tested, as evaluated by the ratio of time ($F_{(3,49)}$=2.2, p=0.0969) and the ratio of visits in the novel arm ($F_{(3,50)}$=0.3, p=0.8109) (Fig. 4A, B), suggesting no loss of spatial

recognition memory. However, it should be noted that MG 1 day significantly increased the time spent in the novel arm, when measured in seconds ($F_{(3,49)}$=3.9, p=0.0128), and the percentage of the time spent in the novel arm in relation to the total time spent in apparatus (sum of time in two familiar arms, novel arm and central area) ($F_{(3,49)}$=3.9, p=0.0139), when compared to all the other groups (data not shown). Data indicate that MG when administrated in a single dose (MG 1 day) could interfere in short-term spatial memory, as detectable using these specific parameters in the Y-maze test.

Biochemical findings

In order to determine whether long-term biochemical alterations occur following ICV administration of MG, we evaluated reactive species formation, GSH content, GLO1 activity and AGEs content in the hippocampus and frontal cortex of these animals, to determine whether oxidative stress and dicarbonyl-related alterations are induced by this treatment. Interestingly, reactive species formation (data not shown) ($F_{(3,52)}$=0.7, p=0.5295 hippocampus; $F_{(3,52)}$=0.6, p=0.5767 cortex) and GSH content (Fig. 6A, B) ($F_{(3,49)}$=3.0, p=0.0358 hippocampus; $F_{(3,46)}$=0.3, p=0.8113 cortex) were not altered in either of the structures studied, when comparing the treated groups with the SHAM group. On the other hand, a significant increase in GLO1 activity was observed following ICV administration of MG for 1 day (Fig. 5C, D) ($F_{(3,50)}$=3.8, p=0.0142 hippocampus; $F_{(3,49)}$=0.6, p=0.5803 cortex) and a significant increase in AGEs content was verify following administration of MG for 6 days ($F_{(3,48)}$=3.4, p=0.0245 hippocampus; $F_{(3,45)}$=0.9, p=0.4303 cortex); these alterations were observed solely in the hippocampus (Fig. 6E, F).

We next investigated whether the astroglial-related parameters, namely GFAP and S100B content, could be altered by ICV administration of MG, since S100B has been proposed to be a marker of astroglial activation in brain disorders (Sen and Belli, 2007) and GFAP elevation is a strong sign of astrogliosis, which can occur in several conditions involving brain injury (Eng et al., 2000). No significant differences was observed in the GFAP ($F_{(3,35)}$=1.1,

p=0.3603 hippocampus; $F_{(3,48)}$=0.7, *p*=0.5198 cortex) and S100B content ($F_{(3,53)}$=0.8, *p*=0.4549 hippocampus; $F_{(3,53)}$=1.4, *p*=0.2518 cortex) in the hippocampus (Fig. 7A, B) and frontal cortex (data not shown) following the infusion of MG. Interestingly, a significant increase in CSF S100B was determined following the administration of MG for 1 day and for 3 days, as compared to the SHAM group (data not shown) ($F_{(3,35)}$=7.6, *p*=0.0005).

Finally, we investigated some hippocampal glutamatergic metabolic parameters and found that the glutamate uptake was significantly reduced when MG was administrated in a single dose (MG 1 day) (Fig. 8A) ($F_{(3,51)}$=3.3, *p*=0.0274). GS activity was not altered by MG infusion in this brain region (Fig. 8B) ($F_{(3,46)}$=0.5, *p*=0.6274).

Discussion

In this study, we evaluated behavioral and biochemical parameters to explore the hypothesis that increased exogenous levels of MG, induced by ICV infusion, could induce cognitive impairment in rats and could be used as an animal model of dementia. Our hypothesis was based on findings from clinical and experimental studies demonstrating that diabetes leads to cognitive impairment (Biessels et al., 2006; Kopf and Frolich, 2009) and dicarbonyl stress, and that increased AGEs levels may mediate this decline (Krautwald and Münch, 2010). Recently, a large body of evidence has indicated that MG and AGEs interfere in cognition processes (Beeri et al., 2011; Huang et al., 2012; Liu et al., 2013; Cai et al., 2014; West et al., 2014). In fact, high consumption of dietary AGEs induces increased levels of circulating AGEs, which are associated with cognitive decline in mice and humans (Cai et al., 2014; West et al., 2014). It has been suggested that serum levels of MG may contribute to declines in attention and cognition and may interfere in processing speed (Beeri et al., 2011; Huang et al., 2012; West et al., 2014). Furthermore, immunohistochemical studies have demonstrated co-localization of GLO1 and AGEs and GLO1 protein has been reported as diminished and AGEs deposits increased in AD, particularly during its late stage (Kuhla et al., 2007).

It is important to mention that we used a battery of behavioral tests to screen for

possible MG-induced changes in learning and memory behavior; batteries of tests have been suggested to be better than using just one test by some authors (Cryan and Holmes, 2005; Kalueff et al., 2007) and ensure superior interpretation of results. Furthermore, the sequence of the behavioral tasks was established as an to impose an increasingly averse environment on the animal, according to the proposal of the test performed. The data obtained for habituation in the OF test, Y-maze and OR tasks, which involve spatial and discriminatory memory, respectively, indicate clearly that MG infusion did not impair learning-memory processes, when evaluated at 3 weeks after infusion using these tasks. A single injection of MG (MG 1 day) generated apparent improvements in short-term spatial memory, as demonstrated by some parameters of the Y-maze test (data not shown). The meaning of this finding is unclear; however, it is possible that an adaptive response of the brain occurs following the infusion of higher concentrations of MG. Furthermore, it is not possible to establish whether MG elevation can improve cognitive function using our protocol of ICV MG infusion together with the distinct tasks performed, as also indicated by the absence of alterations in the discrimination phase in the OR following MG infusion. Elevated levels of MG are known to induce locomotor depression and ataxia (Distler et al., 2012) and these alterations could compromise behavioral evaluation. However, our MG administration study protocol did not cause alterations in locomotor activity, as shown by the behavioral tasks performed and demonstrated by the total distance traveled in the OF (day 1).

The results of the OF test demonstrated that acute high levels of MG (MG 1 day) were able to generate anxiolytic-related behavior in rats. Based on these results, it may be suggested that exogenously acute elevated levels of MG in the brain may cause persistent changes in anxiety-like behavior. This idea is also supported by a mice model in which a persistent (5 weeks) decrease in anxiety-like behavior was observed following chronic endogenous elevation of MG, induced by local inhibition of GLO1 expression with RNA interference (Hovatta et al., 2005). MG has also been reported to have a rapid anxiolytic effect (sixteen hours after the last injection) in mice (Hambsch et al., 2010). These data indicate that the permanent augmentation

of MG (probably more than 6 days of infusion) may be necessary to induce long-term anxiolytic-like behavior in rats.

The role of GLO1 in mental disease is in constant discussion, with a particular focus on dicarbonyl substrate concentration, particularly MG (Hambsch, 2011). The local overexpression of the GLO1 gene in the mouse brain results in increased anxiety-like behavior by reducing MG levels (Hovatta et al., 2005). Our findings regarding the effects of ICV MG infusion support data in the literature that suggest a link between GLO1 and anxiety, making GLO1 a possible target for anxiolytic-drug development (Thornalley, 2006; Distler and Palmer, 2012) via alterations in MG levels in the brain. Aberrant expression of GLO1 has been shown to be involved in other psychiatric disorders, such as major depression, panic disorders and schizophrenia (Hambsch, 2011). These reports indicate that the presence of adequate concentrations of MG in the brain may play an important physiological role in the prevention of brain disorders (Hambsch, 2011).

We were, thus, interested in determining which late biochemical alterations may be induced by MG in the hippocampus and cortex areas, which are brain regions that are sensitive to damage in AD (Fuller et al., 2010). To explore this, we performed biochemical analyses at about 6 weeks after MG ICV injection. MG administration in the ventricle system did not alter the oxidative parameters, as verified by DFCH oxidation and GSH content measurement. However, *in vitro* and *in vivo* studies have reported that MG mediates toxicity in tissues via oxidative stress (Brouwers et al., 2011; Heimfarth et al., 2013; Liu et al., 2013). The absence of evidence of oxidative stress in our study does not rule out the possibility that these biochemical changes occurred acutely prior to measuring these biochemical parameters.

The increased activity of GLO1 in the hippocampus may prevent accumulation of MG-derived AGEs, as observed following the acute elevation of MG (MG 1 day). In the early stage of AD, an upregulation of GLO1 has been reported probably in an attempt to compensate and avoid increases in MG (Kuhla et al., 2005; Kuhla et al., 2007). Consequently, the increase in AGEs has been shown to be prevented by the overexpression of GLO1 (Thornalley et al., 2003). On the other hand, following 6 days of successive MG administration (MG 6 days), there was a

significant increase in AGEs content in the hippocampus, while the activity of GLO1 was preserved, indicating that the GLO1 failed to abolish the augmentation of MG that was induced by lower and consecutive doses of MG.

AGEs have been proposed to contribute to the pathogenesis of AD by cross-linking with cytoskeletal proteins and rendering them insoluble, consequently compromising transport processes and contributing to neuronal dysfunction and death. In addition, extracellular accumulation of AGEs chronically activated micro- and astroglial cells, which constantly produced free radicals and neurotoxic cytokines (Krautwald and Münch, 2010). In AD and other aging-related neurodegenerative diseases, impaired astrocyte functions, as well as recruitment and activation of glial cells occur even before the appearance of pathological and clinical signs of disease (Mrak and Griffin, 2005; Fuller et al., 2010). Glial activation in response to injury stimuli commonly involve changes in GFAP and the S100B protein (Rodrigues et al., 2009; Guerra et al., 2011; Biasibetti et al., 2013). No changes in GFAP nor S100B content were observed in the present study, indicating that ICV MG administration did not induce gliosis. However, gliosis induced by MG and/or AGEs, has been demonstrated by augmentations in GFAP in different studies (Cai et al., 2014; Chu et al., 2014) and, interestingly, overexpression of GLO1 attenuates this astrocyte activation (Berner et al., 2012). In dementia models, CSF S100B was found to be diminished after weeks of brain hypoperfusion or of streptozotocin or okadaic acid administration (Vicente et al., 2009; Costa et al., 2012; Biasibetti et al., 2013). In contrast, in injuries caused by acute ICV LPS infusion, S100B in the CSF increased (Guerra et al., 2011). We found a significant increment in CSF S100B following the administration of MG for 1 and 3 days.

We clearly observed hippocampus susceptibility following ICV MG administration, when compared to frontal cortex. As such, we investigated the effects of MG elevation on some glutamatergic functions in hippocampus. Astrocytes regulate synaptic levels of glutamate from glutamate uptake activity (Fuller et al., 2010), avoiding glutamatergic excitotoxicity, which has been related to damage in AD and other brain disorders (Magistretti et al., 1999; Anderson and Swanson, 2000). Interestingly, a single MG administration (MG 1 day) induced a significant

reduction in glutamate uptake. This finding could suggest that astrocyte transporter activity is impaired, possibly due to the direct and irreversible binding of MG, leading to glutamate excitotoxicity. Whether this alteration is a compensatory response to the exacerbated elevation of MG in order to maintain brain homeostasis is not known, since MG has been described as a $GABA_A$ agonist (Distler et al., 2012). We also evaluated an important enzyme involved in the regulation of glutamine levels in astrocytes, GS. However, no changes in GS activity were observed with the ICV MG infusion.

In this study, we proposed to investigate the effects of ICV infused MG, employing: a) a single administration (MG 1 day), or b) a sustainable administration for 6 subsequent days (MG 6 days), or an intermediary infusion on alternate days (MG 3 days). Our results suggest no impairment in memory processes with our protocol of a short period of administration of MG. However, based on reports suggesting that elevated MG may be indicative of brain cell injury initiated before clinically evident cognitive compromise (Beeri et al., 2011), we think that chronic elevated MG concentrations that persist in the brain tissue could compromise memory functions. Our data, indicating that AGEs content increased at 6 weeks after MG administration, together with the observed susceptibility of the hippocampus to this MG exposure, suggest that cognitive decline could occur as a result of glycation-linked alterations over time. Therefore, we did not rule out the idea that chronic elevation of MG concentrations could be a direct mediator of dementia-related behavior. Our study protocol imposed limitations regarding the time of increased levels of MG. Since GLO1 embryonic deletion is lethal to mice and humans (Rabbani et al., 2014), the modeling of GLO1 by local inhibition of protein expression may be the best alternative to study the chronic effects of MG. More *in vivo* studies will be required to determine, conclusively, the involvement of dicarbonyl-induced glycation and related alterations in the etiology of human neurodegenerative disease (Williams et al., 2011).

Conclusions

In summary, our results suggest that high levels of MG are unable to induce direct cognitive deficits after long-term exposure, but can lead to a sustained reduction in anxiety-

related behavior. Our biochemical findings support the hypothesis that MG induces persistent alterations in the hippocampus. Based on our behavioral and biochemical results, we suggest that MG interferes directly in the brain function and that the time and the levels of exogenous MG determine the different features that can be seen in diabetic patients.

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Figure Captions

Fig. 1. Schematic representation of the experimental protocol. Surgery corresponds to cannula implantation; ICV infusion of MG or vehicle was performed after 5 days of recovery (MG 1 day = one injection at 11th day; MG 3 days = three injections on alternate days – 7th, 9th and 11th; MG 6 days and SHAM = six injections on the subsequent 6 days – 6th - 11th); behavioral tests were performed following this sequence: OF (2 days), OR and Y-maze (24 hours of interval between each task); cerebrospinal fluid, hippocampi and frontal cortex were harvested for biochemical analysis.

Fig. 2. Effect of ICV administration of MG on habituation (A), locomotor activity (B) and anxiety-related behavior (C, D) in the OF task. The rats were exposed for 10 min to the OF on day 1, and 24 h later they were exposed again to the OF on day 2 and the behavioral parameters were evaluated 3 weeks after MG injection. Data are expressed as mean ± S.E.M. Data were analyzed by two-way ANOVA with regard to days of OF and treatments (A), assuming $p<0.05$. Statistical significance is indicated in the graph in bold type. Bonferroni's *post hoc* test was performed and statistical significance is indicated in the graph with letters. Other data were analyzed by one-way ANOVA followed by Duncan's test (B, C, D), assuming $p<0.05$. Means indicated by different letters are significantly different.

Fig. 3. Effect of the ICV administration of MG on performance in the OR task. The investigation time (s) (A) and the discrimination index (B) were evaluated approximately 3 weeks after MG injection. Data are expressed as mean ± S.E.M. Means indicated by different letters are significantly different (one-way ANOVA followed by Duncan's test, assuming $p<0.05$).

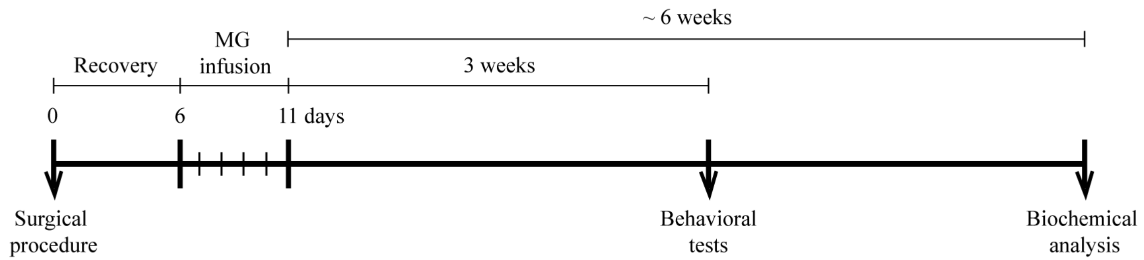
Fig. 4. Effect of the ICV administration of MG on spatial short-term memory, assessed in the Y-maze test. The percentage of total time spent in the novel arm (A) and the percentage of

number visits (entries) in the novel arm (B) during 5 min in the second trial are shown and were evaluated approximately 3 weeks after MG injection. The line in the graphs indicates 33%. Data are expressed as mean ± S.E.M. Data were analyzed by one-way ANOVA followed by Duncan's test, assuming $p<0.05$.

Fig. 5. ICV administration of MG alters GLO1 activity and AGEs content in the hippocampus. Hippocampi and frontal cortex were dissected out and the GSH content (A, B), GLO1 activity (C, D) and AGEs content (E, F) were measured approximately 6 weeks after MG injection. Data are expressed as means ± S.E.M, assuming SHAM value as 100%. SHAM values of GSH levels were 5.5 and 5.1 nmol/mg protein in the hippocampus and frontal cortex, respectively. SHAM values of GLO1 activity were 439 and 379 mU/mg protein in the hippocampus and frontal cortex, respectively. Means indicated by different letters are significantly different (one-way ANOVA followed by Duncan's test, assuming $p<0.05$).

Fig. 6. GFAP and S100B content in the hippocampus of rats submitted to ICV MG injection. Hippocampi were dissected out and the contents of GFAP (A) and S100B (B) were measured approximately 6 weeks after MG injection. Data are expressed as mean ± S.E.M, assuming SHAM value as 100%. SHAM values of GFAP and S100B content are 12.8 and 1.6 ng/µg protein in hippocampus, respectively. Data were analyzed by one-way ANOVA followed by Duncan's test, assuming $p<0.05$.

Fig. 7. Glutamate uptake reduction in the hippocampus of rats submitted to a single injection of MG. Glutamate uptake (A) and GS activity (B) of hippocampal slices were measured approximately 6 weeks after MG injection. Data are expressed as means ± S.E.M, assuming SHAM value as 100%. SHAM values of glutamate uptake are 0.63 nmol/mg protein/min and the GS activity are 0.66 µmol/mg protein/h in hippocampus. Means indicated by different letters are significantly different (one-way ANOVA followed by Duncan's test, assuming $p<0.05$).



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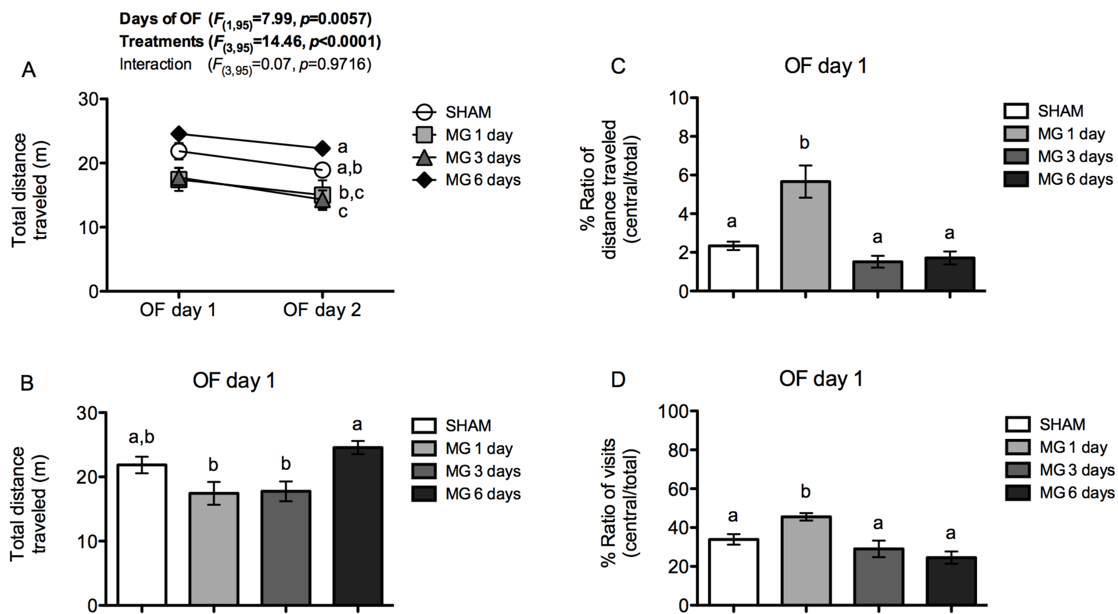

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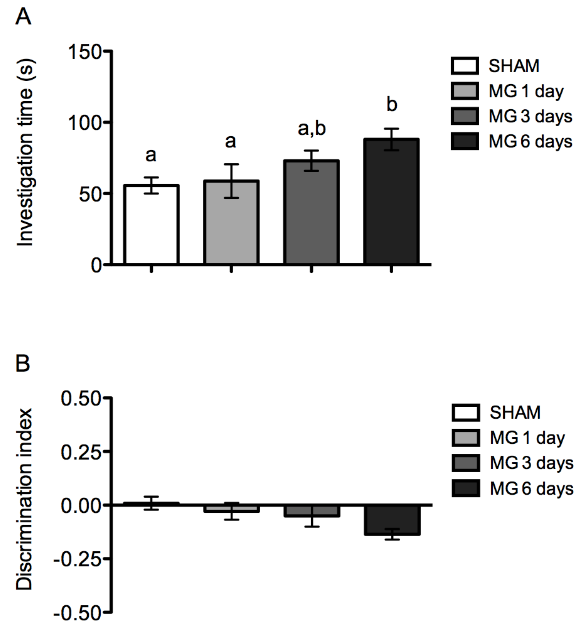

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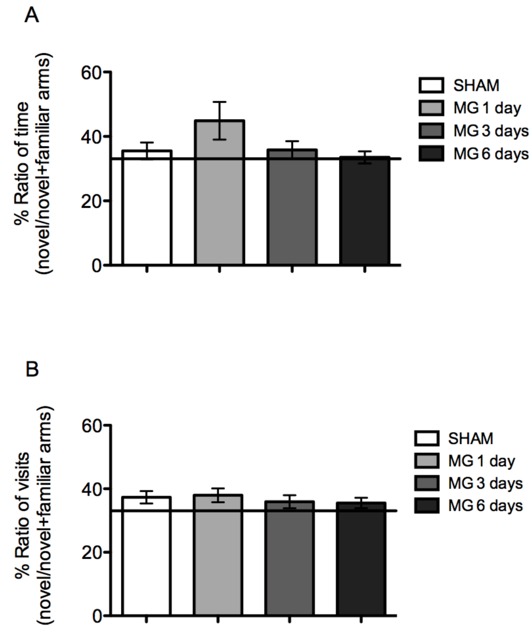

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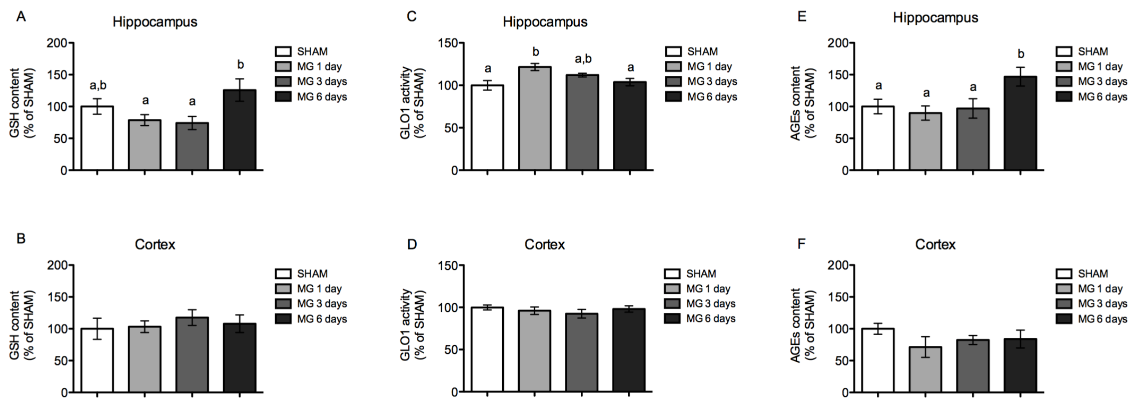

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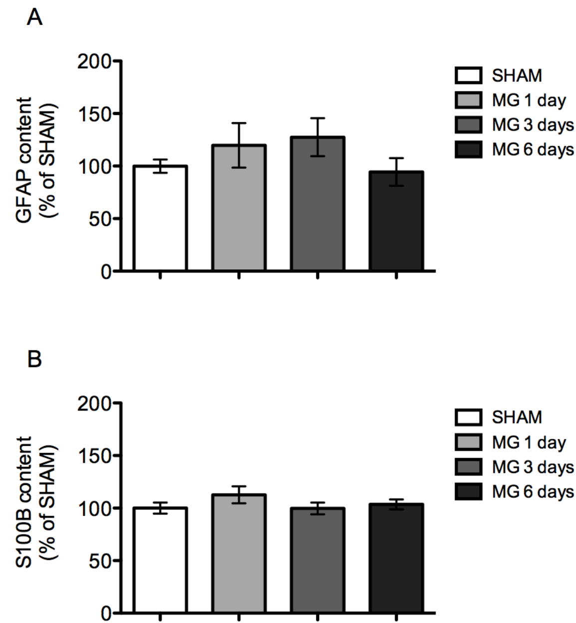

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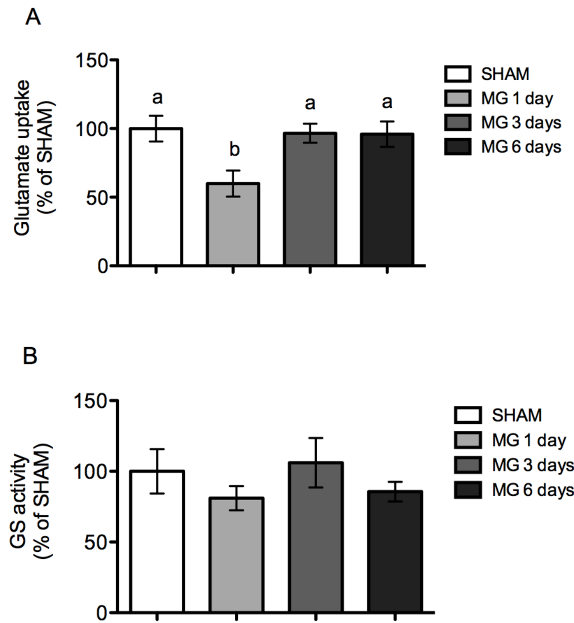

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DISCUSSÃO

Com essa tese foram obtidos diversos resultados oriundos de três artigos científicos, os quais avaliaram efeitos *in vitro, in vivo* e *ex vivo* do MG e também o efeito *in vitro* de outros compostos relacionados com a glicação, glicose e CEL, um AGE derivado da reação de glicação do MG com a lisina.

De acordo com os resultados *in vitro,* obtidos através de culturas primárias de astrócitos e de culturas de linhagem de glioma C6, foi possível verificar diferença em relação a suscetibilidade ao dano causado pelo MG nestas células gliais. Nós observamos que o MG foi capaz de induzir citotoxicidade, com a maior concentração testada – 1 mM, nas culturas gliais estudadas, através do ensaio de incorporação de iodeto de propídio, que representa a ocorrência de morte celular por necrose quando o corante é incorporado pela célula.

Foi observado que o MG (1 mM) induziu morte celular significativa nos astrócitos com um padrão de distribuição da marcação bem diferente do verificado em células C6. Em C6 a distribuição da marcação é difusa e em astrócitos a marcação é acentuada em alguns grupos de células do poço avaliado. Essa divergência do efeito causado pelo MG sugeria o envolvimento das junções GAP. Com o uso de um bloqueador das junções GAP, a carbenoxolona, se comprovou que a morte celular induzida pelo MG em astrócitos é mediada por junções GAP, tendo em vista que a marcação por iodeto de propídio diminuiu em astrócitos através da co-incubação de MG e carbenoxolona e que este efeito não foi observado em culturas de C6, as quais são consideradas controle negativo, por possuírem apenas baixos níveis de junção GAP. Na literatura, até o momento, não existem dados publicados sobre

esse mecanismo de toxicidade do MG em astrócitos que permita discutir esse efeito.

Embora exista essa alteração relevante que sugere presença de necrose, é importante enfatizar que as concentrações testadas não causaram alterações em outros parâmetros, como de viabilidade e integridade celular de células intactas, medidos pela redução do MTT e a incorporação de vermelho neutro. Esses dados, em conjunto, sugerem que, com 24 horas de exposição o MG pode induzir necrose (1 mM) em algumas células avaliadas, mas, ao mesmo tempo, um número expressivo de células em cada poço ainda estava intacta – no que se refere a sua capacidade de realizar endocitose e reduzir o MTT (Liu et al., 1997).

O uso das culturas de astrócitos e C6 também permitiu investigar e elucidar algumas aspectos importantes relacionados com a glicação, como o sistema glioxalase e funções astrocíticas – captação de glutamato, expressão de GFAP e secreção de S100B. Nós demonstramos que os astrócitos possuem maior expressão e atividade da enzima GLO1 comparado com as células C6. Essa diferença está de acordo com o achado de outros grupos, que evidenciaram que células astrogliais possuem um eficiente sistema glioxalase comparado com células neuronais (Amicarelli et al., 2003; Bélanger et al., 2011b). Bélanger e colaboradores (2011b) também observaram que células da linhagem Chinese Hamster ovary (CHO), possuiu atividade da GLO1 mais próxima a dos neurônios, mas ainda significativamente maior que a de neurônios e menor que a de astrócitos.

Outro fator relevante no contexto da eficiência do sistema glioxalase é o conteúdo de GSH, tendo em vista que ela é uma molécula limitante para o

funcionamento da primeira enzima do sistema, a GLO1. Nossos resultados apontam para um maior conteúdo de GSH em astrócitos comparado com as células C6 (14 e 7 nmol/mg proteína, respectivamente) e corroboram com artigos previamente publicados e que possuem condições semelhantes, onde esta diferença também pode ser observada (Guerra et al., 2011; De Souza et al., 2013). Esta diferença no conteúdo de GSH é ainda mais acentuada em C6 quando estas células são expostas ao tratamento com 400 µM de MG, pela diminuição nos níveis de GSH observados desde a primeira hora de exposição a este composto dicarbonil. Entretanto, não foi evidenciada alteração significativa na atividade das enzimas GLO1 e GLO2 em astrócitos e C6. Em trabalho anterior (Hansen et al., 2012), nós havíamos observado uma discreta, mas significativa diminuição na atividade dessas enzimas, obtido através de análise estatística diferente, mas apropriada aos dados. Essa divergência sugere que há suscetibilidade ao dano causado pelo MG em células C6, a qual em longo prazo, pode comprometer ainda mais o sistema glioxalase e causar elevação dos níveis de MG endógenos.

Nós não observamos diferenças significativas no conteúdo de GFAP e secreção de S100B pelo tratamento com MG. Contudo, alterações na organização do citoesqueleto de actina foram evidenciadas através da marcação difusa nos astrócitos após tratamento de 24 horas com MG. Em células C6 essa alteração não foi observada.

Quando outro parâmetro astrocítico foi avaliado, a captação de glutamato, mais uma vez verificou-se um perfil distinto em relação aos astrócitos e as células de glioma C6. Houve redução na captação de glutamato dos astrócitos com 24 horas de tratamento com 400 µM de MG enquanto que

as mesmas condições citadas geraram aumento na captação em C6. Mais interessante é o fato de que esses efeitos observados na captação de glutamato foram prevenidos pela incubação concomitante de aminoguanidina com MG, sugerindo, desta forma, que esse efeito ocorre via glicação nos dois tipos celulares estudados. Um estudo que também foi realizado em cultura de astrócitos de ratos mostrou que o glioxal foi capaz de diminuir a captação de glutamato através da inativação do transportador de glutamato GLT-1. Esse fato foi atribuído à formação de um aduto: GLT-1 CML (carboximetil-lisina) (Kawaguchi et al., 2005). No nosso estudo foi possível descartar a hipótese destas modificações na captação de glutamato serem mediadas por alterações na quantidade desses transportadores, através da medida do conteúdo de transportadores glutamatérgicos predominantemente gliais, GLAST (*Glutamate Aspartate Transporter* ou *Excitatory Amino Acid Transporter 1* - EAAT1) e GLT-1 (*Glial Glutamate Transporter 1* ou EAAT2).

Como parte desta tese foram realizados também ensaios com a incubação de agentes relacionados à glicação em fatias de hipocampo de ratos Wistar, a fim de oportunizar uma melhor compreensão da resposta dos astrócitos quando a conexão com outros tipos celulares é parcialmente mantida. Com este mesmo modelo também se pode avaliar e comparar o efeito da alta concentração de glicose e da CEL, moléculas diretamente relacionadas ao metabolismo do MG.

Através do protocolo experimental realizado, com a incubação das fatias de hipocampo com 50 mM de glicose, 500 µM de MG ou 1 mM de CEL durante 1 hora, foi verificado que a alta concentração de glicose foi capaz de aumentar o conteúdo de GSH e que o MG e a CEL causaram redução significativa na

captação de glutamato e na secreção de S100B em fatias de hipocampo. A fim de elucidar se a ativação do RAGE e as reações de glicação estariam envolvidas neste efeito de redução em dois parâmetros relacionados com funções astrocíticas, a captação de glutamato e a secreção de S100B, foi efetuada a incubação com anticorpo anti-RAGE e com aminoguanidina e L-lisina (um substrato competitivo para glicação). Nenhum desses mecanismos investigados foi capaz de alterar o efeito do MG e da CEL, sugerindo, portanto, que a diminuição observada é independente da ativação de RAGE e de reações de glicação.

A CEL usada em nosso trabalho é um aduto livre de glicação, ou seja, não está ligado à proteína, e foi capaz de induzir as mesmas alterações que o MG em fatias de hipocampo. Neste sentido, um estudo mostrou que o grau de glicação dos AGEs está relacionado com a citotoxicidade desses compostos (Bigl et al., 2008). Como a redução na captação de glutamato e na secreção de S100B causados pela CEL não foi mediada por RAGE e nem prevenida pela L-lisina e aminoguanidina, sugere-se que a CEL pode exercer efeitos intracelulares, que ocorrem por meio de vias não associadas à receptores (Vlassara, 2001). Nós não podemos descartar a hipótese de que a CEL esteja se ligando a outros receptores de AGEs, os quais já foram identificados em células neuronais e gliais (Vlassara, 2001; Ott et al., 2014), embora, ainda pouco estudados.

A redução da captação de glutamato observada pela exposição ao MG e à CEL sugere o comprometimento da atividade dos transportadores glutamatérgicos dos astrócitos. Contudo, através de nossos resultados, o mecanismo pelo qual esse prejuízo ocorre não foi esclarecido. Apesar da

redução da quantidade de glutamato captada, nós não verificamos diferença na atividade da glutamina sintetase com a exposição à elevada concentração de glicose, ao MG e à CEL, indicando que a formação de glutamina não estaria prejudicada. Além disso, foi proposto que os transportadores de glutamato são sensíveis ao estresse oxidativo (Trotti et al., 1998), bem como a atividade da glutamina sintetase (Quincozes-Santos et al., 2014). No entanto, nós não encontramos evidência de indução de estresse oxidativo causada pelo MG ou pela CEL que poderiam explicar a diminuição na captação de glutamato.

Níveis inalterados de transportadores de glutamato foram relatados em estudos com modelo animal de diabetes (Coleman et al., 2004). No entanto, outras pesquisas têm apontado a ativação do receptor NMDA (N-metil-D-aspartato) após o tratamento com MG (De Arriba et al., 2006), indicando que as alterações na transmissão glutamatérgica e excitotoxicidade observadas em ratos diabéticos podem envolver alterações na afinidade e expressão de receptores de glutamato (Gardoni et al., 2002). Nossos resultados sugerem que o comprometimento da captação de glutamato, poderia causar o aumento dos níveis de glutamato na fenda sináptica e, deste modo, gerar disfunção glutamatérgica. Portanto, estes resultados podem ser indicativos de alterações que ocorrem em pacientes diabéticos, uma vez que se sabe que os níveis de MG e CEL estão aumentados nestes indivíduos e estão envolvidos na fisiopatologia do diabetes (Brownlee, 2001).

Permanece a possibilidade de que o aumento dos níveis de glutamato causaria a redução na secreção de S100B, que foi proposta previamente (Tramontina et al., 2006). Esta hipótese é reforçada pelo fato de que a incubação com aminoguanidina e L-lisina não preveniram as alterações

induzidas pelo MG. No entanto, mecanismos distintos podem estar envolvidos nos efeitos do MG e da CEL em fatias de hipocampo.

A S100B é uma proteína que possui efeito trófico (em níveis nM) ou apoptótico (em níveis μM) sobre os neurônios (Donato et al., 2009). Os níveis basais extracelulares encontrados nas nossas preparações são compatíveis com a atividade neurotrófica desta proteína (Nardin et al., 2009). Portanto, nós sugerimos que níveis elevados de MG e CEL afetaram a atividade dos astrócitos, reduzindo os níveis extracelulares de S100B e que, em consequência, essa alteração pode interferir na atividade neuronal e na sobrevivência dessas células.

Neste contexto, diversos grupos têm mostrado que a citotoxicidade do MG é mediada através de apoptose celular, tanto em cultura de neurônios, astrócitos e algumas linhagens celulares quanto em fatias de córtex e hipocampo de ratos (Kuhla et al., 2006; Lee et al., 2009; Heimfarth et al., 2013; Chu et al., 2014). Além disso, foi demonstrado que o aumento dos níveis do MG predispõe à apoptose de neurônios hipocampais e poderia desempenhar um papel importante na neurodegeneração observada na DA (Di Loreto et al., 2008; Tajes et al., 2014), já que o aumento do dano a proteínas através de glicação, oxidação e nitração tem sido relacionado com a morte de células neuronais levando a DA (Ahmed et al., 2005).

Estudos relataram que os astrócitos cultivados em um meio com alta concentração de glicose apresentaram redução nos níveis de GSH (Nardin et al., 2007), sugerindo que o aumento dos níveis de GSH causado pela alta concentração de glicose em fatias do hipocampo pode ser transitório. Além disso, não houve modificação na formação de espécies reativas em fatias de

hipocampo, mesmo com a exposição a 50 mM de glicose. Estes achados permitem propor que a elevação dos níveis de GSH, induzida pela alta concentração de glicose, foi essencial para evitar o estresse oxidativo e o estresse dicarbonil, tendo em vista a necessidade de GSH para o adequado funcionamento do sistema glioxalase. Neste contexto, um estudo verificou que a alta concentração de glicose (em exposição aguda e crônica) gerou aumento do fluxo da via das pentoses e do conteúdo de GSH em astrócitos, impedindo a elevação dos níveis de espécies reativas de oxigênio (Takahashi et al., 2012).

Nós não verificamos alteração significativa na captação de glicose com os tratamentos realizados. Alguns trabalhos realizados em culturas de células indicam o envolvimento de compostos dicarbonil no prejuízo do metabolismo da glicose (De Arriba et al., 2007; Hansen et al., 2012). Contudo, estudo realizado com fatias de córtex cerebral de ratos expostas ao glioxal não tenha encontrado modificações na oxidação de glicose (Schmidt et al., 2010).

Nós presumimos que a exposição a elevados níveis de glicose requer mais tempo para obter efeitos semelhantes aos observados com a incubação de MG e CEL em fatias de hipocampo. Esta diferença pode estar relacionada à menor reatividade da glicose comparada ao MG (Thornalley, 2005) e também pode ser atribuída ao fato de que as concentrações endógenas de MG e CEL gerados durante a exposição a 50 mM de glicose em 1 hora de incubação são menores do que as utilizadas nos ensaios.

Nesta tese nós também avaliamos se o aumento das concentrações exógenas de MG seria capaz de induzir alterações comportamentais e bioquímicas, em longo prazo, em ratos Wistar machos. Foi realizada administração ICV de MG e, após três semanas, tarefas de comportamento

relacionadas à memória e aprendizagem e à ansiedade. Aproximadamente seis semanas após a última infusão de MG foram efetuadas dosagens bioquímicas *ex vivo* em hipocampo, córtex e LCR. A partir deste estudo nós buscamos determinar se o MG poderia causar comprometimento cognitivo em ratos e, assim, ser utilizado como um modelo animal de demência.

A nossa hipótese é respaldada por evidências que indicaram que o MG e os AGEs interferem na cognição (Beeri et al., 2011; Huang et al., 2012; Liu et al., 2013; Cai et al., 2014; West et al., 2014). Tem sido sugerido que os níveis séricos de MG estão relacionados com o declínio na atenção e podem interferir na velocidade de processamento de informações (West et al., 2014), além de estarem associados com o declínio cognitivo (Beeri et al., 2011; Huang et al., 2012). O alto consumo de AGEs pela dieta induziu o aumento dos níveis de AGEs circulantes que estão associados ao declínio cognitivo em ratos e em seres humanos (Cai et al., 2014; West et al., 2014).

Para induzir o aumento do nível exógeno de MG foi realizada infusão ICV, onde a mesma concentração final de MG foi utilizada nos grupos experimentais (3 µmol/µL), conforme o protocolo: a) uma única administração (MG 1 dia), ou b) infusão intermediária - 3 dias alternados (MG 3 dias), ou c) administração sustentada - 6 dias consecutivos (MG 6 dias).

Os resultados que nós obtivemos através das tarefas de habituação realizada no campo aberto, do labirinto em Y e do teste de reconhecimento de objetos que, em conjunto, fornecem um perfil de avaliação da memória espacial e discriminatória, indicaram que após 3 semanas da infusão de MG não houve prejuízo na memória e aprendizagem. Entretanto, com base em alguns parâmetros do teste do labirinto em Y (tempo no braço novo e a relação do

tempo gasto no novo braço em relação ao tempo total gasto no aparato) foi detectado que uma única injeção de MG (MG 1 dia) gerou alterações relacionadas com uma aparente melhora na memória espacial de curto prazo. O significado deste resultado não está claro, mas permite inferir que seria uma resposta adaptativa induzida pelo aumento súbito da concentração exógena de MG. Todavia, a partir do protocolo de infusão e das distintas tarefas realizadas não é possível afirmar que a elevação dos níveis de MG poderia causar algum tipo de melhora na função cognitiva.

À respeito do comportamento relacionado à ansiedade, os resultados obtidos com o teste do campo aberto indicaram que o aumento agudo dos níveis de MG (MG 1 dia) foi capaz de gerar comportamento ansiolítico nos ratos. A partir desses resultados é possível sugerir que seria necessário o aumento preponderante dos níveis exógenos de MG no encéfalo para causar alterações no comportamento relacionado à ansiedade, mesmo após semanas da indução desta elevação. Esta ideia é respaldada por outro estudo em que foi verificada redução no comportamento do tipo ansioso após 5 semanas da indução do aumento dos níveis endógenos de MG (Hovatta et al., 2005). Outra evidência mostrou que o efeito ansiolítico do MG pode iniciar logo depois (16 horas após a infusão ICV) da elevação da concentração exógena de MG no encéfalo (Hambsch et al., 2010). Estes dados, em conjunto, podem indicar que é necessário o aumento permanente de MG (maior do que ocorrido através de seis dias de infusão) para induzir comportamento ansiolítico em longo prazo em ratos. Estes achados fortalecem a ideia de que a apropriada regulação dos níveis de MG no cérebro têm um papel fisiológico importante a fim de evitar desordens cerebrais (Hambsch, 2011).

O papel da GLO1 nas doenças mentais, no que se refere a concentração dos compostos dicarbonil, principalmente o MG, está em constante discussão (Hambsch, 2011). Tem sido sugerida a associação entre GLO1 e ansiedade, tornando a GLO1 um possível alvo para o desenvolvimento de drogas com efeito ansiolítico (Thornalley, 2006; Distler e Palmer, 2012). A superexpressão do gene da GLO1 resultou no aumento do comportamento de ansiedade em ratos através da redução dos níveis de MG (Hovatta et al., 2005). Além disso, foi relatado que a expressão aberrante de GLO1 está envolvida com outras desordens psiquiátricas como a depressão maior, transtorno do pânico e esquizofrenia (Hambsch, 2011).

Os resultados obtidos em nosso estudo não estão relacionados com alterações na atividade locomotora, as quais foram avaliadas em todas as tarefas comportamentais realizadas. A atividade locomotora foi avaliada como parâmetro de controle da realização dos testes comportamentais e também para excluir um possível efeito de depressão locomotora e ataxia, já reportado em estudos anteriores, em resposta à elevação dos níveis de MG (Distler et al., 2012).

Nós realizamos a análise de alguns parâmetros bioquímicos em hipocampo e córtex de ratos, 6 semanas após a administração de ICV de MG, a fim de determinar se o MG era capaz de induzir alterações tardias nestas estruturas que são sensíveis a danos na DA (Fuller et al., 2010). A administração de MG não alterou a formação de espécies reativas, como verificado através da oxidação de DCFH, e nem o conteúdo de GSH. No entanto, estudos *in vitro* e *in vivo* relataram que o MG é capaz de gerar toxicidade através de estresse oxidativo (Brouwers et al., 2011; Heimfarth et al.,

2013; Liu et al., 2013). Portanto, a ausência de modificações nos parâmetros de estresse oxidativo avaliados em nosso estudo não exclui a possibilidade de que o estresse oxidativo tenha ocorrido previamente.

O aumento da atividade da GLO1 no hipocampo pode evitar o acúmulo de AGEs derivados do MG (Thornalley et al., 2003; Kuhla et al., 2005; Kuhla et al., 2007), que foi observado com administração única deste composto (MG 1 dia). Por outro lado, com 6 dias de administração consecutiva de MG (MG 6 dias) houve um aumento significativo no conteúdo de AGEs do hipocampo, enquanto que a atividade da GLO1 estava inalterada em comparação com o grupo SHAM, indicando que a GLO1 não conseguiu conter o dano causado pelo MG quando doses menores foram infundidas durante um número maior de dias.

Foi proposto que os AGEs contribuem com a patogênese da DA por meio de reações de ligação cruzada com proteínas, como as do citoesqueleto, tornando-as insolúveis. Assim, contribuem para a disfunção e morte neuronal. Além disso, o acúmulo de AGEs no espaço extracelular ativa cronicamente células microgliais e astrogliais, que, nesta condição, produzem constantemente radicais livres e citocinas neurotóxicas (Krautwald e Münch, 2010). Na DA e em outras doenças neurodegenerativas relacionadas com o envelhecimento, o prejuízo de funções dos astrócitos, bem como o recrutamento e ativação de células gliais ocorrem mesmo antes do aparecimento dos sinais patológicos e clínicos da doença (Mrak e Griffin, 2005; Fuller et al., 2010). Neste sentido, foi proposto que a ativação glial em resposta à lesões geralmente envolve mudanças nas proteínas GFAP e S100B (Rodrigues et al., 2009; Guerra et al., 2011; Biasibetti et al., 2013).

Em nosso estudo não foram verificadas modificações no conteúdo de GFAP e S100B, indicando que a administração ICV de MG não induziu gliose nos grupos estudados. No entanto, diferentes trabalhos reportaram gliose induzida pelo MG e/ou AGEs, que foi observada pelo aumento no conteúdo de GFAP (Cai et al., 2014; Chu et al., 2014) e a superexpressão de GLO1 foi capaz de atenuar esta elevação (Berner et al., 2012).

Nós observamos um aumento significativo de S100B no LCR com a administração de MG durante 1 e 3 dias. Em estudos com modelos de demência foi observado que a S100B no LCR diminuiu depois de semanas de hipoperfusão cerebral, de administração de estreptozotocina ou de ácido ocadaico (Vicente et al., 2009; Costa et al., 2012; Biasibetti et al., 2013), contudo um aumento da S100B no LCR foi observada após a infusão aguda de LPS ICV (Guerra et al., 2011).

Como nós observamos claramente uma suscetibilidade à alterações induzidas pela administração ICV de MG no hipocampo em relação ao córtex frontal, foi investigado o impacto desta infusão sobre a captação de glutamato e a atividade da enzima glutamina sintetase. Curiosamente, uma única administração de MG (MG 1 dia) induziu redução significativa da captação de glutamato, todavia, não houve mudança na atividade da GS com a infusão ICV de MG nos grupos investigados. Estes resultados podem sugerir que a atividade dos transportadores glutamatérgicos dos astrócitos está prejudicada, devido à ligação irreversível do MG que gera, por consequência, excitotoxicidade glutamatérgica. Outra hipótese é de que esta alteração tenha ocorrido como uma resposta compensatória da elevação exacerbada de MG a

fim de manter a homeostase do cérebro, uma vez que o MG tem sido descrito como um agonista $GABA_A$ (Distler et al., 2012).

Nossos resultados sugerem que não há comprometimento em processos de memória com base no protocolo efetuado, que podemos assumir como um curto período de administração de MG. No entanto, a partir de dados que apontam que o aumento de MG pode ser indicativo de lesão das células cerebrais iniciada antes do comprometimento cognitivo ser clinicamente evidente (Beeri et al., 2011), é possível inferir que se ocorresse a elevação crônica das concentrações de MG no encéfalo poderia existir um comprometimento visível das funções de memória e aprendizagem. Condizentes com esta ideia estão nossos resultados de aumento do conteúdo de AGEs após 6 semanas de exposição ao MG e a suscetibilidade do hipocampo frente às alterações induzidas pelo MG, que nos permitem pensar que o declínio cognitivo pode ocorrer como resultado de alterações associadas à glicação ao longo do tempo. Portanto, não se descarta a ideia de que a elevação crônica das concentrações de MG poderia ser um mediador direto de demência.

O protocolo de estudo que realizamos impõe limitações quanto ao tempo de aumento dos níveis de MG. Sabendo-se que a deleção embrionária do gene da GLO1 é letal para camundongos e humanos (Rabbani et al., 2014), a inibição local da expressão da proteína GLO1 pode ser a melhor alternativa para estudar os efeitos crônicos do MG.

Os dados obtidos com esta tese permitem, em conjunto, discutir alguns aspectos relacionados aos efeitos do MG em parâmetros de estresse oxidativo e específicos de ativação e função astrocítica (Figura 3).

Embora seja um efeito encontrado em diversos estudos, nós não observamos alterações na formação de espécies reativas através do método de oxidação do DCF nas culturas de células gliais estudadas e nas fatias de hipocampo, tanto *in vitro* quanto *ex vivo*. Possivelmente isso se deva à eficiência das células gliais na proteção do efeito citotóxico do MG, isoladas (culturas específicas) ou em contato com outros tipos celulares (fatias de hipocampo). Outros aspectos que podem influenciar no resultado que encontramos são a concentração de MG utilizada e o tempo de incubação com o MG até a realização da técnica. Além disso, pode-se considerar que o MG esteja atuando intracelularmente e seus efeitos não sejam mediados via RAGE, que quando ativado, pode causar aumento de radicais livres (Lapolla et al., 2005b; Vlassara e Striker, 2011).

Tanto em cultura de astrócitos quanto nas fatias de hipocampo *in vitro* e *ex vivo* o conteúdo de GSH permaneceu inalterado frente a exposição ao MG quando comparado com o grupo controle/SHAM de cada desenho experimental. Deste modo, a ausência de alterações na formação de espécies reativas pode ser explicada, pelo menos em parte, pela manutenção dos níveis de GSH, que é um peptídeo fundamental para evitar o estresse oxidativo (Bélanger et al., 2011a).

Evidências apontam que os astrócitos exercem papel fundamental na regulação dos níveis de glutamato na sinapse (Bernardinelli et al., 2004; Fuller et al., 2010), evitando a excitotoxicidade glutamatérgica, que tem sido relacionada aos danos presentes na DA e outros transtornos cerebrais (Anderson e Swanson, 2000). Durante a progressão da DA ocorrem alterações na transmissão glutamatérgica que estão relacionadas com a destruição de

neurônios e elevada ativação dos receptores pós-sinápticos (Scott et al., 2011). Em virtude disto, que a memantina, um fármaco antagonista não-competitivo do receptor NMDA (N-metil-D-aspartato) (Danysz e Parsons, 2012), é utilizada clinicamente na fase intermediária e avançada da DA, diminuindo a toxicidade glutamatérgica associada a essa patologia.

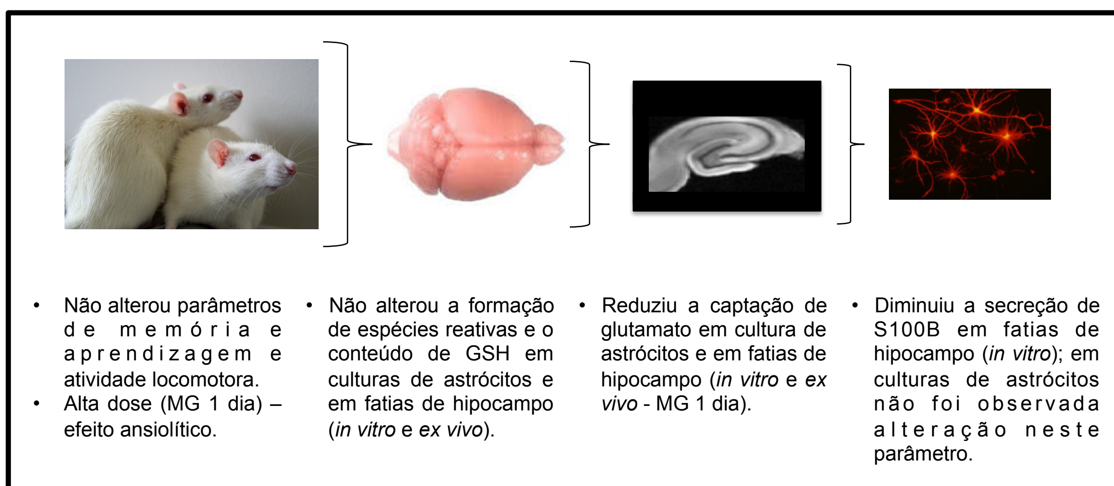

Figura 3. Resumo dos efeitos do MG em parâmetros comportamentais, de estresse oxidativo e específicos de ativação e função astrocítica.

Os resultados que obtivemos indicam que o MG é capaz de reduzir significativamente a captação de glutamato em cultura de astrócitos e em fatias de hipocampo *in vitro* e *ex vivo,* neste último caso somente com a dose única de MG administrado ICV (MG 1 dia). Estes resultados observados em conjunto reforçam a ideia de que o MG, que é um importante mediador de dano no diabetes e na DA, interfere na homeostase cerebral através do prejuízo na remoção do glutamato extracelular. Entretanto, os mecanismos estudados por nós não permitem esclarecer se a redução da captação seria um efeito direto da glicação mediada pelo MG, já que o efeito de prevenção da aminoguanidina

(um composto anti-glicação) foi observado em culturas de astrócito, mas não em fatias de hipocampo.

Também no diabetes foi sugerido o envolvimento dos transportadores e receptores glutamatérgicos na neurotoxicidade e neurodenegeração (Coleman et al., 2010; Lau et al., 2013; Wang et al., 2013). Em modelos animais de diabetes foi verificada redução dos transportadores de glutamato no córtex cerebral e na retina (Lau et al., 2013; Wang et al., 2013) e aumento da expressão dos receptores ionotrópicos (AMPA (alfa-amino-3-hidroxi-metil-5-4-isoxazolpropiônico) e NMDA) (Wang et al., 2013) e redução após 12 semanas de indução do diabetes (Lau et al., 2013), sugerindo que estas alterações no receptores podem ser dependentes da duração do diabetes.

De acordo com a análise realizada em culturas de astrócitos e de glioma C6 o MG, em 24 horas de incubação, não foi capaz de induzir alterações no conteúdo dos transportadores GLAST e GLT-1. Em estudo conduzido com modelo animal de diabetes observou-se aumento da captação de glutamato em homogeneizado cerebral sem modificação na expressão de transportadores glutamatérgicos no hipocampo, no córtex e no cerebelo (Coleman et al., 2010). Portanto, não está claro o mecanismo que leva a redução da captação de glutamato. Com base nos estudos citados anteriormente, parece que há, pelo menos inicialmente, alteração na atividade dos transportadores e ativação de receptores pós-sinápticos.

Nossos resultados sugerem que o MG possa gerar excitotoxicidade glutamatérgica através da diminuição da captação de glutamato e consequente aumento dos níveis deste neurotransmissor na sinapse. Este é um achado importante, especialmente pelo fato de ter sido sugerido que o uso de

moduladores da captação de glutamato seria útil para auxiliar na terapia atual da DA (Scott et al., 2011). No entanto, uma limitação de nosso estudo é que a excitotoxicidade glutamatérgica não foi comprovada pela dosagem dos níveis de glutamato no meio extracelular.

A secreção de S100B reduziu em fatias de hipocampo com a exposição à CEL e ao MG *in vitro*, contudo, não ocorreu alteração neste mesmo parâmetro em culturas de astrócitos expostas ao MG, provavelmente devido a maior resistência dos astrócitos à toxicidade induzida pelo MG quando estas células estão livres da conexão com outros tipos celulares. À respeito da incubação com fatias de hipocampo, é importante lembrar que os oligodendrócitos, são células gliais que também expressam, sintetizam e secretam S100B (Steiner et al., 2008) e poderiam estar envolvidos nos efeitos mediados pelo MG e pela CEL. Neste contexto, foi demonstrado que algumas proteínas, com funções estruturais, metabólicas e regulatórias, de uma linhagem celular de oligodendócritos (OLN-93) são alvos da modificação por AGEs em condições basais (Langer et al., 2006). Portanto, o prejuízo no funcionamento de proteínas, como as do citoesqueleto, pode repercutir em alterações na secreção de S100B.

CONCLUSÕES

Referente aos objetivos 1 e 2:

A partir dos parâmetros utilizados para avaliar a toxicidade do MG frente à exposição de curto (1 hora) e longo (24 horas) prazo nas culturas gliais estudadas, é possível concluir que essas células são mais suscetíveis a alterações após 24 horas de incubação com 400 μM de MG. Além disso, o prejuízo na captação de glutamato induzido pelo MG em astrócitos ocorreu sem alterações na atividade do principal sistema de detoxificação deste composto, o sistema glioxalase. Os dados obtidos a partir de culturas C6 indicam que transportadores de glutamato que não são característicos de astrócitos podem ter sua atividade modificada devido à exposição ao MG e confirmaram que a secreção de S100B e os níveis de GFAP não foram alterados pelo MG em 24 horas. A alta eficiência do sistema glioxalase em culturas de astrócitos é uma característica que foi observada neste estudo em comparação com as células de glioma C6. Esta característica deve ser incluída como uma das funções dos astrócitos, que é relevante por evitar o estresse dicarbonil e, em consequência, a formação de AGEs.

Referente aos objetivos 3 e 4:

Concluiu-se também que a exposição aguda, *in vitro*, ao MG e à CEL, mas não à glicose, foi capaz de induzir efeitos semelhantes em fatias de hipocampo, sugerindo que a alta concentração de glicose é principalmente tóxica por elevar as taxas de compostos glicantes, tais como o MG, e gerar ligações cruzadas com proteínas. A redução na secreção de S100B e

alterações na atividade glutamatérgica ocorreram independente da ativação de RAGE e de reações de glicação. Estas podem ser alterações envolvidas na toxicidade dos agentes glicantes testados *in vivo* e podem contribuir para a disfunção cerebral observada em pacientes diabéticos.

Referente ao objetivo 5:

Os resultados obtidos através dos testes de comportamento sugerem que a elevação dos níveis de MG geradas pela administração ICV não são capazes de induzir prejuízo cognitivo após três semanas da infusão deste composto. Contudo, uma única infusão ICV de MG causou redução no comportamento relacionada com ansiedade. A partir de nossos achados bioquímicos *ex vivo* percebeu-se que o MG causou alterações persistentes no hipocampo dos ratos e essa estrutura apresentou maior suscetibilidade à modificações induzidas pelo MG que o córtex. Em suma, é possível concluir que o MG interfere diretamente em funções cerebrais e que os níveis exógenos de MG determinam as diferentes características que podem ser observadas em pacientes diabéticos.

PERSPECTIVAS

- Realizar dosagem de glutamato no LCR;
- Implementar as técnicas de dosagem de MG e de AGEs que permitam medir a quantidade destes compostos em fluidos corporais e alimentos/rações;
- Avaliar se as alterações observadas *in vitro*, podem ser revertidas pela metformina e pela memantina.

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