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EFEITO DO RESVERATROL EM ASTRÓCITOS: UMA ABORDAGEM SOBRE PARÂMETROS GLIAIS ESPECÍFICOS EM CULTURA E EM FATIAS HIPOCAMPAIS

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“Tudo vale a pena se a alma não é pequena”.

Fernando Pessoa

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RESUMO

O resveratrol (3,5,4'-trihydroxy-*trans*-stilbene) é um polifenol encontrado nas uvas e no vinho tinto, que possui propriedades antioxidantes, antiinflamatórias, bem como cardioprotetoras. Recentemente, cada vez mais estudos sugerem que o resveratrol possa exercer um papel significativo; regulando funções importantes no SNC, especialmente sob condições patológicas. Assim, neste estudo nós investigamos o papel neuroprotetor do resveratrol através de um efeito modulatório sobre parâmetros gliais em cultura primária de astrócitos corticais e em um modelo de fatias hipocampais. Os astrócitos são as células mais abundantes constituintes do SNC e uma de suas funções consiste em exercer um papel protetor contra o estresse oxidativo. Nós observamos que o tratamento com resveratrol por 24 h foi capaz de aumentar a captação de glutamato, o conteúdo de glutationa e secreção de S100B em cultura de astrócitos corticais. Além disso, a pré-incubação com resveratrol confirmou seu potencial não só como antioxidante, mas também modulando as funções gliais mesmo após um insulto oxidativo com 0,1 mM de H_2O_2. Observamos que o resveratrol impediu o aumento das espécies reativas de oxigênio após uma injúria com H_2O_2 e evitou a diminuição da captação de glutamato e do conteúdo de glutationa. Enquanto que a secreção de S100B, 1 h após o dano com H_2O_2, apresentou um aumento transitório que foi revertido pela presença de resveratrol. O oposto ocorreu 24 h após o insulto, quando o H_2O_2 levou a uma diminuição na secreção de S100B, enquanto que na presença do resveratrol foi observado um aumento da proteína no meio extracelular. Um perfil semelhante foi encontrado em fatias hipocampais tratadas com resveratrol após um insulto com 0,1 e 1 mM de H_2O_2. Apesar do resveratrol não ter revertido a diminuição dos níveis extracelulares de lactato e a redução da viabilidade celular induzidos por 1 mM de H_2O_2, o polifenol preveniu o aumento da permeabilidade da membrana observada. O resveratrol também impediu a diminuição do conteúdo de glutationa após a exposição ao H_2O_2. Ainda, a ativação da ERK1/2 provocada por 1 mM de H_2O_2 foi completamente prevenida pelo resveratrol. Entretanto, o resveratrol evitou a diminuição na captação de glutamato apenas após o insulto com 0,1 mM de H_2O_2. Embora os mecanismos através dos quais o resveratrol exerce seus efeitos ainda não estejam completamente esclarecidos, podemos propor que o polifenol apresenta um papel protetor contra injúrias no SNC por modular funções gliais.

ABSTRACT

Resveratrol (3,5,4'-trihydroxy-*trans*-stilbene) is a polyphenol present in grapes and red wine, which has antioxidant, anti-inflammatory and cardioprotective properties. Recently, a great number of reports suggest that resveratrol might be involved in important functions of SNC, especially under pathological conditions. Therefore, in this study, we investigate the neuroprotective role of resveratrol through modulatory effect on glial parameters in primary cortical astrocytes and hippocampal slice preparations. Astrocytes are the most abundant cell type CNS and are involved in a protective role against oxidative stress. We observed that 24 h treatment with resveratrol lead to an increase in glutamate uptake, glutathione content and S100B secretion in astrocyte cultures. Moreover, pre-incubation with resveratrol has confirmed its potential not just as an antioxidant, but also by modulating glial functions even after an oxidative insult with 0,1 mM H_2O_2. Resveratrol avoided the increase of reactive species of oxygen after an insult with H_2O_2 and prevented the decrease in glutamate uptake and glutathione content. Moreover, 1 h after H_2O_2 injury, S100B secretion demonstrated a transitory increase that was reverted by resveratrol. In contrast, 24 h after the oxidative damage exposure it was observed a decrease in S100B caused by H_2O_2 while in the presence of resveratrol there was an increase of S100B in the extracellular. A similar profile was found in hippocampal slices treated with resveratrol after oxidative damage with 0,1 and 1 mM H_2O_2. Even though resveratrol did not changed the decrease in lactate levels and the reduction in cell viability induced by 1 mM H_2O_2, it was able to prevent the increase in cell permeability. Resveratrol also prevented the decrease of glutathione content after H_2O_2. There was also an activation of ERK1/2 by 1 mM H_2O_2 that resveratrol fully prevented. However, resveratrol only reverted the decrease in glutamate uptake induced by 0,1 mM H_2O_2. Although the mechanisms underlying resveratrol effects are not completely clear, we may propose that resveratrol could represent a protective strategy to avoid brain injury by modulating glial functions.

LISTA DE ABREVIATURAS

AGE	Produto Final de Glicação Avançada
ATP	Adenosina Trifosfato
CISGLI	Cisteinilglicina
COA	Coenzima A
COX	Ciclooxigenase
EAAT	Transportador de Aminoácidos Excitatórios
ERK	Cinase Reguladora de Sinal Extracelular
γ-GCS	γ-Glutamilcisteina sintetase
γ-Glucis	γ-Glutamilcisteina
γ-GT	γ-Glutamiltranspeptidase
GABA	Ácido γ-Aminobutírico
GFAP	Proteína Ácida Fibrilar Glial
GLAST	Transportador de glutamato-aspartato
GLT	Transportador de glutamato
GS	Glutamina Sintetase
H_2O_2	Peróxido de Hidrogênio
IL-6	Interleucina-6
iNOS	Óxido Nítrico Sintase Induzível
LDH	Lactato Desidrogenase
LDL	Lipoproteína de Baixa Densidade
MAPK	Proteína Cinase Ativada por Mitógenos

NFkB	Fator de Transcrição Nuclear
NMDA	N-metil-D-aspartato
NO	Óxido Nítrico
NOS	Óxido Nítrico Sintase
PI3K	Fosfatidil-Inositol-3-cinase
PKC	Proteína Cinase C
RAGE	Receptor para AGE
RESV	Resveratrol
SIRT	Sirtuína
SOD	Superóxido Dismutase
SNC	Sistema Nervoso Central
TNFα	Fator de Necrose Tumoral alfa
XC^-	Trocador Glicina/Glutamato Independente de sódio

INTRODUÇÃO

Astrócitos

Os astrócitos são as células mais abundantes do sistema nervoso central (SNC). Embora durante muito tempo estas células tenham sido consideradas como apenas um suporte estrutural para os neurônios, hoje têm seu papel reconhecido por sua capacidade dinâmica, participando não só do desenvolvimento e da manutenção da fisiologia normal, mas também respondendo a injúrias (He e Sun, 2006).

Entre as principais funções exercidas pelos astrócitos estão: formação da barreira hemato-encefálica (Abbott et al., 2006); regulação dos níveis de íons extracelulares (Walz, 1989); produção de fatores tróficos (Schwartz e Mishler, 1990; Eriksen e Druse, 2001), metabolismo de neurotransmissores (Kimelberg e Katz, 1985) e suporte energético para os neurônios (Pellerin, 2005).

Além disso, os astrócitos são ativados em situações de dano no SNC. Uma das características observadas durante a ativação glial é a expressão aumentada de GFAP (proteína ácida glial fibrilar) em um processo chamado astrogliose reativa que ocorre em situações como trauma, desordens genéticas, insultos químicos e doenças neurodegenerativas (Eng et al., 2000).

Estudos recentes ressaltam a importância da interação neurônio-astrócitos, estabelecendo uma comunicação recíproca para a fisiologia do SNC. Os astrócitos participam ativamente nas sinapses, respondendo de maneira seletiva a liberação de neurotransmissores, através da elevação do Ca^{2+} intracelular (Perea e Araque, 2006).

Os astrócitos e o estresse oxidativo

Os astrócitos desempenham um papel fundamental na proteção do SNC contra o estresse oxidativo (Makar et al., 1994; Takuma et al., 2004). O estresse oxidativo pode ser definido como um desequilíbrio entre a produção de espécies reativas de oxigênio e a capacidade antioxidante. Muitos estudos relacionam o estresse oxidativo com o desenvolvimento de diversas condições patológicas que incluem isquemia, inflamação e doenças neurodegenerativas, como por exemplo, as doenças de Alzheimer, Huntington e Parkinson (Hyslop et al., 1995; Halliwell, 2006). Sabe-se que o SNC é particularmente sensível ao dano oxidativo devido ao alto consumo de oxigênio e a conseqüente geração de espécies reativas de oxigênio durante a fosforilação oxidativa (Castagne et al., 1999).

O tripeptídeo glutationa (γ-glutamilcisteinilglicina) constitui uma das principais defesas celulares contra as espécies reativas de oxigênio. A síntese de glutationa ocorre a partir de uma reação catalisada pela enzima γ-glutamilcisteina sintetase (γ-GCS), que utiliza glutamato e cisteína para formar o dipeptídeo γ-glutamilcisteina, que juntamente com a glicina, através uma reação catalisada pela glutationa sintetase, dará origem a glutationa (Figura 1) (Dringen et al., 2000). A cisteína é o substrato limitante para a síntese de glutationa (Wang e Cynader, 2000; Sagara et al., 1993).

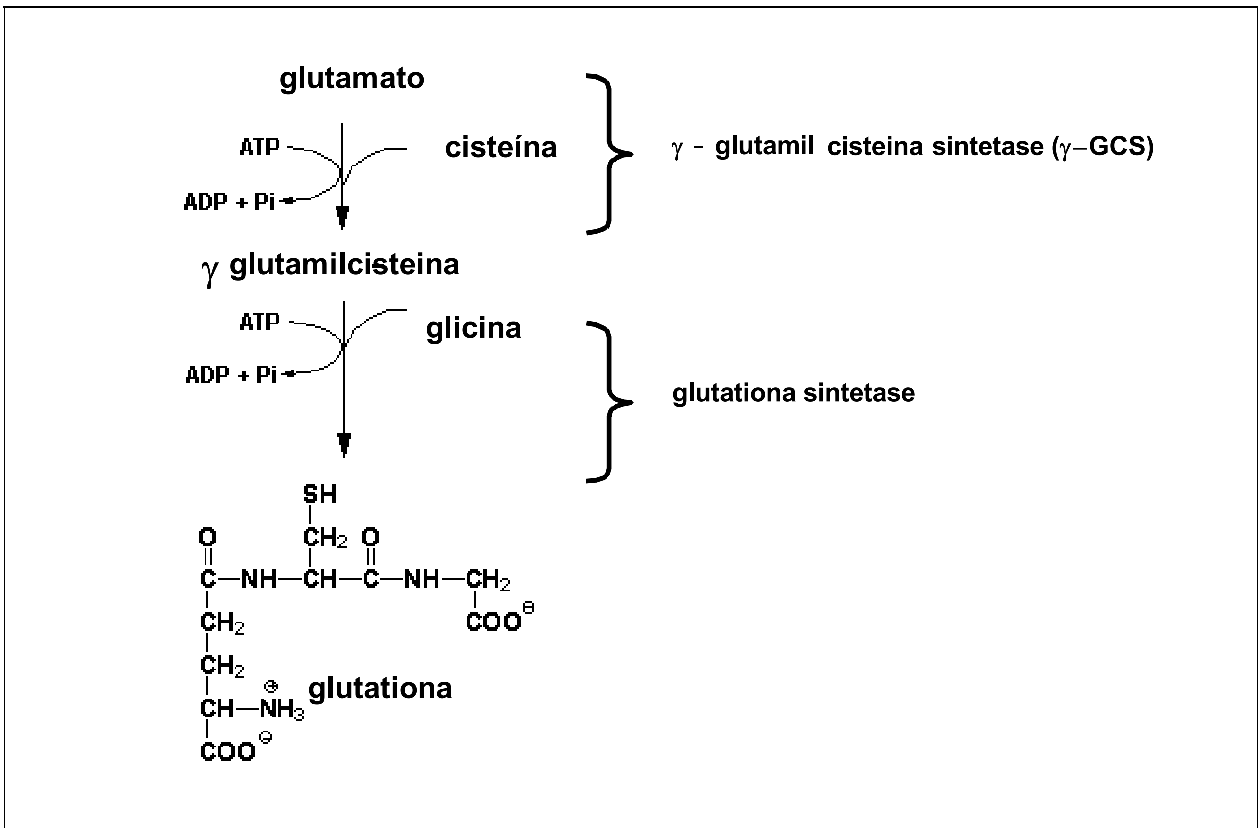

Figura 1: Esquema representativo da síntese de glutationa.

A glutationa pode agir diretamente na detoxificação de espécies reativas de oxigênio de uma forma não enzimática ou ainda ser oxidada a glutationa dissulfeto pela glutationa peroxidase (Dringen et al., 2000).

Os astrócitos apresentam níveis de glutationa mais elevados do que os neurônios (Raps et al., 1989). Além disso, são responsáveis pela manutenção do conteúdo de glutationa neuronal, já que exportam glutationa e precursores como a glutamina (Dringen et al., 2000; Wang e Cynander, 2000). A glutationa extracelular, através de uma reação catalisada pela ectoenzima γ-glutamil transpeptidase (γ-GT), dará origem ao dipeptídeo cisteinilglicina que pode ser

hidrolisado por peptidases, resultando em cisteína e glicina, utilizados como precursores para a síntese de glutationa neuronal (Figura 2) (Dringen et al., 1999).

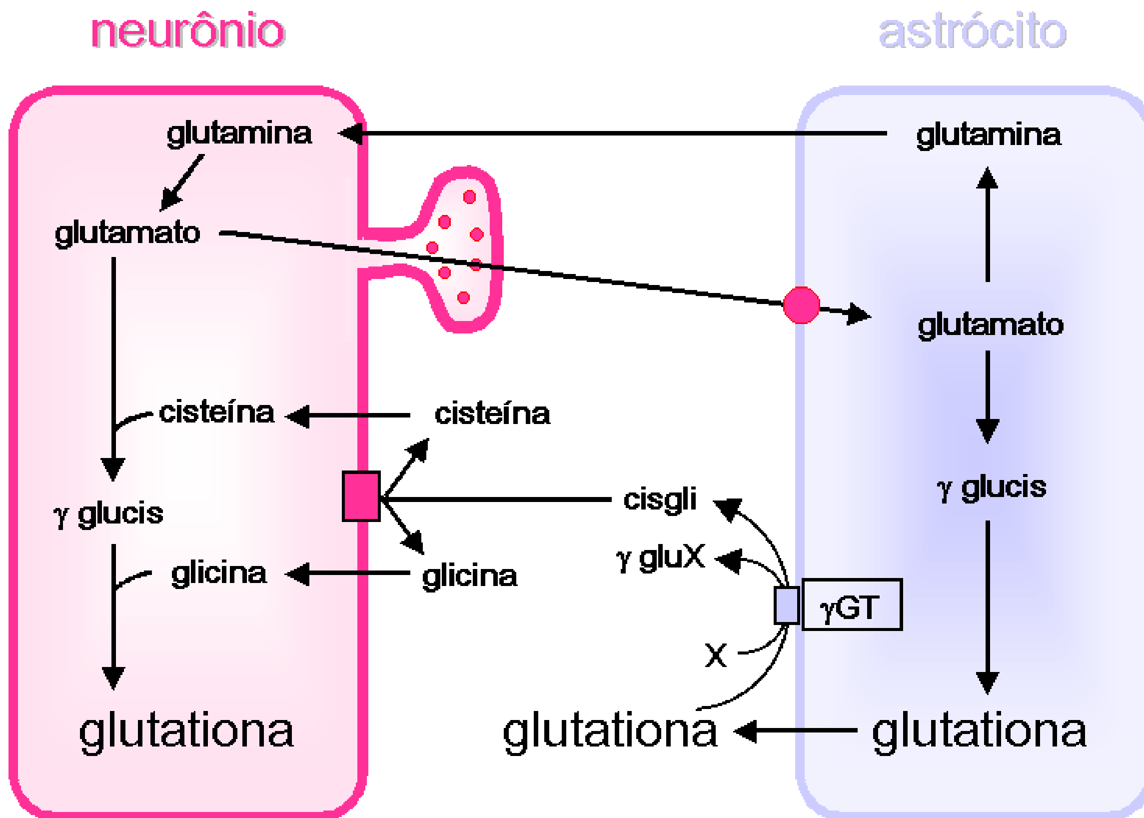

Figura 2: Esquema ilustrativo da proposta de interação metabólica entre neurônio e astrócitos no metabolismo da glutationa. Adaptado de Dringen et al., 2000.

Diversos estudos confirmam a importância dessa troca metabólica entre astrócitos e neurônios na proteção contra danos oxidativos, através da manutenção da glutationa neuronal. Neurônios cultivados na presença de astrócitos apresentam um conteúdo de glutationa mais elevado, sendo desta forma mais resistentes a toxicidade induzida por H_2O_2 (Desagher et al., 1996;

Dringen et al., 1999). Além da glutationa, outros antioxidantes não-enzimáticos como o ácido ascórbico, contribuem para prevenir a formação de espécies reativas de oxigênio (Winkler et al., 1994).

O sistema de defesa antioxidante enzimático inclui as enzimas superóxido dismutase (SOD), catalase, glutationa peroxidase e glutationa redutase. A enzima SOD catalisa a dismutação do íon superóxido para formar peróxido de hidrogênio (H_2O_2). O H_2O_2 é convertido em água e oxigênio molecular por ação da catalase. Durante a ação da glutationa peroxidase na detoxificação do H_2O_2, a glutationa é oxidada e a enzima glutationa redutase é responsável pela regeneração da glutationa reduzida (Dringen et al., 2000). Stewart e colaboradores (2002), demonstraram que além de fornecerem glutationa para o meio extracelular, importante para a proteção e síntese da glutationa neuronal, os astrócitos ainda secretam SOD extracellular, que atua preservando a glutationa contra a oxidação. Sabe-se que a SOD localiza-se predominantemente nos astrócitos, justificando a sua menor suscetibilidade ao estresse oxidativo (Lindenau et al., 2000).

Metabolismo do glutamato em astrócitos

O glutamato é o principal neurotransmissor excitatório do SNC. A ativação de seus receptores é capaz de modular diversas rotas de sinalização envolvidas na plasticidade sináptica e na memória (Winters e Bussey, 2005; Segovia et al., 2001). Entretanto, uma ativação prolongada dos receptores glutamatérgicos pode levar a um processo conhecido como excitotoxicidade, que está envolvida diretamente em uma variedade de processos patológicos como isquemia, estresse oxidativo e doenças neurodegenerativas (Maragakis e Rothstein, 2004; Hynd et

al., 2004; Waxman e Lynch, 2005; Arundine e Tymianski, 2004). Não é surpreendente o fato do sistema glutamatérgico ser considerado alvo para o desenvolvimento de drogas neuroprotetoras (Gegelashvili et al., 2001).

Os astrócitos são considerados as células mais importantes na remoção do glutamato do meio extracelular, protegendo os neurônios contra a excitotoxicidade glutamatérgica. Após a liberação do glutamato em uma sinapse o neurotransmissor é captado através de transportadores de alta afinidade dependentes de Na^+. Já foram clonados e identificados cinco subtipos de transportadores que são expressos de maneira distinta em neurônios e astrócitos (Danbolt, 2001). Os transportadores de glutamato presentes, predominantemente nos astrócitos, são: EAAT1/GLAST e EAAT2/GLT-1 (Storck et al., 1992; Pines et al., 1992). O EAAT3/EAAC1 é expresso em neurônios (Kanai e Hediger, 1992) enquanto que o EAAT4 é encontrado nas células de Purkinje no cerebelo (Fairman et al., 1995) e o EATT5 é descrito somente na retina (Arriza et al., 1997).

A localização dos transportadores de glutamato e da glutamina sintetase nas sinapses glutamatérgicas reforçam a idéia de que os astrócitos protegem os neurônios contra a excitotoxicidade induzida pelo glutamato (Rauen e Wiessner, 2000; Suarez et al., 2002). São principalmente os transportadores presentes nos astrócitos, GLAST e GLT-1, os responsáveis pela remoção do glutamato extracelular, como demonstrado em estudo com ratos “knockout” destes transportadores (Rothstein et al., 1996; Oliet et al., 2001). É interessante observar que em culturas de astrócitos a expressão de GLAST é predominante, enquanto que a expressão de GLT-1 é bastante reduzida. Porém, quando estas células são co-cultivadas com neurônios ambos os transportadores, GLT-1 e GLAST são

expressos (Gegelashvili et al., 1997). Além disso, a comunicação celular parece ser determinante no metabolismo glutamatérgico, pois o bloqueio das junções GAP em co-culturas de astrócitos e neurônios submetidos a um insulto com glutamato provocou um aumento de morte neuronal (Naus et al., 2001; Ozog et al., 2002).

Uma vez captado pelos astrócitos, o glutamato pode ser convertido a glutamina, através da ação de uma enzima astrocítica dependente de ATP chamada glutamina sintetase (Norenberg e Martinez-Hernandez, 1979). Dessa forma, a glutamina formada pode ser liberada e utilizada pelos neurônios para a síntese de glutamato ou ainda GABA. Este ciclo recebeu o nome de ciclo glutamato-glutamina e destaca a importância da interação neurônio-glia (Bak et al., 2006). Entretanto, o glutamato nos astrócitos também apresenta outros destinos metabólicos, como síntese de peptídeos e proteínas, em especial a síntese de glutationa (Dringen e Hirrlinger, 2003). Além disso, as concentrações de glutamato intra e extracelular regulam um trocador cistina/glutamato independente de sódio (Xc^-) promovendo a entrada de cistina, que então será reduzida a cisteína, precursora limitante para a síntese de glutationa (Dringen, 2000; Had-Aissouni et al., 2002). Ainda, o glutamato pode ser convertido a α-cetoglutarato e entrar no ciclo de Krebs, contribuindo para o aumento na produção de energia (Hassel e Sonnewald, 1995). O transporte de glutamato pelos astrócitos, além de evitar a excitotoxicidade, pode estimular a glicólise e a produção de lactato, fornecendo substrato energético para os neurônios (Pellerin e

Magistretti, 1994; Voutsinos-Porche et al., 2003). Um esquema representativo ilustra o metabolismo glutamatérgico na figura 3.

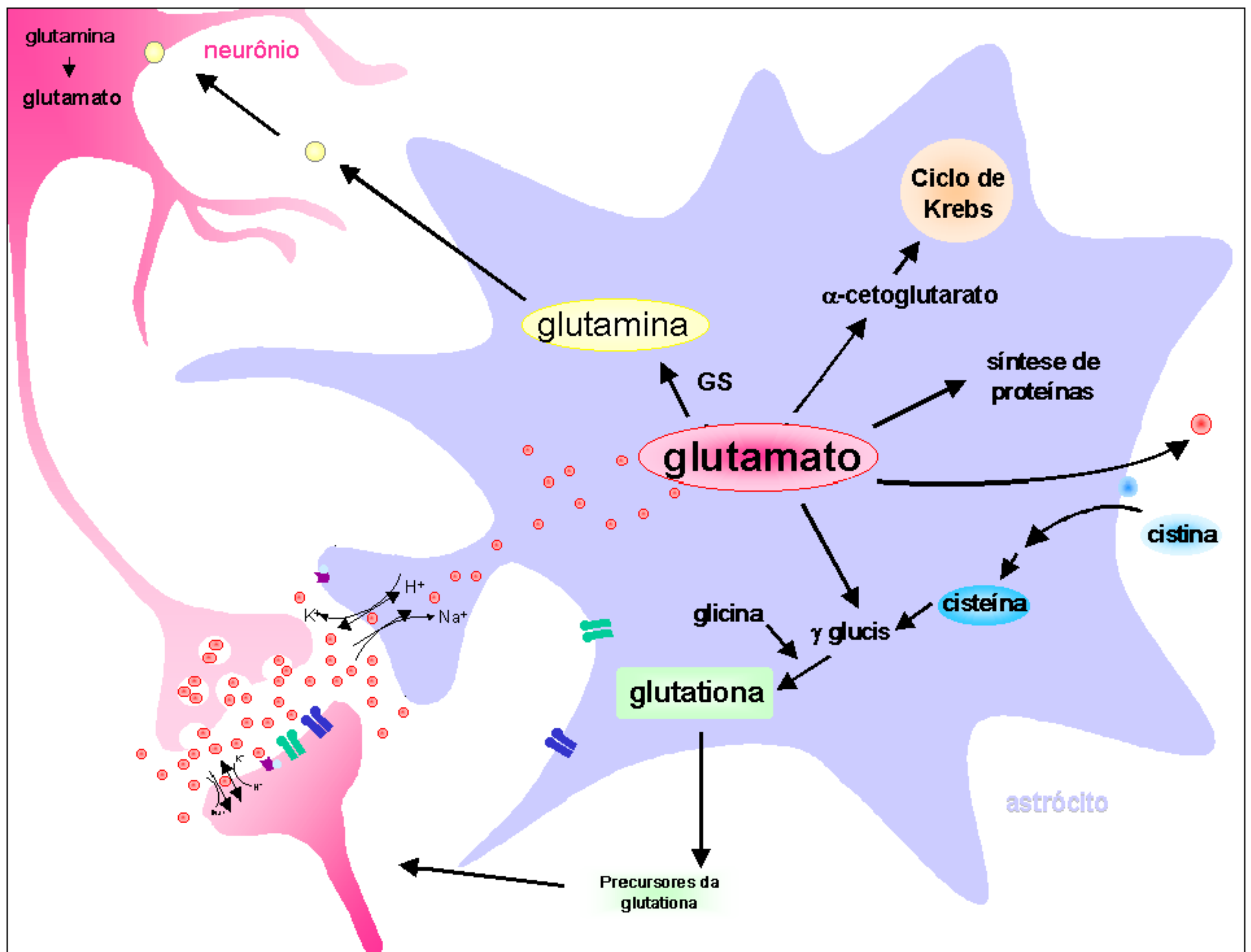

Figura 3: Esquema representativo do metabolismo glutamatérgico nos astrócitos. Adaptado de Had-Aissuoni et al., 2002.

Já está bem estabelecido na literatura que altas concentrações de glutamato extracelular podem induzir morte neuronal pela ativação exacerbada dos receptores glutamatérgicos, principalmente NMDA. Entretanto, o dano celular

induzido pelo glutamato no meio extracelular ocorre não só em neurônios, mas também em astrócitos, levando ao aumento do volume celular (Schneider et al., 1992; Chen et al., 2000). A toxicidade mediada pelo glutamato ocorre devido à inversão do trocador cistina/glutamato independente de sódio (Xc^-) inibindo a entrada de cistina o que leva a depleção da glutationa, gerando maior suscetibilidade neuronal a excitotoxicidade e morte astrocítica por estresse oxidativo (Mawatari et al., 1996; Chen et al., 2000; Had-Aissouni et al., 2002).

Entretanto, dados recentes sugerem que além da alteração no trocador cistina/glutamato independente de sódio (Xc^-), a reversão dos transportadores de glutamato, que também leva a depleção do conteúdo de glutationa devido à diminuição do "pool" de glutamato intracelular, causa uma diminuição da viabilidade dos astrócitos (Re et al., 2006).

É evidente que os astrócitos exercem um papel neuroprotetor no controle da transmissão glutamatérgica através dos transportadores gliais. Sabe-se que as espécies reativas de oxigênio são capazes de inibir a captação de glutamato em culturas de astrócitos (Volterra et al., 1994; Sorg et al., 1997; Trotti et al.,1998). Já foi observado que camundongos knockout para GLT-1 apresentam epilepsia e lesões neuronais devido a altas concentrações extracelulares de glutamato (Tanaka et al., 1997). Portanto, a disfunção glial pode ser a peça chave em diversos quadros de doenças neurodegenerativas, como mostram dados da literatura em que a captação de glutamato é inibida na presença do peptídeo beta-amilóide pela geração de espécies reativas de oxigênio (Harris et al., 1996).

Os astrócitos e a proteína S100B

A S100B é uma proteína de 21 kDa, ligante de cálcio do tipo “EF-hand”. É uma das constituintes de uma família de proteínas chamada S100, assim determinada por serem solúveis em solução 100% de sulfato de amônio (Moore, 1965). No SNC, a S100B é expressa e secretada por astrócitos (Donato, 2001). O mecanismo através do qual a S100B é secretada ainda não está elucidado (Davey et al., 2001), porém estudos demonstraram que sua concentração no meio extracelular pode ser modulada por diversos fatores, tais como: ativação dos receptores adenosina A1, níveis elevados de glutamato, estimulação de receptores serotoninérgicos e aumento de cálcio intracelular (Ciccarelli et al., 1999; Gonçalves et al., 2002; Whitaker-Azmitia et al., 1990; Davey et al., 2001). Estudos *in vitro* revelaram que a concentração de S100B presente no meio extracelular é determinante para seu efeito neurotrófico (concentrações na ordem de nanomolar) ou neurotóxico (concentrações na ordem de micromolar). Entre as funções extracelulares tróficas propostas para a S100B estão a estimulação do crescimento de neuritos, proteção contra estresse oxidativo e o aumento da sobrevivência dos neurônios durante o desenvolvimento. Ao contrário, em níveis micromolares, a S100B é capaz de estimular a expressão de citocinas pró-inflamatórias e induzir a apoptose (Donato 2001; Van Eldik e Wainwright, 2003).

Alguns dos efeitos extracelulares da proteína S100B têm sido associados a um receptor multiligante, pertencente à família das imunoglobulinas, denominado RAGE (Schmidt et al., 2000). Já foi demonstrado que a S100B através da interação com o RAGE é capaz de exercer tanto efeitos neurotróficos quanto neurotóxicos (Huttunen et al., 2000).

A S100B em doses nanomolares ainda apresenta um efeito autócrino sobre os astrócitos promovendo um aumento na proliferação, protegendo contra insultos tóxicos e modulando a ativação da ERK1/2 e do NF-κB (Selinfreund et al., 1991; Reali et al., 2005; Gonçalves et al., 2000; Lam et al., 2001). Além disso, dados do nosso grupo revelam que a S100B estimula a captação de glutamato nos astrócitos, sugerindo sua ação neuroprotetora contra o dano excitotóxico (Tramontina et al., 2006). De fato, já foi observado que a S100B inibe o dano neuronal causado pelo glutamato em culturas mistas de neurônios e astrócitos (Ahlemeyer et al., 2000). E por sua vez, níveis elevados de S100B causam um ativação da iNOS em astrócitos (Hu et al., 1996). Recentemente, Ponath e colaboradores (2007) demonstraram que a secreção de citocinas, como IL-6 e TNF-α, induzidas por S100B em astrócitos é mediada via RAGE.

É interessante destacar que os níveis elevados de S100B são encontrados no soro de pacientes portadores de Síndrome de Down e no líquor de indivíduos com Doença de Alzheimer (Netto et al., 2005; Peskind et al., 2001).

A S100B ainda apresenta um papel intracelular, atuando na modulação da plasticidade do citoesqueleto e da proliferação celular (Donato, 2001; Van Eldik e Wainwright, 2003). Dados no nosso laboratório indicam que a S100B inibe a fosforilação da GFAP, participando desta forma, da reorganização de filamentos intermediários nos astrócitos (Ziegler et al., 1998; Frizzo et al., 2004).

Resveratrol

O resveratrol (3,4',5-trihidroxiestilbeno) é um polifenol presente em pelo menos 72 espécies de plantas com diversas atividades biológicas que incluem propriedades antioxidantes, antiinflamatórias e antitumorais. As raízes de *Polygnum cuspidatum*, erva usada na medicina popular chinesa e japonesa, apresentam os níveis mais elevados de resveratrol (Soleas et al., 1997). Porém, o resveratrol foi identificado pela primeira vez em 1940 nas raízes de *Verartum grandiflorum O. Loes* (Takaoka, 1940).

Em 1976, foi demonstrada a presença de resveratrol na casca das uvas (Langcake e Pryce, 1976), sendo o vinho tinto uma das principais fontes do composto na dieta humana (Siemann e Creasy, 1992). O chamado "Paradoxo Francês" relaciona o consumo moderado de vinho tinto com um menor risco de desenvolver doenças cardiovasculares, apesar de uma dieta rica em gorduras saturadas (Renaud e Lorgeril, 1992). Estudos *in vitro* e *in vivo* sugerem vários benefícios do consumo moderado de vinho tinto, principalmente cardioprotetor, e atribuem em parte esses efeitos a um dos seus principais componentes, o resveratrol (PaceAsckiak et al., 1995; Hung et al., 2000).

A síntese de resveratrol ocorre quase que exclusivamente na casca das uvas, onde é formado pela condensação entre três moléculas de malonil-CoA e uma de 4-coumaroil-CoA através da enzima resveratrol sintase (Figura 4) (King et al., 2006). O resveratrol é uma fitoalexina, que atua no controle da infecção por fungos. Logo, os níveis de resveratrol nas uvas variam de acordo com a composição do solo, exposição ao sol, infecção por fungos, além do processo de fabricação e conservação dos vinhos (Soleas et al., 1997). Por essas razões, os

níveis de resveratrol encontrados nos vinhos tintos variam muito, situando-se em média entre 0,1 e 14,3 mg/l (Baur e Sinclar, 2006). Nos vinhos brancos a quantidade de compostos fenólicos encontrados é cinco vezes menor do que nos vinhos tintos, devido ao tempo menor de contato com a casca durante o processo de fabricação (Waterhouse, 2002).

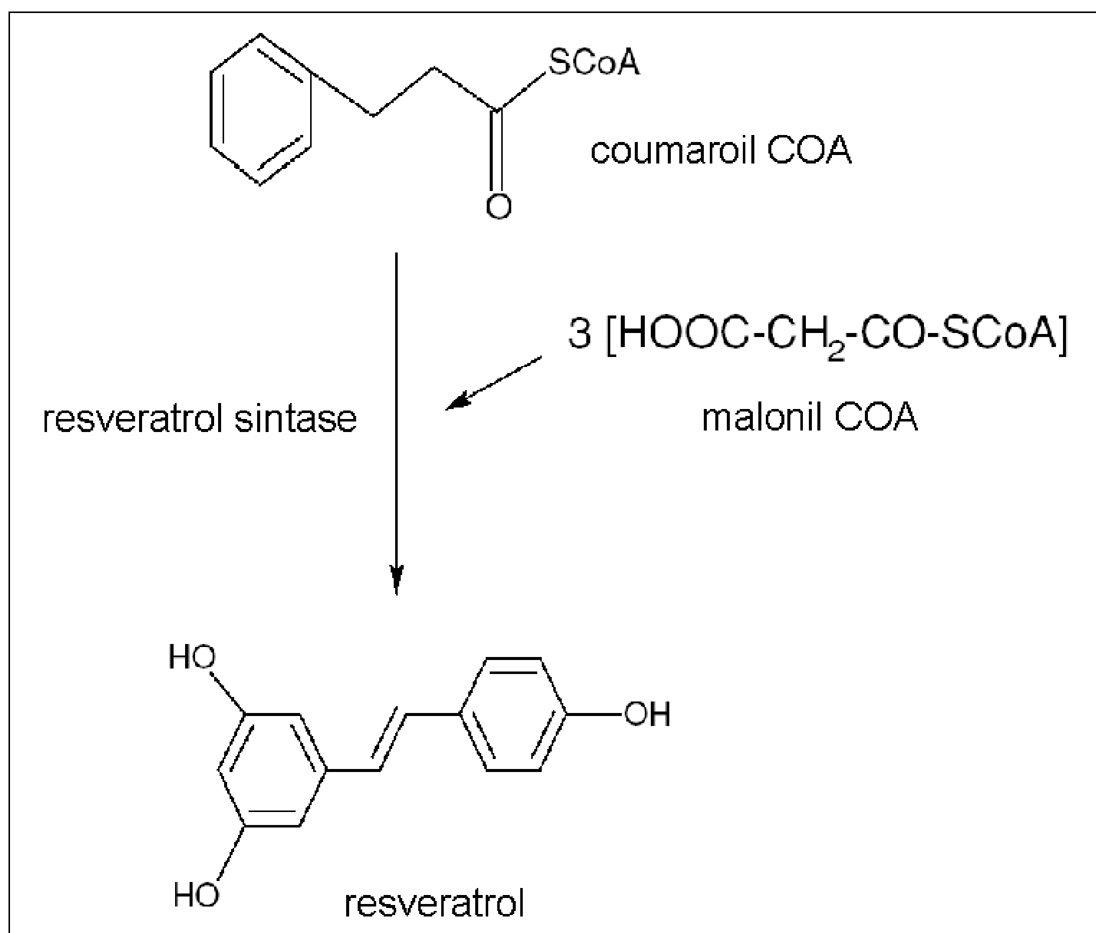

Figura 4: Síntese do resveratrol. Adaptado de King et al., 2006.

Como representado na figura 5, o resveratrol é um estilbeno e a grande maioria dos seus efeitos têm sido atribuída a isoforma *trans*, considerada mais

estável e encontrada em maiores quantidades. A isoforma *cis* é considerada biologicamente menos ativa, embora estudos comparativos recentes demonstrem efeitos semelhantes aos da isoforma *trans* (Yanez et al., 2006; Orallo, 2006). O resveratrol ainda pode ser encontrado na forma de reveratrol-glicosídeo, chamado "piceid".

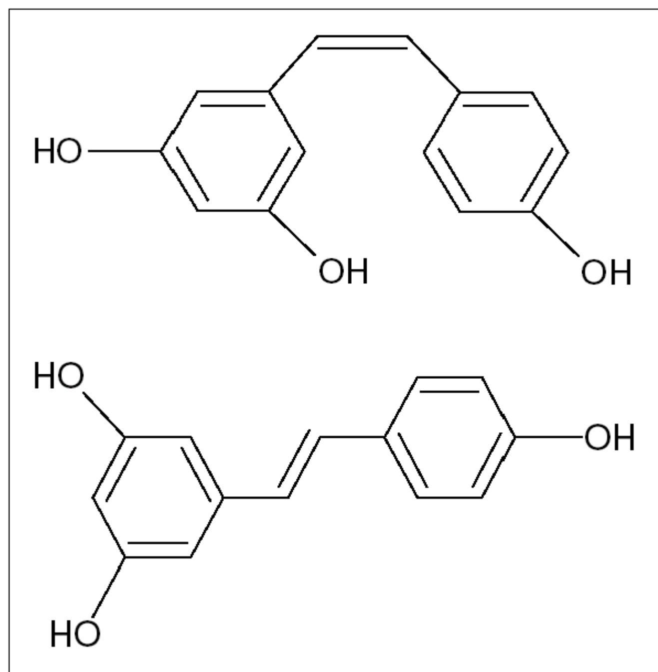

Figura 5: *Cis* e *trans*-resveratrol.

O resveratrol apresenta uma baixa biodisponibilidade e seus principais metabólitos são resveratrol sulfato e resveratrol glucoronídeo (Yu et al., 2002). Vitaglione e colaboradores (2005) demonstraram que o metabólito mais abundante no soro humano é o resveratrol glucoronídeo. As concentrações de resveratrol não conjugado na corrente circulatória e tecidos após a sua ingestão são relativamente baixas, menos que 2 μM no plasma (Gescher e Steward, 2003; Pignatelli et al.,

2006). Nos tecidos o resveratrol foi encontrado principalmente no fígado e nos rins, mas também no cérebro, coração e pulmões (Vitrac et al., 2003).

Lançon e colaboradores (2004) mostraram na linhagem humana hepática HepG2, que o transporte do resveratrol ocorre tanto por difusão passiva como por transporte ativo. E ainda, o resveratrol é capaz de ligar-se a albumina, contribuindo desta forma para sua distribuição no organismo (Jannin et al., 2004).

Diversos estudos *in vivo* demonstram que muitos dos efeitos benéficos do resveratrol podem ser atribuídos a uma associação com outros componentes do vinho, como a quercitina, que impediria a sua modificação metabólica (De Santi et al., 2000).

Propriedades do resveratrol

Uma das mais conhecidas propriedades do resveratrol é a capacidade antioxidante, atenuando a acumulação de espécies reativas de oxigênio. Dados da literatura mostram que resveratrol é capaz de impedir aumento das espécies reativas de oxigênio e a morte por apoptose causada por um insulto oxidativo (Jang e Surh, 2001; Losa, 2003; Osvena et al., 2006; De Salvia et al., 2002). Sabe-se que o resveratrol pode agir como um “scavanger” contra o radical hidroxil e o ânion superóxido (Leonard et al., 2003; Soares et al., 2003). Além disso, o resveratrol é capaz de aumentar a atividade das enzimas envolvidas no estresse oxidativo como SOD, catalase e glutationa peroxidade de forma dose-dependente (Mokni et al., 2007) e o conteúdo de glutationa (Sharma e Gupta 2002; Ates et al., 2007). Já foi demonstrada em linfócitos, após um insulto com H_2O_2, uma maior atividade das enzimas glutationa peroxidase, glutationa-S-tranferase e glutationa

redutase (Yen et al., 2003). Em células endoteliais, o resveratrol através da indução da γ-GCS, enzima limitante da síntese da glutationa, provocou um aumento nos níveis intracelulares do tripeptídeo (Brito et al., 2006).

Outro efeito bastante estudado do resveratrol é a proteção do sistema cardiovascular. Nesse caso, foi proposto que o resveratrol é capaz de inibir a agregação plaquetária tanto *in vivo* quanto e *in vitro* (Bertelli et al., 1995; Wang et al., 2002a), provocar vasodilatação através de um mecanismo envolvendo o óxido nítrico (Li et al., 2006) além de impedir a oxidação de LDL (Fremont et al., 1999).

A partir de 1997 quando Jang e colaboradores demonstraram pela primeira vez um papel antitumoral do resveratrol, diversos estudos buscaram os mesmo efeitos. Estudos *in vivo* mostram que o resveratrol induz apoptose e redução do crescimento tumoral (Carbo et al., 1999). Um dos mecanismos através dos quais o resveratrol afeta a iniciação, promoção e progressão tumoral pode estar associado a sua capacidade antiinflamatória (Jang et al., 1997). O resveratrol inibe a ciclooxigenase (COX), em especial a COX-2, envolvida diretamente nos processos inflamatórios (Murias et al., 2004; Kim et al., 2006a).

Estudos recentes demonstram que o resveratrol modula SIRT1, proteína relacionada à longevidade. Camundongos submetidos a uma dieta hipercalórica, mas com concomitante administração de resveratrol apresentaram um aumento significativo na sobrevida (Baur et al., 2006). Anteriormente, já havia sido relatado o efeito do resveratrol sobre o aumento da longevidade em diversas espécies como *Saccharomyces cerevisiae*, *Caenorhabditis elegans* e *Drosophila melanogaster* (Wood et al., 2004).

Cada vez mais estudos sugerem que o resveratrol possa exercer um papel significativo regulando funções importantes no SNC, especialmente sob condições patológicas (Anekonda, 2006; Dore, 2005). Por exemplo, o tratamento *in vivo* mostra que o resveratrol atravessa a barreira hemato-encefálica e é capaz de proteger contra isquemia cerebral (Wang et al., 2002b; Sinhá et al., 2002). Além disso, também foi demonstrado que o dano neuronal e ativação glial induzidos pelo cainato, um agonista de receptor glutamatérgico, foram minimizados na presença do polifenol (Virgili e Cotestabile, 2000; Wang et al., 2004). Mais recentemente, Gao e colaboradores (2006) relataram que o resveratrol inibe receptores glutamatérgicos pós-sinápticos o que contribuiria para explicar seu efeito protetor contra a excitotoxicidade glutamatérgica. Dados do nosso grupo reforçam a idéia da participação do resveratrol na transmissão glutamatérgica, uma vez que ele promoveu um aumento na captação de glutamato em células de glioma C6 contribuindo para a função protetora dos astrócitos (Dos Santos et al., 2006).

Considerando que a excitoxicidade glutamatérgica é um fator comum em diversas doenças neurodegenerativas, o resveratrol surge como uma molécula promissora em potencial. Estudos demonstram que o resveratrol atenua a neurodegeneração observada na Doença de Huntington como demonstrado em diferentes modelos *in vitro* e *in vivo* revertendo o dano motor e cognitivo (Parker et al., 2005; Kumar et al., 2006). De fato, o papel neuroprotetor do resveratrol sobre neurônios dopaminérgicos frente a insultos relacionados à Doença de Parkinson demonstra sua importância em processos neurodegenerativos (Okawara et al., 2007, Gelinas e Martinoli, 2002).

Evidências epidemiológicas sugerem que o consumo moderado de vinho tinto está relacionado com uma menor incidência da Doença de Alzheimer (Lindsay et al., 2002; Luchsinger et al., 2004). *In vitro*, o resveratrol diminui a toxicidade induzida pelo peptídeo beta-amilóide em células PC12 (Jang e Surh, 2003) e neurônios hipocampais (Han et al., 2004). Além disso, o resveratrol causa um aumento na degradação de peptídeos beta-amilóide possivelmente através do proteassoma, já que inibidores seletivos bloquearam o efeito do polifenol (Marambaud et al., 2005). Han e colaboradores (2004) verificaram que a ação neuroprotetora do resveratrol contra o peptídeo beta-amilóide em neurônios hipocampais depende da ativação da PKC, desencadeando uma rota diretamente associada à sobrevivência celular.

Diversas vias de sinalização, como da proteína cinase ativada por mitógenos (MAPK) e fosfatidil-inositol-3-cinase (PI3-K), têm sido demonstradas como alvos para o resveratrol exercer seus efeitos (Aggarwal et al., 2004). Entretanto, dependendo da concentração e do tipo celular, o resveratrol pode levar a um efeito protetor ou a morte celular (Holme e Pervaiz, 2007). Por exemplo, em células de neuroblastoma SH-SY5Y, o resveratrol em concentrações baixas (até 10 μM), induziu a fosforilação de proteínas da rota das MAPK como ERK1/2, porém em concentrações mais altas (50-100 μM) inibiu a fosforilação dessas cinases (Miloso et al., 1999). Em uma linhagem de carcinoma de tireóide o resveratrol causou uma ativação da ERK1/2 (Shih et al., 2002). Alguns trabalhos mostram que a inibição da ERK1/2 está relacionada ao efeito cardioprotetor do resveratrol (Olson et al., 2005; Chao et al., 2005). Por outro lado, em células

endoteliais, o efeito do resveratrol sobre a função vascular parece ser através da ativação da cascata de sinalização da MAPK (Klinge et al., 2005). Já em um modelo *in vitro* de isquemia em culturas organotípicas de hipocampo, foi demonstrado que o efeito protetor do resveratrol envolve a via PI3-K e não a MAPK (Zamin et al., 2006).

Contudo, os mecanismos através dos quais o resveratrol exerce seu efeito neuroprotetor ainda não estão claros e não envolvem somente a sua capacidade antioxidante, mas um papel modulador de rotas de sinalização (Chen et al, 2005; Han et al., 2004).

OBJETIVOS

Objetivo geral

Investigar o efeito do resveratrol sobre parâmetros gliais.

Objetivos específicos

- ✓ Analisar em culturas primárias de astrócitos corticais o efeito do resveratrol em diferentes concentrações sobre parâmetros gliais, tais como: morfologia, captação de glutamato, atividade da enzima glutamina sintetase, conteúdo de glutationa, secreção de S100B;
- ✓ Estudar o efeito do resveratrol frente a um insulto oxidativo causado pelo H_2O_2 em cultura primária de astrócitos corticais;
- ✓ Avaliar o efeito do resveratrol contra um dano oxidativo causado pelo H_2O_2 em fatias hipocampais.

Artigo 1

Artigo aceito para publicação no periódico Cellular and Molecular Neurobiology.

RESVERATROL INCREASES GLUTAMATE UPTAKE, GLUTATHIONE CONTENT AND S100B SECRETION IN CORTICAL ASTROCYTE CULTURES.

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ORIGINAL PAPER

Resveratrol Increases Glutamate Uptake, Glutathione Content, and S100B Secretion in Cortical Astrocyte Cultures

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Abstract Resveratrol (3,5,4′-trihydroxy-*trans*-stilbene) is a polyphenol present in grapes and red wine, which has antioxidant properties and a wide range of other biological effects. In this study, we investigated the effect of resveratrol, in a concentration range of 10–250 μM, on primary cortical astrocytes; evaluating cell morphology, parameters of glutamate metabolism such as glutamate uptake, glutamine synthetase activity and glutathione total content, and S100B secretion. Astrocyte cultures were prepared of cerebral cortex from neonate Wistar rats. Morphology was evaluated by phase-contrast microscopy and immunocytochemistry for glial fibrillary acidic protein (GFAP). Glutamate uptake was measured using L-[2,3-^{3}H]glutamate. Glutamine synthetase and content of glutathione were measured by enzymatic colorimetric assays. S100B content was determined by ELISA. Typical polygonal morphology becomes stellated when astrocyte cultures were exposed to 250 μM resveratrol for 24 h. At concentration of 25 μM, resveratrol was able to increase glutamate uptake and glutathione content. Conversely, at 250 μM, resveratrol decreased glutamate uptake. Unexpectedly, resveratrol at this high concentration increased glutamine synthetase activity. Extracellular S100B increased from 50 μM upwards. Our findings reinforce the protective role of this compound in some brain disorders, particularly those involving glutamate toxicity. However, the underlying mechanisms of these changes are not clear at the moment and it is necessary caution with its administration because elevated levels of this compound could contribute to aggravate these conditions.

Keywords Astrocyte · GFAP · Glutamate uptake · Glutathione · Resveratrol · S100B

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Introduction

Resveratrol (3,5,4′-trihydroxy-*trans*-stilbene), a polyphenol present in grapes and red wine, has antioxidant and anti-inflammatory proprieties and a wide range of other biological effects. Many studies now attest to the cardioprotective and chemopreventive effects of this compound (Frémont 2000; Baur and Sinclair 2006; Pervaiz 2004). A neuroprotective action of resveratrol has been proposed in many conditions of brain injury (Dore 2005), including

damage mediated by glutamate receptors (Wang et al. 2004; Gao et al. 2006), but the underlying mechanism is not fully understood.

Glutamate is the major excitatory neurotransmitter in the central nervous system and its accumulation is implicated in neurodegenerative disorders. Astrocytes, the main glial cells of the brain, are responsible for major glutamate transport, regulating its extracellular levels through glutamate sodium-dependent transporters (GLAST and GLT-1) (Hertz 2006). Glutamate accumulation in the extracellular space is known to be toxic not only to neurons but also to astrocytes (Matute et al. 2002; Chen et al. 2000). Moreover, astrocytes have a specific enzyme glutamine synthetase (GS) that catalyses the amidation reaction of glutamate to form glutamine, which is exported to neurons, allowing the synthesis of not only glutamate, but also GABA by the glutamate–glutamine cycle (Bak et al. 2006). We recently showed that resveratrol increases glutamate uptake and GS activity in C6 glioma cells (Dos Santos et al. 2006). Moreover, astrocytic glutamate uptake is also essential for maintaining glutathione levels, the main antioxidant of the brain (Dringen 2000). Indeed, neurons depend on astrocytes for precursors to synthesize their own glutathione (Dringen et al. 1999). Brain tissue is particularly vulnerable to oxidative damage, due to its high consumption of oxygen and the consequent generation of high quantities of reactive oxygen species during oxidative phosphorylation (Castagne et al. 1999). Thus, astrocyte antioxidant defense is a key element to physiological brain activity.

Astrocytes are also involved in many other neural functions, such as maintenance of ion homeostasis, energetic metabolism, and secretion of neurotrophic factors. Among these trophic factors is the S100B protein, a calcium-binding protein that is secreted from astrocytes in a glutamate transporter-dependent manner (Tramontina et al. 2006). This protein has neurotrophic activity at nanomolar levels in the extracellular medium (Donato 2001; Van Eldik and Wainwright 2003).

Here, we investigate the potential neuroprotective effects of resveratrol on primary cortical astrocytes, evaluating cell morphology, parameters of glutamate metabolism such as glutamate uptake, glutamine synthetase activity and glutathione total content, and S100B secretion.

Methods

Materials

Resveratrol (approximately of 99% purity), poly-D-lysine, γ-glutamylhydroxamate, and antibody anti-S100B (SH-B1) were purchased from Sigma and polyclonal anti-GFAP from DAKO. L-[2,3-^{3}H]Glutamate was purchased from Amersham (specific activity 33 Ci/mmol). Fetal calf serum (FCS) was purchased from Cultilab (São Paulo, Brazil). Dulbecco's modified Eagle's medium (DMEM) and other materials for cell culture were purchased from Gibco.

Astrocyte cultures

Primary cortical astrocyte cultures were prepared as previously described (Gottfried et al. 1999). Briefly, cerebral cortex of newborn Wistar rats (1- to 2-day-old) were removed, placed in Ca^{2+} and Mg^{2+}-free buffer saline solution pH 7.4, containing (in mM): 137 NaCl; 5.36 KCl; 0.27 Na_2HPO_4; 1.1 KH_2PO_4, and 6.1 glucose. The cortices were cleaned of meninges and mechanically dissociated. After centrifugation at 1,000 rpm for 5 min the pellet was resuspended in DMEM (pH 7.6) supplemented with 8.39 mM HEPES; 23.8 mM $NaHCO_3$; 0.1% fungizone; 0.032% garamycin, and 10% FCS. The cells were plated at a density of 2×10^5 cells per cm^2 onto 24-well plates pre-treated with poly-L-lysine. Cultures were maintained in 5% CO_2/95% air at 37°C and allowed to grow to confluence and used at 15–20 days in vitro.

Drug treatment

Astrocytes were incubated for 24 h in DMEM (pH 7.4) without serum in the absence or presence of resveratrol at concentrations of 10, 25, 50, 100, and 250 μM. In all analyzed parameters, the results obtained with vehicle (0.25% ethanol) were not different from those obtained in basal conditions without ethanol.

Immunocytochemistry and cell morphology

After 24 h, cells treated with resveratrol were fixed for 20 min with 4% paraformaldehyde in phosphate

buffer (PBS): 2.9 mM KH_2PO_4, 38 mM $Na_2HPO_4 \cdot 7H_2O$, 130 mM NaCl, 1.2 mM KCl, 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 0.5% bovine serum albumin and incubated overnight with polyclonal anti-GFAP (Dako, 1:200) for 2 h. Finally, the cells were treated with 0.05% diaminobenzidine (Sigma) containing 0.01% hydrogen peroxide for 10 min (Gottfried et al. 2003). Cells were viewed with a Nikon inverted microscope and images transferred to a computer with a digital camera (Sound Vision Inc., Wayland, MA, USA). All images are representative fields from at least three experiments carried out in triplicate.

Glutamate uptake assay in astrocytes

Glutamate uptake was performed as previously described (Gottfried et al. 2002). Briefly, cortical astrocytes were incubated at 37°C in a Hank's balanced salt solution (HBSS, pH 7.4) containing: 135 mM NaCl; 3.1 mM KCl; 1.2 mM $CaCl_2$; 1.2 mM $MgSO_4$; 0.5 mM KH_2PO_4; 2 mM glucose; 0.1 mM L-glutamate, and 0.33 μCi/ml L-[2,3-^{3}H]glutamate for 7 min. Na^+-free medium was prepared by replacing NaCl with choline chloride. Incubation was terminated by removal of the medium and rinsing the cells twice with ice-cold HBSS. Cells were then resuspended in a lysis solution containing 0.1 N NaOH and 0.01% SDS. Radioactivity was measured with a scintillation counter.

Glutamine synthetase activity

The enzymatic assay was performed according to Petito et al. (1992) with some modifications. Briefly, homogenate (0.1 ml) was added to 0.1 ml of reaction mixture containing (in mM): 10 $MgCl_2$; 50 L-glutamate; 100 imidazole–HCl buffer (pH 7.4); 10 2-mercaptoethanol; 50 hydroxylamine–HCl; 10 ATP and incubated for 15 min at 37°C. The reaction was stopped by the addition of 0.4 ml of a solution containing (in mM): 370 ferric chloride; 670 HCl; and 200 trichloroacetic acid. After centrifugation, the supernatant was measured at 530 nm and compared to the absorbance generated by standard quantities of γ-glutamylhydroxamate treated with ferric chloride reagent.

Total glutathione assay

Total glutathione content was determined by a slightly modified assay, as described previously (Allen et al. 2001; Tietze 1969). Briefly, cells were scraped in phosphate-buffered saline (0.01 M, pH 7.6), 6.3 mmol edetic acid (pH 7.5), and Triton-X (0.05%) and protein was precipitated with 1% sulfosalicylic acid. Supernatant was assayed with 462.6 μM 5,5′-dithiobis-(2-nitrobenzoic acid) (DTNB), 0.5 U/ml glutathione reductase, and 0.3 mM NADPH; reduced DTNB was measured at 412 nm.

Immunocontent of S100B

The S100B concentration was determined in the culture medium at 1, 6, and 24 h. Cells were washed and scraped at 24 h for measurement of intracellular S100B content. ELISA for S100B was carried out as described previously with modifications (Tramontina et al. 2000). Briefly, 50 μl of sample plus 50 μl of Tris buffer were incubated for 2 h on a microtiter plate previously coated with monoclonal anti-S100B (SH-B1, from Sigma). Polyclonal anti-S100 (from DAKO) was incubated for 30 min and peroxidase-conjugated anti-rabbit antibody was then added for a further 30 min. The color reaction with *o*-phenylenediamine was measured at 492 nm.

Cell integrity

Total intracellular lactate dehydrogenase (LDH) activity after resveratrol exposure was determined in lysed cells with 0.2% Triton X-100 by a colorimetric assay kit (from Doles, Goiânia, Brazil).

Protein content

The total protein content was determined by the method of Lowry et al. (1951), using bovine serum albumin as standard.

Statistical analysis

Data from at least three independent experiments are presented as mean ± S.E.M and were analyzed

statistically by one-way analysis of variance (ANOVA) followed by the Tukey's test. Values of $P < 0.05$ were considered to be significant. All analyses were carried out in an IBM compatible PC using the Statistical Package for Social Sciences (SPSS) software.

Results

Cell integrity and morphology in the presence of resveratrol

Phase-contrast images and immunocytochemistry for GFAP show typical polygonal astrocyte morphology (Fig. 1, panels **a** and **b**, respectively). Exposure to 25 μM resveratrol (panels **c** and **d**) had no effect 24 h afterwards, but process-bearing cells (stellation) were observed following 250 μM resveratrol exposure (panels **e** and **f**). Membrane integrity, based on LDH activity assay, was preserved after resveratrol (from 10 to 250 μM) or vehicle per se (0.25% ethanol) exposure (data not shown).

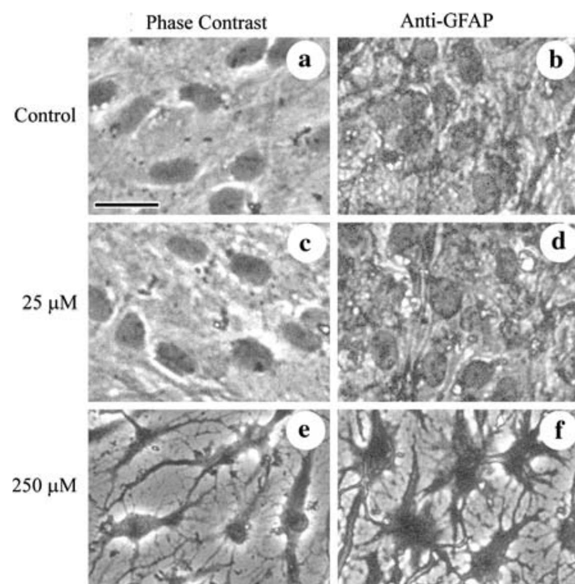

Fig. 1 Phase-contrast microscopy and immunocytochemistry showing morphological changes in the astrocyte cultures after treatment with resveratrol. Cells were cultured in DMEM/10% SFB to confluence and then transferred to serum-free DMEM, incubated for 24 h with resveratrol and fixed with paraformaldehyde and immuno-stained with anti-GFAP as described in Methods section. Representative images showed phase-contrast (panels **a**, **c**, and **e**) and immunocytochemistry for GFAP (panels **b**, **d**, and **f**) from control (0.25% ethanol), 25 and 250 μM resveratrol, respectively. All images are representative fields from at least three independent experiments carried out in triplicate. Scale bar = 50 μm

Resveratrol and glutamate uptake

Basal glutamate uptake under our conditions was about 3.15 nmol/mg protein/min and was not affected by 0.25% ethanol (Fig. 2). Treatment of astrocytes with 25 μM resveratrol for 24 h was able to induce a significant increase in glutamate uptake. Conversely, incubation of astrocytes with a 10-fold higher concentration of resveratrol leads to a significant decrease in glutamate uptake.

Resveratrol and glutamine synthetase

In order to investigate the effect of resveratrol on glutamine synthetase, we measured its activity after 24 h of treatment with resveratrol (Fig. 3). Basal glutamine synthetase activity was about 2.37 μmol/mg protein/h. With 250 μM resveratrol, a significant increase in glutamine synthetase activity was detected, but no effect was observed at lower concentrations.

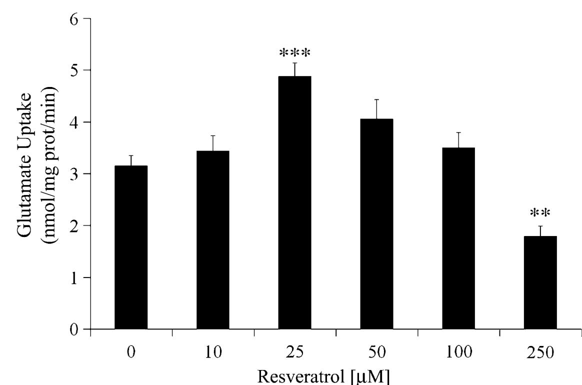

Fig. 2 Effect of resveratrol on glutamate uptake. Cultures of astrocytes were treated for 24 h in the absence or presence of resveratrol at indicated concentrations. Cell culture media were replaced with HBSS and incubated with [^{3}H]-glutamate for 7 min. The data represent the mean ± SEM values from eight independent experiments performed in triplicate. Statistically significant differences from controls (0.25% ethanol), as determined by one-way ANOVA followed by Tukey's multiple variation test, are indicated: $^{**}P < 0.01$ and $^{***}P < 0.001$

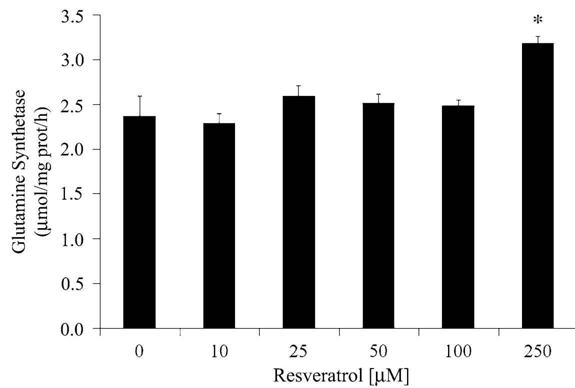

Fig. 3 Influence of resveratrol on glutamine synthetase activity. Cells were treated with increasing concentrations (10, 25, 50, 100, and 250 µM) of resveratrol for 24 h in DMEM without serum. After this time, the incubation medium was removed and GS activity assay was assessed as described in Methods section. The data represent the mean ± SEM values from four independent experiments performed in triplicate. Statistically significant differences from controls (0.25% ethanol), as determined by one-way ANOVA followed by Tukey's multiple variation test, are indicated: *$P < 0.05$

Resveratrol and glutathione content

Next, we examined the effect of resveratrol on glutathione content (Fig. 4). A marked increase in glutathione content was observed when the cells were treated with 25 and 50 µM of resveratrol. Interestingly, resveratrol at a higher concentration (250 µM) tended to decrease glutathione content, but this effect did not reach significance.

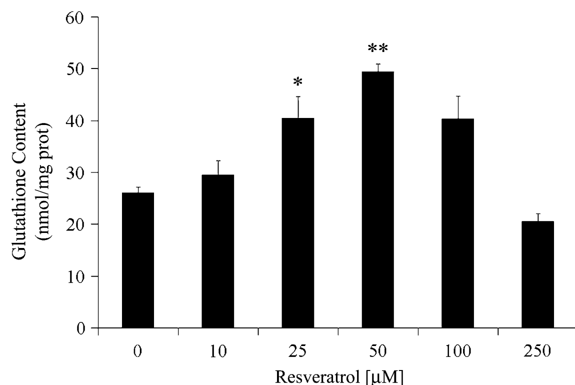

Fig. 4 Effect of resveratrol on glutathione content. After confluence, cells were treated with DMEM without serum in the absence or presence of 10, 25, 50, 100, and 250 µM of resveratrol for 24 h. The data represent the mean ± SEM values of four independent experiments performed in triplicate. Statistically significant differences from controls (0.25% ethanol), as determined by one-way ANOVA followed by Tukey's multiple variation test, are indicated: *$P < 0.05$ and **$P < 0.01$

Resveratrol and S100B secretion

At 24 h of treatment with resveratrol, 50, 100, and 250 µM increased S100B secretion (Fig. 5A). Interestingly, 250 µM resveratrol induced S100B secretion at 6 h and a tendency for increase was observed

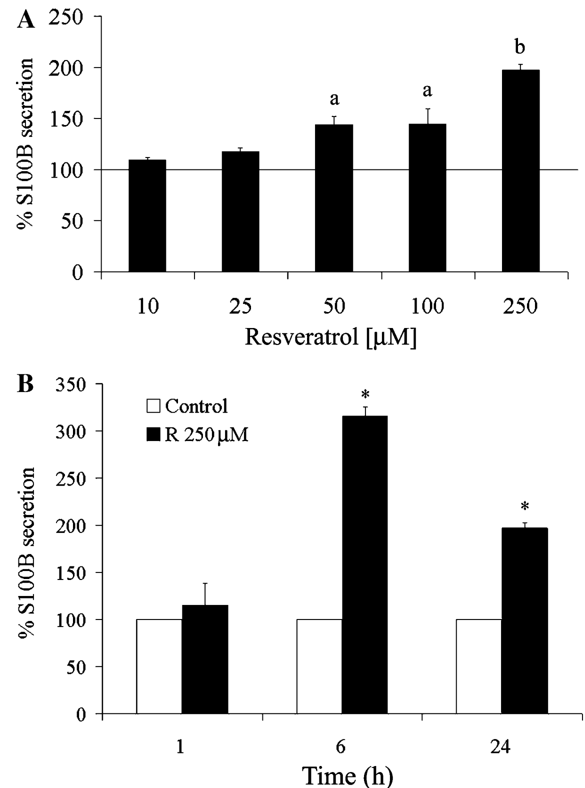

Fig. 5 Effect of resveratrol on S100B secretion in astrocyte culture. In **A**, extracellular S100B was measured by ELISA 24 h after treatment with resveratrol at indicated concentrations in DMEM without serum. Each value is the percentage mean ± SEM from four independent experiments performed in triplicate. Basal secretion (assumed as being 100%) is represented by the continuous line. Different letters indicate statistical difference of extracellular S100B levels from control and other resveratrol concentrations, determined by one-way ANOVA followed by Tukey's multiple variation test, for $P < 0.05$. In **B**, time curve of extracellular S100B in astrocytes exposed to resveratrol (R 250 µM) compared to control in each time. Each value is the percentage mean ± SEM from four independent experiments performed in triplicate. *Significantly different from respective control (Student *t*-test, $P < 0.01$)

during the first hour of exposure of the compound (Fig. 5B).

Discussion

Our data indicate that 24 h exposure to resveratrol was able to modulate basal glutamate uptake in astrocytes. Resveratrol has been shown to be effective against ischemic brain injury and kainic acid-induced seizures or neuronal cell damage in rodents (Wang et al. 2002, 2004). This effect could be related, at least in part, to this effect observed in cultured astrocytes. Glutamate uptake was also increased by resveratrol in C6 glioma cells (Dos Santos et al. 2006).

The neuroprotection imparted by resveratrol has been mainly attributed to its intrinsic antioxidant properties. In vitro studies indicate that glutamate uptake by glial cells is regulated by the surrounding redox environment and that this uptake activity decreases under oxidizing conditions (Trotti et al. 1998). Thus, resveratrol could affect the redox environment of glutamate transporters and favor their activities. Resveratrol, however, is able to induce some cell activities independently of its antioxidant properties (Ovesna and Horvathova-Kozics 2005).

Many antioxidants have anti-inflammatory activity, and resveratrol in particular, due to its structural resemblance with estrogens could provide anti-inflammatory activity by binding to estrogen receptors (Jannin et al. 2004). Moreover, resveratrol has a modulatory activity on cyclooxygenases and nitric oxide synthase (Signorelli and Ghidoni 2005; Kim et al. 2006). Other possible enzyme targets to explain long-term changes induced by resveratrol, for example on glutamate uptake, would be the protein kinases particularly phorbol ester-responsive kinase and MAP kinases (Stewart et al. 2000; Han et al. 2004; Klinge et al. 2005; Guillet et al. 2005). However, the mechanism(s) underlying the long-term changes, particularly in brain need to be clarified.

Astrocytes are the only cells in brain that have the important ability to convert glutamate into glutamine via GS. Glutamine is released to neurons and used for the synthesis of glutamate (and then GABA, in GABAergic neurons). However, glutamate has another important fate in astrocytes, particularly glutathione synthesis (Dringen 2000). Glutamate serves as a substrate per se for glutathione synthesis and as a moiety for exchange by cysteine, another substrate for glutathione synthesis. Interestingly, we did not observe any change in GS activity. Moreover, in concomitantly with the increase in glutamate uptake, an increase in the content of glutathione was seen, reinforcing the antioxidant activity of astrocytes. This effect may also contribute to explain the neuroprotective effect of resveratrol in disorders associated with excitotoxicity and/or oxidative stress. Conversely, astrocytes exposed to a high concentration of resveratrol did not exhibit any increment in glutathione content and, unexpectedly, exhibited an increase in GS activity. Data demonstrating decreased glutamate uptake reinforce the concept of resveratrol as a pro-oxidant compound at elevated concentrations. The effect of elevated resveratrol on GS was surprising, since we observed a decrease in C6 glioma cells (Dos Santos et al. 2006) and many reports describe sensitivity of this enzyme to oxidative stress.

Morphological changes of astrocytes in culture, particularly stellation have been used as a parameter of cell activation (Cechin et al. 2002). For example, adrenaline stimulation of astrocytes (via cAMP) induces cell stellation. Cell stellation in culture has also been observed in stress conditions triggered by many signals, such as beta-hydroxy-butyrate (Leite et al. 2004), ammonia (Leite et al. 2006), and changes in intracellular pH (Cechin et al. 2002). At 250 μM resveratrol, but not at lower concentrations, we observed cell stellation by phase-contrast microscopy and this was confirmed by GFAP immunocytochemistry. Decreased glutamate uptake observed together with cell stellation, at this concentration of resveratrol, indicate strong astrocyte impairment, possibly mediated by the pro-oxidant effect of resveratrol at this elevated concentration.

S100B is a calcium-binding protein expressed and secreted in the central nervous system by astroglia. Besides its intracellular role, S100B works as a cytokine for neighboring cells (astrocytes, neurons, and microglia) depending on its concentration, being neurotrophic at nanomolar levels and apoptotic at micromolar levels (Donato 2001; Van Eldik and Wainwright 2003). We found that resveratrol exposure, 24 h afterwards, increased extracellular S100B in a concentration-dependent manner. Since no parallel loss of LDH was observed at 250 μM

resveratrol, it would be reasonable to conceive that resveratrol affects the mechanism of S100B secretion in astrocytes. Similarly, we found an increase in extracellular S100B in C6 glioma cultures, 24 h afterwards at concentrations of 100 and 250 μM (Dos Santos et al. 2006). Recently, we proposed a negative correlation between glutamate uptake activity and S100B secretion (Tramontina et al. 2006) and our current data reinforce this idea. Moreover, S100B at nanomolar levels appears to protect neurons against glutamate toxicity (see Donato 2001; Van Eldik and Wainwright 2003 for a review). Conversely, persistent and elevated extracellular levels of protein have been associated with neurodegenerative disorders. Thus, it is possible that resveratrol stimulates S100B secretion, in turn stimulating neuronal survival and activity during brain injury and recovery; however, high levels of resveratrol could result in neuronal apoptosis and brain impairment.

Finally, it is important to emphasize that we have investigated resveratrol at concentration ranges from 10 to 250 μM. These values were chosen based on many other recent studies (Wang et al. 2003); however, there is an apparent discrepancy between the concentrations used for in vitro resveratrol efficacy (commonly 5–100 μM) and the extracellular concentrations (less than 2 μM in plasma) (Gescher and Steward 2003). Low levels of resveratrol are due to its fast metabolism in liver and intestinal epithelial cells, mainly due to resveratrol-glucuronide and resveratrol-sulfate (see Signorelli and Ghidoni 2005 for a review). Regardless of this limitation, this study indicated some putative molecular targets of resveratrol in the nervous system, particularly is astrocytes. Other important feature observed is the dual effect of resveratrol on glutamate uptake, depending on its concentration. This also been observed in other biological parameters that are altered by resveratrol, including anti- and pro-oxidant activity and anti- and pro-apoptotic effects (Pervaiz 2004).

In summary, we demonstrate that the neuroprotective activity of resveratrol involves astrocyte activation, as indicated by the in vitro increase of glutamate uptake, glutathione content, and S100B secretion. Our findings reinforce the protective role of this compound in some brain disorders, particularly those involving glutamate toxicity. However, the underlying mechanisms of these changes are not clear at the moment and it is necessary caution with its administration because elevated levels of this compound could contribute to aggravate these conditions.

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Artigo 2

Artigo aceito para publicação no periódico Neurochemical Research.

PROTECTIVE EFFECTS OF RESVERATROL ON HYDROGEN PEROXIDE INDUCED TOXICITY IN PRIMARY CORTICAL ASTROCYTE CULTURES.

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ORIGINAL PAPER

Protective Effects of Resveratrol on Hydrogen Peroxide Induced Toxicity in Primary Cortical Astrocyte Cultures

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Abstract It is well established that the brain is particularly susceptible to oxidative damage due to its high consumption of oxygen and that astrocytes are involved in a variety of important activities for the nervous system, including a protective role against damage induced by reactive oxygen species (ROS). The use of antioxidant compounds, such as polyphenol resveratrol found in red wine, to improve endogenous antioxidant defenses has been proposed for neural protection. The aim of this study is to evaluate the putative protective effect of resveratrol against acute H_2O_2-induced oxidative stress in astrocyte cultures, evaluating ROS production, glutamate uptake activity, glutathione content and S100B secretion. Our results confirm the ability of resveratrol to counteract oxidative damage caused by H_2O_2, not only by its antioxidant properties, but also through the modulation of important glial functions, particularly improving glutamate uptake activity, increasing glutathione content and stimulating S100B secretion, which all contribute to the functional recovery after brain injury.

Keywords Astrocyte · Glutathione · Glutamate uptake · Oxidative stress · S100B protein · Resveratrol

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Introduction

Reactive oxygen species (ROS) are products of general metabolism and play important roles in several physiological cellular functions. Imbalances in the generation of ROS and cellular antioxidant defenses lead to oxidative stress, which results in oxidative damage (see [1] for a review). ROS include hydrogen peroxide (H_2O_2) that is normally produced in the tissues through reactions catalyzed by superoxide dismutase (SOD) and oxidases. H_2O_2 is removed predominantly by the antioxidant enzymes catalase and glutathione peroxidase [2], however, H_2O_2 may also act as a regulator of signal pathways since it has the ability to cross the plasma membrane and increase cytosolic calcium [3, 4].

Oxidative stress has long been associated with the development of pathological conditions in brain tissue such as ischemia, inflammation and degenerative diseases including Alzheimer's disease, Huntington and Parkinsons's disease [5, 6]. It is well established that the brain is particularly susceptible to oxidative damage due to its high consumption of oxygen and high quantities of polyunsaturated fatty acids. In brain, SOD and monoamoniunoxidase A and B (involved in the catecholamine and serotonin catabolism) are the main sources of H_2O_2.

Astrocytes are the most abundant cell type in the brain and involved in a variety of important activities for the nervous system, including a protective role against damage induced by ROS [7]. Glutathione is a major antioxidant of the brain [8] present in higher amounts in astrocytes [9]. In fact, astrocytes are able to uptake cystine, convert cystine to cystein and incorporate cystein in glutathione [10]. Neurons depend on the astrocyte content of cystein and glutathione to synthesize their own glutathione, demonstrating the importance of the interaction between astrocyte

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67 and neurons [11]. Moreover, there is much evidence to 68 demonstrate another crucial role of astrocytes in glutamate 69 metabolism. The impairment of glutamate transporters 70 causes excitotoxicity and leads to increased ROS produc-71 tion and consequent cell damage [12]. In addition, ROS 72 directly impair glutamate transporters in astrocytes and 73 neurons [13].

74 S100B is a calcium-binding protein that is primarily 75 expressed and secreted in the central nervous system by 76 astroglia. This protein, at nanomolar concentrations, 77 stimulates neuronal survival in vitro and is able to protect 78 hippocampal neurons against glutamate toxicity [14]. 79 Although the mechanism of S100B secretion is unknown, it 80 appears to be related to glutamate uptake activity [15] and 81 is affected by oxidative stress [16].

82 Resveratrol (3,5,4′-trihydroxy-*trans*-stilbene) is a 83 polyphenol found in grapes and red wine with diverse 84 established biological activities, such as antioxidant, anti-85 inflammatory, cardioprotective and anticarcinogenic roles 86 [17]. Recently, a number of studies have focused on the 87 neuroprotective effects of resveratrol, demonstrating that 88 this compound attenuates β-amyloid toxicity [18] and 89 protects against cerebral ischemic injury [19] and kainic 90 acid-induced excitotoxicity [20]. However, little is known 91 about the effect of resveratrol on astrocytes. The aim of this 92 study is to evaluate the putative protective effect of res-93 veratrol against acute H_2O_2-induced oxidative stress in 94 astrocyte cultures, evaluating ROS production, glutamate 95 uptake activity, glutathione content and S100B secretion.

96 Experimental procedure

97 Material

98 Resveratrol (3,5,4′-trihydroxy-*trans*-stilbene, approxi-99 mately 99% purity), poly-D-lysine, γ-glutamylhydroxa-100 mate and anti-S100B antibody (SH-B1) were purchased 101 from Sigma. L-[2,3-^{3}H]glutamate was purchased from 102 Amersham (specific activity 33 Ci/mmol). Fetal calf serum 103 was purchased from Cultilab (São Paulo, Brazil). 104 Dulbecco's modified Eagle's medium (DMEM) and other 105 materials for cell culture were purchased from Gibco. DCF 106 was provided from Molecular probe. H_2O_2 was obtained 107 from Merck.

108 Astrocyte cultures

109 Primary cortical astrocyte cultures were prepared as pre-110 viously described [21]. Briefly, cerebral cortices of new-111 born Wistar rats (1–2-day-old) were removed, placed in 112 Ca^{2+} and Mg^{2+}-free buffer saline solution, pH 7.4, con-113 taining (in mM): 137 NaCl; 5.36 KCl; 0.27 Na_2HPO_4; 1.1 114 KH_2PO_4 and 6.1 glucose. The cortices were cleaned of 115 meninges and mechanically dissociated. After centrifuga-116 tion at 1,000 rpm for 5 min the pellet was resuspended in 117 DMEM (pH 7.6) supplemented with 8.39 mM HEPES; 118 23.8 mM $NaHCO_3$; 0.1% fungizone; 0.032% garamycin 119 and 10% fetal calf serum (FCS). The cells were plated at a 120 density of 2×10^5 cells per cm^2 onto 24 well-plates pre-121 treated with poly-L-lysine. Cultures were maintained in 5% 122 CO_2/95% air at 37 °C and allowed to grow to confluence 123 and used at 15–20 days in vitro.

124 Immunocytochemistry for GFAP

125 Cells in basal conditions were fixed for 20 min with 4% 126 paraformaldehyde in phosphate buffer (PBS): 2.9 mM 127 KH_2PO_4, 38 mM $Na_2HPO_4 7H_2O$, 130 mM NaCl, 1.2 mM 128 KCl, rinsed with PBS and permeabilized for 10 min in PBS 129 containing 0.2% Triton X-100. Fixed cells were then 130 blocked for 60 min with PBS containing 0.5% bovine 131 serum albumin and incubated overnight with polyclonal 132 anti-GFAP (Dako, 1:200) for 2 h. Finally, the cells were 133 treated with 0.05% diaminobenzidine (Sigma) containing 134 0.01% hydrogen peroxide for 10 min [22]. Cells were 135 viewed with a Nikon inverted microscope and images 136 transferred to a computer with a digital camera (Sound 137 Vision Inc., USA).

138 H_2O_2 treatment

139 Prior to H_2O_2 insult, cell cultures were pre-incubated in 140 serum-free DMEM for 30 min, containing or not resvera-141 trol at a concentration of 50 µM. Media were then replaced 142 and cells were incubated with serum-free DMEM con-143 taining, or not, freshly made 100 µM H_2O_2 for another 144 30 min. Cells were then maintained in serum-free DMEM 145 for 24 h and possible resulting changes were evaluated at 146 1 h and 24 h after H_2O_2 exposure. Importantly, cells pre-147 incubated with resveratrol were supplemented with this 148 compound during all replacements, i.e., during H_2O_2 insult 149 and afterwards. In all analyzed parameters, the results ob-150 tained with vehicle (0.25% ethanol) were not different from 151 those obtained in basal conditions without ethanol.

152 Evaluation of intracellular ROS production

153 Intracellular ROS production was detected using the non-154 fluorescent cell permeating compound, 2′–7′-dichlorofluo-155 rescein diacetate (DCF-DA). DCF-DA is hydrolyzed by 156 intracellular esterases and then oxidized by ROS to a 157 fluorescent compound 2′–7′-dichlorofluorescein (DCF). 158 Astrocytes were treated with DCF-DA (10 µM) for 30 min 159 at 37°C and rinsed with DMEM without serum. Cells were 160 viewed with a Nikon inverted microscope using a TE-FM

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Epi-Fluorescence accessory and images transferred to a computer with a digital camera (Sound Vision Inc, USA). All images are representative fields from at least three experiments carried out in triplicate. In another set of experiments, 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.

Glutamate uptake assay in astrocytes

Glutamate uptake was performed as previously described [21]. Briefly, cortical astrocytes were incubated at 37°C in a Hank's balanced salt solution (HBSS, pH 7.4) containing: 135 mM NaCl; 3.1 mM KCl; 1.2 mM $CaCl_2$; 1.2 mM $MgSO_4$; 0.5 mM KH_2PO_4; 2 mM glucose, 0.1 mM L-glutamate and 0.33 μCi/ml L-[2,3-^{3}H]glutamate for 7 min. Na^+-free medium was prepared by replacing NaCl with choline chloride. Incubation was terminated by removal of the medium and rinsing the cells twice with ice-cold HBSS. Cells were then resuspended in a lysis solution containing 0.1 N NaOH and 0.01% SDS. Radioactivity was measured with a scintillation counter.

Total glutathione assay

Total glutathione content was determined by a slightly modified assay, as described previously [23, 24]. Briefly, cells were scraped in phosphate buffered saline (0.01 M, pH 7.6), 6.3 mM EDTA (pH 7.5) and Triton-X (0.05%) and protein was precipitated with 1% sulfosalicylic acid. Supernatant was assayed with 462.6 μM 5,5′-dithiobis-(2-nitrobenzoic acid) (DTNB), 0.5 U/ml glutathione reductase, and 0.3 mM NADPH; reduced DTNB was measured at 412 nm.

Immunocontent of S100B

The S100B concentration was determined in the culture medium at 1 h and 24 h. ELISA for S100B was carried out as described previously with modifications [25]. Briefly, 50 μl of sample plus 50 μl of Tris buffer were incubated for 2 h on a microtiter plate previously coated with monoclonal anti-S100B (SH-B1, from Sigma). Polyclonal anti-S100 (from DAKO) was incubated for 30 min and peroxidase-conjugated anti-rabbit antibody was then added for a further 30 min. The color reaction with *o*-phenylenediamine was measured at 492 nm.

Other measurements

Protein content was measured by Lowry's method using BSA as a standard [26]. Extracellular S100B content was referred to as ''secretion,'' based on cell integrity measurement (data not shown) with LDH activity by a colorimetric commercial kit (from Doles, Brazil) and Trypan blue exclusion assay [27].

Statistical analysis

Data from at least three independent experiments are presented as means ± S.E.M and were analyzed statistically by one-way analysis of variance (ANOVA) followed by the Tukey's test. Values of $P < 0.05$ were considered to be significant. All analyses were carried out in an IBM compatible PC using the Statistical Package for Social Sciences (SPSS) software.

Results

Resveratrol inhibited intracellular ROS production induced by H_2O_2

Immunocytochemistry for GFAP showed typical polygonal flat morphology and astrocyte purity >95% (Fig. 1A, panel a). In order to investigate the effect of resveratrol on astrocytes exposed to 100 μM H_2O_2, we previously characterized the changes in intracellular ROS production using DCF. One hour after the insult with H_2O_2 we observed an increase in ROS production, as expected (Fig. 1A, panel c), whilst addition of 50 μM resveratrol prevented this alteration (Fig. 1A, panel d). These observations at 1 h after H_2O_2 exposure were confirmed by a quantification of DCF using a microplate reader (Fig. 1B). Twenty-four hours afterwards, no alteration was observed (data not shown).

Effect of resveratrol on glutathione content after H_2O_2 insult

H_2O_2 (at 100 μM) induced a decrease in glutathione levels after 1 h in astrocytes (Fig. 2A); conversely, resveratrol increased glutathione content. Moreover, resveratrol (at 50 μM) not only prevented the H_2O_2 effect, but increased glutathione at 1 h after H_2O_2. The H_2O_2-induced decrease in glutathione was not observed at 24 h; however, the resveratrol-induced glutathione increment was maintained at this time (Fig. 2B).

Effect of resveratrol on glutamate uptake after H_2O_2 insult

We observed that 100 μM H_2O_2 decreased glutamate uptake at 1 h after insult. Resveratrol (at 50 μM), added together with H_2O_2, was able not only to prevent this decrease, but to induce an increment in glutamate uptake

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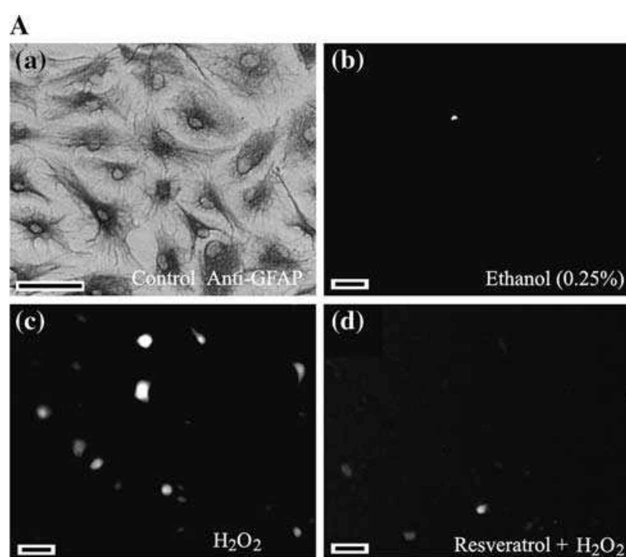

B

Ethanol (0.25%)	H_2O_2	Resveratrol + H_2O_2
106.0 ± 8.5	151.6 ± 4.8***	54.8 ± 6.7**

Fig. 1 Effect of resveratrol on intracellular ROS accumulation 1 h after H_2O_2 induced damage in astrocyte cultures. In **A**, cells were transferred to serum-free DMEM containing, or not, resveratrol (50 μM) for 30 min and then exposed or not to H_2O_2 (100 μM) for another 30 min. The level of intracellular ROS was measured with DCF-DA. Panel a depicts immunocytochemistry for GFAP under basal conditions. Representative fluorescent microscope images of primary astrocyte cultures in basal conditions (panel b) or exposed to H_2O_2 (panel c) or exposed to H_2O_2 in presence of resveratrol (panel d). All images are representative fields of at least three independent experiments carried out in triplicate. Scale bar = 50 μm. In **B**, the fluorescence was measured in a fluorescence microplate reader (excitation 485 nm and emission 520 nm). The basal condition without ethanol was assumed as being 100%. Statistically significant differences from basal, by one-way ANOVA followed by Tukey's multiple variation test, are indicated: ** $P < 0.01$, *** $P < 0.001$

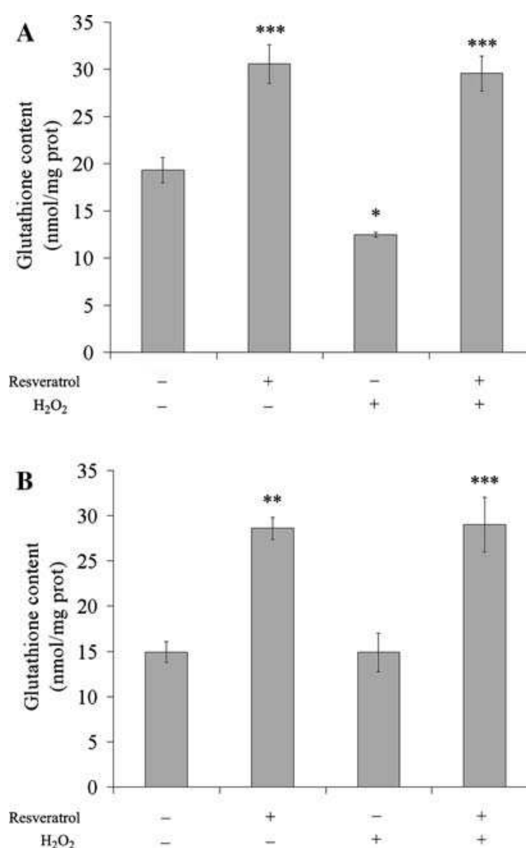

Fig. 2 Glutathione content in primary astrocyte cultures treated with resveratrol. Cells were transferred to serum-free DMEM containing or not resveratrol (50 μM) for 30 min and then exposed or not to H_2O_2 (100 μM) for another 30 min. Glutathione content was measured by a colorimetric assay with DTNB 1 (in **A**) and 24 h (in **B**) after H_2O_2 exposure. The data represent the mean ± SEM values of 4 independent experiments performed in triplicate. Statistically significant differences from controls, as determined by one-way ANOVA followed by Tukey's multiple variation test, are indicated: * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$

(Fig. 3A). Resveratrol per se did not increase glutamate uptake activity. Glutamate uptake activity was recovered 24 h after H_2O_2 insult (Fig. 3B). Interestingly, the combined exposure of resveratrol and H_2O_2 resulted in a persistent increase in glutamate uptake at 24 h after insult.

Effect of resveratrol on S100B secretion after H_2O_2 insult

About 100 μM H_2O_2 exposure caused an increase in S100B secretion at 1 h and 50 μM resveratrol, *per se*, was not able to change basal secretion. H_2O_2 insult in the presence of resveratrol, however, resulted in a decrease in S100B (Fig. 4A). Measuring extracellular levels of S100B, we found a decrease 24 h after H_2O_2 exposure. Conversely, resveratrol addition resulted in an increment of extracellular S100B at 24 h, even after H_2O_2 exposure (Fig. 4B).

Discussion

During the last decade, several studies have demonstrated resveratrol to be a potent antioxidant that protects against different ROS [17]. Furthermore, other important proprieties of resveratrol have been also reported, such as anti-inflammatory properties, modulation of diverse signaling pathways and anti-carcinogenic effects [17]. In the brain, resveratrol shows promise as a compound that may be useful in neurodegenerative processes and acute situations of injury, where the astrocytes act as potential therapeutic targets [28, 29].

Many studies in non-neural cell preparations have shown that increased ROS, induced by a brief H_2O_2 insult, was prevented in the presence of resveratrol (e.g., [30, 31]). In addition, resveratrol attenuates intracellular ROS accumulation and prevents H_2O_2-induced apoptosis in PC12

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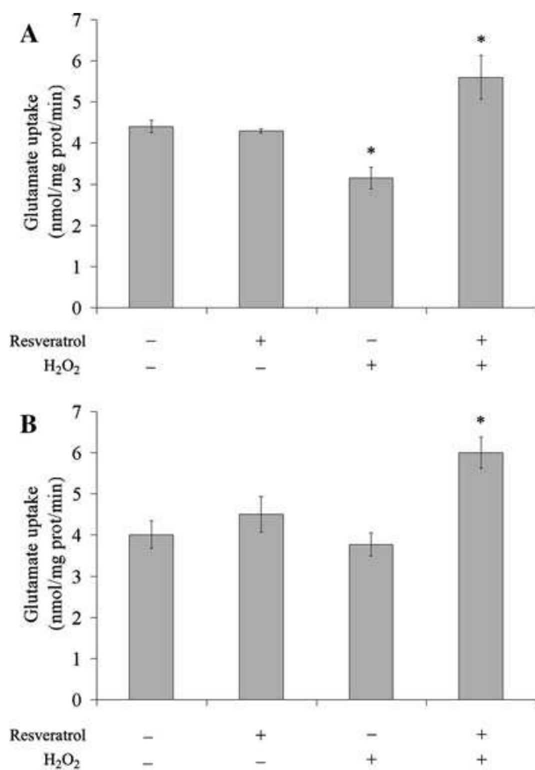

Fig. 3 Effect of resveratrol on glutamate uptake in astrocyte culture 1 h (**A**) and 24 h (**B**) after the insult with H_2O_2. Cells were transferred to serum-free DMEM containing or not resveratrol (50 µM) for 30 min and then exposed or not to H_2O_2 (100 µM) for another 30 min. After 1 (panel A) or 24 h (panel B) cell culture media were replaced with HBSS and incubated with [^{3}H]-glutamate for 7 min. The data represent the mean ± SEM values from 4 to 5 independent experiments performed in triplicate. Statistically significant differences from controls, as determined by one-way ANOVA followed by Tukey's multiple variation test, are indicated: * $P < 0.05$

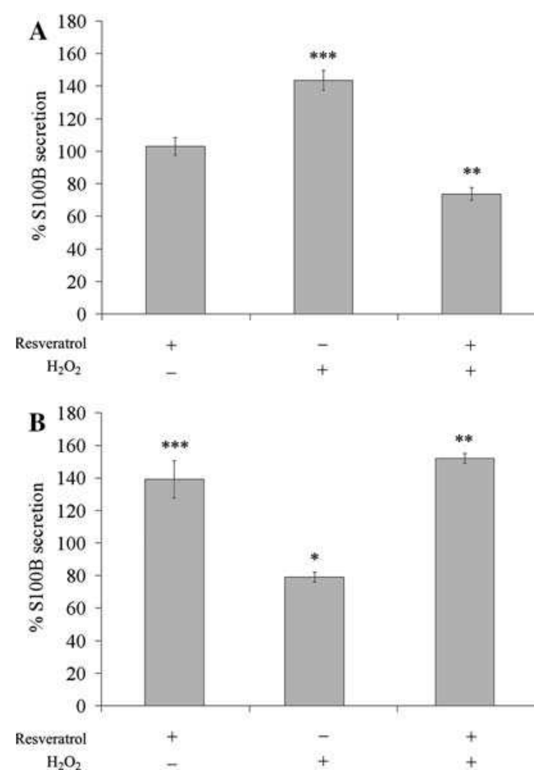

Fig. 4 Effect of resveratrol on S100B secretion in astrocyte culture after the insult with H_2O_2. Cells were transferred to serum-free DMEM containing or not resveratrol (50 µM) for 30 min and then exposed or not to H_2O_2 (100 µM) for another 30 min. Extracellular S100B was measured by ELISA 1 h (in panel **A**) and 24 h (in panel **B**) after insult with H_2O_2. Control S100B secretion presented mean values of 0.41 and 1.92 ng/ml at 1 h and 24 h, respectively; these values were assumed as 100%. Each value is the percentage mean ± SEM of 4–5 independent experiments performed in triplicate. Different letters indicate statistical difference of extracellular S100B levels from the control, determined by one-way ANOVA followed by Tukey's multiple variation test: * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$

cells [32]. In agreement with these studies, we observed a decrease in DCF oxidation when primary astrocyte cultures were treated with resveratrol. It is important to emphasize that H_2O_2 exposure occurred for exactly 30 min and that medium was then replaced before DCF assay and other measurements. Astrocytes, the most abundant glial cells, have an important protective role against ROS in CNS. Astrocytes can rapidly remove H_2O_2 and release antioxidant and trophic substances, protecting neurons against oxidative stress [33]. Moreover, astrocytes are involved in numerous other functions including synthesis and secretion of neurotrophic substances, uptake of neurotransmitters, and are essential for metabolic energy support of neurons [34]. It is important to mention that we used ethanol as a vehicle (at 0.25%) for resveratrol and no apparent oxidative stress was caused by ethanol per se in primary astrocyte culture, confirming the ethanol resistance of these cells, which could even protect neurons against ethanol-induced damage [35].

Several brain disorders are accompanied by a decrease in glutathione and other indications of oxidative stress [36]. Resveratrol was able to induce a fast and persistent increment of glutathione in primary astrocyte cultures. As expected, H_2O_2 induced a decrease in total glutathione content, which was prevented in cells that were pre-incubated and incubated in the presence of resveratrol. Glutathione constitutes a non-enzymatic scavenger and substrate for glutathione peroxidases, furthermore, astrocytes export glutathione precursors for neuronal synthesis [37]. Our data indicate an important regulation of glutathione content by resveratrol, and the provision of protection against H_2O_2 injury.

Astrocytes are key elements that regulate glutamate levels in the synaptic cleft. It has been well characterized that glutamate transporters are very sensitive to oxidative stress and this vulnerability is commonly associated to

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neurodegenerative diseases and some acute brain injuries [13]. Previous studies have reported that H_2O_2 significantly affects glutamate transporter activity in cultured rat cortical astrocytes [e.g., 38]. Resveratrol at 50 μM was able to improve glutamate uptake activity during H_2O_2 insult, but was unable to alter basal glutamate uptake. Our results corroborate the idea that resveratrol provides protection in the presence of oxidative damage induced by H_2O_2. Recently, resveratrol was demonstrated to increase basal glutamate uptake in C6 glioma cells [16]. Reasons for this difference are unclear at this moment, but could be due to differences in glutamate transporters [39] and/or the redox environment of these cultures. Regardless of this aspect, our results indicate the beneficial effect of resveratrol on glutamate uptake activity. This effect cannot be attributed exclusively to the antioxidant property of resveratrol, since it persists 24 h afterwards. The long-term effect of this compound could involve changes in glutamate activity, mediated by protein kinases [18, 40].

Another possible effect that may be afforded by resveratrol neuroprotection is its ability to induce S100B secretion. Many clinical studies have suggested the elevation of peripheral S100B as a marker of brain damage [41, 42]. However, based on the neuroprotection observed in neural cultures of this protein (see [43] for a review) and on its transitory extracellular increment in acute brain injury it has been proposed that, far from being a negative determinant of outcome, S100B may improve functional recovery following acute brain injury [42].

It should be noted that S100B secretion was transiently increased (first hour) after H_2O_2 insult. Interestingly, H_2O_2-induced S100B secretion showed a contrasting profile in our experiment, dependent on the presence of resveratrol. When oxidative damage was induced in astrocytes treated with H_2O_2, an increase in S100B secretion was detected one hour after; conversely, when astrocytes were pre-incubated with resveratrol a decrease was observed in S100B secretion. Resveratrol did not change S100B secretion during the first hour of insult, but induced a late increment (24 h afterwards) of extracellular levels of S100B. In C6 glioma, a late increment (48 h afterwards) in the S100B secretion also was induced by resveratrol [16].

Recently, we proposed that an increment of glutamate influx (as observed in excitotoxic conditions) could result in a decrease in S100B secretion [15]. In agreement, H_2O_2-induced damage could explain the opposite variations in S100B secretion and glutamate uptake, observed under our experimental conditions during the first hour. However, the mechanism by which pre-incubation with resveratrol decreases S100B secretion, stimulated by H_2O_2, during the first hour is not clear at the moment. The limited knowledge about the mechanism of S100B secretion, as well as the molecular signaling involved contributes to maintain this doubt. However, it may be postulated that resveratrol stimulates S100B secretion, during the long-term, which, in turn, stimulates neuronal survival and activity during brain injury and recovery.

Resveratrol has also been shown to be beneficial against a number of brain disorders, including spinal cord and cerebral ischemia, Parkinson disease, amyotrophic lateral sclerosis, diabetes mellitus, epilepsy, brain tumors and aging [19, 44, 45]. In the same vein, our results sustain and corroborate the benefits of resveratrol in CNS. Moreover, this study complements previous results with C6 glioma cells. Some limitations of this study, however, should be considered; firstly, further studies involving co-culture with neurons are necessary to confirm the protection of neurons mediated by astrocytes; secondly, the concentration of resveratrol used in this study is apparently elevated when compared to its concentration in tissues (less than 2 μM). In this investigation, based on recent studies, we used 50 μM [16, 46]. Some authors have proposed that it would be reasonable to conceive that resveratrol at levels ≤ 25 μM could be potentially useful in experimental conditions [45]. It is important to mention that we found that 25 μM resveratrol also induced a similar increment in glutathione content in primary astrocyte cultures treated for 24 h [28]. Finally, a limitation to be considered is the exogenous addition of H_2O_2 as a model of oxidative stress in cell culture; however, the quantity of exogenous or generated H_2O_2 appropriate for mimicking the pathological and/or physiological conditions in which this compound is involved remains questionable in the several models utilized to study oxidative stress [47].

In conclusion, the results presented here attest the ability of resveratrol to counteract oxidative damage caused by H_2O_2, not only via its antioxidant properties, but also through the modulation of important astrocytic functions, particularly improving glutamate uptake activity, increasing glutathione content and stimulating S100B secretion. All these changes favor the neural functional recovery after brain injury.

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DISCUSSÃO

Durante a última década, diversos estudos têm demonstrado a relevância do resveratrol como antioxidante. Além disso, outras propriedades importantes também têm sido relatadas como, por exemplo, atividades antiinflamatórias, cardioprotetoras e antitumorais além da modulação de várias rotas de sinalização (Baur e Sinclair, 2006). No SNC, o resveratrol pode representar uma molécula promissora, sendo útil nos processos neurodegenerativos e situações de injúria, e os astrócitos por sua vez, são alvos terapêuticos em potencial. Os resultados obtidos neste trabalho contribuem para avaliar os efeitos do resveratrol, em condições basais e também após insulto oxidativo, sobre parâmetros relacionados à atividade glial. Recentemente, alguns trabalhos sugerem um papel neuroprotetor do resveratrol, já que este composto é capaz de atenuar a toxicidade induzida pelo peptídeo β-amilóide (Han et al., 2004), proteger contra a isquemia cerebral (Wang et al., 2002b) e a excitotoxicidade induzida pelo cainato (Virgili e Contestabile, 2000; Wang et al., 2004). Contudo, os mecanismos e alvos de ação onde o resveratrol exerce seus efeitos ainda não foram completamente elucidados.

Os astrócitos estão envolvidos em diversas funções, tais como: síntese e secreção de fatores tróficos, metabolismo de neurotransmissores, suporte energético e metabólico para os neurônios, além de um papel fundamental na proteção do SNC contra o estresse oxidativo (Takuma et al., 2004).

Na tentativa de avaliar o efeito do resveratrol frente a um insulto oxidativo em cultura primária de astrócitos corticais, utilizamos um modelo de exposição ao

H_2O_2. Embora a adição exógena de H_2O_2 não seja fisiológica, diversos trabalhos utilizam esta prática, a fim de compreender melhor os mecanismos envolvidos numa condição de estresse oxidativo (Gottfried et al., 2002). Sabe-se que o H_2O_2 participa da sinalização celular, é importante para a resposta celular em condições normais e também está presente em diversas condições patológicas (Forman, 2007; Hyslop et al., 1995).

Outro aspecto interessante a ser considerado é a interação entre astrócitos e neurônios. Em culturas primárias de astrócitos, por exemplo, a expressão de GLAST é predominante enquanto que a expressão de GLT-1 é bastante reduzida. Porém, quando estas células são co-cultivadas com neurônios, ambos os transportadores, GLT-1 e GLAST são expressos (Gegelashvili et al., 1997). Dessa forma, o modelo de fatias hipocampais é bastante apropriado, pois preserva as interações entre astrócitos e neurônios e tem sido utilizado para investigar a captação de glutamato (Thomazi et al., 2004) e a secreção de S100B (Fontella et al., 2005; Buyukuysal, 2005). Além disso, permite avaliar o papel neuroprotetor do resveratrol na presença de mais de um tipo celular, comparando com os resultados prévios obtidos com cultura primária. Utilizamos em fatias hipocampais, um modelo de insulto oxidativo com 0,1 e 1 mM de H_2O_2.

Estudos anteriores em células não neurais mostraram que o resveratrol impediu o aumento das espécies reativas de oxigênio após um insulto com H_2O_2 (Osvena et al., 2006; De Salvia et al., 2002). E ainda, o acúmulo de espécies reativas de oxigênio e a apoptose induzida pelo H_2O_2 em células PC12 foram atenuados pelo resveratrol (Jang e Surth, 2001). Em astrócitos observamos um

perfil semelhante, houve diminuição da oxidação do DCF, 1 h após o insulto com H_2O_2 nas células pré-incubadas com resveratrol.

Conteúdo de Glutationa

Como mencionado anteriormente, a glutationa constitui uma das principais defesas não enzimáticas contra as espécies reativas de oxigênio. O tratamento dos astrócitos com resveratrol em baixas concentrações (25 e 50 μM) induziu um aumento rápido (1 h) e persistente (24 h) no conteúdo de glutationa. E ainda, como esperado encontramos uma diminuição do conteúdo de glutationa após o insulto com H_2O_2, que foi prevenido na presença do resveratrol. Estes resultados sugerem que o resveratrol é capaz de aumentar síntese de glutationa intracelular, independente de sua atividade antioxidante, impedindo o dano oxidativo causado pelo H_2O_2. De fato, Brito e colaboradores (2006) demonstraram que em células endoteliais o resveratrol provocou um aumento no conteúdo de glutationa através da indução da γ-GCS, enzima limitante da síntese do tripeptídeo. Outros trabalhos também relatam o aumento no conteúdo de glutationa, como Sharma e Gupta (2002) que demonstraram que o resveratrol preveniu o déficit cognitivo e o estresse oxidativo causado pela administração intracerebroventricular de estreptozotocina em ratos. Dessa forma, o resveratrol pode exercer um efeito protetor não apenas sobre os astrócitos, mas também sobre os neurônios que dependem de precursores gliais para a síntese de sua própria glutationa (Dringen et al., 1999). Além disso, o glutamato contribui não só como substrato para a glutationa, mas também seus níveis intracelulares determinam a atividade do

trocador cistina/glutamato independente de sódio (Xc^-), permitindo a entrada de cistina (Dringen e Hirrlinger, 2003). Nos experimentos realizados com fatias hipocampais, verificamos um aumento no conteúdo de glutationa na presença do resveratrol. Observamos ainda que o resveratrol impediu o decréscimo do conteúdo de glutationa induzido por H_2O_2.

Captação de glutamato

Nossos resultados obtidos com culturas primárias de astrócitos corticais demonstraram que o resveratrol é capaz de modular a captação basal de glutamato. Na concentração de 25 μM de resveratrol observamos um aumento na captação de glutamato após 24 h de tratamento. Resultado semelhante foi obtido em um trabalho recente do nosso laboratório quando observamos também um aumento na captação de glutamato em células de glioma C6 tratadas com resveratrol (Dos Santos et al., 2006). Interessantemente, já foi demonstrado um papel do resveratrol na proteção contra dano neuronal e ativação glial induzidas pelo cainato, um agonista de receptor glutamatérgico (Wang et al., 2004) e podemos atribuir este efeito, pelo menos em parte, a um aumento no transporte glial de glutamato. Podemos inferir que a participação dos astrócitos na transmissão glutamatérgica é neuroprotetora, impedindo o acúmulo do neurotransmissor no meio extracelular, o que é comprovado pela distribuição dos transportadores gliais nas sinapses glutamatérgicas (Rauen e Wiessner, 2000).

Outro aspecto relevante a ser considerado, é que os transportadores de glutamato são bastante sensíveis ao estresse oxidativo (Trotti et al., 1998). Já foi

demonstrado que o H_2O_2 é capaz de afetar a captação de glutamato em cultura de astrócitos (Volterra et al., 1994). Nossos resultados reforçam essa evidência demonstrando que o H_2O_2 leva a uma diminuição da captação de glutamato 1 h após o insulto e que o resveratrol é capaz de não apenas reverter como causar um aumento no transporte de glutamato. Possivelmente, a proteção exercida pelo resveratrol possa ser atribuída a sua atividade antioxidante, porém não exclusivamente já que o aumento na captação de glutamato persiste 24 h após o insulto. Sabe-se que o resveratrol é capaz de modular a atividade de proteínas cinases, como a PKC, (Klinge et al., 2005; Han et al., 2004) e dessa forma poderia estar envolvido na regulação do metabolismo glutamatérgico através de seus transportadores (Guillet et al., 2005).

Um perfil similar ao obtido com culturas de astrócitos foi encontrado nas fatias tratadas com resveratrol, frente ao insulto oxidativo causado pelo H_2O_2. O resveratrol foi capaz de aumentar a captação de glutamato nestas fatias. Sabe-se que os transportadores presentes nos astrócitos, GLAST e GLT-1, são os principais responsáveis pela remoção do glutamato extracelular (Rothstein et al.,1996). Além disso, o resveratrol preveniu a diminuição na captação de glutamato causada por 0,1 mM de H_2O_2. No entanto, o mecanismo relacionado à ação neuroprotetora do resveratrol necessita ser mais bem estudado a fim de distinguir quais rotas metabólicas estão envolvidas no seu efeito.

S100B

A S100B é uma proteína ligante de cálcio e expressa por astrócitos. Intracelularmente, a S100B está envolvida na modulação do citoesqueleto e da

proliferação celular (Donato, 2001; Van Eldik e Wainwright, 2003). No meio extracelular a S100B age como uma citocina dependendo da sua concentração, pode ser trófica (em concentrações nanomolar) ou tóxica (em concentrações micromolar). Após 24 h de tratamento com resveratrol encontramos um aumento de secreção S100B dependente de concentração. Nossos dados indicam que o resveratrol afeta o mecanismo de secreção da S100B já que não houve alteração na medida de LDH. Resultados em células de glioma C6 apresentaram um perfil bastante semelhante (Dos Santos et al., 2006). A secreção de S100B 1 h após o insulto com H_2O_2 apresentou um aumento transitório que foi revertido pela presença de resveratrol. O conteúdo extracelular de S100B após 1 h de tratamento não foi diferente do controle, quando os astrócitos foram expostos somente ao resveratrol. O oposto ocorreu 24 h após o insulto, quando o H_2O_2 induziu diminuição na secreção de S100B, enquanto que na presença do resveratrol observamos um aumento da proteína no meio extracelular. O mecanismo de secreção da S100B ainda não foi elucidado e, logo não é claro o motivo pelo qual o resveratrol diminui a secreção de 100B 1 h após o insulto com H_2O_2. Recentemente, uma correlação negativa entre a captação de glutamato e a secreção de S100B foi proposta pelo nosso grupo (Tramontina et al., 2006). Além disso, a S100B em concentrações nanomolares protege os neurônios contra a toxicidade induzida pelo glutamato (Ahlenmeyer et al., 2000) e estimula a captação de glutamato (Tramontina et al., 2006).

A concentração da S100B no meio extracelular das fatias hipocampais, assim como nas culturas de astrócitos tratados por 1 h com resveratrol, não sofreu alterações quando as fatias foram incubadas na presença do polifenol. Além disso,

ambas as concentrações de H_2O_2 (0,1 e 1 mM) induziram decréscimo na concentração extracelular de S100B, efeito não prevenido pelo resveratrol. Nossos dados indicam que o estresse oxidativo prejudica a secreção de S100B e conseqüentemente sua atividade neurotrófica. Considerando os resultados obtidos com cultura de astrócitos, o resveratrol parece induzir um aumento mais tardio da secreção de S100B que poderia por sua vez estimular a sobrevivência neuronal após a injúria cerebral. No entanto, o efeito do resveratrol sobre a secreção de S100B merece investigações futuras.

Outros resultados

Este estudo demonstrou ainda em fatias hipocampais que 1 mM de H_2O_2 alterou o metabolismo mitocondrial e causou um aumento de lactato no meio extracelular. O resveratrol por sua vez, não foi capaz de reverter os danos metabólicos causados pelo H_2O_2 nesta concentração. Entretanto, níveis elevados de H_2O_2 tornaram a membrana plasmática mais vulnerável, de acordo com o ensaio feito com azul de Trypan e este efeito foi bloqueado pela presença de resveratrol. O aumento da fosforilação da ERK induzida pelo H_2O_2 também foi prevenido pela pré-incubação com resveratrol. Cabe mencionar que a rota da MAPK está envolvida diretamente com a morte neuronal e astrocítica decorrente de estímulos neurotóxicos, como o H_2O_2 (Lee et al., 2005). Dados recentes da literatura demonstram que em alguns tipos celulares não neurais, o resveratrol reverte a fosforilação da ERK induzida por estímulos tóxicos (Kim et al., 2006b,; Olson et al., 2005; Chao et al., 2005). Entretanto, em neuroblastoma SH-SY5Y o resveratrol estimulou a fosforilação da ERK em concentrações de até 10 μM e

provocou o efeito oposto em concentrações mais altas 50-100 μM (Miloso et al, 1999). Recentemente, foi demonstrado em culturas organotípicas de hipocampo de ratos, que 6 h após a privação de oxigênio e de glicose o resveratrol estimulou a fosforilação da ERK (Zamin et al., 2006). É importante ressaltar que a sinalização celular depende do tipo celular e do insulto envolvido.

<u>Limitações do estudo</u>

Cabe salientar que os astrócitos expostos a uma concentração elevada de resveratrol (250 μM) apresentaram efeitos opostos nos parâmetros avaliados, demonstrando uma atividade pró-oxidante. Não observamos alterações morfológicas nos astrócitos tratados com resveratrol em baixas doses, enquanto que na concentração de 250 μM após 24 h ocorreu a formação de processos ao redor do corpo celular, analisado por microscopia de contraste de fase e confirmada por imunocitoquímica para GFAP. Sabe-se que alterações morfológicas dos astrócitos em cultura podem representar ativação celular (Cechin e al., 2002). Diversos estímulos que podem induzir formação de estresse oxidativo, como β-hidroxibutirato, amônia e alterações no pH intracelular, induzem modificação na morfologia celular (Leite et al., 2004; Leite et al., 2006; Cechin et al., 2002). Encontramos ainda, diminuição na captação de glutamato em culturas tratadas com 250 μM de resveratrol, o que em conjunto com a alteração morfológica, indica um comprometimento das funções astrocíticas. Além disso, não observamos aumento no conteúdo de glutationa, mas surpreendentemente encontramos um aumento na atividade da enzima glutamina sintetase. Esta

enzima, predominantemente astrocítica, responsável pela conversão de glutamato em glutamina, é descrita como sendo bastante sensível ao estresse oxidativo e não apresentou alteração na presença de resveratrol em doses mais baixas. Ainda, em células de glioma C6 relatamos um efeito oposto, ou seja, diminuição da atividade da glutamina sintetase frente a uma concentração elevada de resveratrol (Dos Santos et al., 2006). O aumento persistente da S100B é outra alteração observada quando as células foram tratadas com resveratrol na concentração de 250 μM, o que tem sido associado com desordens neurodegenerativas podendo levar a morte neuronal.

Sabe-se que as concentrações de resveratrol que apresentaram propriedades neuroprotetoras descritas na literatura estão na faixa de 10 a 100 μM (Wang et al., 2003). Porém, a quantidade de resveratrol obtida pela dieta atinge concentrações menores que 2 μM no plasma (Gescher e Stweward, 2003). Níveis baixos de resveratrol podem ser atribuídos ao rápido metabolismo intestinal e hepático, principalmente pela formação de resveratrol glucoronídeo e resveratrol sulfato (Signorelli e Ghidoni, 2005). É importante considerar que avaliamos um efeito agudo do resveratrol e, portanto doses menores por períodos maiores talvez resultem nos mesmos efeitos. Ainda, modelos *in vivo* comprovam a capacidade do resveratrol mesmo atingindo baixas concentrações de exercer seu papel neuroprotetor (Wang et al., 2002). Outra limitação deste estudo consiste na adição exógena de H_2O_2 como modelo de estresse oxidativo em cultura de células e em fatias, já que a concentração apropriada para mimetizar uma condição patológica ou fisiológica ainda é questionável (Forman, 2007).

Conclusões:

Por fim, nossos dados demonstram que a atividade neuroprotetora do resveratrol envolve a participação dos astrócitos, como indicado pelo aumento *in vitro* da captação de glutamato, do conteúdo de glutationa e da secreção de S100B em cultura de astrócitos. E ainda, que o resveratrol é capaz de reverter os danos causados por um insulto oxidativo por H_2O_2 em culturas de astrócitos,

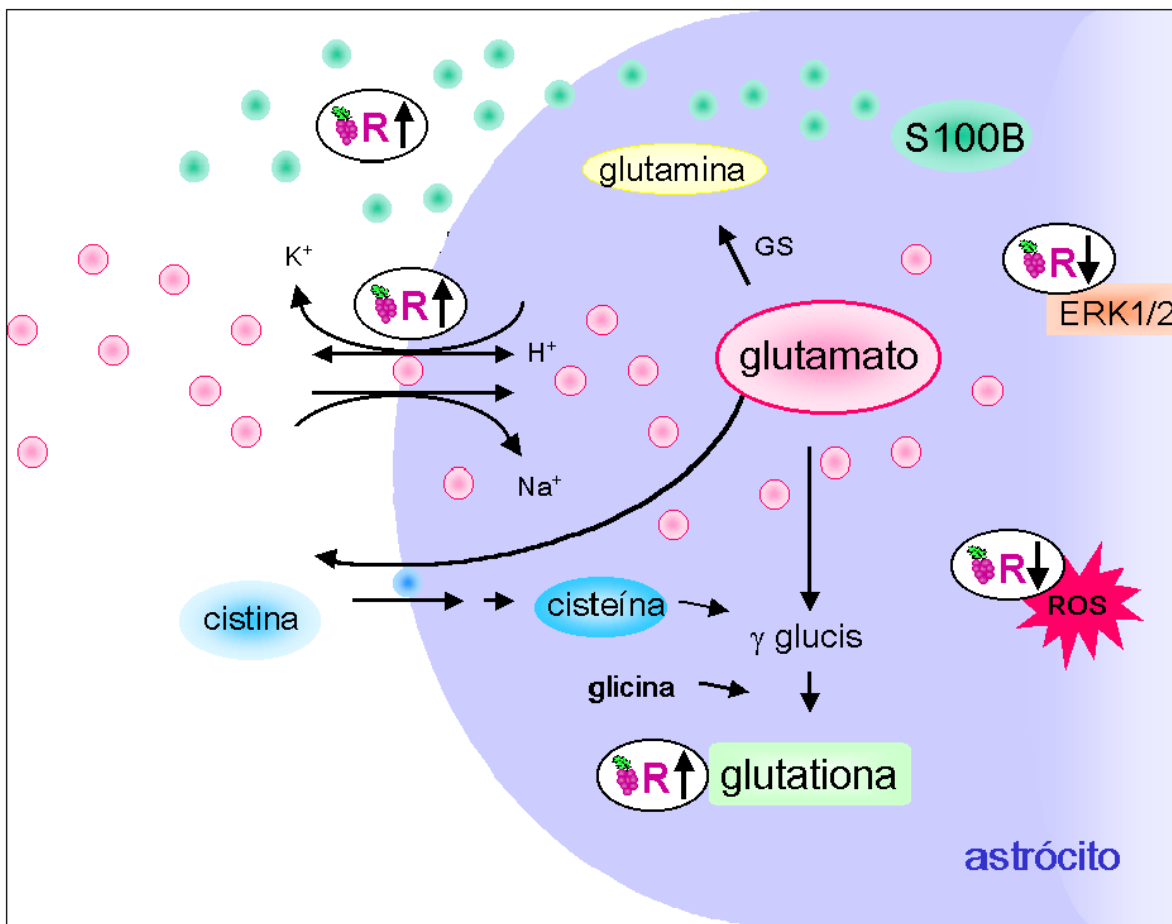

Figura 6: Sumário dos efeitos neuroprotetores do resveratrol.

diminuindo o acúmulo de espécies reativas de oxigênio, aumentando a captação de glutamato, o conteúdo de glutationa e a secreção de S100B. Este efeito do resveratrol sobre parâmetros gliais frente ao insulto com H_2O_2, também foi observado em um modelo de fatias hipocampais. Entretanto, utilizando uma concentração mais alta de H_2O_2 o resveratrol preveniu apenas alguns parâmetros como o aumento da permeabilidade da membrana, a queda no conteúdo de glutationa e a ativação da ERK, causados pelo insulto oxidativo.

Portanto, estudos adicionais são necessários para compreender melhor os efeitos do resveratrol e seus mecanismos de ação no SNC. Apesar disso, podemos propor um papel neuroprotetor do resveratrol frente a insultos oxidativos e desordens cerebrais, principalmente aquelas relacionadas com a toxicidade do glutamato.

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