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

# EFEITO DO RESVERATROL SOBRE PARÂMETROS BIOQUÍMICOS ASTROGLIAIS

André Quincozes dos Santos

Orientadora: Profa. Carmem Gottfried

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Tese apresentada ao Programa de Pós-Graduação em Ciências Biológicas – Bioquímica da Universidade Federal do Rio Grande do Sul, como requisito parcial à obtenção do grau de Doutor em Bioquímica.

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Dedico esta tese à minha família.

"Dans les champs de l'observation le hazard ne favorise que les esprits préparés."

"O acaso só favorece a mente preparada."

Louis Pasteur

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**SUMÁRIO**

# PARTE I

**RESUMO**

A espécie redox ativa resveratrol (*3,5,4'-trihidroxi-trans-estilbeno*), uma fitoalexina encontrada nas uvas e no vinho tinto, apresenta importantes efeitos biológicos como atividade antioxidante, anti-inflamatória, antitumoral e cardioprotetora. O sistema nervoso central (SNC) também é alvo do resveratrol que por sua vez pode atravessar a barreira hematoencefálica e exercer efeito neuroprotetor, modulando importantes funções neurogliais. Fisiologicamente os astrócitos controlam importantes funções cerebrais, principalmente, relacionadas ao metabolismo glutamatérgico, à plasticidade sináptica e neuroproteção; e em situações neuropatológicas, relacionadas ao estresse oxidativo. Assim, neste estudo nós investigamos o efeito do resveratrol sobre importantes parâmetros gliais em células C6 frente a dois modelos de insulto oxidativo: (I) 1 mM de $H_2O_2$/0,5 h e (II) 0,1 mM de $H_2O_2$/6 h. Avaliamos, nessas condições, o perfil das principais defesas antioxidantes enzimáticas celulares, a genotoxicidade celular e possíveis vias de sinalização que possam contribuir para o melhor entendimento do mecanismo de ação do resveratrol no SNC. Nós demonstramos que o resveratrol modula a captação de glutamato; a atividade da enzima glutamina sintetase; os níveis intra e extracelulares de glutationa; a secreção da proteína S100B; as enzimas SOD, CAT e GPx; a peroxidação lipídica; a frequência de micronúcleos; as enzimas heme oxigenase 1 e iNOS e o fator de transcrição NFκB, de maneira dependente das condições oxidativas do meio e do ambiente redox celular. Dessa forma, nossos resultados mostram que o resveratrol apresenta comportamentos anti e pró-oxidante e que sua ação neuroprotetora pode estar relacionada a modulação da atividade glial e da heme oxigenase 1. Enfim, o resveratrol pode representar um potencial agente terapêutico em patologias que envolvam déficits no sistema glutamatérgico associado ao estresse oxidativo.

**ABSTRACT**

The redox active compound, resveratrol (3,5,4 '-trihydroxy-trans-stilbene), a phytoalexin, found in grapes and red wine, has a wide range of biological effects, such as, antioxidant, antiinflammatory, anticancer and cardioprotective. The central nervous system (CNS) is also the target of resveratrol which is able to trespass the blood-brain barrier and exerts neuroprotective effects by modulating important glial parameters. Physiologically, astrocytes control important brain functions, mainly related to glutamatergic metabolism, synaptic plasticity and neuroprotection; and in neurophatology events related to oxidative stress. In this study, we investigated the effect of resveratrol on important parameters in C6 astoglial cells against oxidative insult in two models: (I) 1 mM $H_2O_2$/0.5 h and (II) 0.1 mM $H_2O_2$/6 h. We evaluated under these conditions, the activity of the major cellular enzymatic antioxidant defenses, the possible genotoxicity and cell signaling pathways that may contribute to better understanding the mechanism of resveratrol in the CNS. Resveratrol modulates glutamate uptake; glutamine synthetase activity; intracellular and extracellular levels of glutathione; S100B secretion; the enzymes SOD, CAT and GPx; lipid peroxidation; the frequency of micronuclei; the enzymes heme oxygenase 1 and iNOS and the transcription factor NFκB depending upon the oxidant conditions of the milieu and the cellular redox environment. Thus, our results show that resveratrol presents anti and pro-oxidant effects and their neuroprotective action may be related to modulation of glial function and heme oxygenase 1. Finally, resveratrol can represent a potential therapeutic agent against pathologies associated to glutamatergic system and oxidative stress.

**LISTA DE ABREVIATURAS**

| | |
|---|---|
| ATP | Adenosina Trifosfato |
| CAT | Catalase |
| CoA | Coenzima A |
| COX | Ciclooxigenase |
| DCFH-DA | 2’ 7’-Diclorofluoresceína diacetato |
| DMSO | Dimetilsulfóxido |
| EAAT | Transportador de Aminoácidos Excitatórios |
| EGTA | Ácido Etileno Glicol Tetracético |
| ERN | Espécies Reativas de Nitrogênio |
| ERO | Espécies Reativas de Oxigênio |
| GABA | Ácido γ-Aminobutírico |
| GFAP | Proteína Glial Fibrilar Ácida |
| GLAST | Transportador Glutamato-aspartato |
| GLT-1 | Transportador de Glutamato Tipo 1 |
| GS | Glutamina Sintetase |
| GSH | Glutationa |
| GPx | Glutationa Peroxidase |
| $H_2O_2$ | Peróxido de Hidrogênio |
| iNOS | Óxido Nítrico Sintase induzível |
| LDH | Lactato Desidrogenase |
| LDL | Lipoproteína de Baixa Densidade |
| L-NAME | $N^{\omega}$-nitro-L-arginina metil éster |
| LPA | Ácido Lisofosfatídico |
| MDA | Malondialdeído |
| NFκB | Fator Nuclear da Cadeia κ de Linfócitos B |
| NMDA | N-metil-D-aspartato |

| | |
|---|---|
| NO | Óxido Nítrico |
| NOS | Óxido Nítrico Sintase |
| Nrf2 | Fator Nuclear Eritróide 2 |
| $O_2^{\bullet-}$ | Ânion Superóxido |
| $OH^{\bullet}$ | Radical Hidroxila |
| $ONOO^-$ | Peroxinitrito |
| PI3K | Fosfatidil-Inositol-3-Cinase |
| PKC | Proteína Cinase C |
| RESV | Resveratrol |
| SIRT | Sirtuína |
| SOD | Superóxido Dismutase |
| SNC | Sistema Nervoso Central |
| $Xc^-$ | Trocador Cistina/Glutamato |

## INTRODUÇÃO

### 1. Células Gliais

Neurônios e células gliais são os dois principais tipos celulares do Sistema Nervoso Central (SNC) e apresentam íntima interação celular e molecular. Neurônios são as células especializadas na condução de impulsos eletroquímicos e as células gliais muito mais do que suporte, garantem proteção e nutrição aos neurônios, além de participarem na neurotransmissão sináptica (Bacci et al, 1999; Vallejo et al). As células gliais são divididas em três categorias principais: (1) astrócitos e oligodendrócitos, ambos de origem ectodérmica, constituindo a macroglia; (2) a microglia, de origem mesodérmica; e (3) células ependimais, de origem ectodérmica (Vallejo et al; Young, 1991). Células astrogliais foram as células alvo deste estudo.

### 1.1. Astrócitos

O médico e patologista alemão, Rudolph Virchow foi quem descreveu pela primeira vez, em 1846, a neuroglia, do inglês *nerve glue* (por parecer-se com uma cola entre os neurônios), como um conjunto de células, anatomicamente distinto do neuronal e do conectivo do SNC (Young, 1991). Foi preciso quase um século para que as células gliais passassem a ser reconhecidas como um tecido funcional (Kettenmann & Verkhratsky, 2008). Atualmente, os astrócitos são reconhecidos por sua capacidade dinâmica participando não apenas da manutenção e fisiologia normal, mas também respondendo a injúrias (Wang & Bordey, 2008). Os astrócitos constituem aproximadamente 50% do número total de células do SNC. Eles são divididos em dois tipos: os protoplasmáticos, na substância cinzenta (frequentemente

ramificados e com largas expansões) e os fibrosos, na substância branca (com menos ramificações, cilíndricas e longas) (Young, 1991).

As principais funções desempenhadas pelos astrócitos no SNC são: (1) manutenção da homeostase de íons extracelulares, especialmente o $K^+$ (Wang & Bordey, 2008); (2) metabolismo de neurotransmissores, particularmente o glutamato e o GABA (ácido gama-aminobutírico) (Anderson & Swanson, 2000; Chen & Swanson, 2003; Wang & Bordey, 2008); (3) formação e manutenção da barreira hematoencefálica (Wang & Bordey, 2008); (4) suporte metabólico através do ciclo glutamato-glutamina e do metabolismo da glicose e glicogênio (Magistretti, 2006; Pellerin, 2005; Wang & Bordey, 2008); (5) síntese e liberação de fatores tróficos e substâncias neuroativas para os neurônios e para outros astrócitos (Pellerin, 2005); (6) participação na resposta imune encefálica (Wang & Bordey, 2008); (7) participação na resposta a injúrias ao SNC, tornando-se reativos (gliose reativa[1]) (Eng et al, 2000; Wang & Bordey, 2008); (8) regulação do espaço extracelular através da variação do volume astrocítico (Kimelberg et al, 1992); (9) atuam como guias na migração de neurônios nos estágios iniciais do desenvolvimento (Paixao & Klein); (10) atuam como progenitores neurais em zonas neurogênicas (Wang & Bordey, 2008).

Estudos recentes ressaltam a importância da comunicação recíproca neurônio-astrócito para o SNC. Atualmente, sabe-se que os astrócitos constituem o terceiro elemento da sinapse (sinapse tripartite), respondendo de maneira seletiva à liberação de neurotransmissores (Perea & Araque, 2006). Nesse contexto, a compreensão bioquímica de tais eventos e a descoberta de

[1] Gliose reativa se refere à hiperplasia e/ou hipertrofia das células gliais após injúrias ao SNC formando uma "cicatriz" no local da lesão (Wang DD, Bordey A (2008) The astrocyte odyssey. *Prog Neurobiol* **86**(4)**:** 342-367).

moléculas com potencial terapêutico sobre tais funções torna-se extremamente relevante para a fisiologia do SNC.

### 1.2. Células Astrogliais C6

A linhagem C6 tem sua origem datada da década de 60 e constitui-se numa das linhagens mais amplamente utilizadas em estudos neuroquímicos. Ela foi obtida após injeções em ratos do agente alquilante *N-nitrosometiluréia* e C6 se refere ao sexto clone pós injeção do agente alquilante (Benda et al, 1968).

Esta linhagem é morfologicamente similar a glioblastomas, quando injetada no cérebro de ratos neonatos (Auer et al, 1981). Os glioblastomas representam menos de 2% das neoplasias malignas humanas, porém apresentam prognóstico bastante ruim, decorrente principalmente da excitotoxicidade glutamatérgica (Mourad et al, 2005).

Embora com algumas limitações, as células astrogliais C6 constituem um bom modelo de estudos para avaliação e regulação do crescimento celular, liberação de fatores tróficos e vias de transdução de sinal (Kim et al, 2006). Além disso, a linhagem C6 apresenta-se marcada positivamente para dois importantes marcadores gliais: GFAP (do inglês Glial Fibrillary Acidic Protein) e S100B (Benda et al, 1971).

Por apresentar características de uma célula astrocítica, as células astrogliais C6 também são utilizadas para investigação de características bioquímicas e metabólicas que envolvam funções desempenhadas por astrócitos (Cechin et al, 2005; Feng & Zhang, 2004; Mangoura et al, 1989). As células astrogliais C6 utilizadas neste estudo apresentam forte marcação para

a proteína GFAP e vários trabalhos do nosso grupo mostram que elas exercem importantes funções astrocíticas (dos Santos et al, 2006; Funchal et al, 2005; Funchal et al, 2007; Quincozes-Santos et al, 2008; Quincozes-Santos et al, 2009a; Quincozes-Santos et al, 2009b).

### 1.3. Marcadores Gliais

Como anteriormente citado, os astrócitos participam de vários processos fisiológicos e metabólicos responsáveis por manter a homeostase do SNC. Insultos físicos e funcionais provocam rápidas alterações nas células gliais e este fenômeno é denominado gliose reativa, que é caracterizado entre outras alterações, por um aumento na expressão de marcadores gliais tais como GFAP e S100B (Baydas et al, 2003).

#### 1.3.1. Proteína Glial Fibrilar Ácida (GFAP)

A GFAP é uma proteína estrutural e constitui a subunidade protéica de filamentos intermediários do tipo III do citoesqueleto glial (Rodnight et al, 1997). Inicialmente isolada de lesões cerebrais (placas) de pacientes com esclerose múltipla (Eng et al, 2000), a GFAP é reconhecida e amplamente utilizada como marcador de astrócitos.

A GFAP possui peso molecular aparente de 50 kDa, sendo composta por três regiões distintas. Uma região amino-terminal não-helicoidal com 35 resíduos de aminoácidos e uma estrutura em conformação β que contém oito resíduos de arginina, caracterizando esta região como básica. Uma região carboxi-terminal não-helicoidal que contém cerca de 50 resíduos de aminoácidos e uma estrutura globular que pode estar envolvida em interações da GFAP com outras proteínas. A região central é formada por uma extensa

hélice cuja sequência de aminoácidos é conservada em relação a outras proteínas de filamentos intermediários (Battu et al, 2005; Eng et al, 2000 ; Martinez et al, 1998).

### 1.3.2. S100B

A S100B é um membro da família de proteínas S100, encontrada principalmente na forma homodimérica (com cerca de 21 kDa). É uma proteína ligante de cálcio do tipo EF-hand (hélice-alça-hélice) com dois sítios de ligação ao cálcio por monômero (Donato, 2001). Foi isolada há mais de 40 anos (Moore, 1965) a partir de um extrato de cérebro bovino. Posteriormente verificou-se que este extrato continha duas proteínas muito similares, a S100A1 e a S100B, sendo esta já identificada em tecidos extracerebrais. Hoje são conhecidos 21 membros da família S100 presentes nos mais diversos tipos celulares de vertebrados (Donato, 2003) os quais possuem aproximadamente 50% de homologia na sua sequência de aminoácidos (Zimmer & Van Eldik, 1989). As proteínas da família S100 receberam esta denominação por serem solúveis mesmo em 100% de sulfato de amônio.

A S100B é produzida e secretada, principalmente, por astrócitos e exerce efeitos autócrinos e parácrinos sobre outras células gliais e neurônios. Ela também é expressa em outros tipos celulares como: adipócitos, células satélites musculares, mioblastos e oligodendrócitos (Goncalves et al; Riuzzi et al, 2006; Steiner et al, 2008). Seus efeitos biológicos intracelulares são devidos a sua interação com outras proteínas, afetando assim suas atividades. A S100B está envolvida na regulação e diferenciação celular; na fosforilação de proteínas; na manutenção da integridade do citoesqueleto e na atividade e

metabolismo de enzimas (Donato, 2001; Donato, 2003; Donato et al, 2008; Frizzo et al, 2004). Já extracelularmente, a S100B apresenta efeitos biológicos dependentes da concentração, ou seja, em concentrações na ordem nanomolar apresenta efeitos tróficos promovendo crescimento de neuritos, aumentando a sobrevivência neuronal e protegendo neurônios contra excitotoxicidade glutamatérgica (Goncalves et al, 2008; Tramontina et al, 2006a; Tramontina et al, 2006b; Van Eldik & Wainwright, 2003). Porém, em concentrações micromolares exerce efeitos neurotóxicos induzindo apoptose (Van Eldik & Wainwright, 2003).

Culturas primárias de astrócito e células astrogliais C6 são comumente utilizadas para estudar a expressão e a secreção de S100B, podendo apresentar mecanismos diferentes relacionados aos efeitos sobre a S100B (Castets et al, 1997; Nardin et al, 2007; Van Eldik & Zimmer, 1987).

### 1.4. Metabolismo do Glutamato

O glutamato é o principal neurotransmissor excitatório do SNC de mamíferos e exerce um importante papel na plasticidade neural e neurotoxicidade. A regulação dos níveis extracelulares de glutamato determina suas ações fisiológicas ou excitotóxicas (Danbolt, 2001). O glutamato medeia importantes processos vitais como: (1) cognição, memória e aprendizado; (2) formação e eliminação de sinapses; (3) migração, diferenciação e morte neuronal (Anderson & Swanson, 2000; Danbolt, 2001; Lujan et al, 2005). Além disso, o glutamato está relacionado ao metabolismo energético do SNC e com a síntese e liberação de glutamina e do antioxidante glutationa (GSH).

Os receptores glutamatérgicos têm papel fundamental na plasticidade neural e exercem os papéis sinalizadores do glutamato nas células neurais (Hertz & Zielke, 2004). As concentrações de glutamato extracelular devem ser mantidas baixas para manutenção da homeostasia no SNC, já os níveis de glutamato intracelular, geralmente, são baixos e podem ser considerados inertes (Danbolt, 2001).

Existem duas classes distintas de receptores glutamatérgicos: os ionotrópicos e os metabotrópicos (Lujan et al, 2005). Os receptores ionotrópicos são assim denominados por serem canais iônicos permeáveis a cátions e são subdivididos em N-metil-D-aspartato (NMDA), ácido α-amino-3-hidróxi-5-metil-isoxazolenopropionato (AMPA) e cainato. Os receptores metabotrópicos (mGlu) pertencem a uma família de receptores que estão acoplados a proteínas G (Lau & Tymianski; Lujan et al, 2005).

### 1.4.1. Captação de Glutamato

O glutamato, após ser sintetizado, pode ser utilizado como neurotransmissor. Nesse caso, ele é transportado dentro de vesículas sinápticas por um sistema de transporte vesicular e liberado para o espaço extracelular (fenda sináptica), onde interage com seus receptores ionotrópicos e/ou metabotrópicos. Esses receptores estão localizados nas membranas celulares pré- e pós-sinápticas de dendritos, terminais nervosos e células gliais. Após, o glutamato deve ser removido da fenda sináptica e esse processo é realizado principalmente pelos transportadores glutamatérgicos dependentes de sódio localizados nos astrócitos. Esse processo é chamado captação de glutamato e representa o mecanismo mais eficiente para manter normais os

níveis de glutamato extracelular (Anderson & Swanson, 2000; Danbolt, 2001; Foran & Trotti, 2009; Gallo & Ghiani, 2000; Newsholme et al, 2003; Trotti et al, 1998).

Três grupos de transportadores glutamatérgicos são conhecidos: os dependentes de sódio (EAATs – do inglês: Excitatory Amino Acid Transporters), sendo que EAAT1/GLAST, EAAT2/GLT-1 e EAAT3/EAAC1 estão presentes nas membranas das células gliais; EAAT4, nas células de Purkinje e EAAT5, nas células da retina (Danbolt, 2001). Além destes, existem os transportadores vesiculares que são independentes de sódio (VGLUTs) e o trocador cistina-glutamato ($Xc^-$) (Banerjee et al, 2008; Danbolt, 2001; Moriyama & Omote, 2008).

Os primeiros estudos sobre transportadores gliais de glutamato em células astrogliais C6 mostraram que elas apresentavam apenas o transportador EAAT3/EAAC1 (Palos et al, 1996). Porém, estudos posteriores demonstraram que as células C6 podem apresentar os três principais transportadores glutamatérgicos gliais (EAAT1-3) (Bianchi et al, 2008; Bianchi et al, 2006; Takano et al, 2001; Vanhoutte et al, 2009).

Inúmeras patologias como epilepsia, esquizofrenia, dor crônica e Alzheimer (Coyle, 2006a; Coyle, 2006b; Gispen & Biessels, 2000; Marino & Conn, 2006; Moult, 2009; Sultana & Butterfield, 2008), estão associadas a níveis alterados de glutamato extracelular. Assim a busca de moléculas que melhorem os sistemas de transporte glutamatérgico pode representar um avanço no tratamento de doenças com fisiopatologia associada ao metabolismo do glutamato.

### 1.4.2. Ciclo glutamato-glutamina

Após ser captado pelos astrócitos, o glutamato é convertido em glutamina pela ação da enzima glutamina sintetase – GS (EC 6.3.1.2), em um processo dependente de ATP. A glutamina é o aminoácido mais abundante encontrado na corrente sanguínea (0,7 mM) com inúmeras funções desde a metabólica e detoxificadora à manutenção dos níveis de neurotransmissores (glutamato e GABA) (Hertz & Zielke, 2004; Newsholme et al, 2003).

O ciclo glutamato-glutamina caracteriza-se pela liberação de glutamato, a partir das vesículas neuronais, na fenda sináptica, onde é captado pelos astrócitos e convertido a glutamina, que retorna em sentido oposto (figura 1). Após ser captada pelos neurônios, a glutamina é convertida novamente a glutamato pela enzima glutaminase (EC 3.5.1.2) e este é então armazenado em vesículas sinápticas podendo, posteriormente, ser liberado. Uma adequada regulação entre astrócitos e neurônios através do ciclo glutamato-glutamina é essencial para a homeostasia glutamatérgica (Banerjee et al, 2008; Danbolt, 2001; Magistretti, 2006; Newsholme et al, 2003; Pellerin et al, 2002).

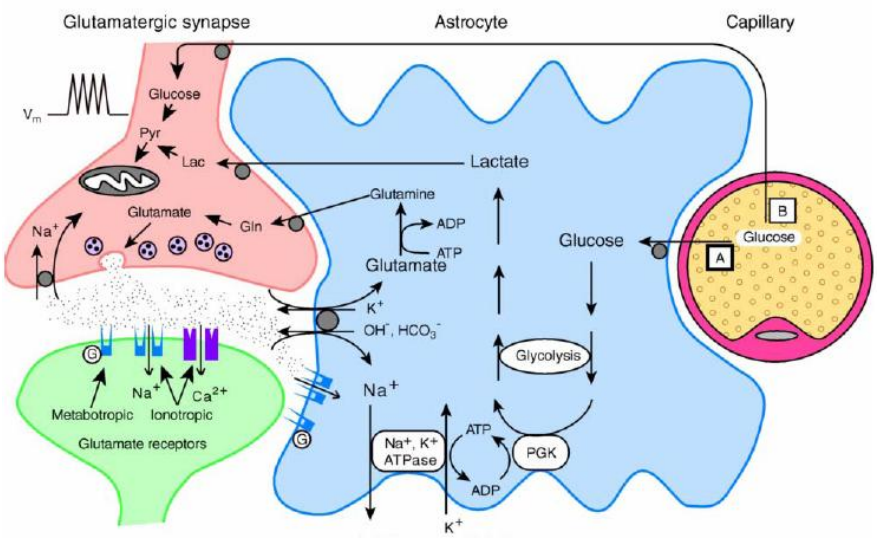


**Figura 1**. Representação esquemática de uma sinapse glutamatérgica. Adaptado de Magistretti et al, 1999.

### 1.4.3. Síntese de glutationa

Os astrócitos desempenham um papel fundamental na proteção do SNC contra o estresse oxidativo (Markiewicz & Lukomska, 2006; Wang & Bordey, 2008). A glutationa (GSH) constitui-se na principal defesa antioxidante do SNC e é produzida e secretada por astrócitos (Dringen, 2000; Pope et al, 2008). A GSH pode reagir diretamente contra espécies reativas de oxigênio (ERO) de forma não-enzimática ou sofrendo oxidação, formando glutationa dissulfeto (GSSG) pela ação da enzima glutationa peroxidase (GPx) (Pope et al, 2008).

O tripeptídeo GSH consiste de três aminoácidos (γ-glutamil-cisteinil-glicina) e sua síntese é um dos destinos do glutamato nos astrócitos. A GSH é sintetizada usando glutamato e cisteína como substrato da enzima γ-glutamil-cisteíno-sintetase formando o dipeptídeo γ-glutamil-cisteinil, o qual se combina com glicina numa reação catalisada pela glutationa sintetase, sendo ATP substrato de ambas as enzimas (figura 2). A cisteína é o substrato limitante para a síntese de GSH (Banerjee et al, 2008; Dringen, 2000; Pope et al, 2008).

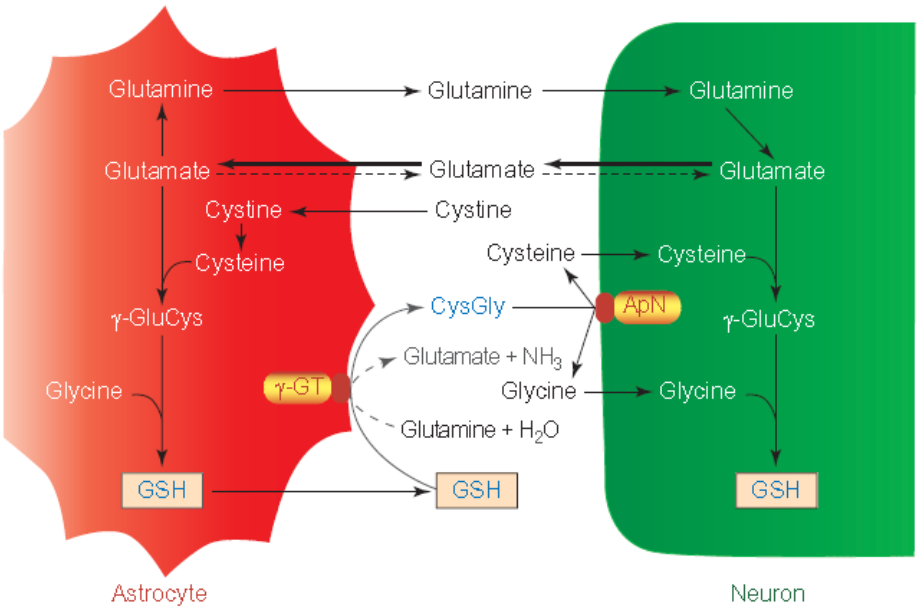


**Figura 2**. Representação da síntese intra e extracelular de glutationa. Hertz & Zielke, 2004.

Os astrócitos apresentam maiores níveis de GSH que os neurônios e estes são dependentes da GSH astrocítica para sua síntese. A GSH é secretada pelos astrócitos e extracelularmente sofre ação da enzima γ-glutamil transpeptidase (γGT) formando o dipeptídeo cisteinil-glicina e glutamato (ver figura 2). O dipeptídeo é clivado por outra ectopeptidase, a aminopeptidase N (ApN) em cisteína e glicina que são captadas pelos neurônios e utilizadas para síntese da GSH neuronal (Hertz & Zielke, 2004; Pope et al, 2008).

O trocador cistina/glutamato independente de sódio ($Xc^-$) também está envolvido na síntese de GSH, uma vez que este trocador promove a entrada de cistina para dentro da célula que é reduzida a cisteína, precursora limitante da síntese de GSH (Dringen, 2000). Assim, o trocador $Xc^-$, juntamente com os demais transportadores glutamatérgicos são críticos para manutenção dos níveis de GSH, representando proteção contra o estresse oxidativo (Lewerenz et al, 2006).

Dessa forma, a busca de substâncias que melhorem as defesas antioxidantes ou garantam proteção contra diminuição do conteúdo de GSH pode representar melhorias em doenças que caracteristicamente diminuem os níveis de GSH, como Parkinson e Alzheimer.

## 2. Radicais livres e espécies reativas de oxigênio

O termo radical livre (RL) se refere a uma estrutura química com um elétron desemparelhado, ou seja, ocupando um orbital atômico ou molecular sozinho. Isso o torna muito instável, extremamente reativo e com uma enorme capacidade de combinar-se inespecificamente a moléculas integrantes da

estrutura celular ou derivados de cada uma delas (Halliwell, 2006b; Halliwell, 2007).

Muitos RL existem *in vivo* e podem apresentar efeitos tóxicos ou fisiológicos (Forman, 2007). Fisiologicamente esses radicais são importantes para a função celular (Bergendi et al, 1999; Vargas & Johnson, 2009), para a atividade de neutrófilos na eliminação de infecções (Fortin et al) e em processos de sinalização celular (Forman, 2007).

Também em condições fisiológicas do metabolismo celular aeróbio o oxigênio molecular ($O_2$) é reduzido à água ($H_2O$). No entanto, uma parcela desse oxigênio utilizado na cadeia respiratória mitocondrial não é completamente reduzido, podendo ser convertido em RL como o radical superóxido ($O_2^{\bullet -}$) e o radical hidroxila ($OH^{\bullet}$), sendo que em condições patológicas há um aumento da produção desses radicais (Halliwell, 2007).

O termo espécies reativas de oxigênio (ERO) é frequentemente usado para incluir não apenas RL de oxigênio como radical superóxido ($O_2^{\bullet -}$) e hidroxila ($OH^{\bullet}$), mas também espécies que não são propriamente um RL, mas derivadas de $O_2$, como o peróxido de hidrogênio ($H_2O_2$) que são capazes de gerar RL (Halliwell, 2007). Além dessas, existem ainda as espécies reativas de nitrogênio (ERN), sendo o óxido nítrico ($NO^{\bullet}$) e o peroxinitrito ($ONOO^-$) os principais representantes (Halliwell & Whiteman, 2004).

Quando há produção excessiva de RL, eles podem oxidar biomoléculas como lipídios, proteínas e DNA (Halliwell, 2001). Assim, em relação aos efeitos maléficos das reações oxidantes ao organismo podemos citar a lipoperoxidação, a oxidação de lipoproteínas de baixa densidade (LDL), danos a proteínas com alteração de sua funcionalidade e alterações no DNA,

podendo causar mutações somáticas ou alterações na transcrição gênica (Halliwell, 2001; Halliwell & Whiteman, 2004).

**3. Estresse Oxidativo**

O termo estresse oxidativo se refere a uma alteração no equilíbrio entre a produção de espécies reativas e defesas antioxidantes (Halliwell, 2006a; Halliwell, 2007; Sies, 1991). Esse desequilíbrio envolve o balanço entre a produção de pró e antioxidantes favorecendo a formação de moléculas pró-oxidantes.

O estresse oxidativo pode promover adaptação, dano ou morte celular. No processo de adaptação as células podem compensar o estresse oxidativo por ERO e ERN aumentando a síntese de antioxidantes. Quando isso não é possível, ocorre dano a biomoléculas, onde caso não haja um sistema de reparo adequado ocorrerá morte celular (Halliwell, 2001; Halliwell, 2007).

Inúmeras doenças têm fisiopatologia associada ao estresse oxidativo, como por exemplo, Alzheimer, Huntington e Parkinson (Halliwell, 2006a). O prognóstico dessas doenças é agravado pelo processo de excitotoxicidade glutamatérgica mediada por receptores tipo NMDA que estão intimamente relacionados ao estresse oxidativo (Coyle & Puttfarcken, 1993; Gardoni et al, 2002). Além disso, na doença de Alzheimer ocorre ativação astrocítica e microglial que leva ao aumento de ERO e ERN. O NO é formado numa reação catalisada pela óxido nítrico sintase (NOS) e sofre regulação dos receptores glutamatérgicos (Coyle & Puttfarcken, 1993). Ele está envolvido nos danos ao metabolismo oxidativo, decorrentes de hipóxia e isquemia, e na disfunção neuronal que resulta em diminuição da memória espacial após hipoperfusão

cerebral crônica, um distúrbio presente antes, durante e após o desenvolvimento inicial da doença de Alzheimer (de la Torre & Aliev, 2005). É também precursor do peroxinitrito, um radical associado a várias doenças neurodegenerativas.

Para evitar os efeitos danosos das espécies reativas, existem importantes mecanismos de defesa antioxidante. Entre estes, destacam-se as enzimas antioxidantes: superóxido dismutase (SOD), catalase e glutationa peroxidase. A SOD catalisa a dismutação do superóxido ($O_2^{\bullet -}$) para formar $H_2O_2$, que é convertido à água e $O_2$ pela ação da catalase. A SOD localiza-se predominantemente nos astrócitos (Barbeito et al, 2004), que secretam SOD extracelular evitando a oxidação da GSH a GSSG (Pope et al, 2008). A GSH é uma importante defesa antioxidante não-enzimática, que juntamente com α-tocoferol, ácido ascórbico e bilirrubina sequestram espécies reativas de oxigênio e nitrogênio evitando assim o estresse oxidativo. Dessa forma, a busca de moléculas antioxidantes pode representar importantes avanços terapêuticos em doenças cuja patogenia esteja correlacionada ao estresse oxidativo.

## 4. Resveratrol

Nos últimos anos, o uso de produtos naturais, alimentos funcionais e uma dieta equilibrada, muitas vezes envolvendo restrição calórica, tem sido alvo de inúmeros estudos por apresentarem importantes efeitos benéficos à saúde. Além disso, com o aumento da expectativa média de vida populacional observamos aumento da incidência de doenças crônicas, merecendo destaque as doenças cardiovasculares, neurodegenerativas e demência. Assim, o

melhor entendimento dos mecanismos de ação dessas moléculas com potencial terapêutico para tais patologias têm sido amplamente estudado.

Nesse cenário, podemos destacar a espécie redox ativa resveratrol, um polifenol encontrado em inúmeras plantas que apresenta importantes efeitos benéficos à saúde (Pervaiz, 2003). Os polifenóis são divididos em duas categorias principais: flavonóides e não-flavonóides. Os flavonóides são os polifenóis mais abundantes e são encontrados em extratos de plantas e frutas; já os estilbenos[2] não-flavonóides são a menor classe, porém com muitos efeitos biológicos e entre eles, destaca-se o resveratrol (Dore, 2005).

O resveratrol foi primeiramente isolado em raízes de *Veratum grandiflorum* O. Loes, em 1940, e posteriormente em 1963 em raízes de *Polygonum cuspidatum*, uma planta utilizada na medicina tradicional chinesa e japonesa. Além dessas plantas, o resveratrol está presente em mais de setenta espécies vegetais, como no amendoim e também em frutas vermelhas – morango, amora e framboesa (Dudley et al, 2009), sendo também muito abundante em cascas e sementes de uvas de *Vitis vinifera* (Baur & Sinclair, 2006) e consequentemente nos vinhos tinto e branco (Soleas et al, 1997a).

O principal significado do resveratrol na biologia da planta consiste em protegê-la de ataques externos, especialmente infecções fúngicas e radiações UV, como também de privação de nutrientes, uma propriedade que inclui o resveratrol na classe das fitoalexinas, também conhecida como antibiótico das plantas (Baur & Sinclair, 2006; Langcake & Pryce, 1977).

São encontrados duas isoformas de resveratrol, os isômeros c*is* e *trans* (figura 3), sendo que as uvas apresentam principalmente o isômero *trans* e o

[2] Os estilbenos são compostos que contêm grupamento 1,2-difeniletileno (Dore, 2005).

vinho apresenta ambos isômeros. O processo de isomerização ocorre pela ação da luz e o isômero *trans* (3,5,4'-triidroxi-*trans*-estilbeno) é o principal responsável pelos efeitos biológicos do resveratrol em mamíferos (Soleas et al, 1997a).

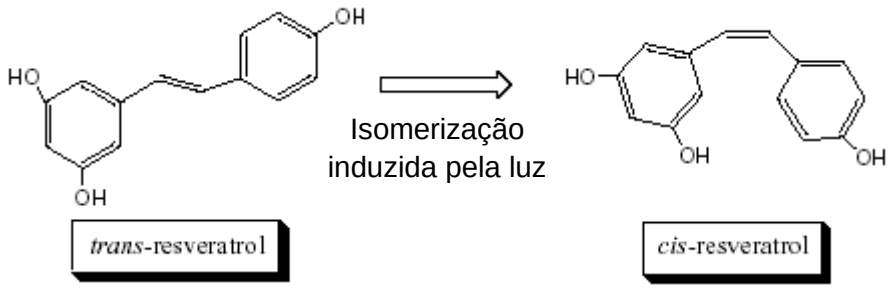


**Figura 3.** Isomerização do resveratrol**.** Adaptado de Doré, 2005

O resveratrol é sintetizado nas cascas das uvas a partir da condensação de três moléculas de malonil-CoA e uma de 4-coumaroil-CoA, pela ação da enzima resveratrol sintase (figura 4) (Soleas et al, 1997b). Sendo o resveratrol uma fitoalexina, seus níveis podem variar em função da presença de fungos, cultivo da uva, composição dos solos, exposição ao sol, localização geográfica e também com o processo de fabricação e conservação dos vinhos (Soleas et al, 1997a). Nos vinhos tintos a concentração de resveratrol varia de 0,1 – 14,3 mg/L, já nos vinhos brancos esse valor é reduzido em cinco vezes, devido ao menor tempo de contato com a casca durante o processo de fabricação (Baur & Sinclair, 2006; Dudley et al, 2009). Os vinhos tintos produzidos na Serra Gaúcha apresentam uma das mais elevadas concentrações de resveratrol, devido à alta umidade do solo, que favorece a proliferação de fungos e, consequentemente, aumenta a produção da fitoalexina resveratrol na planta.

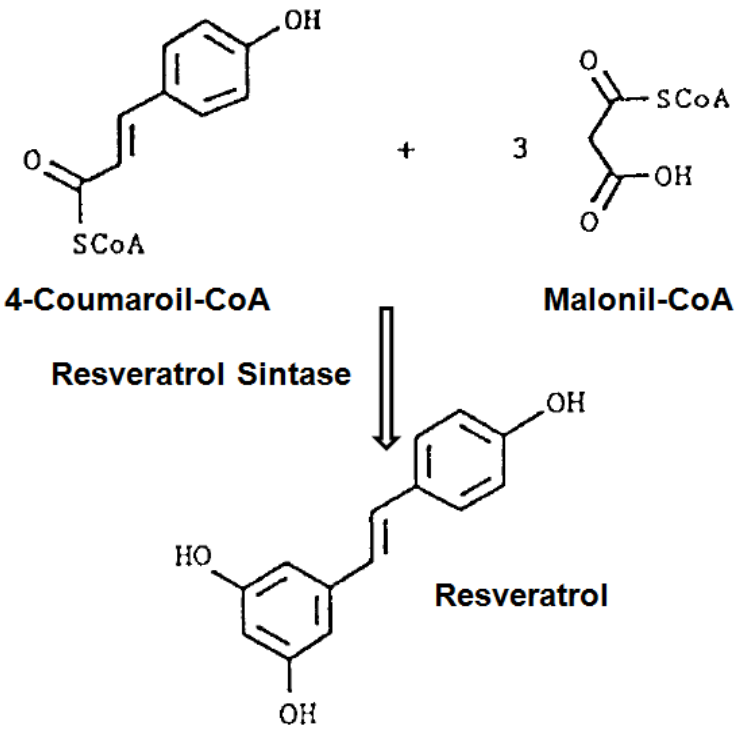


**Figura 4**. Representação esquemática da síntese de resveratrol. Adaptado de Soleas et al, 1997.

Há inúmeras lendas relatando o consumo e produção de vinhos pela humanidade. A Bíblia relata que Noé, após desembarcar de sua arca plantou um vinhedo do qual fez vinho. Já os gregos realizavam “Simpósios” que literalmente significa “bebendo junto” e dessa forma se reuniam para beber vinho em ambientes de alegre convívio (Soleas et al, 1997b). Os gregos também fizeram relatos do uso medicinal do vinho, onde Hipocrates fez observações sobre suas propriedades que são citadas em textos da história da medicina, e Galeno escreveu um tratado denominado “De antídotos” sobre o uso de preparações a base de vinhos e ervas usados como antídotos de venenos (Soleas et al, 1997b).

A partir de 1979, o vinho tinto, uma das principais fontes de resveratrol, vem ganhando destaque especial devido ao estudo de Leger et al (St Leger et al, 1979) que demonstrou que o consumo de vinho estaria correlacionado negativamente com a incidência de doenças cardíacas isquêmicas. Em 1992, outro estudo atribuiu ao vinho a baixa incidência de doenças cardiovasculares na população do sul da França, uma região onde tradicionalmente se consome uma dieta rica em gorduras saturadas. Esta controvérsia foi chamada de "Paradoxo Francês" (Renaud & de Lorgeril, 1992).

### 4.1. Efeitos biológicos do resveratrol

O resveratrol apresenta inúmeros efeitos biológicos, muitos independentes de sua atividade antioxidante, onde ele atua como "scavenger" (sequestrador) de ERO. Além disso, embora o resveratrol não tenha um receptor específico, ele modula várias vias de sinalização, atuando em vários receptores a nível celular e molecular (Baur & Sinclair, 2006). Ele é absorvido e metabolizado pelas células, sendo seus principais metabólitos o resveratrol glicuronídeo e o resveratrol sulfato (Wenzel & Somoza, 2005), e também se liga a albumina e lipoproteínas, exercendo assim alguns de seus efeitos biológicos (Jannin et al, 2004). A administração oral de resveratrol em humanos apresenta dois picos plasmáticos do mesmo, indicando que há recirculação entero-hepatica do resveratrol (Boocock et al, 2007).

Entre os mais significativos efeitos do resveratrol e primeiramente estudados destaca-se a sua atividade cardioprotetora (Miller & Rice-Evans, 1995). Este efeito envolve a diminuição da oxidação da lipoproteína de baixa densidade (Fremont et al, 1999), ação vasodilatadora, provavelmente via óxido

nítrico (Das & Das; Lekakis et al, 2005) e inibição da agregação plaquetária (Bertelli et al, 1995). Este efeito pode ser devido a ação inibitória do resveratrol sobre as ciclooxigenases, COX 1 e COX 2 (Baur & Sinclair, 2006), sendo que essa ação caracteriza um importante efeito biológico do resveratrol, a atividade anti-inflamatória, onde ele reduz as inflamações agudas e crônicas, evitando todas as características do processo inflamatório (Chen et al, 2005).

O resveratrol também exerce efeito antitumoral, inibindo os três estágios da carcinogênese: iniciação, promoção e progressão (Jang et al, 1997). Uma das hipóteses para esse efeito envolve a inibição da COX 2 pelo resveratrol (Murias et al, 2004). Ele também modula várias vias de sinalização que regulam o ciclo celular e apoptose (Signorelli & Ghidoni, 2005), e recentemente foi associado ao aumento da longevidade em mamíferos, envolvendo uma família de proteínas denominadas Sirtuínas (Baur et al, 2006).

O SNC também é alvo terapêutico do resveratrol, pois ele atravessa a barreira hemato-encefálica e exerce importantes efeitos em situações normais ou patológicas (Baur & Sinclair, 2006). Muitos desses estudos são em modelos de isquemia ou doenças neurodegenerativas, como Parkinson e Alzheimer, onde o resveratrol exerce importantes efeitos neuroprotetores, mediados por sua ação antioxidante (Sharma & Gupta, 2002), ou por modulação de proteínas como heme oxigenase 1 (HO1) (Sakata et al), óxido nítrico sintase (NOS) (Bastianetto et al, 2000) e SIRT1 (Della-Morte et al, 2009). Este polifenol ainda modula fatores de transcrição – fator nuclear da cadeia κ de linfócitos B (NFκB) (Lukiw et al, 2008; Sarkar et al, 2009); e vias de sinalização como a proteína cinase ativada por mitógeno (MAPK) (de Almeida et al, 2008; Sarkar et al, 2009; Zhou et al) e fosfatidil-inositol-3-cinase (PI3K) (Zamin et al, 2006).

Várias doenças neurológicas têm fisiopatologia associada ao processo de excitotoxicidade glutamatérgica que também está relacionado ao estresse oxidativo (Coyle & Puttfarcken, 1993; Fukui et al; Gao et al, 2006; Virgili & Contestabile, 2000). Nesse contexto Gao et al, 2006, demonstraram que o resveratrol é capaz de inibir receptores glutamatérgicos, dados em acordo com Virgili & Contestabile, 2000, que mostraram que o resveratrol diminui o dano neuronal induzido por cainato. Fukui et al, 2010, por sua vez, evidenciaram que resveratrol protege células neuronais contra estresse oxidativo induzido por glutamato.

Nosso grupo tem mostrado que o resveratrol modula importantes funções gliais como captação de glutamato, atividade da glutamina sintetase e níveis de GSH (de Almeida et al, 2007; dos Santos et al, 2006). Sendo assim, o resveratrol surge como um potencial agente farmacológico relacionado ao estresse oxidativo, à excitotoxicidade glutamatérgica, mediando então importantes efeitos relacionados à atividade glial, representando uma nova estratégia terapêutica a fisiopatologias do SNC.

## OBJETIVOS

### Objetivo geral

Avaliar o efeito do resveratrol sobre parâmetros gliais em células C6 visando o melhor entendimento do seu mecanismo de ação, verificando se esta resposta é dependente do estado redox celular.

### Objetivos específicos

1. Avaliar o efeito genotóxico do resveratrol *per se* e após insulto oxidativo com $H_2O_2$, assim como a viabilidade celular e o dano a lipídios de membrana.
2. Investigar o efeito do resveratrol *per se* sobre o metabolismo do glutamato e mediante insulto oxidativo com $H_2O_2$.
3. Estudar os efeitos do resveratrol sobre sistemas de defesa antioxidante e os prováveis mecanismos envolvidos nessa resposta.

# PARTE II

## CAPÍTULO I

Objetivo 1 – Avaliar o efeito genotóxico do resveratrol *per se* e após insulto oxidativo com $H_2O_2$, assim como a viabilidade celular e o dano a lipídios de membrana.

**Artigo publicado no periódico Toxicology in Vitro**

### Actions of redox-active compound resveratrol under hydrogen peroxide insult in C6 astroglial cells

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ABSTRACT

The mechanisms by which resveratrol (3,5,4′-*trihydroxy-stilbene*) imparts neural effects is not well understood. We previously demonstrated that, depending upon the concentration of resveratrol and the cell type, this compound exerts anti-or pro-oxidant effects. In the present study, we investigated the effects of resveratrol on $H_2O_2$-mediated genotoxicity in C6 astroglial cells (I – 1 mM $H_2O_2$/30 min or II – 0.1 mM $H_2O_2$/6 h), evaluated by micronucleus assay, lipid peroxidation (TBARS) and membrane integrity. $H_2O_2$ increased micronuclei to 1.5 (I) and 1.7-fold (II), compared to control cells. This DNA damage was prevented (I) or partially prevented (II) by resveratrol. Oxidative insult also increased TBARS, 52% in I and 38% in II, $P < 0.05$. These effects were prevented by resveratrol in I and increased in II (70% of increase). Present data contribute to the understanding of resveratrol effects under oxidative stress damage.



## 1. Introduction

There is an intense interest regarding the role of nutrition and specific diet constituents in the etiology and prevention of neurological disorders (Joseph et al., 2009). The polyphenolic compound, resveratrol (3,5,4′-trihydroxy-stilbene), is a naturally occurring phytochemical that was detected in 1963 in the dried roots of *Polygonum cuspidatum* (Itadori tea), traditionally used in Chinese and Japanese medicines as an anti-inflammatory agent (Nonomura et al., 1963). About 10 years later, this compound was also detected in grapevines (*Vitis vinifera*) (Langcake and Pryce, 1977) and, last decade, in wine in 1992 (Siemann and Creasy, 1992).

Resveratrol has been proposed as a pharmacological tool for neuroprotection against neuronal injury, including age-associated chronic diseases (Harikumar and Aggarwal, 2008; Lukiw et al., 2008), ischemic brain damage (Dong et al., 2008) and cerebral models of stroke (Sinha et al., 2002). However, the mechanisms by which resveratrol imparts its effects are not well understood. It has been demonstrated that its anti-oxidant properties could be due to a direct scavenging effect or the result of the activation of pathways that up regulate the natural anti-oxidant cell defenses (Baur and Sinclair, 2006). In addition, depending on the concentration of resveratrol and the cell type, the compound has shown pro-oxidant effects (de la Lastra and Villegas, 2007; Quincozes-Santos et al., 2007, 2009).

Numerous studies have reported increased reactive oxygen species (ROS), such as hydrogen peroxide ($H_2O_2$) and oxidative stress, in various neurodegenerative disorders (Nakamura and Lipton, 2007). Excessive ROS can lead to lipid, protein and DNA oxidation, causing cell damage to some cellular constituents. As a result, irreparable DNA damage is involved in carcinogenesis, aging and other degenerative diseases (Perticone et al., 1997).

We have recently demonstrated in C6 astroglial cells, the genoprotective effects of resveratrol against $H_2O_2$-induced DNA damage, assessed by the alkaline single-cell gel electrophoresis assay. In addition, we have also shown that resveratrol *per se* induced a slight time and dose-dependent DNA damage (Quincozes-Santos et al., 2007). The rat glial cell line C6 possesses important chemical and functional analogy to normal rat brain astrocytes (Ren et al., 2000). This lineage has served as a useful astroglial cell model, including in studies on oxidative stress with responsive effects to redox-active compounds (Han et al., 1997; Slamon and Pentreath, 2000).

In the present study, we investigated the effects of resveratrol on $H_2O_2$-mediated genotoxicity in C6 astroglial cells, determined through micronucleus assay. The membrane lipid damage was also measured by production of thiobarbituric acid-reactive substances (TBARS). Cell viability and integrity were also evaluated by MTT assay and LDH leakage.

## 2. Experimental procedures

### 2.1. Cell culture

The C6 astroglial cells were obtained from the American Type Culture Collection (Rockville, MD, USA) and were maintained,

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essentially, according to the procedure previously described (dos Santos et al., 2006). The C6 lineage cells was originally derived from rat brain tumors induced by *N*,*N*′-nitroso-methylurea (Benda et al., 1968). This cell line has oligodendrocytic, astrocytic, and neuronal properties (Parker et al., 1980) and is a widely used astrocyte-like cell line (Feng and Zhang, 2004; Mangoura et al., 1989). After 50 passages, cells start to express glial fibrillary acidic protein (GFAP) and assume astrocyte characteristics. Late passages cells (99% GFAP positive) were seeded in flasks and cultured in DMEM (GIBCO) (pH 7.4) containing 5% fetal bovine serum (FBS), 2.5 mg/mL Fungizone® and 100 U/L gentamicin. Cells were kept at a temperature of 37 °C in an atmosphere of 5% $CO_2$/95% air. Exponentially-growing cells were detached from the culture flasks using 0.05% trypsin/ethylene-diaminetetracetic acid (EDTA) and seeded at $5 \times 10^3$ cells/cm$^2$ in 24- or 6-well plates.

### 2.2. Cell treatments

After pre-confluence (micronucleus assay) or confluence (other assays), the culture medium was removed by suction and cells were pre-treated in the absence or presence of 100 μM of *trans*-resveratrol (Sigma) for 1 h, in serum-free DMEM (pH 7.4). After pre-treatment, incubation medium was maintained and $H_2O_2$ was added as follows: model I – 1 mM $H_2O_2$ for 30 min (a high concentration of $H_2O_2$ in a short time – acute effect) and model II – 0.1 mM $H_2O_2$ for 6 h (a less concentration of $H_2O_2$ in a larger time). During incubations, cells were maintained at 37 °C in an atmosphere of 5% $CO_2$/95% air. Control cells were exposed to 0.25% ethanol vehicle. For all parameters analyzed, the results obtained with vehicle were not different from those obtained under basal conditions without ethanol. For the micronucleus assay, the treatment was carried out as follows: cells were incubated with resveratrol at different concentrations (10, 100 and 250 μM) or 100 μM ascorbic acid (AA, Sigma) for 1, 6, 12, 24 and 48 h. This concentration of ascorbic acid was used as a positive control of micronuclei induction established for C6 cells in our lab (Bobermin, personal communication). Each experiment was measured in triplicate and repeated three times ($N = 3$).

### 2.3. Cell viability assays

#### 2.3.1. Colorimetric MTT assay

Cell viability assay was performed by the colorimetric [3(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide] (MTT-Sigma) assay (Hansen et al., 1989). Yellow MTT is reduced to purple formazan, due to the metabolic activity of living cells. MTT (0.05 mg/mL – Sigma) was added to cells at analysis time points, according to the experimental design and allowed to metabolize for 30 min at 37 °C. Blue crystals were dissolved in 300 μL dimethylsulfoxide (DMSO, Merck). Optical density was measured using a spectrophotometric microtiter plate reader at 560 nm and 620 nm.

#### 2.3.2. Lactate dehydrogenase (LDH) assay

LDH is a cytosolic enzyme that is released into the cytoplasm upon membrane integrity loss. After different treatments, LDH activity (oxidation of lactate to pyruvate in the presence of NAD, which is reduced to NADH) was evaluated in the culture medium using a colorimetric assay (Kit from Doles, GO, Brazil). Optical density was measured using a spectrophotometric microtiter plate reader at 492 nm.

#### 2.3.3. Micronucleus assay

Micronuclei are chromosome segments that were not incorporated into nucleus during mitosis. The *in vitro* micronucleus assay permits the detection of substances that cause chromosomal breaks and thus result in incorrect separation of chromosomes in mitosis. The micronucleus assay was performed, as described (Reyes et al., 2001) with modifications. Firstly, cells were cultured in a 24-well plate on coverslips, and incubated for 1, 6, 12, 24 or 48 h in conditions described above and in the absence or presence of resveratrol (10, 100 or 250 μM). Models I and II of oxidative insult were also employed. The concentrations of resveratrol used in these experiments were the same as those reported previously (Quincozes-Santos et al., 2007). After treatment, cells were incubated with 2 μg/ml cytochalasin B for 18 h, fixed with chilled methanol/glacial acid (3:1) for 5 min and stained with Giemsa. In each experiment, two slides per variable were analyzed by evaluating the presence of micronucleus (see the representative micronuclei at Fig. 1) in 1000 binucleated cells/slide.

### 2.4. Thiobarbituric acid-reactive substances (TBARS) measurement

Lipid peroxidation can be evaluated by the thiobarbituric acid reactive substance assay. This method evaluates the oxidative stress assayed for malondialdehyde, the last product of lipid breakdown caused by oxidative stress. The assay was performed, as previously described (Esterbauer and Cheeseman, 1990). Briefly, 100 μL of cellular suspension were added to 200 μL of cold 10% trichloroacetic acid and 300 μL of 0.67% TBA in 7.1% sodium sulfate in a boiling water bath for 1 h. The mixture was placed in cold water for 3 min. Afterwards 400 μL of butyl alcohol were added and samples were centrifuged at 5000*g* for 5 min. Pink-stained TBARS was determined in resulting supernatants in a spectrophotometric microtiter plate reader at 532 nm. A calibration curve was performed using 1,1,3,3-tetramethoxypropane. Data were expressed as nmol TBARS/mg protein.

#### 2.4.1. Protein determination

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

#### 2.4.2. Statistical analysis

Data from the experiments are presented as means ± SD. Resveratrol and different doses of $H_2O_2$ were analyzed statistically by two-way analysis of variance (ANOVA) followed by the Tukeýs test. Values of $P < 0.05$ were considered to be significant. Data from the micronucleus assay were analyzed statistically by one-way ANOVA followed by the Tukey's test. All analyses were performed using statistical package for the social sciences (SPSS) software in a PC-compatible computer.

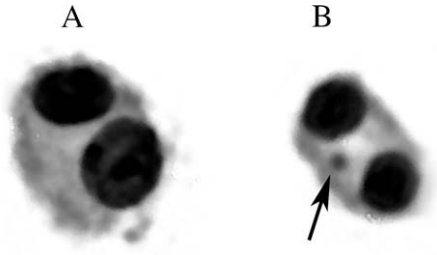


**Fig. 1.** Representative images of binucleated C6 cells, analysed by cytochalasin-B blockage of cytokinesis. Images were viewed with a Nikon inverted microscope, using a TE-FM Epi-Fluorescence accessory and images were transferred to a computer with a digital camera (Sound Vision Inc., Wayland, MA). A, indicates an acentric chromosome fragment (centromere negative micronucleus). B, indicates micronucleus (arrow) containing a whole chromosome (centromere positive micronucleus).

## 3. Results

Firstly, we evaluated integrity and metabolic activity of the cells in models I and II of oxidative insult by measuring LDH content and MTT reduction, respectively (Table 1). No significant changes were observed in these parameters, compared to control conditions.

After different treatments, the effect of resveratrol on chromatine damage was measured by micronucleus assay. As shown in Table 2, only 250 μM resveratrol induced an increase (30%) in micronucleated cells ($P < 0.05$) after 24 h of treatment. The micronucleus frequency was not measured at 48 h with 250 μM of resveratrol, due to the presence of cell death and in agreement with our previous results (dos Santos et al., 2006). In order to compare the effect of resveratrol with a well studied redox-active compound, we incubated cells with 100 μM vitamin C (ascorbic acid). In contrast to ascorbic acid, resveratrol (up to 100 μM) did not significantly alter the micronuclei frequency, compared to control cells.

In order to broaden and enrich the studies regarding the effect of resveratrol on $H_2O_2$-induced genotoxicity in astroglial cells, we decided to investigate chromosome damage under oxidative insult in the presence or absence of 100 μM resveratrol. An increase in the genotoxic damage is associated with an increased overall risk of cancer (Hagmar et al., 1998). The micronucleus is a well known marker of genotoxicity and any reduction in the frequency of the genotoxic endpoint gives an indication of the antigenotoxicity of a particular compound (Siddique and Afzal, 2009). Data from Fig. 2 show that $H_2O_2$ induced an increase in the number of micronuclei to 1.5 ($F_{(3,36)} = 12.590$) and 1.7 ($F_{(3,36)} = 15.530$)-fold in models I and II, respectively ($P < 0.05$), compared to control conditions. In addition, the $H_2O_2$-induced nuclear damage was completely prevented by resveratrol in model I ($F_{(3,36)} = 9.270$, Fig. 2A) and partially prevented in model II ($F_{(3,36)} = 10.710$, Fig. 2B), $P < 0.05$.

As there are significant changes in lipid peroxidation under oxidative stress, we have estimated the lipid peroxidation in terms of quantifying malondialdehyde (MDA). Lipid peroxidation products of poly unsaturated fatty acids (PUFAs) are considered of importance for genotoxic effects (Siddique and Afzal, 2009). As shown in Fig. 3A, in model I, resveratrol decreased basal TBARS levels (about 12%) (from 6.1 ± 0.41 to 5.4 ± 0.23 nmol/mg protein), $F_{(3,36)} = 11.250$ ($P < 0.05$). As expected, under oxidative insult, there was an increase in TBARS to 9.3 ± 0.61 nmol/mg protein ($F_{(3,36)} = 11.250$, $P < 0.05$). This effect was completely prevented by resveratrol ($F_{(3,36)} = 13.550$, $P < 0.001$). In model II (Fig. 3B), resveratrol did not change TBARS levels, however under oxidative insult there was an increase of about 38% compared to control values (from 7.4 ± 0.62 to 10.2 ± 0.73 nmol/mg protein), $F_{(3,36)} = 19.650$ ($P < 0.001$). This effect was significantly increased by resveratrol (12.6 ± 0.95 nmol/mg protein).

**Table 1**
Effects of resveratrol and $H_2O_2$ on C6 cell viability.

| Treatments | Cell viability assays (% of control) | | | |
|---|---|---|---|---|
| | MTT | | LDH | |
| | Model I | Model II | Model I | Model II |
| Control | 100 | 100 | 100 | 100 |
| Resv. | 99 ± 4.9 | 108 ± 5.3 | 99 ± 4.2 | 98 ± 5.6 |
| $H_2O_2$ | 97 ± 6.1 | 90 ± 5.7 | 97 ± 3.3 | 96 ± 3.7 |
| Resv. + $H_2O_2$ | 98 ± 5.8 | 95 ± 5.6 | 98 ± 4.3 | 99 ± 4.7 |

Cells were pre-incubated for 1 h in the presence of 100 μM resveratrol, followed by the addition of 1 mM $H_2O_2$ for 30 min (model I) or 0.1 mM $H_2O_2$ for 6 h (model II). After treatments, extracellular LDH activity and MTT reduction were measured, as described in the Material and Methods section. Data (% of control 0.25% ethanol) represent means ± SD from three experimental determinations performed in triplicate.

**Table 2**
Effects of resveratrol and ascorbic acid (AA) on micronucleus frequency in C6 cells.

| Time (h) | Micronuclei (arbitrary unit) | | | | |
|---|---|---|---|---|---|
| | Resveratrol (μM) | | | | AA (μM) |
| | 0 | 10 | 100 | 250 | 100 |
| 1 | 1.0 ± 0.05 | 1.1 ± 0.05 | 1.1 ± 0.04 | 1.1 ± 0.04 | 1.3 ± 0.07* |
| 6 | 1.0 ± 0.06 | 1.1 ± 0.05 | 1.1 ± 0.05 | 1.2 ± 0.05 | 1.5 ± 0.05* |
| 12 | 1.0 ± 0.06 | 1.0 ± 0.04 | 1.0 ± 0.05 | 1.0 ± 0.05 | 1.3 ± 0.06* |
| 24 | 1.0 ± 0.09 | 1.0 ± 0.09 | 1.2 ± 0.07 | 1.3 ± 0.08* | 1.8 ± 0.08* |
| 48 | 1.0 ± 0.10 | 1.0 ± 0.12 | 1.1 ± 0.12 | | |

Cells were incubated for different times (1–48 h) in the presence of resveratrol (10, 100 and 250 μM) or ascorbic acid (100 μM). Data (fold of control 0.25% ethanol – arbitrary unit) represent the means ± SD from three experimental determinations performed in triplicate.

* Significant differences compared to control from each incubation time. $P < 0.05$.

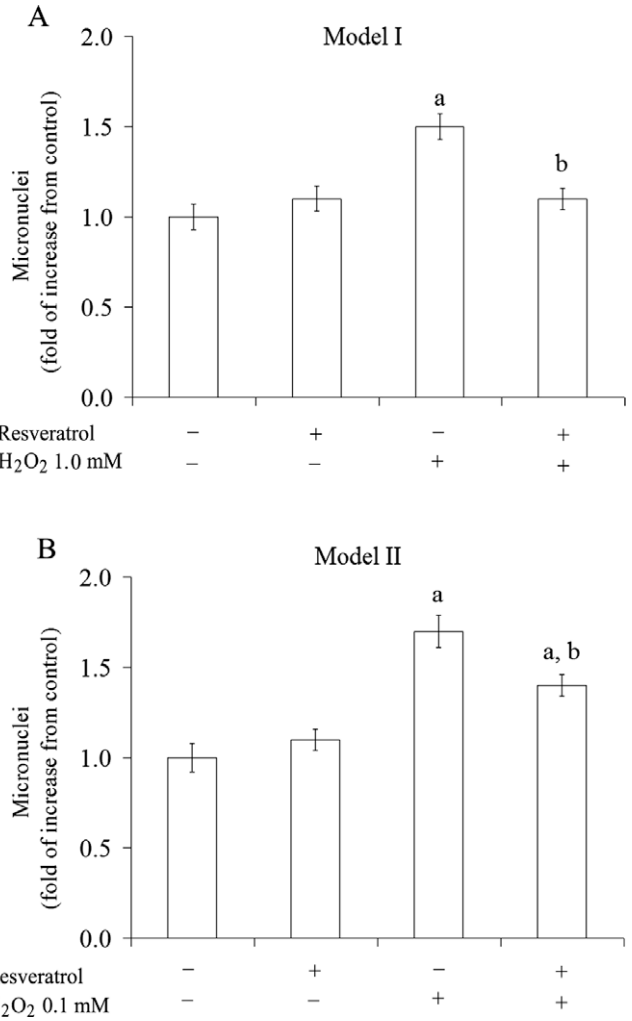


**Fig. 2.** Effects of resveratrol and $H_2O_2$ on micronucleus frequency in C6 astroglial cells. Cells were pre-incubated for 1 h in the presence of 100 μM resveratrol, followed by the addition of 1 mM $H_2O_2$ for 30 min (model I) or 0.1 mM $H_2O_2$ for 6 h (model II). Data represent means ± SD from three experimental determinations performed in triplicate. (A) Significant differences from control values; (B) significant differences from $H_2O_2$. $P < 0.05$.

## 4. Discussion

In the present study we demonstrate that, depending upon the oxidative conditions, resveratrol may have different effects on astroglial cells. This study was performed in two *in vitro* models

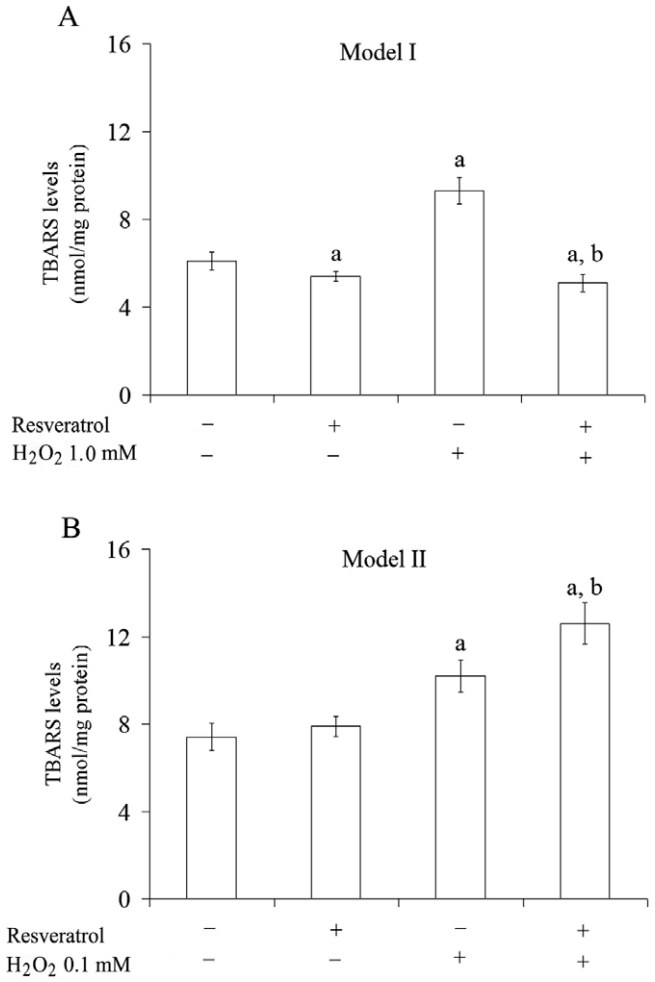


**Fig. 3.** Effects of resveratrol and $H_2O_2$ on TBARS levels in C6 astroglial cells. Cells were pre-incubated for 1 h in the presence of 100 μM resveratrol, followed by the addition of 1 mM $H_2O_2$ for 30 min (model I) or 0.1 mM $H_2O_2$ for 6 h (model II). Data represent means ± SD from three experimental determinations performed in triplicate. (A) Significant differences from control values; (B) significant differences from $H_2O_2$. $P < 0.001$.

of oxidative injury, where cell integrity and viability were evaluated by LDH release and MTT reduction capacity assays, respectively. The useful of these models for study is indicated by oxidative stress because there are not significant changes in cell integrity and viability.

Due to the inefficiency of our endogenous defense systems as well as the existence of some physiopathological situations, such as cigarette smoke, inflammation and ischaemia/reperfusion, ROS can be produced in excess, and increasing amounts of dietary anti-oxidants will be needed for diminishing the cumulative effect of oxidative damage over an individual's life span (Sun et al., 2008). The nervous tissue is rich in both unsaturated fatty acids and iron that coupled with its high aerobic metabolic activity, makes it particularly susceptible to oxidative damage (Vieira de Almeida et al., 2008). Natural polyphenols, including resveratrol, are considered to be chemopreventive agents able to prevent cell damage caused by reactive oxygen species, targeting not only lipids and proteins but also DNA in living cells (Murias et al., 2005). Therefore, their potential pharmacological or toxicological effects in the brain, under physiological or pathological conditions, especially in glial cells, deserve special attention. Among neural cells, astrocytes are no longer considered to be passive cells that merely provide structural support for neurons, as was believed in the past. Recent evidence has demonstrated new roles for astrocytes in both neuroprotection and neurodegeneration (Wang and Bordey, 2008). In this context, targeting astrocytes as mediators for neuroprotective drugs may be a promising strategy.

We have demonstrated, in astroglial cells, that resveratrol is able to modulate important neurotrophic functions under different oxidative conditions (de Almeida et al., 2007; Vieira de Almeida et al., 2008). In addition, our studies (Quincozes-Santos et al., 2009), in agreement with others (de la Lastra and Villegas, 2007), have demonstrated that resveratrol can exhibit anti- or pro-oxidant properties, depending upon the concentration and oxidant conditions. We have used two different models (I and II) of oxidative insult. In model I, $H_2O_2$ exposure induces an intense (1 mM $H_2O_2$) and acute (30 min) damage. In model II, $H_2O_2$ exposure induces a less intense (0.1 mM $H_2O_2$), but lasting (6 h) damage. Resveratrol usually shows an anti-oxidant effect in model I and a pro-oxidant effect in model II, with regard to astroglial functions, such as glutamate uptake, glutamine synthetase activity and glutathione content. We have also shown a protective and toxic effect of resveratrol against $H_2O_2$-induced DNA damage, as evaluated by the comet assay (Quincozes-Santos et al., 2007).

In the present study, we evaluated the presence of micronuclei by the micronucleus assay, a technique that has emerged as one of the preferred methods for assessing genotoxicity (Fenech, 2007). There is now growing evidence that, under physiological conditions, resveratrol additionally contributes to the maintenance of genome stability (Gatz and Wiesmuller, 2008). In this context, the study of DNA damage by this polyphenol is relevant and our data contribute to the proposal that glial cells are targets of resveratrol, which could be involved in the therapeutic response of this polyphenol, to improve neuroglial plasticity.

In both models studied, resveratrol was able to protect cells against $H_2O_2$-induced genotoxicity, in agreement with previous data from the comet assay (Quincozes-Santos et al., 2007). Ascorbate, at the same concentration as that of resveratrol, increased the number of micronuclei. This effect could be due to the induction of ROS in the presence of this compound with subsequent DNA damage, as previously demonstrated (Halliwell, 2008).

Oxidative stress and free radical production can promote oxidative modification of lipids, as quantified by the measurement of end products of lipid peroxidation screening by the TBARS assay. In model I, resveratrol was able to significantly prevent $H_2O_2$-induced TBARS. However, in model II, this polyphenol had an opposite effect, increasing the effect of $H_2O_2$. It is interesting to observe that resveratrol *per se* was able to decrease TBARS in astroglial cells, compared to control cells. The level of lipid peroxidation in cells is controlled by various cellular defense mechanisms consisting of enzymatic and non-enzymatic scavenger systems (Halliwell and Gutteridge, 1984). A protective effect of resveratrol against lipid peroxidation suggests that this polyphenol is able to modulate these cell defense mechanisms.

The present study shows that the cell response to resveratrol is dependent upon the oxidant conditions of the milieu. Different data indicate that the mechanistic basis of the neuroprotective activity of resveratrol does not only rely on the general free radical trapping or anti-oxidant activity *per se* in neurons, but also on glial cell signaling modulation. In addition, oxidation of polyphenols produces $O_2^{\cdot-}$, $H_2O_2$ and a complex mixture of semiquinones and quinones (Halliwell, 2008), all of which share potentially cytotoxic properties. Therefore, a clearer appreciation of the potential therapeutic utility of anti-oxidants may emerge only when the complexity of their effects on mechanisms that interact to determine the extent of oxidative damage *in vivo* are more fully defined and understood. In this context, clinical trials are therefore warranted with dietary resveratrol and also administered in combination with other anti-oxidants. However, since no data are available to

indicate whether resveratrol is pro-oxidant *in vivo* in the human peripheral and cerebral tissues, caution is necessary with its therapeutic use.

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## CAPÍTULO II

Objetivo 2 – Investigar o efeito do resveratrol *per se* sobre o metabolismo do glutamato e mediante insulto oxidativo com $H_2O_2$.

**Artigo publicado no periódico Neurotoxicity Research**

### The Janus Face of Resveratrol in Astroglial Cells

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DOI 10.1007/s12640-009-9042-0

# The Janus Face of Resveratrol in Astroglial Cells

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**Abstract** Astroglial cells are key modulators of neuropathology events. Resveratrol, a redox-active compound present in grapes and wine, has a wide range of biological effects. The aim of this study was to investigate whether resveratrol is able to prevent hydrogen peroxide ($H_2O_2$)-induced oxidative damage in C6 astroglial cells. We found that following a short oxidative insult (Model I—1 mM $H_2O_2$/30 min), resveratrol increased glutamate uptake (60%), glutamine synthetase (GS) (139%), glutathione (GSH) (120%), and S100B secretion (24%); and attenuated DCFH oxidation (34%) as compared to $H_2O_2$ values. Under less intense (0.1 mM $H_2O_2$), but lasting (6 h) insult (Model II), resveratrol had an opposite effect, potentiating the $H_2O_2$-induced decrease in glutamate uptake (from 34 to 63%), in GS (from 22 to 50%), in GSH (from 22 to 54%), and also potentiating DCFH oxidation (from 24 to 38%). The transcription factor, NF-$\kappa$B, was activated in both models. Cell morphology alterations were also observed in the presence of $H_2O_2$ with process-bearing cells, accompanied by cell body retraction and actin reorganization. This effect was not prevented by resveratrol, but was prevented by lysophosphatidic acid (LPA), a specific upstream positive regulator of Rho A. In summary, these findings showed that resveratrol, a redox-active compound, was able to modulate important neurotrophic function of astroglial cells under different oxidative conditions.

**Keywords** C6 · Glutamate · Glutamine synthetase · GSH · $H_2O_2$ · Neuroprotection · S100B

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## Introduction

The redox-active compound, resveratrol (3,5,4′-trihydroxy-*trans*-stilbene), a phytoalexin found mainly in grapes and red wine, has a wide range of biological effects (Soleas et al. 1997; Fremont 2000; Baur and Sinclair 2006; de la Lastra and Villegas 2007). Resveratrol has antioxidant effects, but it is not yet clear whether this is primarily a direct scavenging effect or the result of the activation of pathways that upregulate the natural antioxidant defenses of cells (Baur and Sinclair 2006). Nevertheless, depending on the concentration of the resveratrol and the cell type, it has also been shown that this polyphenol can exhibit both anti- and pro-oxidant properties (dos Santos et al. 2006; de la Lastra and Villegas 2007; Quincozes-Santos et al. 2007).

Many studies have demonstrated that resveratrol blocks the multistep process of carcinogenesis at various stages: tumor initiation, promotion, and progression (Jang et al. 1997). The anti-inflammatory, cardioprotective (Pervaiz 2004) and neuroprotective (Virgili and Contestabile 2000; Sinha et al. 2002) effects have been attributed to resveratrol, and more recent studies have suggested that this compound acts as an anti-aging agent in treating age-related human disease (Delmas et al. 2005). Resveratrol also improves the health and survival of mice on a high-calorie diet (Baur et al. 2006). Furthermore, while direct neuroprotective effects of resveratrol against oxidative

stress have been demonstrated in vitro, the mechanisms of these neuroprotective effects are not fully understood.

Brain tissue is particularly vulnerable to oxidative damage, possibly due to its high consumption of oxygen and the consequent generation of high quantities of reactive oxygen species (ROS) (Castagne et al. 1999; Dringen et al. 2005), including hydrogen peroxide ($H_2O_2$) and organic hydroperoxides. The detoxification of peroxides is well established for cultured astrocytes. $H_2O_2$ disappears from the medium of these cultures in a reaction following first-order kinetics in the minute range (Dringen and Hamprecht 1997).

Glial cells are the most abundant cells in the human brain and have long been considered as passive supporting cells for neurons (He and Sun 2007). Astrocytes are the main class of glial cells, serving a wide range of adaptive functions in the mammalian nervous system. They interact with neurons, providing structural, metabolic, and trophic support for them. In pathological circumstances, astrocytes have the potential to induce neuronal dysfunction, but they can also play a protective role, by releasing neurotrophic factors (Markiewicz and Lukomska 2006). The C6 lineage cells were originally derived from rat brain tumors induced with *N*-nitrosomethylurea (Benda et al. 1968). This cell line is able to assume oligodendrocytic, astrocytic, and neuronal properties (Parker et al. 1980). After 50 passages, cells start to express glial fibrillary acidic protein and assume astrocyte characteristics. After 100 passages, cells are widely used as an astrocyte-like cell line to study cell functions such as glutamate uptake, glutamine synthetase (GS) activity, S100B secretion, and oxidative stress (Mangoura et al. 1989; Feng and Zhang 2004; Funchal et al. 2005; dos Santos et al. 2006; Quincozes-Santos et al. 2007).

Glutamate is the major excitatory neurotransmitter in the central nervous system and its accumulation is implicated in neurodegenerative disorders. Astroglial cells are responsible for major glutamate transport and regulate extracellular levels of glutamate (Hertz 2006). The impairment of glutamate transporters causes excitotoxicity and leads to increased ROS production and consequent cell damage (Had-Aissouni et al. 2002). Moreover, astrocytes have a specific enzyme glutamine synthetase (EC 6.3.1.2) that catalyses the amidation reaction of glutamate to form glutamine, which is then exported to neurons, allowing the synthesis of not only glutamate, but also gamma-amino-butyric acid (GABA) by the glutamate–glutamine cycle (Bak et al. 2006). Moreover, astrocytic glutamate uptake is also essential for maintaining glutathione (GSH) levels, the main antioxidant of the brain (Dringen 2000). Indeed, neurons depend on astrocytes for precursors to synthesize their own glutathione. S100B is a calcium-binding protein that is produced and secreted from astroglial cells, constitutively and in response to injury (Goncalves et al. 2008). This protein has neurotrophic activity at nanomolar levels in the intracellular medium (Donato 2001; Van Eldik and Wainwright 2003) and its secretion is impaired in excitotoxic conditions (Tramontina et al. 2006). Nuclear factor-kappa B (NF-$\kappa$B) target genes mediate cell response to injury, including inflammation, proliferation, and apoptosis, as well as encode negative regulators of NF-$\kappa$B. Detected in the cytoplasm of almost all cell types, NF-$\kappa$B is modulated in response to several external stimuli, including cytokines, growth factors, glutamate, polyphenols (e.g., resveratrol), and ROS (Kundu and Surh 2004; Czyz 2005).

In the present investigation, we evaluated whether resveratrol could alter astroglial parameters, such as glutamate uptake, GS activity, GSH levels and NF-$\kappa$B in C6 astroglial cells, under two models of oxidative insult by $H_2O_2$—1 mM for 30 min or 0.1 mM for 6 h. We also observed $H_2O_2$-induced alterations in cell morphology and cytoskeleton reorganization.

## Materials and Methods

### Materials

Resveratrol, monoclonal anti-S100B (SH-B1), standard GSH, *o*-phthaldialdeyde, $\gamma$-glutamylhydroxamate, propidium iodide (PI), and cell culture materials were purchased from Sigma (St. Louis, MO, USA) except Dulbecco's Eagle's medium (DMEM), which was purchased from Gibco BRL (Carlbad, CA, USA), fetal bovine serum (FBS) from Cultilab (Campinas, SP, Brazil). L-[$^3$H]glutamate was purchased from Amersham International (UK), polyclonal anti-S100 was from Dako, and rhodamine-labeled phalloidin was from Molecular Probes (OR, USA). All other chemicals were purchased from common commercial suppliers.

### Maintenance of Cell Line

The C6 glioma cell line was obtained from the American Type Culture Collection (Rockville, Maryland, USA) and was maintained essentially according to the procedure previously described (dos Santos et al. 2006). The cells were seeded in flasks and cultured in DMEM (pH 7.4) containing 5% fetal bovine serum (FBS), 2.5 mg/ml Fungizone® and 100 U/l gentamicin. Cells were kept at a temperature of 37°C in an atmosphere of 5% $CO_2$/95% air. Exponentially growing cells were detached from the culture flasks using 0.05% trypsin/ethylene-diaminetetracetic acid (EDTA) and seeded in 24-well plates ($10 \times 10^3$ cells/well) or 6-well plates ($10 \times 10^4$ cells/well).

### Resveratrol and Hydrogen Peroxide Treatments

After cells reached confluence, the culture medium was removed by suction and cells were pre-incubated in the absence or presence of 100 μM of resveratrol for 1 h, in serum-free DMEM (pH 7.4). After this time, the medium was maintained and $H_2O_2$ was added as follows: Model I—1 mM $H_2O_2$ for 30 min and Model II—0.1 mM $H_2O_2$ for 6 h. During incubations, cells were maintained at 37°C in an atmosphere of 5% $CO_2$/95% air. Control cells were exposed to 0.25% ethanol vehicle. For all parameters analyzed, the results obtained with vehicle were not different from those obtained under basal conditions without ethanol.

### Glutamate Uptake Assay

Glutamate uptake was performed as previously described (Gottfried et al. 2002) with some modifications. Briefly, C6 glioma cells were incubated at 37°C in a Hank's balanced salt solution (HBSS) containing (in mM): 137 NaCl, 5.36 KCl, 1.26 $CaCl_2$, 0.41 $MgSO_4$, 0.49 $MgCl_2$, 0.63 $Na_2HPO_4 \cdot 7H_2O$, 0.44 $KH_2PO_4$, 4.17 $NaHCO_3$, and 5.6 glucose, adjusted to pH 7.4. The assay was started by the addition of 0.1 mM L-glutamate and 0.33 μCi/ml L-[2,3-$^3$H] glutamate. The incubation was stopped after 10 min by removal of the medium and rinsing the cells twice with ice-cold HBSS. The cells were then lysed in a solution containing 0.5 M NaOH. Radioactivity was measured in a scintillation counter. Sodium-independent uptake was determined by using *N*-methyl-D-glucamine instead of sodium chloride. Sodium-dependent glutamate uptake was obtained by subtracting the non-specific uptake of the total uptake to obtain the specific uptake. In order to investigate the involvement of PKC in glutamate uptake, we used the PKC activator, phorbol-12-myristate-13-acetate (PMA), as described (Lortet et al. 1999). Cells were exposed to 10 nM PMA during the entire time of incubation.

### Glutamine Synthetase Activity

The enzymatic assay was performed as previously described (dos Santos et al. 2006). Briefly, homogenate (0.1 ml) was added to 0.1 ml of the reaction mixture containing (in mM): 10 $MgCl_2$, 50 L-glutamate, 100 imidazole-HCl buffer (pH 7.4), 10 2-mercaptoethanol, 50 hydroxylamine-HCl, and 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 absorbance of the supernatant was measured at 530 nm and compared to the absorbance generated by standard quantities of γ-glutamylhydroxamate treated with ferric chloride reagent.

### Glutathione (GSH) Content Assay

GSH levels were assessed as previously described (Browne and Armstrong 1998). C6 glioma cell homogenates were diluted in 10 volumes of 100 mM sodium phosphate buffer, pH 8.0, containing 5 mM EDTA and the protein was precipitated with 1.7% meta-phosphoric acid. Supernatant was assayed with *o*-phthaldialdeyde (1 mg/ml methanol) at room temperature for 15 min. Fluorescence was measured using excitation and emission wavelengths of 350 and 420 nm, respectively. A calibration curve was performed with standard GSH solutions (0–500 μM). GSH concentrations were calculated as nmol/mg protein.

### S100B Immunocontent Assay

S100B measurement was carried out by ELISA, as we previously described (Leite et al. 2008). Briefly, 50 μl of sample plus 50 μl of Tris buffer were incubated for 2 h on a microtiter plate previously coated with monoclonal anti-S100B (SH-B1). Polyclonal anti-S100B was incubated for 30 min and the peroxidase-conjugated anti-S100 was then added and incubated for 30 min. The color reaction with *o*-phenylenediamine was measured at 492 nm. The standard S100B curve ranged from 0.1 to 4 ng/ml.

### Nuclear Extraction and Electrophoretic Mobility Shift Assay (EMSA) for the Detection of Nuclear NF-κB

In order to prepare nuclear extracts, C6 glioma cells were collected by centrifugation at 750×*g* for 5 min and resuspended in 200 μl of hypotonic buffer consisting of (in mM): 10 HEPES (pH 7.9), 1.5 $MgCl_2$, 10 KCl, 0.2 phenylmethylsulfonyl fluoride, and 0.5 dithiothreitol, supplemented with 1 μg/ml pepstatin A and 1 μg/ml leupeptin. Samples were kept on ice for 15 min, 12 μl of 10% Nonidet P-40 was then added and the swollen cells were disrupted by vortex (15 s). Nuclei were isolated by centrifugation at 14,000×*g* for 30 s and resuspended in 70 μl of high salt buffer consisting of (in mM): 10 HEPES (pH 7.9), 420 NaCl, 1.5 $MgCl_2$, 10 KCl, 1 phenylmethylsulfonyl fluoride, and 1 dithiothreitol, supplemented with 1 μg/ml pepstatin A and 1 μg/ml leupeptin. Samples were then incubated for 40 min on an ice-bath to release soluble proteins from the nuclei (Han and Brasier 1997), followed by centrifugation at 14,000×*g* for 10 min. Supernatant containing soluble nuclear proteins was stored at −80°C until experiments. DNA single strand oligonucleotides containing NF-κB binding sequences (5′-CGACACCCC TCGGGAATTCCCCCACTGGGCC-3′; 5′-GCTGTGGGG AGCCCTTAAGGGGGTGACCCGG-3′) were labeled using the Biotein 3′ End DNA Labeling Kit (Pierce Biotechnology, Rockford, IL, USA). Biotin-labeled DNA

probes were generated by annealing nucleotide templates. Electrophoretic mobility shift assays (EMSA) were performed, as described (Ballard et al. 1990). Biotin-labeled probes were incubated with 2 μg of nuclear extracts from C6 glioma cells obtained as described above. Binding reactions were carried out for 30 min on ice in the presence of 1 μg poly (dI–dC), 5 μg BSA, 5 mM DTT, 50–100 mM NaCl or KCl, 20 mM HEPES, and 1 mM EDTA. Protein–DNA complexes were resolved via 6% polyacrylamide gel electrophoresis (PAGE) under non-denaturing conditions in a TRIS/Borate/EDTA buffer. Resolved DNA probes were transferred to positively charged nylon membranes and incubated with streptavidin conjugated to Horseradish peroxidase. Membranes were incubated with Chemiluminescent Nucleic Acid Detection Module (Pierce Biotechnology) and exposed to X-ray films.

### Cell Morphology, Integrity, and Actin-Labeling Studies

Morphological studies were performed using phase contrast optics and cells were photographed or used in fixed labeling studies. Cellular damage was assessed by fluorescent image analysis (Nikon inverted microscope using a TE-FM Epi-Fluorescence accessory) of PI uptake (PI, at 7.5 μM) (dos Santos et al. 2006), at 37°C in an atmosphere of 5% $CO_2$/95% air in DMEM. Optical density was determined with the Optiquant version 02.00 software (Packard Instrument Company).

For actin-labeling studies, pre-confluent cells cultured on coverslips were fixed for 20 min with 4% paraformaldehyde in phosphate-buffered saline (PBS; 2.9 mM $KH_2PO_4$, 38 mM $Na_2HPO_4 \cdot 7H_2O$, 130 mM NaCl, and 1.2 mM KCl), rinsed with PBS, and permeabilized for 10 min in PBS containing 0.2% Triton X-100, followed by incubation with 2.5 U/ml rhodamine-labeled phalloidin in PBS for 20 min and washed twice with PBS. C6 cells were analyzed and photographed with a Nikon microscope using a TE-FM Epi-Fluorescence accessory. In order to evaluate whether the effect of $H_2O_2$ on the rearrangement of actin fibers was mediated by the Rho signaling pathway, cells were treated with 2 μM lysophosphatidic acid (LPA), which is a specific upstream positive regulator of Rho A (Cechin et al. 2002; Guasch et al. 2003).

### Evaluation of Intracellular ROS Production

Intracellular ROS production was detected using the nonfluorescent cell permeating compound, 2′-7′-dichlorofluorescein diacetate (DCFH-DA). DCFH-DA is hydrolyzed by intracellular esterases to dichlorofluorescin (DCFH), which is trapped within the cell. This nonfluorescent molecule is then oxidized to fluorescent dichlorofluorescin (DCF) by the action of cellular oxidants. C6 cells were treated with DCFH-DA (10 μM) for 30 min at 37°C. Following DCFH-DA exposure, the cells were 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 (Vieira de Almeida et al. 2008).

### Propidium Iodide (PI) Uptake

Cellular damage on $H_2O_2$ time course was assessed by PI uptake as previously described (Quincozes-Santos et al. 2007). Cells were treated with 7.5 μM PI concomitantly with $H_2O_2$ addition and maintained until the end of incubation, after which cells were analyzed and photographed with a Nikon inverted microscope using a TE-FM Epi-Fluorescence accessory. Optical density was determined with the Optiquant version 02.00 software (Packard Instrument).

### Protein Determination

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

### Statistical Analysis

Data from the experiments are presented as mean ± S.E. Resveratrol and different doses of $H_2O_2$ were analyzed statistically by two-way analysis of variance (ANOVA) followed by the Tukey's test. The evaluation of intracellular ROS production by $H_2O_2$ treatment (concentration–time curve) was analyzed statistically by one-way ANOVA followed by the Tukey's test. Values of $P < 0.05$ were considered to be significant (*a* indicates differences from control and *b* indicates differences from $H_2O_2$). All analyses were performed using Statistical Package for the Social Sciences (SPSS) software in a PC-compatible computer.

## Results

### Effects of Resveratrol on Glutamate Uptake in C6 Astroglial Cells under $H_2O_2$ Oxidative Insult

In Model I (Fig. 1a), glutamate uptake was significantly increased in the presence of resveratrol ($F_{(3,36)} = 21.273$) as compared to control data (0.25 nmol/mg protein/min), in agreement with previous results (dos Santos et al. 2006). Under oxidative insult, resveratrol was also able to increase the glutamate uptake by 36% ($F_{(3,36)} = 21.273$) and by 62% ($F_{(3,36)} = 25.718$), as compared to control and to $H_2O_2$ values, respectively. Hydrogen peroxide did not affect the glutamate uptake.

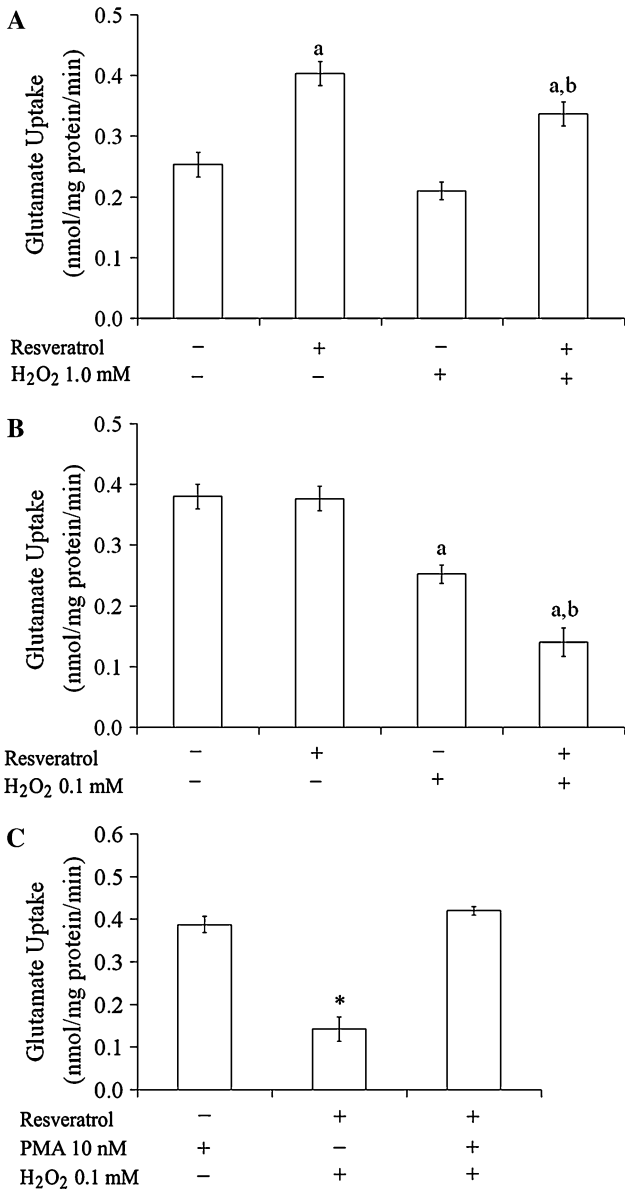


**Fig. 1** Effects of resveratrol on glutamate uptake under $H_2O_2$ oxidative insult. Cells were pre-incubated for 1 h in the presence of 100 μM resveratrol, followed by the addition of (**a**) 1 mM $H_2O_2$ for 30 min or (**b**, **c**) 0.1 mM $H_2O_2$ for 6 h. In **c**, cells were exposed to 10 nM PMA during the entire time of incubation. Data represent means ± S.E. from the three experimental determinations performed in triplicate. **a** Significant differences from control values and **b** significant difference from $H_2O_2$. * indicates the significant difference from PMA ($P < 0.001$)

In Model II (Fig. 1b), resveratrol did not interfere with glutamate transport, as compared to control data (0.38 ± 0.02 nmol/mg protein/min). Oxidative insult significantly decreased the glutamate uptake by 34% ($F_{(3,36)} = 9.649$); resveratrol was unable to prevent this effect, but potentiated $H_2O_2$-decreased glutamate uptake, reducing it by 63% as compared to control values. In order to investigate a possible involvement of PKC in this effect, cells were co-incubated with the PKC activator, PMA. As observed in Fig. 1c, PMA completely prevented the glutamate uptake decrease induced by the co-incubation of resveratrol with $H_2O_2$. It is important to mention that PMA values (0.40 ± 0.05 nmol/mg protein/min) did not differ from control and resveratrol data.

## Effects of Resveratrol on Glutamine Synthetase Activity in C6 Astroglial Cells under $H_2O_2$ Oxidative Insult

In agreement with previous results (dos Santos et al. 2006), in Model I (Fig. 2a), GS activity was significantly higher (about 68%) in the presence of resveratrol ($F_{(3,36)} = 139.973$). This effect was maintained even under oxidative condition ($F_{(3,36)} = 45.765$), as compared to control data (0.38 ± 0.02 μmol/mg protein/h). Oxidative insult did not affect GS activity.

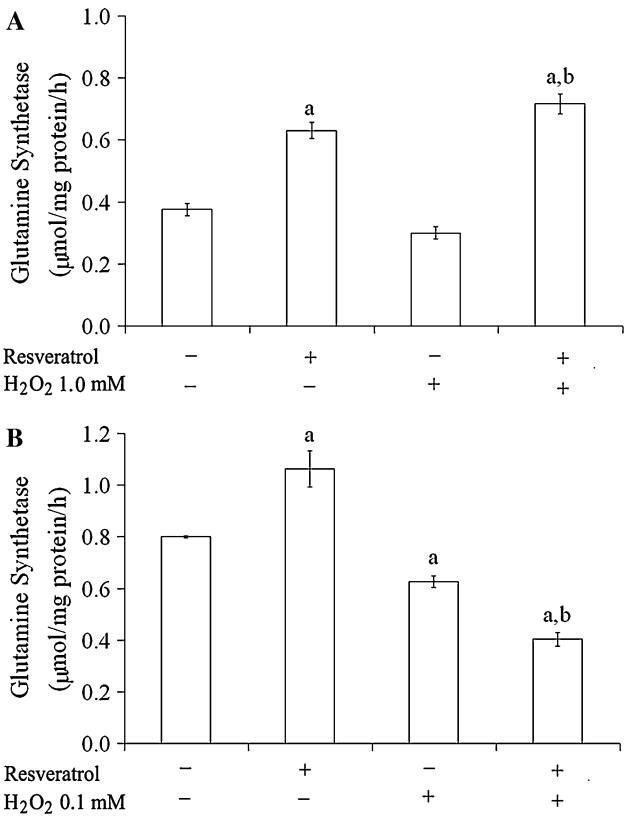


**Fig. 2** Effects of resveratrol on GS activity under $H_2O_2$ oxidative insult. Cells were pre-incubated for 1 h in the presence of 100 μM resveratrol followed by the addition of (**a**) 1 mM $H_2O_2$ for 30 min or (**b**) 0.1 mM $H_2O_2$ for 6 h. Data represent means ± S.E. from three experimental determinations performed in triplicate. **a** Significant differences from control values; **b** significant difference from $H_2O_2$ ($P < 0.001$)

In Model II (Fig. 2b), resveratrol increases GS activity by 37% ($F_{(3,36)} = 9.884$), as compared to control data (0.8 ± 0.004 μmol/mg protein/h). Oxidative insult significantly decreases GS activity by 22% ($F_{(3,36)} = 9.884$). This effect was potentiated by resveratrol, reducing it by 50% ($F_{(3,36)} = 9.884$) as compared to control conditions (similar to those observed with glutamate uptake in Fig. 1b) and reducing it by 36% ($F_{(3,36)} = 19.577$), as compared to $H_2O_2$ values.

## Effects of Resveratrol on Glutathione (GSH) Levels in C6 Astroglial Cells under $H_2O_2$ Oxidative Insult

In Model I (Fig. 3a), resveratrol significantly increased the GSH content (by approximately 20%, $F_{(3,36)} = 103{,}712$), as compared to control data (5.7 ± 0.18 nmol GSH/mg protein). Oxidative insult significantly decreased the GSH content by 27% ($F_{(3,36)} = 103{,}712$). In addition to preventing the $H_2O_2$-induced decrease in GSH content, resveratrol also increased the GSH content to levels above control values (about 60%, $F_{(3,36)} = 103{,}712$).

In Model II (Fig. 3b), resveratrol did not alter the GSH content as compared to control data (20.3 ± 0.29 nmol GSH/mg protein). Oxidative insult significantly decreased the GSH content by 22% ($F_{(3,36)} = 152.966$). This effect was potentiated by resveratrol, reducing it by 54% ($F_{(3,36)} = 152.966$), as compared to control conditions and reducing it by 42% ($F_{(3,36)} = 87{,}950$) as compared to $H_2O_2$ values.

## Effects of Resveratrol on S100B Secretion in C6 Astroglial Cells under $H_2O_2$ Oxidative Insult

In Model I (Fig. 4a), resveratrol increased S100B secretion (by approximately 10%, $F_{(3,36)} = 69.000$), as compared to control data (0.1 ± 0.0013 ng/ml). Secretion of this protein was not affected by $H_2O_2$ insult. However, this oxidative condition induced a positive resveratrol response,

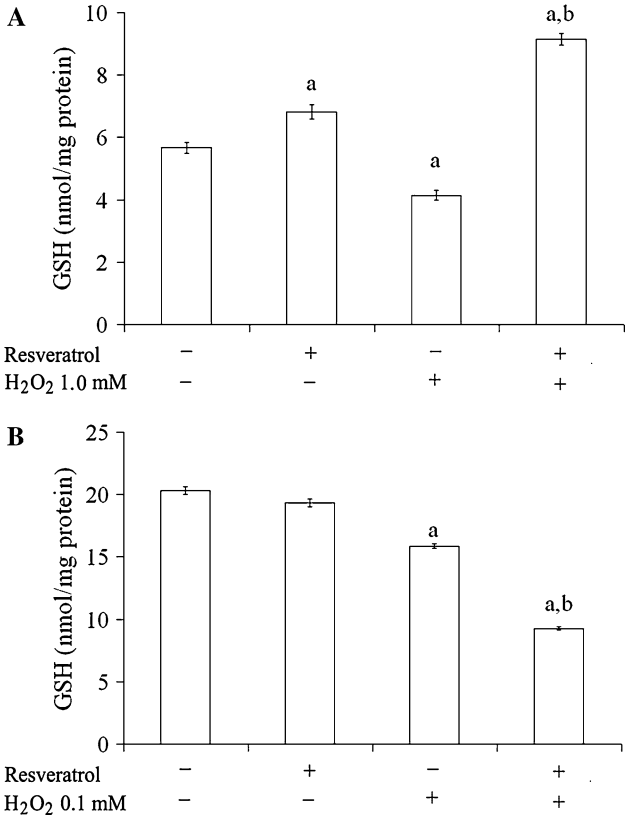


**Fig. 3** Effects of resveratrol on glutathione (GSH) content under $H_2O_2$ oxidative insult. Cells were pre-incubated for 1 h in the presence of 100 μM resveratrol followed by the addition of (**a**) 1 mM $H_2O_2$ for 30 min or (**b**) 0.1 mM $H_2O_2$ for 6 h. Data represent means ± S.E. from the three experimental determinations performed in triplicate. **a** Significant differences from control values and **b** significant difference from $H_2O_2$ ($P < 0.001$)

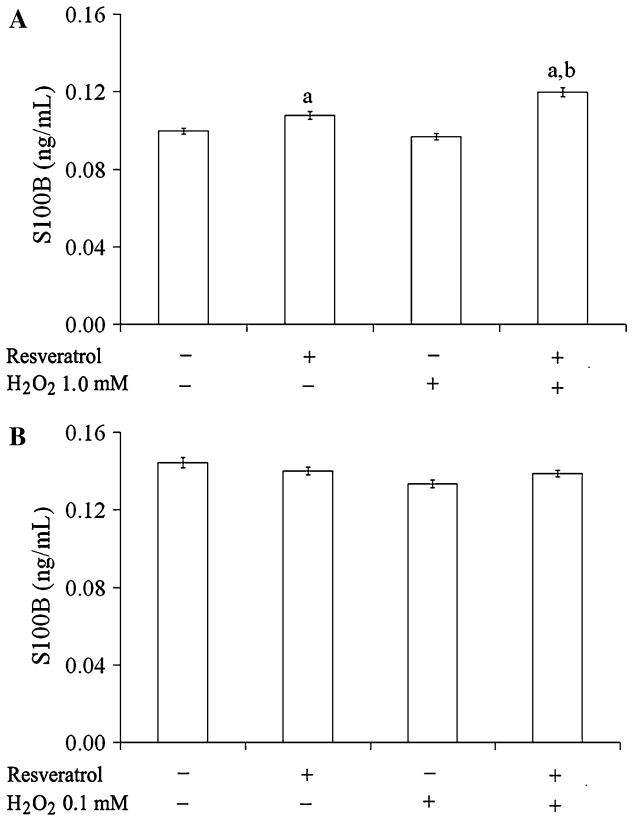


**Fig. 4** Effects of resveratrol on S100B secretion under $H_2O_2$ oxidative insult. Cells were pre-incubated for 1 h in the presence of 100 μM resveratrol followed by the addition of (**a**) 1 mM $H_2O_2$ for 30 min or (**b**) 0.1 mM $H_2O_2$ for 6 h. Data represent means ± S.E. from the three experimental determinations performed in triplicate. **a** Significant differences from control values and **b** significant difference from $H_2O_2$ ($P < 0.001$)

significantly increasing S100B secretion by 20% ($F_{(3,36)} = 69.000$), as compared to control values. In Model II (Fig. 4b), S100B secretion was not altered in all parameters investigated.

## Effects of Resveratrol on Nuclear NF-κB in C6 Astroglial Cells under $H_2O_2$ Oxidative Insult

Mobility shift assays for NF-κB activation were performed to evaluate the response of C6 lineage cells to resveratrol, in the presence or absence of oxidizing conditions. In Model I (Fig. 5a), both resveratrol and $H_2O_2$ induced a twofold activation of NF-κB, compared to the control condition. Under oxidative conditions, resveratrol resulted in a fourfold higher nuclear NF-κB, as compared to control conditions. This effect was higher than the induction observed by resveratrol alone.

In Model II (Fig. 5b), resveratrol also induced a twofold greater NF-κB activation, as compared to control conditions, although oxidative insult did not affect nuclear NF-κB. However, after $H_2O_2$ insult, resveratrol induced a fourfold increase in NF-κB activation.

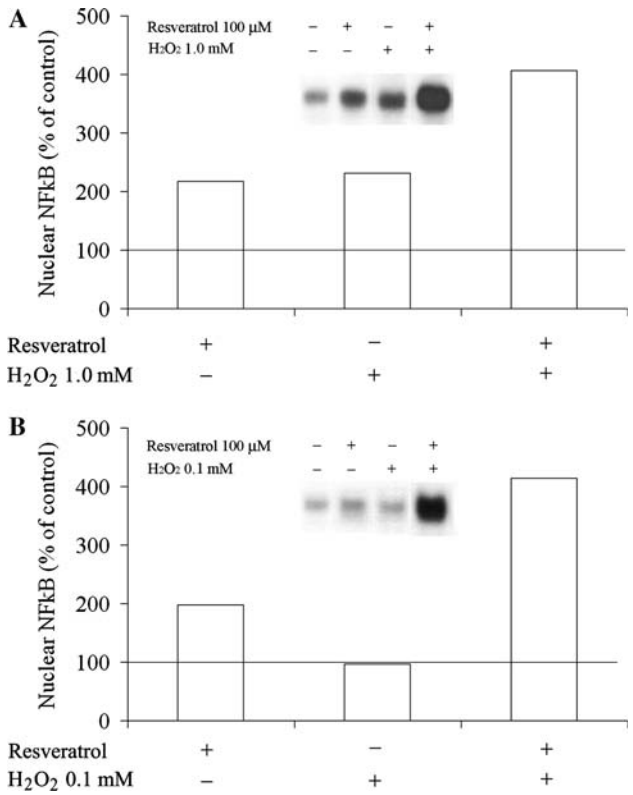


**Fig. 5** Effects of resveratrol on nuclear NF-κB under $H_2O_2$ oxidative insult. Cells were pre-incubated for 1 h in the presence of 100 µM resveratrol followed by the addition of (**a**) 1 mM $H_2O_2$ for 30 min or (**b**) 0.1 mM $H_2O_2$ for 6 h. Bands in the insets are representative images from EMSA of the NF-κB-inducible complex. Values were obtained after quantification of the bands shown in the *inset*. *Line* indicates control (0.25% ethanol) values as 100%. Data are representative of the two experimental determinations

## Effects of Resveratrol and $H_2O_2$ on Morphology and Integrity of the C6 Astroglial Cells

C6 cells were morphologically analyzed by phase contrast microscopy (Fig. 6a, b) and by fluorescence microscopy (Fig. 7a, b) to investigate actin cytoskeleton reorganization. PI-labeled representative images in Fig. 6 (left panels) demonstrate that there was no significant loss of membrane integrity in the presence of $H_2O_2$. Under basal culture conditions, as demonstrated previously (dos Santos et al. 2006), C6 glioma cells showed a fusiform and flattened appearance (Figs. 6a1, b1, 7a1, b1). This characteristic shape was altered by $H_2O_2$, resulting in process-bearing cells accompanied by cell body retraction and actin reorganization (Figs. 6a3, b3, 7a3, b3). Interestingly, LPA was able to prevent $H_2O_2$-induced actin reorganization, maintaining the same characteristics of the actin cytoskeleton observed in control cells (Fig. 7a4, b4). Figure 7b5 is a representative image of cell body retraction and process formation found in the presence of 0.1 mM $H_2O_2$. Figure 7b6 illustrates the flattened appearance maintained by LPA under oxidative insult.

## Effects of Resveratrol and $H_2O_2$ on ROS Production in C6 Astroglial Cells

The ability of $H_2O_2$ to generate ROS was investigated by the oxidation of DCFH (Fig. 8a). A significant linear increasing fluorescence activity could be observed depending on $H_2O_2$ concentration and the incubation time. The ROS generation showed a linear increase at 100 µM and at 1 mM $H_2O_2$ after 3 h and 30 min of incubation, respectively. It is important to mention that the final incubation points (5 and 6 h) regarding to 1 mM $H_2O_2$ were omitted in the Fig. 8a, due to the partial loss of cell membrane integrity (12 and 15%, respectively—data not shown). All other points maintained the same membrane integrity as the control cells (data not shown), quantified by PI uptake assay. As observed at Fig. 8b (Model I), in the presence of resveratrol, there was a decrease on DCFH oxidation compared to control cells ($F_{(3,36)} = 13.275$). In addition, resveratrol was able to prevent the increase of ROS production induced by $H_2O_2$ ($F_{(3,36)} = 22.470$) to lowest values than that of control conditions. In Model II of oxidative insult, resveratrol is still able to decrease DCFH oxidation compared to control cells ($F_{(3,36)} = 10.412$) but in the presence of $H_2O_2$, resveratrol potentiated ROS production compared to $H_2O_2$ alone ($F_{(3,36)} = 6.398$).

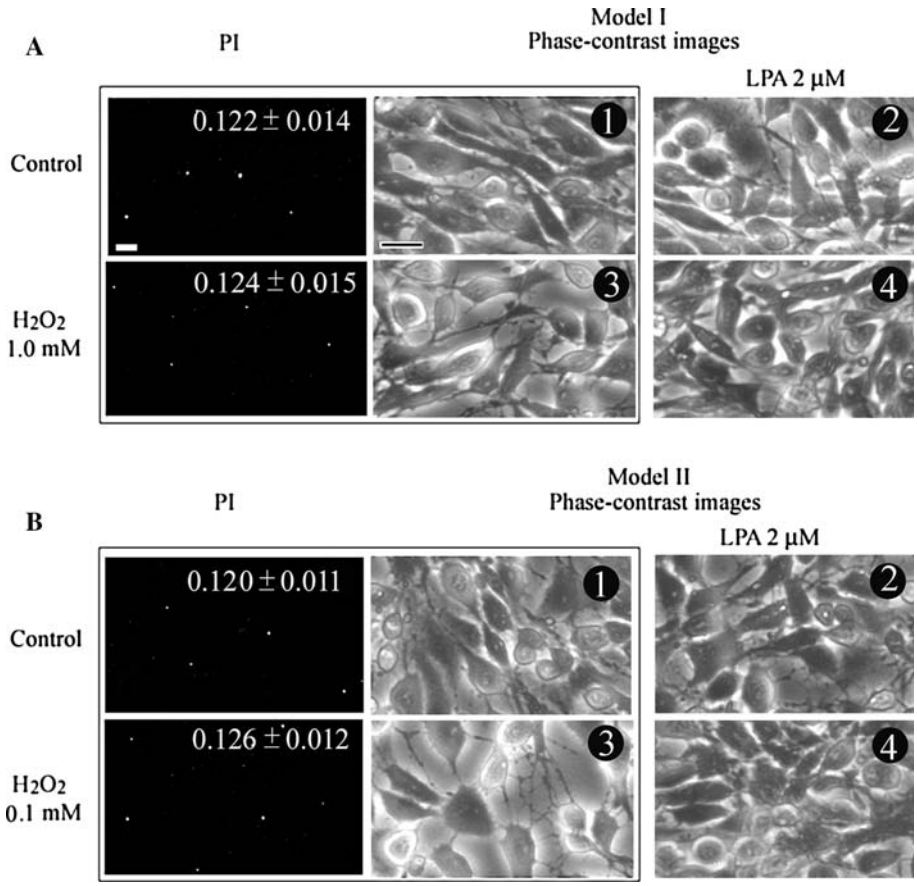


**Fig. 6** Effects of $H_2O_2$ on morphology and integrity in C6 astroglial cells. Representative photomicrographs of cells exposed to resveratrol and LPA under oxidative insult. Cells were pre-incubated for 1 h in the presence of 100 μM resveratrol followed by the addition of (**a**) 1 mM $H_2O_2$ for 30 min and (**b**) 0.1 mM $H_2O_2$ for 6 h. Cells were fixed and images recorded by a Nikon inverted microscope coupled to a digital camera. *Left panels* show representative fluorescent images of PI uptake (obtained with a Nikon inverted microscope coupled to a TE-FM Epi-Fluorescence accessory). All images are representative fields from at least three experiments carried out in triplicate. Scale bar: 50 μm

## Discussion

ROS accumulation may induce the oxidative modification of cellular macromolecules (lipid, proteins, and nucleic acids) with potential deleterious effects on mutagenesis, oncogenesis, and aging (Halliwell 2006). Moreover, ROS are key events in the development of neuronal injury in several acute and long-term neurodegenerative diseases. In this context, we evaluated the effect of resveratrol on astroglial mechanisms related to neuroprotection, under two models of $H_2O_2$ insult established by our group (Quincozes-Santos et al. 2007). Model I of $H_2O_2$ exposure was used to induce an intense (1 mM $H_2O_2$) and acute (30 min) damage. Model II of $H_2O_2$ exposure was used to induce a less intense (0.1 mM $H_2O_2$), but lasting (6 h) damage.

One of the biological activities that have been ascribed to resveratrol involves its antioxidant potential. Recently, we showed that cells pretreated with 100 μM resveratrol (for 1 h) were protected from $H_2O_2$-induced genotoxicity (Quincozes-Santos et al. 2007). In addition, this polyphenol was able to stimulate the glutamate uptake in C6 astroglial cells up to 100 μM (dos Santos et al. 2006). High-affinity glutamate uptake systems, mainly in astrocytes, represent the major mechanism for controlling the extracellular levels of glutamate and to maintain them below neurotoxic values (McBean and Roberts 1985). Defects in glutamate uptake function have been reported both in acute (Silverstein et al. 1986) and long-term (Rothstein et al. 1992) neurodegenerative pathologies associated with oxidative stress. Therefore, we have previously suggested that resveratrol could affect the redox environment of glutamate transporters and favor their activities, at least in a range of 0.1–100 μM (dos Santos et al. 2006). In this study, we show that in Model I of oxidative insult, resveratrol is still able to increase the glutamate uptake; however, after 6 h of $H_2O_2$ insult (Model II), this compound can also act on the oxidizing direction, impairing glutamate transport. No significant cell death under PI assay was seen in parallel to uptake inhibition, suggesting that the reduction observed in glutamate transport was not primarily due to cell damage, but some more specific mechanism, such as the involvement of protein kinases. It has been demonstrated that resveratrol is able to modulate the PKC family (Slater et al. 2003; Atten et al. 2005), in turn influencing glutamate transporters (Bull et al. 2007). Based on this information, we used the PKC activator, PMA. Interestingly, the resveratrol-potentiated glutamate uptake decrease under oxidizing

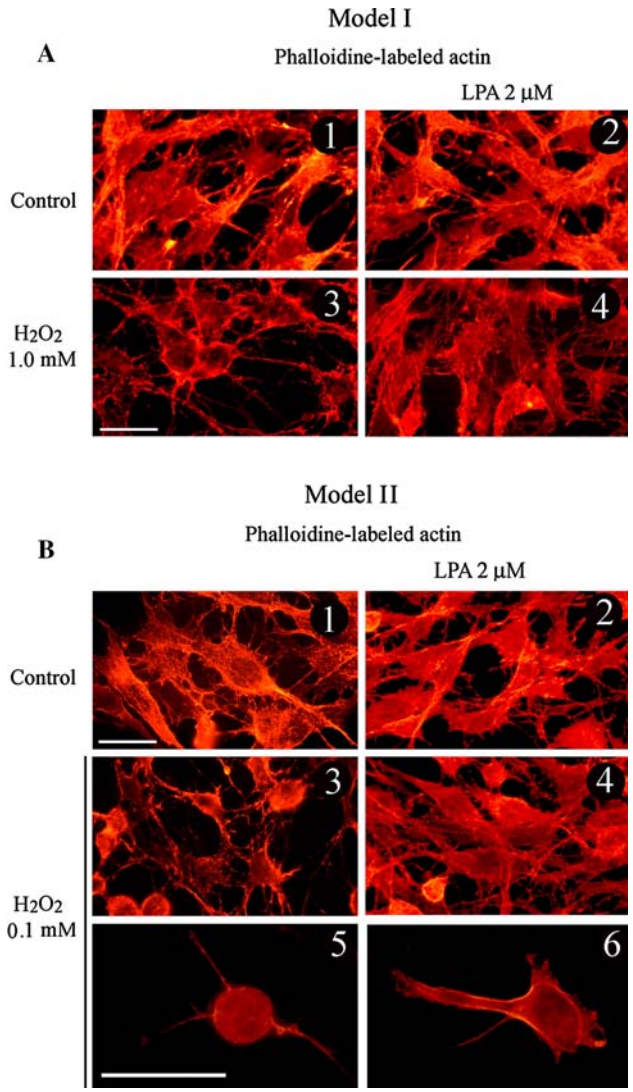


**Fig. 7** Effects of $H_2O_2$ on actin reorganization in the C6 astroglial cells. Representative images of cells exposed to resveratrol and LPA under oxidative insult. Cells were cultured on circular coverslips and pre-incubated for 1 h in the presence of 100 μM resveratrol followed by the addition of (**a**) 1 mM $H_2O_2$ for 30 min; (**b**) 0.1 mM $H_2O_2$ for 6 h. Cells were fixed and images recorded by a Nikon microscope coupled to a TE-FM Epi-Fluorescence accessory. All images are representative fields from at least three experiments carried out in triplicate. Scale bar: 50 μm

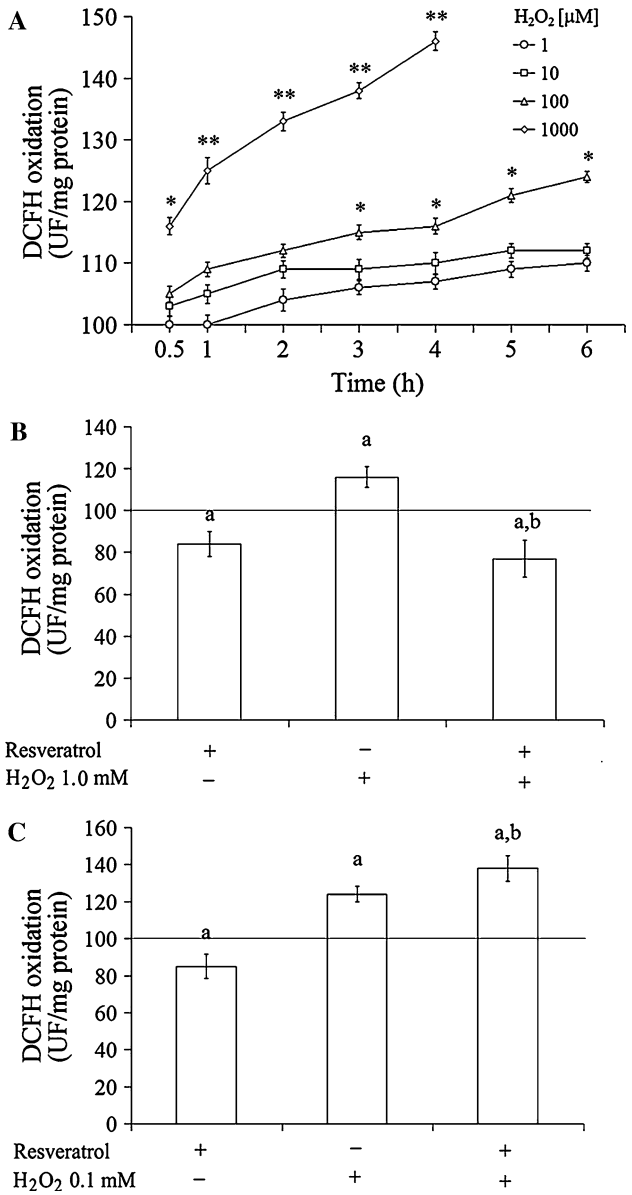


**Fig. 8** Effects of resveratrol and $H_2O_2$ on DCFH oxidation in C6 astroglial cells. Cells were incubated up to 6 h in the absence or presence of $H_2O_2$ (**a**) or pre-incubated for 1 h in the presence of 100 μM resveratrol followed by the addition of (**b**) 1 mM $H_2O_2$ for 30 min or (**c**) 0.1 mM $H_2O_2$ for 6 h. *Line* indicates control values (0.25% ethanol) as 100% (**b**–**c**). Data represent means ± S.E. from three experimental determinations performed in triplicate. **a** $*P < 0.05$; $**P < 0.01$; **b**, **c** "a" significant differences from control values; "b" significant differences from $H_2O_2$ ($P < 0.05$)

conditions (Model II of $H_2O_2$ insult) was completely prevented in the presence of PMA, suggesting the involvement of PKC in this effect.

Although glutamate is usually thought of as the major excitatory neurotransmitter in the brain, it is important to note that glutamate has many other fates in the brain, including oxidation for energy, incorporation into proteins, and formation of glutamine, GABA, and GSH (McKenna 2007). With regard to astrocytes, after uptake, glutamate can be converted into glutamine by the enzyme, GS, essential for the glutamate–glutamine cycle. Glutamine, in turn, can be released back to neurons and used for the re-synthesis of glutamate (and then GABA, in GABAergic neurons). We have demonstrated that resveratrol (1–100 μM) increases GS activity in a concentration-dependent manner in C6 cells (dos Santos et al. 2006). Here, we demonstrate that, in Model I, resveratrol was able to efficiently increase the GS activity in the presence of $H_2O_2$ and that, in Model II, this polyphenol

had an opposite effect, potentiating the decrease in GS activity induced under oxidative insult. We can assume that under an intense but fast $H_2O_2$ insult (Model I), resveratrol is able to prepare C6 cells, to remove more glutamate from the extracellular space, and also to convert them into glutamine, improving the glutamate/glutamine cycle. In contrast, at longest time of incubations under oxidizing conditions, in the presence of $H_2O_2$, resveratrol could impair some astroglial functions, by potentiating oxidant conditions, resulting in a pro-oxidant effect.

As glutamate also serves as a substrate per se for glutathione synthesis and as a moiety for exchange by cysteine, another substrate for glutathione synthesis (Dringen 2000), we measured the effect of resveratrol on GSH content. Resveratrol is both a free-radical scavenger and a potent antioxidant due to its ability to promote the activities of a variety of antioxidant enzymes (de la Lastra and Villegas 2007) and also the tripeptide GSH (Vieira de Almeida et al. 2008). As previously demonstrated with primary astrocyte cultures (de Almeida et al. 2007), C6 astroglial cells are also able to induce a fast increment of GSH content in the presence of resveratrol. As expected, $H_2O_2$ induced a decrease in GSH content, which was prevented in cells that were incubated in the presence of resveratrol in Model I. Several brain disorders are accompanied by a decrease in glutathione and other indications of oxidative stress (Schulz et al. 2000). As astrocytic GSH constitutes a non-enzymatic scavenger and substrate for glutathione peroxidases and also a precursor for neuronal GSH synthesis, our data indicate an important regulation of GSH content by resveratrol, and the provision of protection against $H_2O_2$ injury. In Model II, resveratrol potentiated $H_2O_2$-induced GSH decrease, as well as glutamate uptake and GS activity. As discussed above, glutamate is a precursor for GSH and, probably, a decrease in its transporter in astroglial cells is responsible for the decrease in GSH content and, subsequently, lowers activity, as observed in Model II.

Another possible effect that may be afforded by resveratrol neuroprotection is its ability to induce S100B secretion. Based on the neuroprotection observed in neural cultures of this protein (Donato 2001) and on its transitory extracellular increment in acute brain injury, it has been proposed that S100B may improve functional recovery following acute brain injury (Kleindienst and Ross Bullock 2006). Both resveratrol and $H_2O_2$ could affect S100B secretion via redox-sensitive kinases (de Souza et al. 2009). Interestingly, resveratrol per se was able to increase S100B secretion by C6 cells 1.5 h afterward (Model I of peroxide insult), but not 7 h afterward (Model II).

We observed that resveratrol was able to transiently activate NF-$\kappa$B pathway. NF-$\kappa$B is a quite complex element in the cell signaling network, playing an important role in the transcription, including a close and very versatile regulatory connection between oxidative stress and inflammatory response (Gloire et al. 2006; Nakano et al. 2006; Papa et al. 2006). Our results suggest that NF-$\kappa$B translocation to nucleus is increased by resveratrol in C6 astroglial cells, in both models of oxidative insult. However, the possible physiological and therapeutic significance of these findings remains to be established. In our oxidative models, we also observed that $H_2O_2$-induced cell morphology alterations and that resveratrol were not able to prevent this effect (Quincozes-Santos et al. 2007). A recent study on astrocytes showed that $H_2O_2$ promoted cytoskeleton reorganization (Zhu et al. 2005). Here, in order to investigate a possible mechanism involved in the cytoskeleton reorganization induced by $H_2O_2$ in C6 cells, we used LPA, a classical upstream activator of the small GTPase RhoA, which has been widely used to demonstrate the involvement of RhoA in actin cytoskeleton changes (Hall 1998; Kranenburg and Moolenaar 2001; Cechin et al. 2002; Guasch et al. 2003). Disruptions in actin organization may modulate important astrocyte functions, participating in $H_2O_2$-induced astroglial and brain damage during development (Zhu et al. 2005). Interestingly, LPA completely prevented cell morphology alteration induced under oxidative insult. This result clearly indicates, for the first time to our knowledge, that $H_2O_2$ promotes cytoskeleton reorganization by negative modulation of RhoA activity. As resveratrol was not able to prevent $H_2O_2$-induced morphology alterations, we can assume that this polyphenol, at least in this astroglial model, did not interfere in RhoA activity.

As the results presented here indicate a dual effect of resveratrol, preventing or potentiating the $H_2O_2$ effect, we investigated ROS production by measuring DCFH oxidation, in the presence of resveratrol in both oxidative insults studied. The time course of $H_2O_2$ indicates that lower concentrations (1–10 μM) were not able to significantly increase ROS production. However, higher concentrations, as used in Models I and II, induced a significant increase in ROS production. Resveratrol prevented this effect at Model I and, as expected, potentiated $H_2O_2$-induced ROS production at Model II. It is important to mention that resveratrol alone did not exert a pro-oxidant effect; on the contrary, it surprisingly decreased the DCFH oxidation as compared to control conditions. It suggests that this polyphenol, in the presence of a strong oxidant compound as $H_2O_2$, could be probably metabolized to oxidant molecules (Murias et al. 2005) potentiating $H_2O_2$ effect.

Resveratrol is emerging as an important focus of research in studies of both aging and neuroprotection against age-related diseases. Understanding the influence of resveratrol on glial changes in the structure and function of cellular processes is critical for the elucidation of the molecular basis of the action of this polyphenol. In

summary, we demonstrated different effects of resveratrol on important astroglial functions, depending upon oxidizing conditions. The neuroprotective role of resveratrol appears to involve astrocyte activation, as indicated by the in vitro increase in glutamate uptake, GS activity, GSH content, and S100B secretion. These in vitro results suggest that caution is necessary with its therapeutic use because elevated levels of this compound, particularly during oxidative insult, could contribute to aggravate neural conditions.

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## CAPÍTULO III

**Artigo de revisão aceito para publicação no periódico Annals of the New York Academy of Sciences**

# Resveratrol Modulates Astroglial Functions: Neuroprotective Hypothesis

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# Resveratrol modulates astroglial functions: neuroprotective hypothesis

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**Resveratrol, a redox active compound present in grapes and wines, have a wide range of biological effects including cardioprotective, chemopreventive, and anti-inflammatory activity. The central nervous system has been unfolded to be a target of resveratrol that is able to trespass the blood–brain barrier and display neuroprotective effects. Astrocytes are one of the most functionally diverse groups of cells in the nervous system, intimately associated with glutamatergic metabolism, transmission, synaptic plasticity, and neuroprotection. In this review, we focus on the resveratrol properties and response to oxidative insult on important astroglial parameters involved in brain plasticity, such as glutamate uptake, glutamine synthetase activity, glutathione content, and secretion of the trophic factor S100B.**

**Keywords:** astrocytes; resveratrol; glutamatergic metabolism; neuroprotective; oxidative stress

## Resveratrol from plants to mammal's brain-targeting

### *Plants*

Many natural components of diet have been investigated in recent years. Among these, the antioxidants are the molecules which present numerous biological effects in different cell types and tissues.[1–3] Nowadays, the redox active compound resveratrol (3,4′,5-trihydroxy-*trans*-stilbene) is one of the most studied antioxidant, first found in roots of white hellebore and later in roots of *Polygonum cuspidatum*.[4] In plants, it is a phytoalexin found, mainly, in grapes, grape juice, wine, and berries.[5,6] In ancient medicine, Hippocrates made some observations on the medicinal properties of wine and also Galen reported that preparations of the base wine and herbs were used as antidotes to poisons.[7] Red wine has gained particular attention according to the French paradox described in southern France which showed an inverse correlation between intake of a diet rich in lipids and a low incidence of heart disease.[8–10] Among the wines with the highest concentration of resveratrol stand out wines from southern of Brazil, which due to high humidity of the soil; naturally have a higher amount of the phytoalexin resveratrol.[11]

### *Mammals*

Since the first reported detection of resveratrol in grapevines in 1976, a plethora of beneficial effects have been described in mammals, including cardioprotective, chemopreventive, and anti-inflammatory activity.[5,6,10,12–15] Many papers give the most diverse actions of resveratrol in its direct antioxidant and scavenger effect or by modulate and improve cellular antioxidant defenses.[6,13] One of the signaling pathways modulated by resveratrol includes the sirtuin protein family (SIRT1–7 in mammals). This is a conserved family of $NAD^+$-dependent deacetylases (class III histone deacetylases), which exerts effects related to the lifespan extension in diverse species.[16] In mammalian cells, resveratrol induces SIRT1-dependent effects that are consistent with improve cellular function, implicated to play a role in a number of age-related

human diseases. The effects of resveratrol on sirtuins may explain its effect in longevity.[13,17]

### *Brain-targetin*

The central nervous system (CNS) has been unfolded to be also target of resveratrol which is able to trespass the blood–brain barrier and display neuroprotective effects.[18] Among neuroprotective role, we highlight the benefic effect of resveratrol described in animal models of Alzheimer's and Parkinson's diseases[19] and ischemia.[20]

### *Resveratrol, oxidative stress, and brain pathology*

From epidemiological studies, the polyphenol resveratrol is recognized as a component which offers many health benefits as they may protect cell constituents against oxidative damage and, therefore, limits the risk of diseases such as atherosclerosis and cancer by directly acting on reactive oxygen species (ROS) or by stimulating endogenous defense systems.[6,9,21–23] Oxidative stress has strong implications for many human diseases and has been connected with neurodegenerative disorders.[24] Brain cells have the capacity to produce peroxides, particularly hydrogen peroxide ($H_2O_2$) in large amounts.[25] Also, $H_2O_2$ concentrations of up to 100 $\mu$M have been reported for brain in a microdialysis study.[26] In this context, the defense of glial cells against peroxide-mediated oxidative damage is essential for maintaining brain functions.

### *Astroglial plasticity*

The last 25 years has seen an exponential increase in knowledge of the neuroglial plasticity.[27] Astroglial cells have been implicated in numerous ways of brain metabolism, especially by the fact as they influence neuronal function particularly at the level of synapses.[27–31] Numerous studies demonstrated that astrocytes play a significant role in neurodegenerative disorders[32–34] and exert a fundamental protective function against oxidative stress because of their effects on the metabolism of the antioxidant glutathione (GSH) and the defence against ROS.[35]

Primary astrocytes and C6 astroglial cell cultures are good models to study glial function, signaling pathways, and mechanisms of peroxide disposal by brain cells.[36–41] In such cultures; however, the influence of other types of brain cells on the antioxidant potential is lacking, in contrast to the *in vivo* situation. Nevertheless, in spite of the fact that comparison of the *in vitro* results with the *in vivo* condition is limited, mainly because astrocyte cells in cultures are two-dimensional and the astroglia *in situ* exist in a three-dimensional matrix, an enormous amount of molecular information has been learnt from the study of astroglial cultures (primary and lineage cells), particularly the investigation of molecular mechanisms which underlie glutamate metabolism and much of it is applicable *in vivo*.

Glutamate is the major excitatory neurotransmitter in the CNS and plays an important role in neural plasticity and neurotoxicity.[42] The modulation of extracellular glutamate determines its physiological and excitotoxic actions. The main mechanism responsible for the maintenance of low-extracellular concentrations of glutamate is performed by a family of glutamate transporter proteins, which use the electrochemical gradients across the plasma membranes as driving forces for uptake.[27] In astrocytes, glutamate is converted into glutamine by the enzyme glutamine synthetase (EC 6.3.1.2).[43] Glutamine is released by astrocytes and taken up by neurons to be again converted to glutamate; this system is called glutamate–glutamine cycle.[44] The interaction between presynaptic and postsynaptic neurons together with astrocytes characterizes the tripartite synapse.[45] Glutamate uptake is also important for maintaining levels of GSH, the major antioxidant of the brain. GSH is a tripeptide formed by amino acids cysteine, glutamate, and glycine, where the sulfhydryl group (SH) of cysteine serves as a proton donor and is responsible for the biological antioxidant effect of GSH.[44] Moreover, GSH secreted from astrocytes serves as the basis for the synthesis of GSH neuronal.[43,46] A large variety of neurological and psychiatric disorders, including depression, anxiety disorders, schizophrenia, chronic pain, epilepsy, Alzheimer's and Parkinson's diseases show in its pathophysiology impairments in glutamatergic system.[47]

## Resveratrol and astroglial plasticity

### *Resveratrol modulates glutamate metabolism*

As astroglial cells are responsible for the uptake of the main extracellular glutamate, our group has been studied the effect of resveratrol on glutamate metabolism in primary culture, cell lines, and acute hippocampal slices.[38,48–51] First, we demonstrated in C6 astroglial cells that resveratrol increased glutamate uptake with doses range from 0.1 to 250

μM.[48] After, in agreement with these results we demonstrated that resveratrol increases glutamate uptake in both primary astrocyte culture and hippocampal slices. In all designed studies the increase in glutamate uptake was around 50% compared to the control condition, except for an opposite effect obtained with the highest concentration of resveratrol (250 μM) which decreased glutamate uptake in primary cell culture, indicating a hormetic phenomenon.[52,53] The concept of chemical hormesis states that chemicals are able to display opposite effects at low and higher levels.[54]

### *Astroglial effects of resveratrol are influenced by redox condition*

The influence of redox condition of the milieu on the effect of resveratrol, summarized in Tables 1–3, was undertaken by two models of oxidative insult:[38] (I) higher concentration of hydrogen peroxide (1 mM), but short-time of exposure (30 min/acute); and (II) lower concentrations of hydrogen peroxide (0.1 mM) and longest time of incubation (up to 6 h). We observed an interesting dual effect of 100 μM resveratrol mostly protecting cells against $H_2O_2$-induced damage in model I and potentiating it in model II, suggesting a prooxidant effect. In both model, $H_2O_2$ decreased glutamate uptake and resveratrol completely prevented this effect in model I and strongly potentiated $H_2O_2$ insult in model II (Table 1).[38] The benefic effect of resveratrol on glutamate metabolism mediated by modulation of important astroglial cell activities is promising; however, this dual effect of resveratrol observed *in vitro* needs to be extended to *in vivo* conditions under different animal models of stress to be better clarified.

**Table 1.** Effects of resveratrol on glutamate uptake

| | C6 astroglial culture | | Primary astrocyte culture[51] | Acute hippocampal slice[50] |
|---|---|---|---|---|
| | I[38] | II[38] | | |
| RSV | ⇧ | = | ⇧ | ⇧ |
| $H_2O_2$ | = | ⇩ | ⇩ | ⇩ |
| RSV + $H_2O_2$ | ⇧ | ⇩⇩ | ⇧ | ⇧ |

RSV, resveratrol; I (1 mM $H_2O_2$/30 min); II (0.1 mM $H_2O_2$/6 h). Arrows indicate increase or decrease of glutamate uptake compared to control conditions. Two arrows indicate potentiating effect.

**Table 2.** Effects of resveratrol on glutamine synthetase activity

| | C6 astroglial culture | |
|---|---|---|
| | I[38] | II[38] |
| RSV | ⇧ | ⇧ |
| $H_2O_2$ | = | ⇩ |
| RSV + $H_2O_2$ | ⇧ | ⇩⇩ |

RSV, resveratrol; I (1 mM $H_2O_2$/30 min); II (0.1 mM $H_2O_2$/6 h). Arrows indicate increase or decrease of glutamate uptake compared to control conditions. Two arrows indicate potentiating effect.

As glutamatergic system has been involved in several brain pathologies[55,56] the modulation of glutamate uptake by resveratrol may represent important pharmacological advances. Besides, our studies suggest that resveratrol can be influenced by surrounding redox environment.

The effect of resveratrol against glutamate excitotoxicity shown in this review may explain the efficacy of resveratrol in protecting brain disorders such as Alzheimer's and Parkinson's diseases, stroke and ischemia injury. In addition, resveratrol has been able to protect organotipic hippocampal culture against ischemia.[57] Thus, resveratrol may represent the new therapeutic potential in protecting brain disorders involving glutamate and oxidative stress.

### *Resveratrol modulates major glutamate destinations in astrocyte*

As resveratrol increased glutamate uptake by astrocyte, we have been investigated two major destinations of glutamate in glial cells: (1) the conversion of glutamate to glutamine by measuring the activity of the astrocyte marker enzyme GS and (2) the amount of GSH, the main antioxidant defense of the CNS. Glutamate–glutamine cycle is defined as carrying glutamine from astrocytes to neurons and glutamate in the opposite direction.[58,59] After uptake by astrocytes, glutamate is converted to glutamine, which in turn, returned to neurons to be reconverted to glutamate.[59] GSH synthesis is a mainly astrocytic process, and astroglial GSH exported to the extracellular space is essential for providing neurons with the GSH precursors.[60]

**Table 3.** Effects of resveratrol on glutathione content

| | C6 astroglial culture | | Primary astrocyte culture[51] | Acute hippocampal slice[50] |
|---|---|---|---|---|
| | I[38] | II[38] | | |
| RSV | ⇧ | = | ⇧ | ⇧ |
| $H_2O_2$ | ⇩ | ⇩ | ⇩ | ⇩ |
| RSV + $H_2O_2$ | ⇧ | ⇩⇩ | ⇧ | ⇧ |

RSV, resveratrol; I (1 mM $H_2O_2$/30 min); II (0.1 mM $H_2O_2$/6 h). Arrows indicate increase or decrease of glutamate uptake compared to control conditions. Two arrows indicate potentiating effect.

Resveratrol, was able to increase the activity of the enzyme GS in both C6 astroglial cells[38,48] and primary culture of astrocytes,[49] indicating an important role in glutamate–glutamine cycle (Table 2). Glutamine levels are related with cellular redox variations and have been decreased under catabolic stress.[44] ROS appear to be a key pleiotropic modulator which may be involved in different pathways, leading to modifications of macromolecules, such as proteins and lipids.[24,61] The activity of the enzyme GS was impaired by oxidative stress and resveratrol was able to prevent this effect. As glutamine is an important source of glutamate, it also helps to maintain GSH levels after injuries in CNS.[62,63] There are many models of study for understanding the pathophysiology of the Alzheimer's disease and one of them involves the intracerebroventricular streptozotocin administration.[64] In this model there is a decrease in GSH levels and resveratrol was able to restore the amount of this antioxidant displaying an important *in vivo* effect of resveratrol in dementia.

Resveratrol increased intracellular GSH in astroglial cell culture[38] and hippocampal slices.[49] However, under oxidative insult resveratrol also display dual effect depending on the redox condition of the milieu (Table 3), similar to the effect observed with glutamate uptake. In an intense (1 mM $H_2O_2$) and acute (30 min) oxidative insult resveratrol prevented $H_2O_2$ -induced GSH decrease, but after 6 h of oxidative insult resveratrol display pro-oxidant effect, potentiating GSH decrease.[38]

In summary, we have been demonstrated that resveratrol may have important role in neuroprotection by increasing glutamate uptake, GS activity, and GSH levels. Neurons are unable to take up



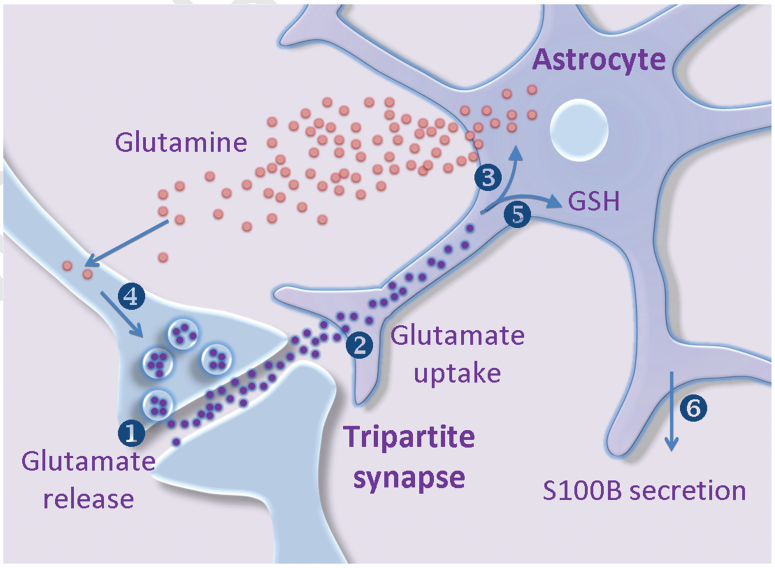


**Figure 1.** Hypothesis for the influence of resveratrol on neuroglial cells. After the release of glutamate at the synaptic cleft 1, resveratrol may improve glutamate uptake by astrocytes 2; stimulating the enzyme GS to convert glutamate into glutamine 3, which in turn is able to be released to the extracellular fluid, taken up by neurons and reconverted into glutamate 4. Also, resveratrol may stimulate another important fate of glutamate in astrocytes, particularly the synthesis of the tripeptide L-γ-glutamyl-L-cysteinyl-glycine or glutathione (GSH) 5 and promote the secretion of the trophic factor S100B 6.

extracellular GSH, but they can make use of cysteinyl-glicine and cysteine, two molecules derived from extracellular GSH. So the neurons need GSH from astrocytes to synthesize it and resveratrol can modulate glutamate–glutamine cycle, through GS activity and GSH levels.

## Hypothesis for the resveratrol on the tripartite synapse

There has been a lack of studies demonstrating the effect of polyphenolic compounds on neuroglial communication and signaling. Astrocytes are one of the most functionally diverse groups of cells in the nervous system, intimately associated with glutamatergic metabolism and transmission, S100B secretion and, thus, with synaptic plasticity and neuroprotection.[27,28,33,65] S100B is trophic factor produced and secreted by astrocytes, involved in neuronal survival and activity during brain injury and recovery.[66–68] Emerging evidence indicates that signaling between perisynaptic astrocytes and neurons at the tripartite synapse plays an important role when neural circuits are formed and refined.[45] Given the role of glutamate in CNS injury, it is important the development of strategies to reduce glutamate-mediated excitotoxicity in psychotic disorders. Among neural cells, astrocytes are more resistant to oxidative stress and provide a protective role for neurons, mainly due to their higher GSH content. Hence, resveratrol modulation of glutamate metabolism *in vitro* is an important key to clarify how effectively this polyphenol could act *in vivo*. As resveratrol was able to induce *in vitro* a significantly increase in glutamate uptake; GS activity; GSH levels and S100B secretion it indicate that astrocytes can be targets of resveratrol *in vivo* to improve brain pathologies.

## Conclusions and future directions

In summary, in spite of vast progress has been made in understanding the resveratrol effects in the brain, our knowledge still rudimentary because the majority of the experiments were performed in cell culture or in brain slices. The number of unanswered questions generated by this scenario highlights the relevance of further studies regarding to the effect of resveratrol on neuroglial plasticity. This knowledge is important to be translated into effective treatments for neural pathologies, including Parkinson's and Alzheimer's diseases. The results found *in vitro* needs to be extended to studies *in vivo* with different doses and redox conditions to get a clear picture of the resveratrol effect to human health.

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## Conflicts of interest

The author declares no conflict of interest.

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## CAPÍTULO IV

Objetivo 3 – Estudar os efeitos do resveratrol sobre sistemas de defesa antioxidante e os prováveis mecanismos envolvidos nessa resposta.

### Manuscrito a ser submetido

### Modulation of antioxidant defenses by resveratrol in the C6 astrocyte cell line

André Quincozes-Santos, Larissa Daniele Bobermin, Alexandra Latini, Moacir Wajner, Carlos-Alberto Gonçalves, Diogo Onofre Souza, Carmem Gottfried

**Modulation of antioxidant**

**defenses by resveratrol in the C6 astrocyte cell line**

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**ABSTRACT**

Resveratrol, a redox-active compound present in grapes and wine display wide range biological effects, including neuroglial modulation and neuroprotection. As astrocytes are key modulators of neuropathology events, frequently associated to oxidative stress, this study sought to determine the effect of resveratrol on neuroglial parameters against $H_2O_2$-induced oxidative stress. Hence, we used two models of oxidative insult: (I) 1 mM $H_2O_2$/0.5 h and (II) 0.1 mM $H_2O_2$/6 h. Resveratrol presented anti- and pro-oxidants effects modulating NO levels; SOD, CAT and GPx activity and GSH content. Resveratrol also stimulated heme oxygenase 1 followed by iNOS decrease. Taken together, these findings shows a potential pathway by which resveratrol can provide protection of astroglial cells against oxidative stress induced by $H_2O_2$.

**Keywords:** resveratrol, astroglial cells, oxidative stress, heme oxygenase 1, neuroprotection

## 1. INTRODUCTION

Oxidative stress defined as an imbalance between cellular oxidants and antioxidants/antioxidant enzymes favors the accumulation of the former and occurs when redox homeostasis is tipped towards an overbalance of reactive species (RS) due to deficiencies in their removal and/or impairment of cellular defense mechanisms (Halliwell, 2006). Reactive oxygen species (ROS), such as hydrogen peroxide ($H_2O_2$), are constitutively generated during normal cellular metabolism and at physiological levels, play important roles in signaling pathways regulating cell proliferation, differentiation and activation of transcription factors (Dringen et al., 1998, Dringen et al., 2005).

Heme oxygenase (HO) the rate-limiting enzyme in heme degradation has two isoforms; the inducible HO1 and the constitutive HO2 (Dore, 2005). HO1 is able to inhibit inducible nitric oxide synthase (iNOS) (Wakabayashi et al.), an isoform of nitric oxide synthase that catalyze the synthesis of NO from L-arginine. NO interacts with many intracellular targets to trigger an array of signal transduction pathways, resulting in stimulatory or inhibitory output signals. NO also becomes noxious if it is produced in excess. Furthermore, if a cell is in a pro-oxidant state, NO can undergo oxidative-reductive reactions to form toxic compounds (these belong to a family known as “reactive nitrogen species” or RNS), which cause cellular damage (Coyle and Puttfarcken, 1993, Calabrese et al., 2007). It has frequently been suggested that naturally occurring polyphenols help to regulate the ROS/RNS imbalance (Halliwell, 2007).

The polyphenol resveratrol (3,5,4’-trihydroxy-stilbene), a redox active compound is a phytoalexin found in a wide variety of dietary sources including grapes and peanuts (Soleas et al., 1997, Fremont, 2000, Pervaiz, 2003, Delmas

et al., 2005) and is also present in wines, especially red wines (de la Lastra and Villegas, 2007). In this context, resveratrol appears as a molecule with high antioxidants properties, possible by its properties of direct scavenging effect and/or activation of pathways that upregulate natural antioxidant defenses (Baur and Sinclair, 2006). Many studies have shown that resveratrol can prevent or slow the progression of a wide variety of illnesses, including cancer (Jang et al., 1997) and cardiovascular disease (Pervaiz, 2004, Ungvari et al., 2009) and improves health and survival of mice under high-calorie diet (Baur et al., 2006). Moreover, it has been demonstrated that resveratrol also has beneficial effects in neurological diseases (Virgili and Contestabile, 2000, Sinha et al., 2002, Baur and Sinclair, 2006, dos Santos et al., 2006, Quincozes-Santos et al., 2009) and is able to inhibit β-amyloid peptide aggregation (Riviere et al., 2010). Whilst direct protective effects of resveratrol against oxidative stress have been demonstrated in neuroglial cells (de Almeida et al., 2008, Vieira de Almeida et al., 2008, Quincozes-Santos et al., 2009), the mechanisms of these neuroprotective effects are not fully understood.

Glial cells are the most abundant cells in the human brain maintaining the homeostasis throughout the normal central nervous system (CNS). Astrocytes have been shown to be involved in the regulation of the brain microenvironment, in particular as regards neurotransmitter and ionic homeostasis, metabolic support of neurons, regulation of energy metabolism, synaptic transmission and neuronal excitability, synaptic generation, detoxification, free-radical scavenging, metal sequestration, developmental and maintenance of the blood-brain barrier, guidance of neuronal migration and immune function (Markiewicz and Lukomska, 2006). Astrocytes express

numerous receptors that enable them to respond to various neuroactive compounds, including neurotransmitters, neuropeptides, growth factors, cytokines, small molecules and toxins. These receptors not only to participate in signal processing, but also to function as sentinels (Nedergaard et al., 2003, Barbeito et al., 2004). The C6 cell lineage (98% GFAP positive after 100 passages) are widely used as an astrocyte-like cell line to study cell function, *e.g.*, glutamate uptake, glutamine syntethase (GS) activity, S100B secretion and oxidative stress (dos Santos et al., 2006, de Almeida et al., 2007, Quincozes-Santos et al., 2009).

In the present study, we investigated the effect of resveratrol in C6 astroglial cells, under two independents models of oxidative insult, namely model I ($H_2O_2$ – 1 mM for 0.5 h) and model II (0.1 mM for 6 h) on neurochemical parameters. Nitric oxide (NO) levels, immunocontent of iNOS and HO1, total antioxidant reactivity (TAR), the activities of the main antioxidant enzymes, superoxide dismutase (SOD), catalase and glutathione peroxidase (GPx), the intra and extracellular content of the main non-enzymatic antioxidant glutathione (GSH) and creatine kinase activity were assessed. Additionally, the rate of DCFH oxidation was measured to evaluate the effects of extracellular calcium (with the use of EGTA) in model I and II of oxidative insult.

## 2. EXPERIMENTAL PROCEDURES

### 2.1. Materials

Resveratrol, chemical reagents and cell culture materials were purchased from Sigma (St. Louis, MO, USA), except for Dulbecco's Eagle's medium (DMEM) which was purchased from Gibco BRL (Carlbad, CA, USA)

and fetal bovine serum (FBS) from Cultilab (Campinas, SP, Brazil). All other chemicals were purchased from local commercial suppliers.

**2.2. Maintenance of cell culture**

The C6 astroglial cells were obtained from the American Type Culture Collection (Rockville, MD, USA) and were maintained essentially according to the procedure previously described by Dos Santos et al., 2006 (dos Santos et al., 2006). The cells were seeded in flasks and cultured in DMEM (pH 7.4) containing 5% fetal bovine serum (FBS), 2.5 mg/mL Fungizone ® and 100 U/L gentamicin. Cells were kept at a temperature of 37°C in an atmosphere of 5% $CO_2$/95% air. Exponentially growing cells were detached from the culture flasks using 0.05% trypsin/ethylene-diaminetetracetic acid (EDTA) and seeded 10 X $10^3$ cells/$cm^2$ in 96-, 24- or 6-well plates.

**2.3. Resveratrol and hydrogen peroxide treatments**

After cells reached confluence, the culture medium was removed and cells were pre-incubated in the absence or presence of 100 µM of resveratrol for 1 h, in serum-free DMEM (pH 7.4). After this time, the medium was maintained and $H_2O_2$ was added as follows: model I – 1 mM $H_2O_2$ for 30 min and model II – 0.1 mM $H_2O_2$ for 6 h. During incubations, cells were maintained at 37°C in an atmosphere of 5% $CO_2$/95% air. Control cells were exposed to 0.25% ethanol vehicle. For all parameters analyzed, the results obtained with vehicle were not different from those obtained under basal conditions without ethanol. To test the effect of L-NAME in models I and II we pre-treated cells with 500 µM of L-NAME for 1 h.

**2.4. Nitric oxide production**

Nitric oxide was determined by measurement of nitrite (a stable oxidation product of NO), based on the Griess reaction. The Griess reagent was prepared by mixing equal volumes of 1% sulfanilamide in 0.5 N HCl and 0.1% N-(1-naphthyl) ethylenediamine in deionized water. The assay was performed as described (Hu et al., 1996) with modifications. Briefly, cells were cultured on 96-well plate and after treatment, the Griess reagent was added directly to the cell culture and the incubation was maintained under reduced light at room temperature during 15 min. Samples were analyzed at 550 nm on a microplate spectrophotometer. Controls and blanks were run simultaneously. Nitrite concentrations were calculated using a standard curve prepared with sodium nitrite (0–50 µM).

**2.5. Western blot analysis**

Equal amounts of proteins from each sample were boiled in sample buffer (0.0625 M Tris-HCl, pH 6.8, 2% (w/v) SDS, 5% (w/v) β-mercaptoethanol, 10% (v/v) glycerol, 0.002% (w/v) bromophenol blue) and electrophoresed in 10% (w/v) SDS-polyacrylamide gel. The separated proteins were blotted onto a nitrocellulose membrane. Equal loading of each sample was confirmed with Ponceau S staining (Sigma). The following polyclonal antibodies were used anti-iNOS (1:10000; Sigma) and anti-HO-1 (1:3000; Santa Cruz). After incubating overnight with the primary antibody at room temperature, filters were washed and incubated with peroxidase-conjugated anti-rabbit immunoglobulin (IgG) at a dilution of 1:1 000 for 1 h. The chemiluminescence signal was detected using an ECL (Amersham), after the films were scanned and bands were quantified using the Scion Image software.

**2.6. Total antioxidant reactivity (TAR)**

TAR, which represents the reactivity or quality of the tissue antioxidants, was determined by measuring the luminol chemiluminescence intensity induced by 2,2'-azo-bis-(2-amidinopropane) (ABAP) (Lissi et al., 1995). The background chemiluminescence was measured by addicting 4 ml 2 mM ABAP (in 0.1 M glycine buffer, pH 8.6) into a glass scintillation vial. Ten microliters of luminol (4 mM) were added to each vial and the chemiluminescence was determined. This was considered to be the basal value. Fifty microliters of cell homogenates were then added and the chemiluminescence was measured during 60 s. The supernatant addition reduces the chemiluminescence and this rapid reduction in luminol intensity is considered measure of TAR capacity. A calibration curve was carried out with standard trolox solutions (10-20 µM). TAR values were calculated as nmol trolox/mg protein and expressed as percentage of controls.

**2.7. Superoxide dismutase (SOD) activity**

SOD activity was determined using the RANSOD kit from Randox (Autrim, United Kingdom). The method is based on the formation of red formazan from the reaction of 2-(4-iodophenyl)-3-(4-nitrophenol)-5-phenyltetrazolium chloride and superoxide radical produced in the incubation medium from the xanthine and xanthine oxidase reaction system, which is assayed spectrophotometrically at 505 nm. The inhibition of the produced chromogen is proportional to the activity of the SOD present in the sample. A 50% inhibition is defined as one unit of SOD and the specific activity is represented as U/mg protein.

**2.8. Catalase (CAT) activity**

CAT activity was assayed by the method of Aebi (1984) (Aebi, 1984) by measuring the absorbance decrease at 240 nm in a reaction medium containing

20 mM $H_2O_2$, 0.1% Triton X-100, 10 mM potassium phosphate buffer, pH 7.0, and 50 µg protein. One unit (U) of the enzyme is defined as 1 µmol of $H_2O_2$ consumed per minute and the specific activity is reported as U/mg protein.

**2.9. Glutathione peroxidase (GPx) activity**

GPx activity was measured by the method of Wendel (1981) (Wendel, 1981) using *tert*-butyl-hidroperoxide as substrate. GPx activity was determined by monitoring NADPH (0.1 mM) disappearance at 340 nm in a medium containing 2 mM GSH, 0.15 U/ml glutathione reductase, 0.4 mM azide and 0.5 mM *tert*-butyl-hidroperoxide. One GPx unit is defined as 1 µmol of NADPH consumed per minute and the specific activity is represented as U/mg protein.

**2.10. Glutathione (GSH) intra- and extracellular levels measurement**

GSH levels were measured according to Browne and Armstrong (1998) (Browne and Armstrong, 1998). C6 astroglial cells homogenates and extracellular medium were diluted in 10 volumes of 100 mM sodium phosphate buffer, pH 8.0, containing 5 mM EDTA and protein was precipitated with 1.7% meta-phosphoric acid. Supernatant was assayed with *o*-phthaldialdeyde (1 mg/mL methanol) at room temperature for 15 min. Fluorescence was measured using excitation and emission wavelengths of 350 and 420 nm, respectively. A calibration curve was performed with standard GSH solutions (0-500 µM). GSH concentrations were calculated as nmol/mg protein and expressed as percentage of controls.

**2.11. Creatine Kinase (CK) Activity**

The cells were homogenized with a 0.9% saline solution and an aliquot containing approximately 1 mg protein was pre-incubated for 15 min at 37°C in a mixture containing the following final concentrations: 7 mM phosphocreatine,

9 mM $MgSO_4$, 60 mM Tris–HCl buffer, pH 7.5 in a final volume of 0.1 mL. Incubation was started by the addition of 3.2 mM ADP plus 0.8 mM reduced glutathione. The reaction was stopped after 10 min by the addition of 10 mmol *p*-hydroxymercuribenzoic acid. The reagent concentrations and the incubation time were chosen to assure linearity of the enzymatic reaction. Appropriate controls were carried out to measure chemical hydrolysis of phosphocreatine. The creatine formed was estimated according to the colorimetric method of Hughes (1962) (Hughes, 1962). The color was developed by the addition of 0.1 mL 20% *α*-naphthol and 0.1 mL 20% diacetyl in a final volume of 1 mL and read after 20 min at 540 nm. All assays were performed in triplicate and the mean was used for the calculations. Results were obtained as *μ*mol of creatine formed/min/mg protein.

**2.12. DCFH oxidation**

Intracellular ROS production was detected using the nonfluorescent cell permeating compound, 2'-7'-dichlorofluorescein diacetate (DCFH-DA). DCFH-DA is hydrolyzed by intracellular esterases to dichlorofluorescin (DCFH), which is trapped within the cell. This nonfluorescent molecule is then oxidized to fluorescent dichlorofluorescin (DCF) by action of cellular oxidants. C6 astroglial cells were pre-incubated with 2 mM EGTA (ethylene glycol tetraacetic acid) for 30 min before treatments described in item 2.3 and maintained until the end of incubations. After C6 cells were treated with DCFH-DA (10 μM) for 30 min at 37°C. Following DCFH-DA exposure, the cells were 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 (Vieira de Almeida et al., 2008).

### 2.13. Protein determination

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

### 2.14. Statistical analysis

Data from the experiments are presented as mean ± S.E.M. Resveratrol and different doses of $H_2O_2$ were analyzed statistically by two-way analysis of variance (ANOVA) followed by the Tukey´s test. Values of $P < 0.05$ were considered to be significant. All analyses were performed using Statistical Package for the Social Sciences (SPSS) software in a PC-compatible computer (*a* indicates differences from control, *b* indicates differences from $H_2O_2$, *c* refers differences from L-NAME and EGTA treatments).

## 3. RESULTS

### 3.1. Resveratrol and L-NAME decrease nitric oxide (NO) levels

The production of NO was measured by the formation of nitrite, expressed in µM. To test whether nitrite accumulation was dependent on NOS activity, we examined the effect of resveratrol in the presence of the NOS inhibitor, L-NAME. In model I of oxidative insult (Figure 1), resveratrol and L-NAME decreased nitrite levels (from 13 ± 0.9 to 11 ± 0.7 µM and from 13 ± 0.9 to 11.3 ± 0.9 µM), respectively compared to control conditions. Oxidative insult increased nitrite formation up to 15%, which was completely prevented by resveratrol and L-NAME. The co-incubation of resveratrol and L-NAME decreased the nitrite levels up to 22%.

In model II, resveratrol and L-NAME also decreased nitrite levels (from 14.1 ± 0.7 to 12.3 ± 0.8 µM and from 14.1 ± 0.7 to 12.4 ± 0.6 µM), respectively compared to control conditions. As expected, oxidative insult significantly

increased nitrite levels up to 20%. Resveratrol, in this oxidative insult induced a positive response, decreasing nitrite levels to lower values than control conditions. As in model I, resveratrol plus L-NAME were able to decrease nitrite levels by 22%.

### 3.2. Role of iNOS activity on resveratrol and in models I and II of oxidative insult

To verify the role of NO in models I and II we determine the involvement of iNOS activity. Resveratrol and oxidative insult modulated iNOS. In models I and II (Figure 2) $H_2O_2$-induced oxidative stress increases iNOS activity (about 50%) compared to control conditions. When the cells were pre-treated with resveratrol under oxidative insult in both models resveratrol decreases iNOS activity compared to oxidative insult.

### 3.3. Effect of resveratrol on heme oxygenase 1 under $H_2O_2$ oxidative insult

Recent studies have shown that resveratrol modulates HO1. Here we demonstrated that resveratrol increases HO1 in both models compared to control conditions (Figure 3). However, $H_2O_2$-oxidative insult induces a slight decrease in HO1 in model II compared to control conditions. Resveratrol under model I of oxidative insult increases HO1 protein levels; but under model II of oxidative insult decreases HO1 (about 20%), potentiating the effect induced by $H_2O_2$.

### 3.4. Resveratrol increase total antioxidant reactivity (TAR)

In model I (Fig. 4), resveratrol increased (50%) the total antioxidant reactivity (TAR) compared to control conditions. Under oxidative insult ($H_2O_2$) the TAR levels were reduced about 15%. Resveratrol was able to prevent $H_2O_2$ effect increasing TAR levels up to 55%, completely prevented this effect. In

model II, resveratrol also increased the total antioxidant reactivity (45%). Oxidative insult significantly decreased the TAR levels and resveratrol reestablishes these values to control conditions.

**3.5. Effects of resveratrol on superoxide dismutase (SOD), catalase and glutathione peroxidase (GPx) activity**

The superoxide dismutase (SOD) activity was increased by resveratrol (2.7 ± 0.15 to 3.1 ± 0.15 U/mg protein) compared to control conditions, Table 1. Under oxidative insult there was a decrease in SOD activity (from 2.7 ± 0.15 to 1.9 ± 0.15 U/mg protein), which was partially prevented by resveratrol. In model II, resveratrol increased the SOD activity about 20% (3.0 ± 0.15 to 3.6 ± 0.15 U/mg protein). The $H_2O_2$ insult significantly decreased the SOD activity. This effect was potentiated by resveratrol decreasing it by 65% compared to control conditions and reducing it by 30% when compared to $H_2O_2$ values.

Table 1 also displays the catalase activity. In model I, under oxidative insult the catalase activity decrease (14.2 ± 1.35 to 12.8 ± 1.15 U/mg protein) compared to control conditions. Resveratrol *per se* did not affect the catalase activity, but was able to prevent $H_2O_2$-insult.

In model II, 0.1 mM of $H_2O_2$ for 6 h, decrease the catalase activity about 20% compared to control values and resveratrol did not prevent the reduce induced by $H_2O_2$-insult, on the contrary, decrease the catalase activity about 15% compared the controls conditions.

GPx activity was not affected by resveratrol in both models (I and II), Table 1. In model I, oxidative insult also did not affect GPx activity compared to control conditions and under oxidative conditions resveratrol decreased GPx

activity (20%). In model II, resveratrol and oxidative insult did not affect GPx activity compared to control conditions.

### 3.6. Effects of resveratrol on intra and extracellular glutathione (GSH) levels

In model I (Table 2) resveratrol increased GSH intracellular (26%) and extracellular (13%) levels compared to control conditions. Oxidative insult decreased GSH content by 28%. Resveratrol was able to prevent the $H_2O_2$ effect increasing GSH intracellular (50%) and extracellular (10%) levels compared to control conditions. In model II resveratrol *per se* only increased GSH extracellular levels (19%) and resveratrol potentiated $H_2O_2$-induced decreasing GSH intra and extracellular levels, about 48% and 20%, respectively, compared to ethanol conditions.

### 3.7. Effects of resveratrol on creatine kinase (CK) activity

Figure 5 shows the CK activity. Oxidative insult decreased CK activity by 15 % (from 1.5 ± 0.05 to 1.3 ± 0.043) in model I and it two fold (from 3.0 ± 0.065 to 1.7 ± 0.05) in model II. Resveratrol *per se* did not affect CK activity; however, was able to prevent $H_2O_2$ effect in model I.

### 3.8. Effects of resveratrol on DCFH oxidation

The production of ROS was investigated by DCFH oxidation. In model I (Figure 6) resveratrol decreased DCFH oxidation compared to control conditions. In addition, resveratrol was able to prevent the increase of ROS production induced by $H_2O_2$ to lowest values than that of control conditions. In model II of oxidative insult, resveratrol is still able to decrease DCFH oxidation compared to control cells but in the presence of $H_2O_2$, resveratrol potentiated ROS production compared to $H_2O_2$-insult.

We also demonstrated the effect of EGTA (calcium chelator) on DCFH oxidation under two models of oxidative insult. EGTA in both models did not affect DCFH oxidation. The co-incubation between EGTA + $H_2O_2$ (EGTA oxidative insult) in models I and II increased about 10% DCFH oxidation and between EGTA + resveratrol 100 µM decreased by 20% DCFH oxidation compared to control conditions. However, when we incubated EGTA + $H_2O_2$ + resveratrol 100 µM concomitantly we observed two different effects, in model I there was a decreased DCFH oxidation compared to control conditions and EGTA oxidative insult. In model II, there was an increased DCFH oxidation compared to control and compared to EGTA oxidative insult, showing the pro-oxidant effect. This result suggests that calcium was involved in ROS production induced in models I and II.

**4. DISCUSSION**

We recently demonstrated that resveratrol modulates glial function (dos Santos et al., 2006), attenuates oxidative-induced DNA damage (Quincozes-Santos et al., 2007) and attenuates or potentiating genotoxicity in C6 astroglial cells (Quincozes-Santos et al.). We also have been demonstrated that resveratrol presents anti- and pro-oxidant effects in glial functions and oxidative conditions (Quincozes-Santos et al., 2009).

Here we observed that resveratrol decreased levels of nitric oxide (NO), a multifunctional, a short-lived molecule implicated in the pathogenesis of neurodegenerative disorders like Alzheimer's and Parkinson's diseases (Calabrese et al., 2007). NO is involved in several cellular functions, including neurotransmission, regulation of blood-vessel tone and the immune response. NO is synthesized during the stoichiometric conversion of L-arginine to L-

citrulline in the presence of oxygen and nicotinamide adenine dinucleotide phosphate (NADPH), which is catalyzed by NOS (Moncada et al., 1991). NO production is associated with cognitive function, its role spanning from the induction and maintenance of synaptic plasticity to the control of sleep, appetite, body temperature and neurosecretion (Calabrese et al., 2007). Thus resveratrol may have a protective effect against diseases related to levels of NO.

L-NAME ($N^{\omega}$-nitro-L-arginine methyl ester) a non-selective NOS inhibitor (Hu et al., 1996) was used to investigate whether the effect of resveratrol against $H_2O_2$-induced NO increase was via NOS. In fact, under oxidant conditions resveratrol and L-NAME potentiated NO decrease compared to single resveratrol effect. Thus, it is feasible that resveratrol actually modulates the activity of NOS, accordingly to previous works, which demonstrates that resveratrol acts through the induction of iNOS mRNA (Hattori et al., 2002).

Resveratrol *per se* also was able to modulate the activity of HO1. Recently, Sakata et al (Sakata et al.), showed that HO1 might be a unique candidate by which resveratrol can induce an endogenous cellular pathway which leads to building cellular and/or organ resistance to stress, indicating neuroprotective effect. In agreement with Sakata, we showed here that resveratrol, via HO1, have anti- and pro-oxidant effects and thus could explain the actions of resveratrol on CNS (Bastianetto and Quirion). Furthermore, there is a relationship between HO1 and iNOS. The transcription factor Nrf2 regulates the transcription of HO1 which acts as a scavenger of NO (Wakabayashi et al.). Excess of NO acts as a positive signal to increase HO1, which in turn is able to scavenge NO and block the activity of iNOS to prevent further NO production. Resveratrol, under oxidative conditions induced a decrease in iNOS and an

increase in HO1 (just in model I), thus HO1 can also be critical to signaling antioxidant response of resveratrol. However, in model II resveratrol potentiated the effect of $H_2O_2$, probably via PKC, in agreement with epigallocatechin data which play effect on HO1 via delta isoform of PKC (Bastianetto and Quirion, Ogborne et al., 2008) and, as we recently demonstrated (Quincozes-Santos et al., 2009), the PKC-dependent effect of resveratrol on the potentiation of $H_2O_2$-induced glutamate uptake decrease.

The TAR levels represent the antioxidant capacity of cells and resveratrol was able to increase about 50 % and 45 % TAR levels in models I and II respectively. These findings suggest that resveratrol display antioxidant effect, protecting cells against oxidative damage. In the two models of oxidative insult, $H_2O_2$ decrease TAR levels. The effects elicited by resveratrol have been mainly attributed to its intrinsic antioxidant properties; however, it has been demonstrated that it is also able to modulate diverse cell activities independently of its antioxidant properties (Ovesna and Horvathova-Kozics, 2005).

The concentrations of free radicals are determined by the balance between their rates of production and their rates of clearance by various antioxidant compounds and enzymes such as SOD, GPx and catalase. Resveratrol prevented completely the $H_2O_2$-effect reestablishing SOD activity which in turn can decrease superoxide radicals (Pope et al., 2008, Spanier et al., 2009). In this context modulation of SOD activity by resveratrol is very important to the neuroprotective effect associated to oxidative stress. However, we also observed an increase in catalase activity in the model I compared to oxidative insult, showing that the hydrogen peroxide formed is decomposed to

water (Rodriguez et al., 2004, Smith-Pearson et al., 2008). Resveratrol decreases the GPx activity under $H_2O_2$-induced oxidative stress improving the levels of GSH, the main neuronal antioxidant produced by astrocytes. As many redox-active compounds modulate major enzymatic defenses against free radical action (Khan et al., Sebai et al., Kasdallah-Grissa et al., 2007, Gelain and Moreira, 2008, Meng et al., 2008), the anti or pro-oxidant role is determined by the set of their actions and cell type studied (de la Lastra and Villegas, 2007, Halliwell, 2007).

We have been showed that resveratrol modulates astroglial parameters related to glutamate metabolism (Quincozes-Santos et al., 2009) including the increase in glutamate uptake and GSH levels. GSH is secreted by astrocytes and serves as a substrate to neuronal GSH synthesis (Dringen, 2000, Pope et al., 2008).

We also showed that although resveratrol did not modify creatine kinase (CK) activity *per se*, it was able to prevent $H_2O_2$-induced CK activity decrease. As CK consists of a family of enzymes involved in high-energy consuming tissues such as brain and skeletal muscle and it also is very sensitive to oxidative damage (oxidation and nitration) (Andres et al., 2008), this indicates that resveratrol can display *in vivo* function on CK activity in pathologies related to redox imbalance.

Besides, resveratrol decreased DCFH oxidation, showing antioxidant properties; however, $H_2O_2$-oxidative insult increased DCFH oxidation and resveratrol under model I of oxidative insult prevents totally this effect and in model II potentiated this effect. This suggests that this polyphenol may exert different effects on cell systems under different oxidative conditions and in the

presence of a strong oxidant compound could be probably metabolized to oxidant molecules potentiating $H_2O_2$-oxidative effect (Murias et al., 2005).

Here we demonstrated that resveratrol is also able to modulate $Ca^{2+}$ levels. The intracellular $Ca^{2+}$ increase is associated to oxidative stress and neuronal death via glutamate receptor (Dawson et al., 1992, Coyle and Puttfarcken, 1993, Banerjee et al., 2008). The activation of NMDA receptor could generate NO, mediated by $Ca^{2+}$-activation of NOS (Coyle and Puttfarcken, 1993). Then, resveratrol could display protection against neurotoxicity mediated by glutamate and pathology associated to oxidative stress. Resveratrol under oxidative insult inhibited iNOS, and decreases DCFH oxidation, blocking $Ca^{2+}$ levels, emerging as an important molecule which provides protection against neurotoxicity.

In summary, our results show that the redox active compound resveratrol modulates important functions related to maintenance of the cellular redox state. In addition to its antioxidant properties, resveratrol display pro-oxidant effects, depending of the oxidant conditions of the milieu. The main conclusions of this study are depicted in Figure 7. The NO-, iNOS-, HO1-inhibition; the modulation on the SOD, CAT, GPx and GSH content induced by resveratrol is a relevant data, concerning neurodegenerative diseases, due to their association with oxidative stress. Current research reveals the different potential applications of resveratrol-formulations for treatment-, reverse strategies and preventive care in health.

**Legends of Figures.**

**Fig. 1 Effects of resveratrol and L-NAME on nitrite levels.** Cells were pre-incubated for 1 h in the presence of 100 µM resveratrol or 500 µM L-NAME, followed by the addition of 1 mM $H_2O_2$ for 0.5 h, co-incubated with resveratrol/L-NAME (model I); of 0.1 mM $H_2O_2$ for 6 h, co-incubated with resveratrol/L-NAME (model II) or resveratrol and L-NAME. Data represent means ± S.E.M of three experimental determinations performed in triplicate, analyzed statistically by two way ANOVA followed by Tukey's test. (a) Significant differences from control (0.25% ethanol); (b) significant differences from $H_2O_2$ and (c) significant differences between resveratrol and L-NAME. For model I – a: $F_{(3,36)}$= 10.515, ($P < 0.05$); b: $F_{(3,36)}$= 10.890, ($P < 0.05$); c: $F_{(3,36)}$= 12.110, ($P < 0.05$). For model – II: a: $F_{(3,36)}$= 19.270, ($P < 0.001$); b: $F_{(3,36)}$= 16.630 ($P < 0.001$); c: ($F_{(3,36)}$=12.215, ($P < 0.05$).

**Fig. 2 Effects of resveratrol on Inducible Nitric Oxide Synthase**. Cells were pre-incubated for 1 h in the presence of 100 µM resveratrol before the addition of $H_2O_2$. Oxidative damage was induced with 1 mM $H_2O_2$ for 0.5 h, co-incubated with resveratrol (model I) or with 0.1 mM $H_2O_2$ for 6 h, co-incubated with resveratrol (model II). Data represent means ± S.E.M of three experimental determinations performed in triplicate. (a) Significant differences from control (0.25% ethanol); (b) significant differences from $H_2O_2$. For model I – a: $F_{(3,36)}$= 43.212, ($P < 0.001$); b: $F_{(3,36)}$= 14.486, ($P < 0.001$). For model II – a: $F_{(3,36)}$= 37.708, ($P < 0.001$); b: $F_{(3,36)}$= 13.440 ($P < 0.05$).

**Fig. 3 Effects of resveratrol on Heme Oxygenase 1**. Cells were pre-incubated for 1 h in the presence of 100 µM resveratrol before the addition of $H_2O_2$. Oxidative damage was induced with 1 mM $H_2O_2$ for 0.5 h, co-incubated with resveratrol (model I) or with 0.1 mM $H_2O_2$ for 6 h, co-incubated with resveratrol (model II). Data represent means ± S.E.M of three experimental determinations performed in triplicate. (a) Significant differences from control (0.25% ethanol); (b) significant differences from $H_2O_2$. For model I – a: $F_{(3,36)}$= 31.510, ($P < 0.001$); b: $F_{(3,36)}$= 23.190, ($P < 0.001$). For model II – a: $F_{(3,36)}$= 33.700, ($P < 0.001$); b: $F_{(3,36)}$= 16.980 ($P < 0.05$).

**Fig 4 Effects of resveratrol on total antioxidant reactivity (TAR)**. Cells were pre-incubated for 1 h in the presence of 100 µM resveratrol, followed by the addition of 1 mM $H_2O_2$ for 0.5 h, co-incubated with resveratrol (model I) or of 0.1 mM $H_2O_2$ for 6 h, co-incubated with resveratrol (model II). Data represent means ± S.E.M of three experimental determinations performed in triplicate. (a) Significant differences from control (0.25% ethanol); (b) significant differences from $H_2O_2$. For model I – a: $F_{(3,36)}$= 18.440, ($P < 0.001$); b: $F_{(3,36)}$= 27.310, ($P < 0.001$). For model II – a: $F_{(3,36)}$= 15.215, ($P < 0.001$); b: $F_{(3,36)}$= 14.300 ($P < 0.05$).

**Fig 5 Effects of resveratrol on creatine Kinase (CK) activity**. Cells were pre-incubated for 1 h in the presence of 100 µM resveratrol, followed by the addition of 1 mM $H_2O_2$ for 0.5 h, co-incubated with resveratrol (model I) or of 0.1 mM $H_2O_2$ for 6 h, co-incubated with resveratrol (model II). Data represent means ± S.E.M of three experimental determinations performed in triplicate. (a) Significant differences from control (0.25% ethanol); (b) significant differences from $H_2O_2$. For model I – a: $F_{(3,36)}$= 10.115, ($P < 0.05$); b: $F_{(3,36)}$= 10.220, ($P < 0.05$). For model II – a: $F_{(3,36)}$= 11.755, ($P < 0.05$).

**Fig 6 Effects of resveratrol on DCFH oxidation**. Cells were pre-incubated for 1 h in the presence of 100 µM resveratrol, followed by the addition of 1 mM $H_2O_2$ for 0.5 h, co-incubated with resveratrol (model I) or of 0.1 mM $H_2O_2$ for 6 h, co-incubated with resveratrol (model II). Cells were pre-incubated for 30 min with EGTA (2 mM) before model I and II treatments. Data represent means ± S.E.M of three experimental determinations performed in triplicate. (a) Significant differences from control (0.25% ethanol); (b) significant differences from $H_2O_2$ and (c) significant differences from EGTA. For model I – a: $F_{(3,36)}$= 20.376, ($P < 0.001$); b: $F_{(3,36)}$= 22.470, ($P < 0.01$); c: $F_{(3,36)}$= 20.130, ($P < 0.01$). For model II – a: $F_{(3,36)}$= 10.412, ($P < 0.05$); b: $F_{(3,36)}$= 9.770 ($P < 0.05$); c: ($F_{(3,36)}$= 13.290, ($P < 0.05$).

**Figure 7 Possible targets of resveratrol in astroglial cells**. $H_2O_2$-induced ROS increase is prevented by resveratrol, followed by modulation of antioxidant

enzymes and GSH. ROS impairs iNOS, SOD, CAT and GPx activity and decrease GSH. Resveratrol induces HO1 followed by iNOS activity inhibition and consequently NO levels attenuation. Thus, resveratrol is able to confer protection against $H_2O_2$-induced oxidative stress in astroglial cells via HO1 activation.

**Table 1. Effect of resveratrol on SOD, catalase and GPx activity.**

| Treatments | SOD (U/mg protein) | | Catalase (U/mg protein) | | GPx (U/mg protein) | |
|---|---|---|---|---|---|---|
| | I | II | I | II | I | II |
| Control | 2.7±0.15 | 3.0±0.15 | 14.2±1.35 | 16.5±1.8 | 14.4±1.2 | 15.1±1.2 |
| Resv | 3.1±0.15 (a) | 3.6±0.16 (a) | 14.4±1.15 | 17.9±1.1 | 15.4±1.2 | 16.6±1.2 |
| $H_2O_2$ | 1.9±0.15 (a) | 1.6±0.12 (a) | 12.8±1.15 (a) | 13.5±1.6 (a) | 13.0±0.8 | 13.9±1.3 |
| Resv + $H_2O_2$ | 2.9±0.15 (b) | 1.1±0.13 (a,b) | 14.3±1.15 (b) | 14±0.9 (a) | 11.6±1.2 (a) | 14.1±1.4 |

Cells were pre-incubated for 1 h in the presence of 100 μM resveratrol before the addition of $H_2O_2$. Oxidative damage was induced with 1 mM $H_2O_2$ for 0.5 h, co-incubated with resveratrol (model I) or with 0.1 mM $H_2O_2$ for 6 h, co-incubated resveratrol (model II). All data represent means ± S.E.M. of three experimental determinations performed in triplicate. (a) Significant differences from control (0.25% ethanol); (b) significantly different from $H_2O_2$. Statistical analysis for SOD activity, for model I – a: $F_{(3,36)}$= 10.120, ($P < 0.05$); b: $F_{(3,36)}$= 12.310, ($P < 0.05$) and for model II – a: $F_{(3,36)}$= 11.790, ($P < 0.05$); b: $F_{(3,36)}$= 13.300 ($P < 0.05$). Statistical analysis for catalase activity, for model I – a: $F_{(3,36)}$= 13.520, ($P < 0.05$); b: $F_{(3,36)}$= 17.390, ($P < 0.05$) and for model II – a: $F_{(3,36)}$= 9.990, ($P < 0.05$). Statistical analysis for GPx activity, for model I – a: $F_{(3,36)}$= 14.340, ($P < 0.05$).

**Table 2. Effect of resveratrol on GSH levels.**

| Treatments | GSH intracellular | | GSH extracellular | |
|---|---|---|---|---|
| | I | II | I | II |
| Control | 100±3.2 | 100±3.3 | 100±4.0 | 100±4.1 |
| Resv | 126±4.1 (a) | 110±3.0 | 113±5.1 (a) | 119±4.7 (a) |
| $H_2O_2$ | 72±3.0 (a) | 78±2.9 (a) | 90±3.7 | 84±4.3 (a) |
| Resv + $H_2O_2$ | 150±5.2 (a,b) | 52±2.3 (a,b) | 110±5.3 (b) | 80±5.0 (a) |

Cells were pre-incubated for 1 h in the presence of 100 μM resveratrol before the addition of $H_2O_2$. Oxidative damage was induced with 1 mM $H_2O_2$ for 0.5 h, co-incubated with resveratrol (model I) or with 0.1 mM $H_2O_2$ for 6 h, co-incubated resveratrol (model II). All data represent means ± S.E.M. of three experimental determinations performed in triplicate. (a) Significant differences from control (0.25% ethanol); (b) significantly different from $H_2O_2$. Statistical analysis for GSH intracellular, for model I – a: $F_{(3,36)}$= 48.310, ($P < 0.001$); b: $F_{(3,36)}$= 76.290, ($P < 0.001$) and for model II – a: $F_{(3,36)}$= 35.230, ($P < 0.001$); b: $F_{(3,36)}$= 43.400 ($P < 0.001$). Statistical analysis for GSH extracellular, for model I – a: $F_{(3,36)}$= 13.500, ($P < 0.05$); b: $F_{(3,36)}$= 14.640, ($P < 0.05$) and for model II – a: $F_{(3,36)}$= 18.215, ($P < 0.01$).

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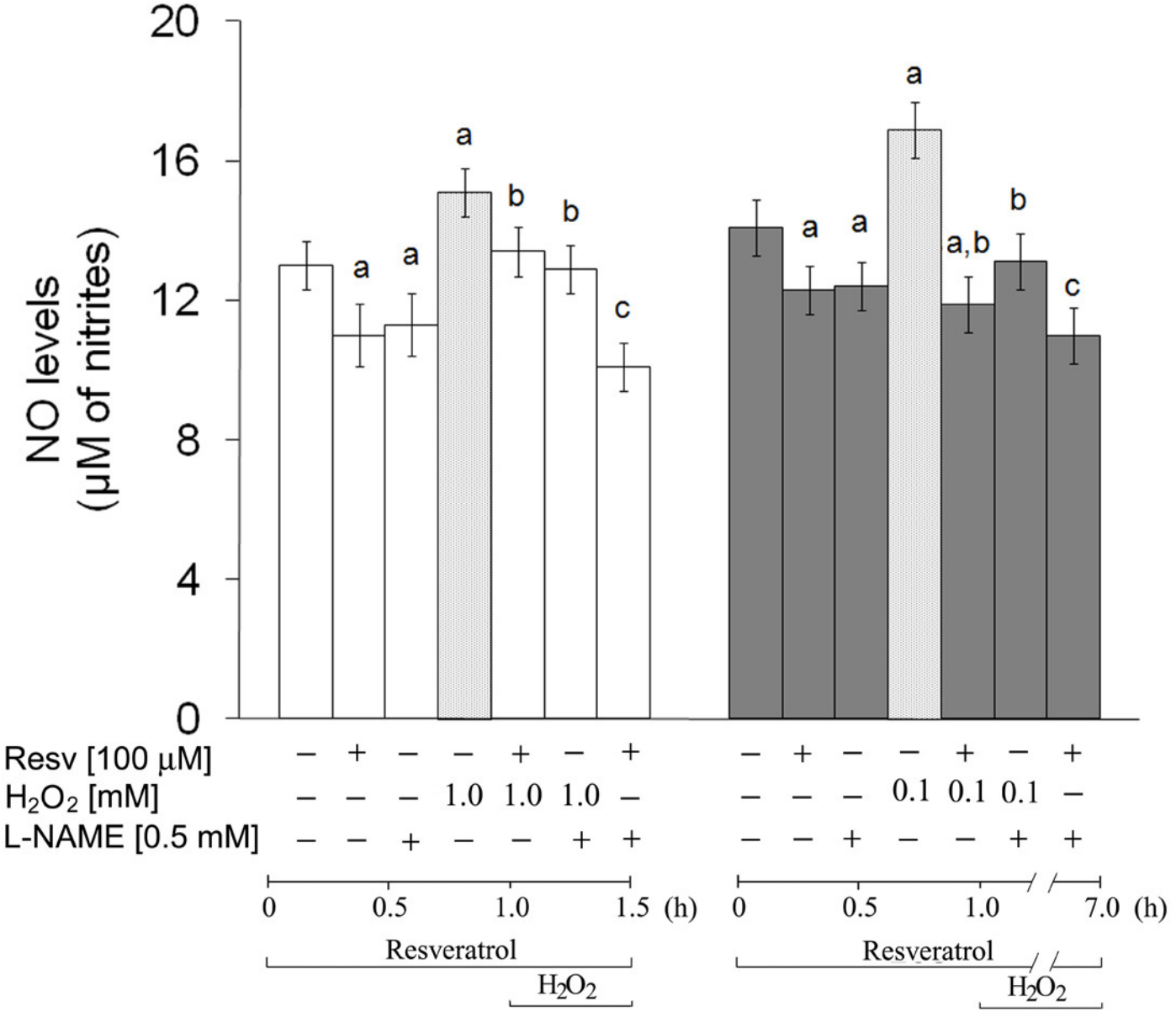
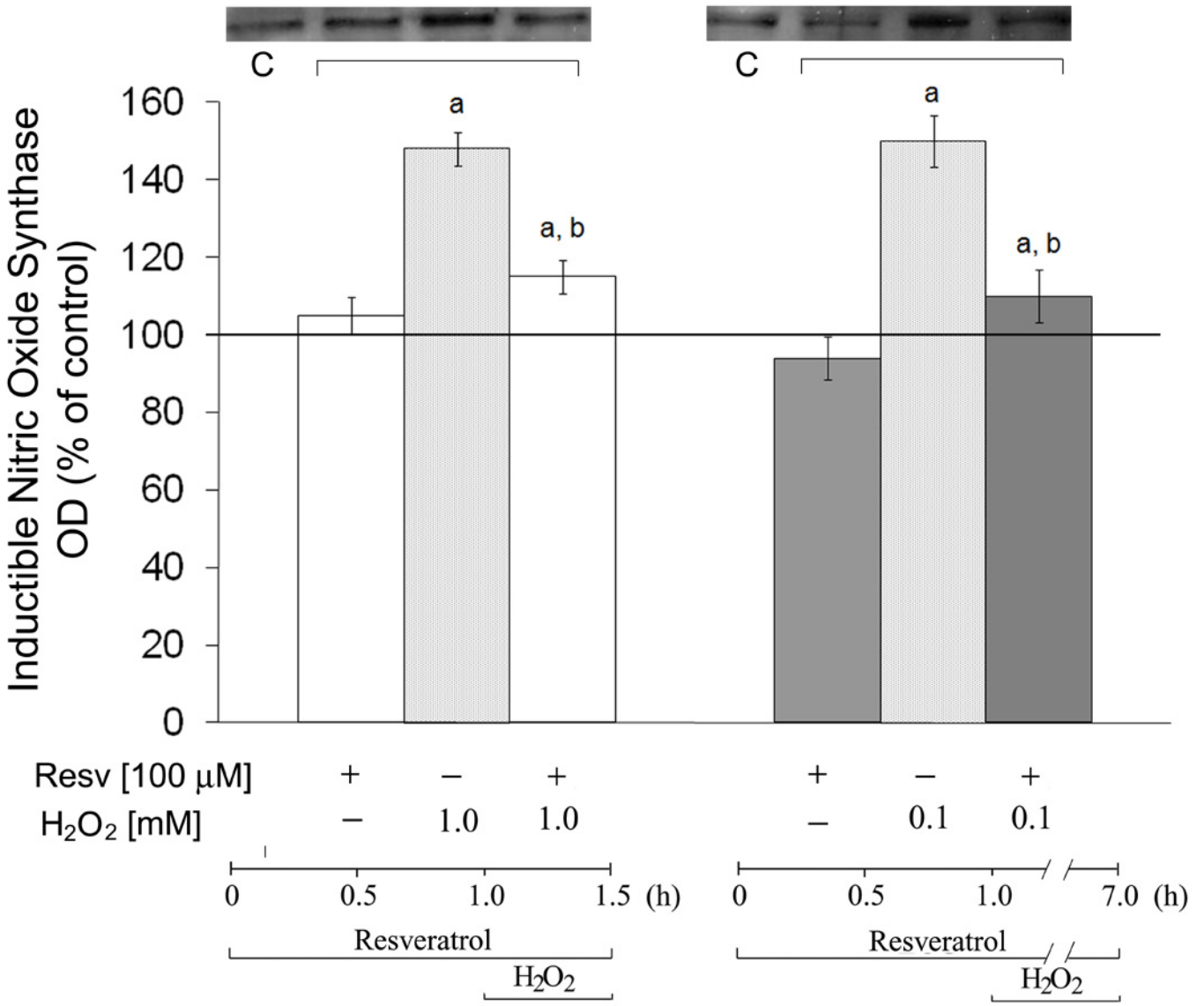
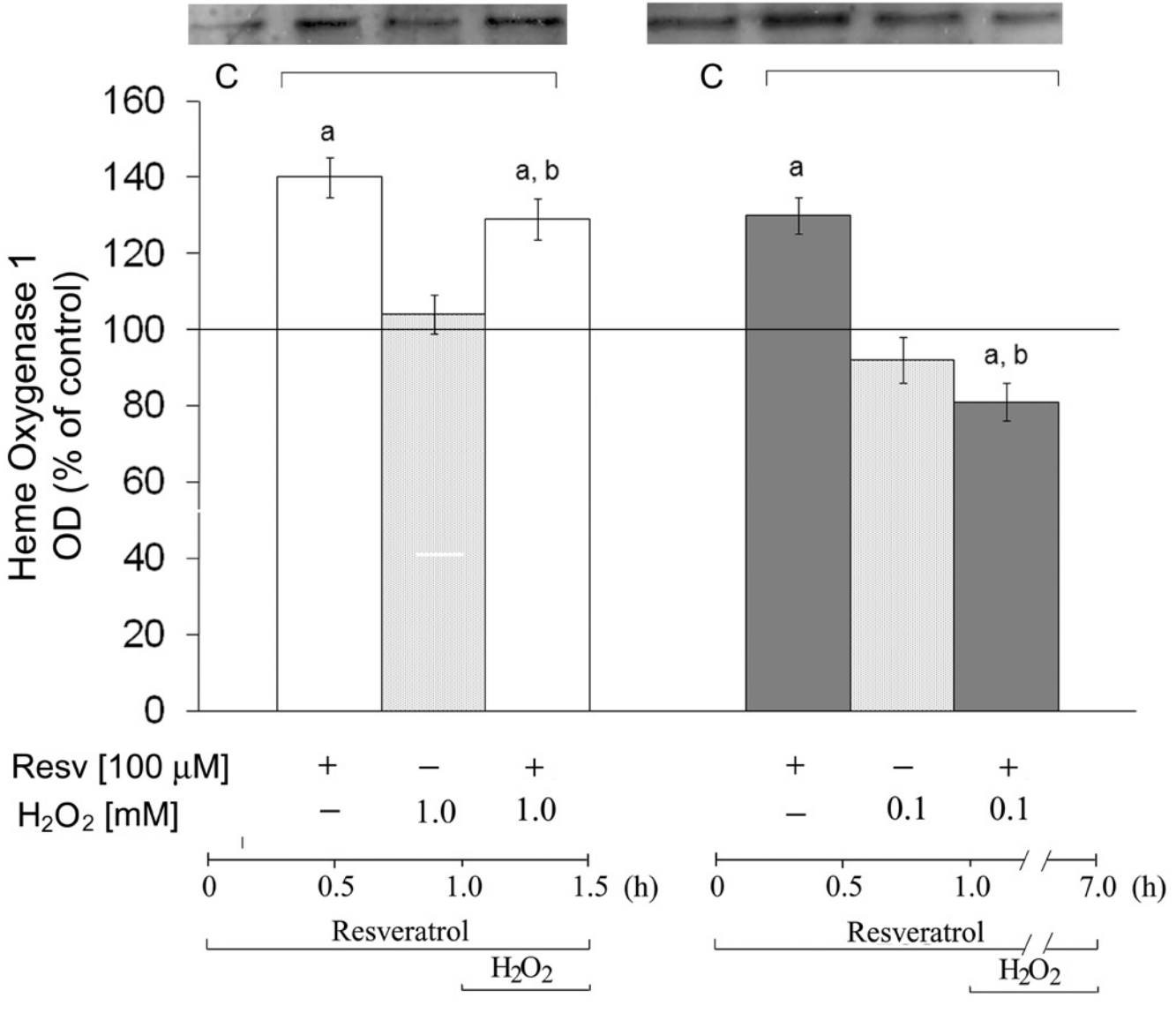
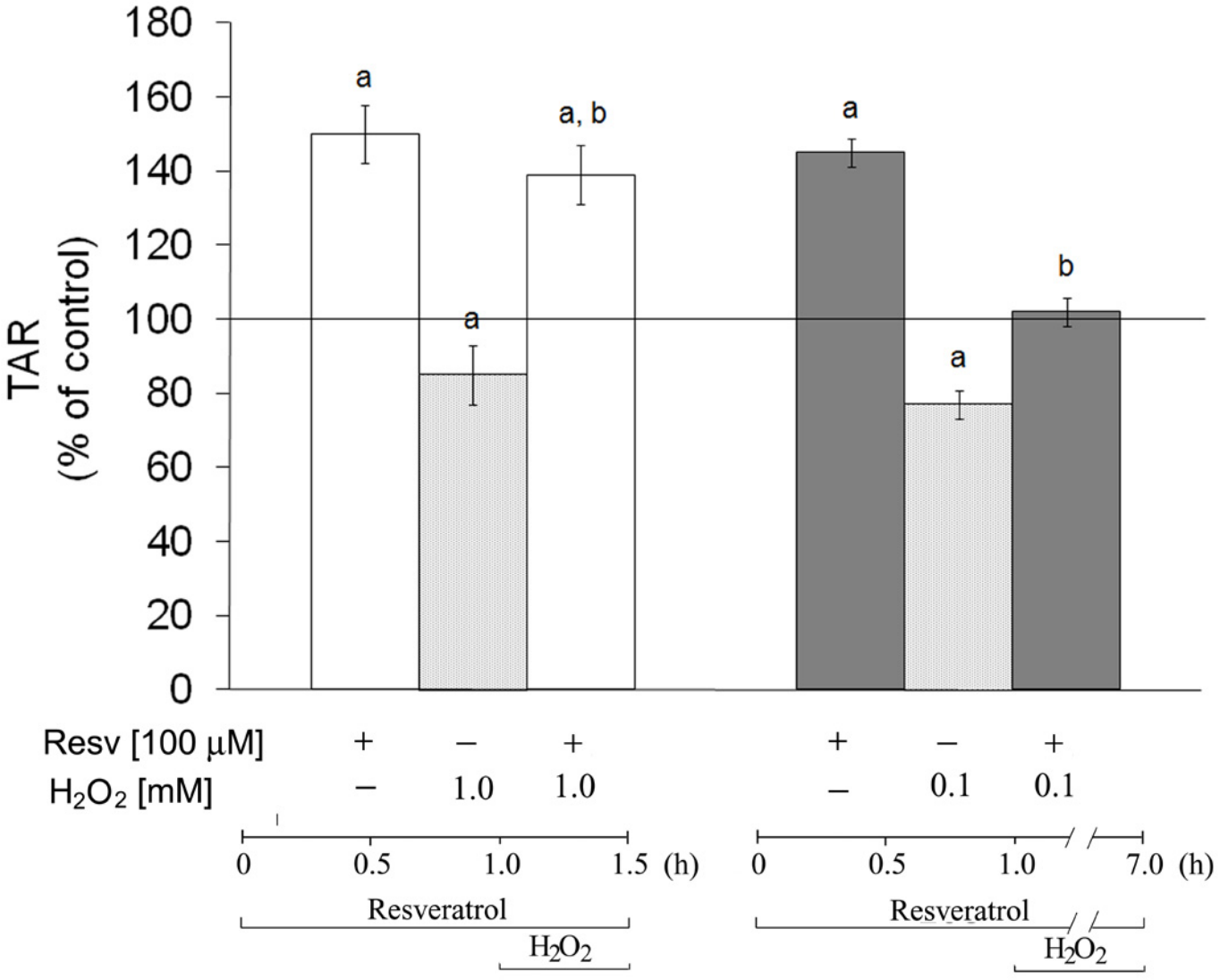
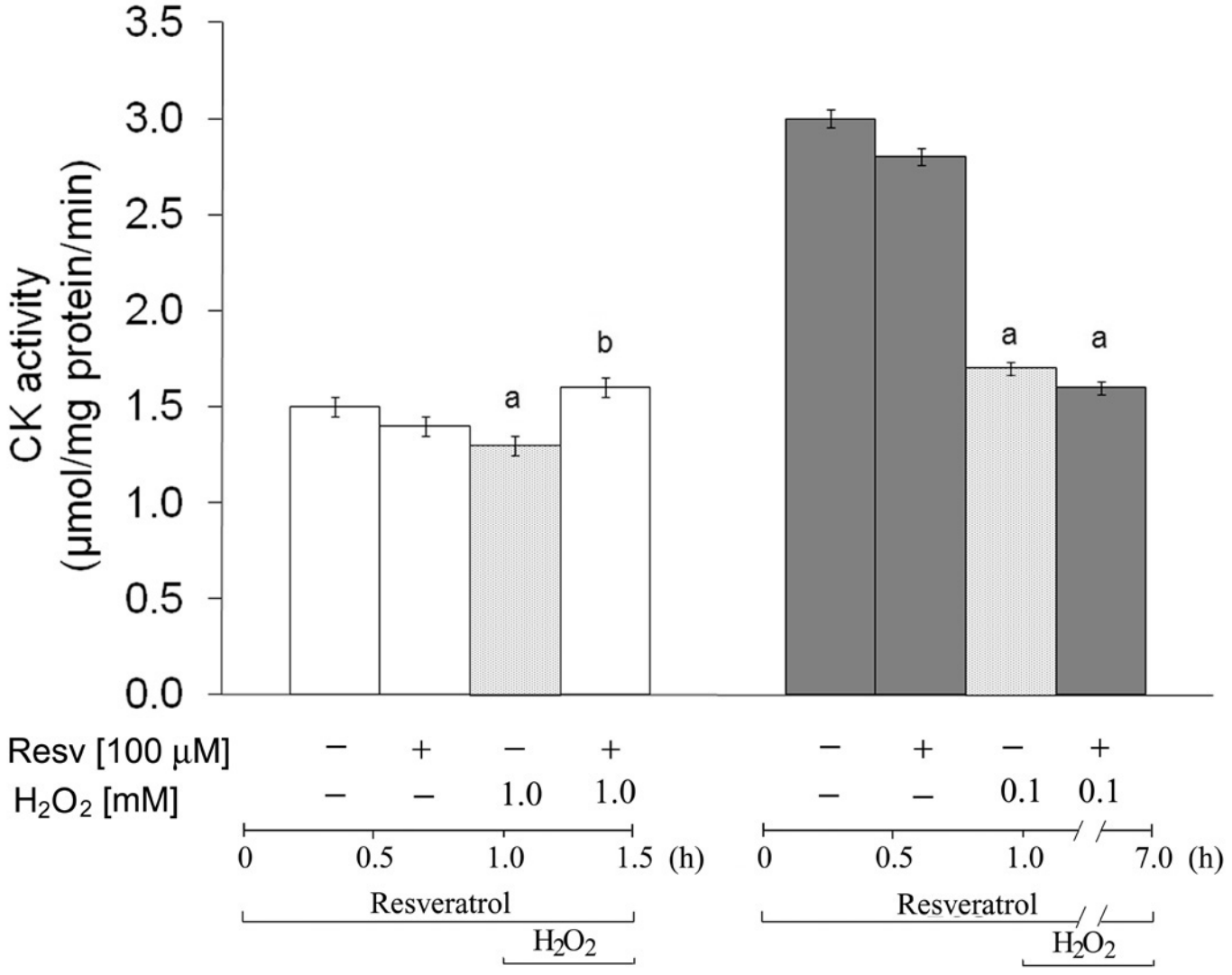

NO levels
(µM of nitrites)
20
16
12
8
4
0
a
a
a
b
b
c
a
a
a
a,b
b
c
Resv [100 µM] – + – – + – + – + – – + – +
H2O2 [mM] – – – 1.0 1.0 1.0 – – – – 0.1 0.1 0.1 –
L-NAME [0.5 mM] – – + – – + + – – + – – + +
0 0.5 1.0 1.5 (h)
0 0.5 1.0 7.0 (h)
Resveratrol
H2O2
Resveratrol
H2O2


C
C
Inductible Nitric Oxide Synthase
OD (% of control)
160
140
120
100
80
60
40
20
0
a
a, b
a
a, b
Resv [100 μM]
+
–
+
+
–
+
$H_2O_2$ [mM]
–
1.0
1.0
–
0.1
0.1
0
0.5
1.0
1.5 (h)
0
0.5
1.0
7.0 (h)
Resveratrol
$H_2O_2$
Resveratrol
$H_2O_2$


C
C
160
140
120
100
80
60
40
20
0
Heme Oxygenase 1
OD (% of control)
a
a, b
a
a, b
Resv [100 μM]
+
–
+
+
–
+
H2O2 [mM]
–
1.0
1.0
–
0.1
0.1
0
0.5
1.0
1.5 (h)
0
0.5
1.0
7.0 (h)
Resveratrol
H2O2
Resveratrol
H2O2


TAR
(% of control)
180
160
140
120
100
80
60
40
20
0
a
a, b
a
a
b
a
Resv [100 μM]
+
–
+
+
–
+
H2O2 [mM]
–
1.0
1.0
–
0.1
0.1
0
0.5
1.0
1.5
(h)
0
0.5
1.0
7.0
(h)
Resveratrol
H2O2
Resveratrol
H2O2


CK activity
(μmol/mg protein/min)
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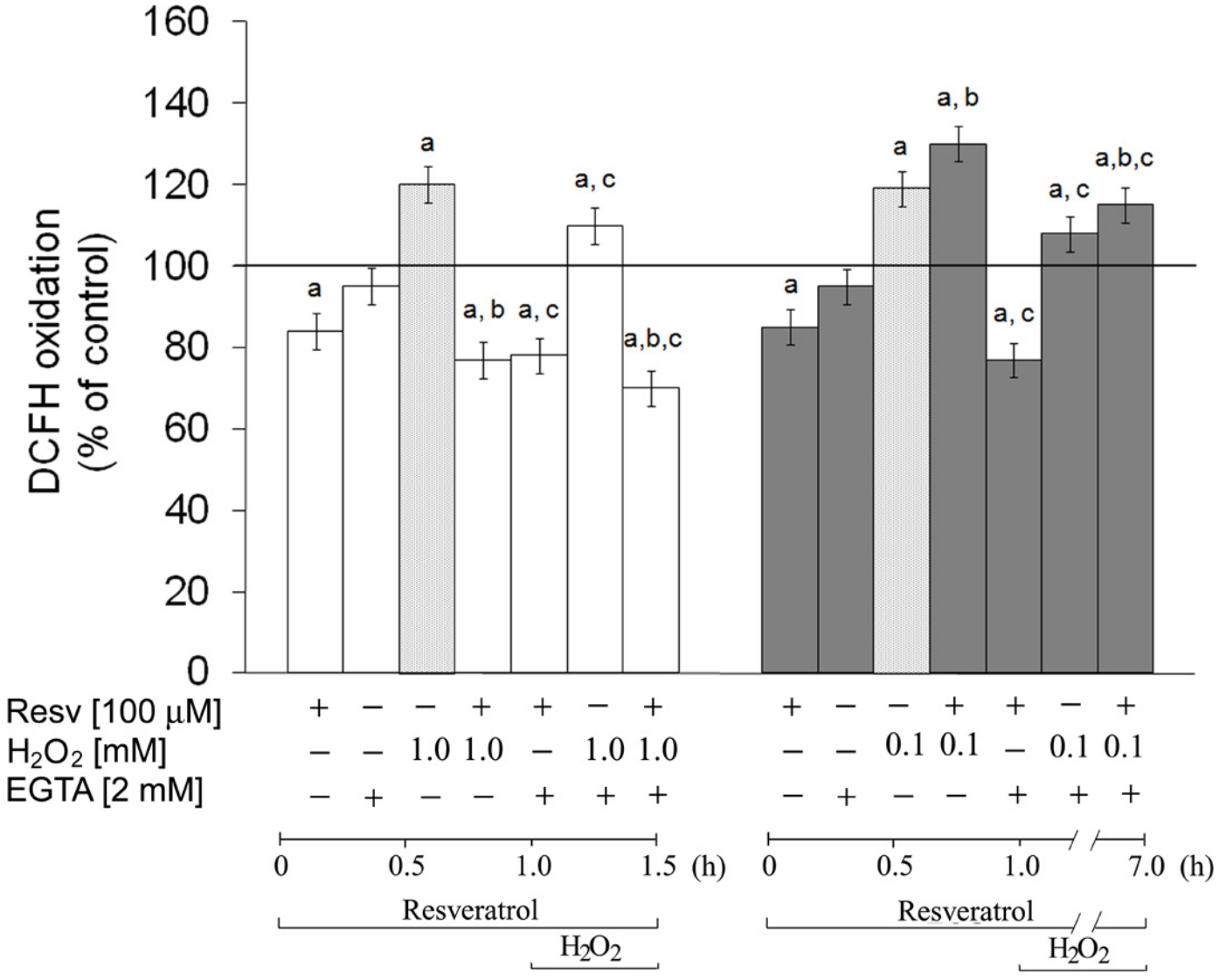
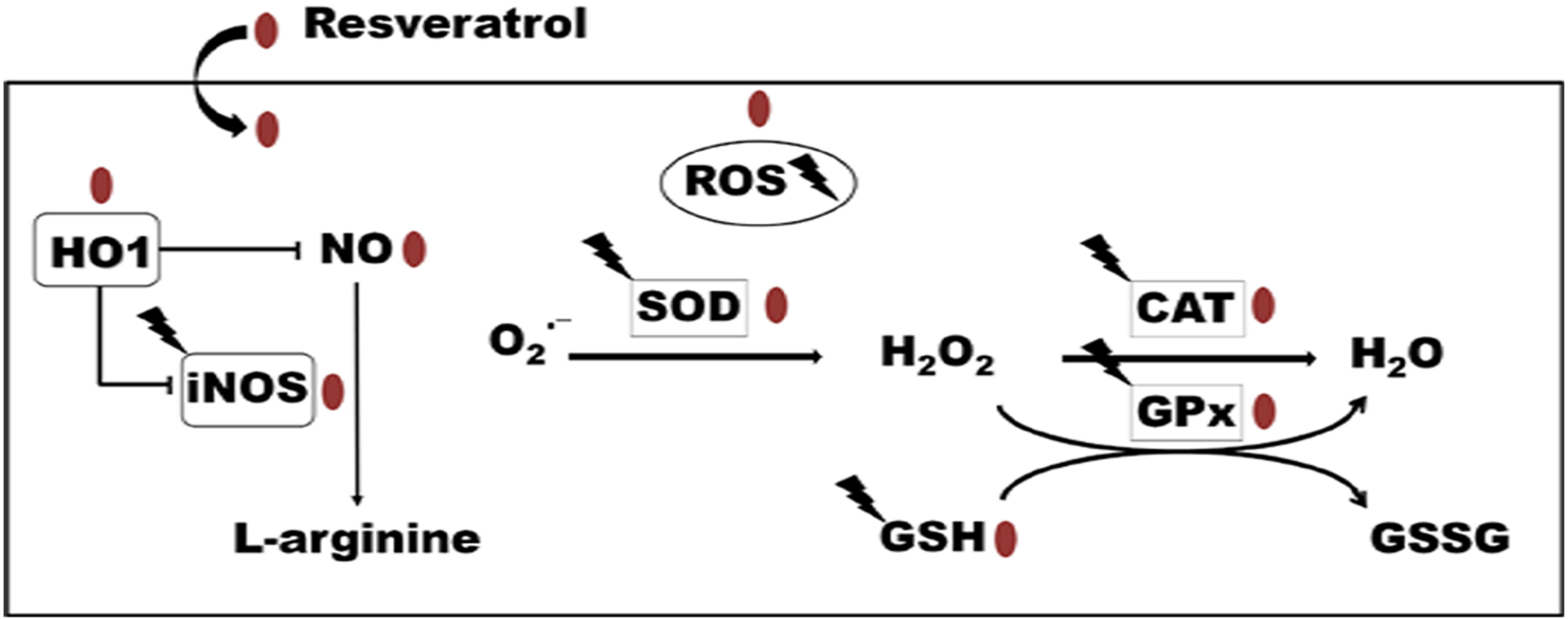
3.0
2.5
2.0
1.5
1.0
0.5
0.0
a
b
a
a
Resv [100 μM]
H2O2 [mM]
− + − +
− − 1.0 1.0
− + − +
− − 0.1 0.1
0 0.5 1.0 1.5 (h)
0 0.5 1.0 7.0 (h)
Resveratrol
H2O2
Resveratrol
H2O2


DCFH oxidation
(% of control)
160
140
120
100
80
60
40
20
0
a
a
a, b
a, c
a, c
a,b,c
a
a
a, b
a
a, c
a, c
a,b,c
Resv [100 µM]
H2O2 [mM]
EGTA [2 mM]
+ − − + + − +
− − 1.0 1.0 − 1.0 1.0
− + − − + + +
+ − − + + − +
− − 0.1 0.1 − 0.1 0.1
− + − − + + +
0 0.5 1.0 1.5 (h)
0 0.5 1.0 7.0 (h)
Resveratrol
H2O2
Resveratrol
H2O2


Resveratrol
HO1
NO
iNOS
L-arginine
ROS
SOD
$O_2^{\bullet -}$
$H_2O_2$
CAT
GPx
$H_2O$
GSH
GSSG

# PARTE III

## DISCUSSÃO

Nas últimas décadas foram estudadas muitas propriedades biológicas de produtos naturais. Consequentemente, muitos destes compostos apresentaram um excelente potencial terapêutico, entre eles o resveratrol. Entre os inúmeros efeitos biológicos do resveratrol, encontrado principalmente em uvas e no vinho tinto (Pervaiz, 2003), destacam-se as atividades antioxidante, cardioprotetora, anti-inflamatória, antitumoral e neuroprotetora (Baur & Sinclair, 2006).

Para o SNC o resveratrol apresenta-se como uma molécula bastante promissora, sendo eficaz em modelos de doenças neurodegenerativas, como Parkinson e Alzheimer e também isquemia cerebral (Han et al, 2004; Khan et al; Sharma & Gupta, 2002; Zamin et al, 2006). Porém, o mecanismo pelo qual o resveratrol age ainda não foi totalmente elucidado, embora existam inúmeros estudos mostrando que ele atua direta e/ou indiretamente sobre várias vias de sinalização celular (Bastianetto & Quirion; Baur & Sinclair, 2006; Roy et al, 2009; Sarkar et al, 2009) e alvos específicos que trariam então seus efeitos benéficos.

Como os astrócitos exercem um papel fundamental na homeostasia do SNC, a busca de moléculas que modulem funções gliais, pode representar avanços na terapêutica de fisiopatologias que afetam o cérebro. Nesse contexto, nosso grupo tem demonstrado que o resveratrol modula importantes funções gliais, como captação de glutamato, atividade da glutamina sintetase e níveis de glutationa (de Almeida et al, 2007; dos Santos et al, 2006). Corroborando com esses achados, esta tese teve como objetivo avaliar o efeito do resveratrol sobre parâmetros astrogliais em células C6 visando o melhor entendimento do seu mecanismo de ação.

Assim, este trabalho foi divido em quatro capítulos, sendo que em três utilizamos resveratrol com $H_2O_2$ como modelo de insulto oxidativo, a fim de melhor compreender os mecanismos envolvidos numa condição de estresse oxidativo. Estes modelos foram chamados de Modelo I: 30 minutos de exposição a 1 mM de $H_2O_2$ e Modelo II: 6 horas de exposição a 0,1 mM de $H_2O_2$, ambos com pré-tratamento de 100 µM de resveratrol, salientando que o resveratrol é mantido por todo período de tratamento, 1:30 h para modelo I e 7 h para modelo II. A partir de dados apresentados no mestrado que versavam sobre o efeito do resveratrol sobre o DNA avaliado pelo ensaio cometa, observamos que o resveratrol *per se* induzia dano ao DNA dependente da dose e do tempo de exposição (exemplo: 250 µM – 24 h exposição). Entretanto, frente ao insulto oxidativo com $H_2O_2$ o resveratrol prevenia total ou parcialmente o dano ao DNA (Quincozes-Santos et al, 2007). Como o ensaio cometa avalia dano recente ao DNA, que pode ser reparado pelo sistema de reparo celular (Maluf & Erdtmann, 2000), nós também avaliamos o dano permanente ao DNA através da técnica de micronúcleos (Maluf et al, 2001).

O estudo em nível cromossomal do dano ao DNA é essencial na determinação da genotoxicidade de muitos compostos e isso pode estar relacionado ao processo de carcinogênese (Umegaki & Fenech, 2000). A técnica de micronúcleos é uma das metodologias preferidas na determinação da perda ou quebra cromossomal e se baseia na contagem de 1000 células binucleadas (Fenech, 2000).

Observamos que a frequência de micronúcleos se altera em função de tempo e dose de resveratrol, pois como observado na tabela 2 – capitulo I, 10 e 100 µM de resveratrol em diferentes tempos que variaram de 1 a 48 h não

apresentaram alterações significativas na frequência de micronúcleos, já 250 µM de resveratrol após 24 h de tratamento aumentou significativamente em 20% a frequência de micronúcleos ($P < 0,05$). O tempo de 48 h não pode ser avaliado, pois essa concentração de resveratrol induziu morte celular, provavelmente por necrose em células C6 (dos Santos et al, 2006).

Para verificar se outro antioxidante apresentaria dano cromossomal, testamos 100 µM de ácido ascórbico (1 a 24 h), uma concentração utilizada amplamente como antioxidante (Davitashvili et al, 2009; Halliwell, 2009; Kim et al, 2005; Pope et al, 2008). Para nossa surpresa, essa concentração de vitamina C induziu aumento significativo na frequência de micronúcleos a partir de 1 h comparado ao controle. Este efeito pode ser devido ao ácido ascórbico aumentar a produção de ERO, gerando dano ao DNA (Halliwell, 2007).

Já em relação ao resveratrol, nosso estudo mostrou que o mesmo apresenta ação em nível cromossomal nas células astrogliais C6, mais uma vez confirmando que estas células são alvos deste polifenol e que a baixa frequência de micronúcleos observada pode estar relacionada ao papel protetor do resveratrol na manutenção da estabilidade genômica, assim como, na prevenção do câncer, um dos efeitos mais proeminentes dessa molécula (Gatz et al, 2008; Gatz & Wiesmuller, 2008).

Também observamos nos modelos I e II que $H_2O_2$ induziu aumento significativo ao redor de 50% quando comparado aos níveis do controle na frequência de micronúcleos. O dano oxidativo induzido foi totalmente prevenido por resveratrol no modelo I e parcialmente, no modelo II. Como os micronúcleos são marcadores de genotoxicidade, uma redução nesses níveis observados com o resveratrol é indicativo da antigenotoxicidade da molécula

(Siddique & Afzal, 2009; Siddique et al). É importante mencionar que o estresse oxidativo acompanhado de dano genotóxico está associado ao aumento de risco de câncer (Droge, 2002).

De acordo com Quincozes-Santos et al, 2007, os modelos I e II de insulto oxidativo não apresentavam morte celular observada pelo ensaio de incorporação de iodeto de propídio. Para confirmar esses resultados, avaliamos a integridade celular e atividade metabólica mitocondrial pelos ensaios de liberação da enzima citosólica lactato desidrogenase (LDH) e redução do MTT, respectivamente, e não observamos diferenças significativas quando comparamos os tratamentos aos controles (tabela 1 – capítulo I), confirmando ausência de morte celular.

Como o aumento de radicais livres pode gerar dano tanto em proteínas, lipídios e DNA, e já demonstramos que $H_2O_2$ causa dano transitório e permanente ao DNA, prevenido total ou parcialmente pelo resveratrol, nosso próximo objetivo foi verificar a presença de peroxidação lipídica através das substâncias reativas ao ácido tiobarbitúrico (TBARS). Este ensaio baseia-se na quantificação de malondialdeído (MDA), que é um produto da oxidação de ácidos graxos poliinsaturados. Observamos que o resveratrol *per se* apenas no modelo I diminui os níveis de TBARS (figura 3 – capítulo I), mantendo-os iguais às condições controle após 7 h de tratamento. Como esperado frente ao insulto oxidativo há um aumento nos níveis de TBARS que foi totalmente prevenido por resveratrol no modelo I. Já no modelo II o resveratrol potencializou o efeito causado por 0,1 mM de $H_2O_2$, aumentando ainda mais os níveis de TBARS.

Fisiologicamente, os níveis de peroxidação lipídica podem ser controlados por mecanismos de defesa enzimática e não-enzimática (Halliwell,

2007). Como a peroxidação de ácidos graxos poliinsaturados está associada a efeitos genotóxicos, o fato de o resveratrol *per se* apresentar efeito protetor contra peroxidação lipídica pode indicar que este polifenol regule mecanismos de defesa celular. É bem descrito na literatura científica que antioxidantes e outros derivados da dieta podem tratar e/ou melhorar danos oxidativos (Berger, 2005).

Porém, em relação aos modelos I e II de insulto oxidativo, o resveratrol apresentou comportamentos distintos. Para o modelo I observamos um efeito antioxidante, enquanto, para o modelo II, um comportamento pró-oxidante. Em relação ao $H_2O_2$ é interessante salientar que ele é rapidamente decomposto em células astrogliais (Dringen et al, 1998; Dringen et al, 2005), podendo assim gerar radicais livres e degradar então biomoléculas (Halliwell & Whiteman, 2004). Aliado a isso, a oxidação de polifenóis também pode gerar radicais livres e esses podem exercer efeitos pró-oxidantes dependentes do meio de cultivo e condições experimentais (Halliwell, 2008). Essas seriam algumas das explicações para a potencialização pelo resveratrol do efeito oxidante induzido por $H_2O_2$.

A partir dos resultados do capítulo I, começamos a observar o resveratrol não apenas como molécula antioxidante, mas também como pró-oxidante. De acordo com dados do nosso grupo, o resveratrol modula importantes funções gliais relacionadas aos destinos do glutamato. Como algumas dessas funções são dependentes de sistemas oxidantes, resolvemos avaliar parâmetros bioquímicos em células gliais frente aos modelos I e II de insulto oxidativo.

Sendo o glutamato o principal neurotransmissor excitatório do SNC e as células gliais as mais importantes na manutenção da homeostasia glutamatérgica, resolvemos testar a captação de glutamato frente aos modelos I e II de insulto oxidativo.

O resveratrol aumentou a captação de glutamato, por modular positivamente os transportadores glutamatérgicos e foi capaz de prevenir a diminuição induzida por $H_2O_2$ no modelo I. Já no modelo II, o resveratrol potencializou a queda observada na captação de glutamato frente ao insulto oxidativo e este efeito foi mediado pela proteína cinase C (PKC).

Como já descrito anteriormente por Trotti et al, (Trotti et al, 1998), os transportadores de glutamato são bastante sensíveis ao estresse oxidativo, resultando em queda na captação de glutamato, podendo isso contribuir para a neurotoxicidade glutamatérgica. Provavelmente, o efeito do resveratrol, não seja somente pela sua ação antioxidante, mas talvez por uma ação direta no sistema glutamatérgico. Também já foi demonstrado que o peróxido de hidrogênio diminui a captação de glutamato (Volterra et al, 1994) e a queda na captação ainda pode estar relacionada a cinases, como a PKC. Nesse contexto, o comportamento pró-oxidante do resveratrol foi totalmente revertido utilizando-se um ativador da PKC (figura 1 – capítulo II). É importante mencionar que resveratrol *per se* é capaz de modular a atividade da PKC (Slater et al, 2003).

Em células astrogliais C6 o EAAT3 é o transportador mais bem descrito na manutenção dos níveis extracelulares de glutamato (Palos et al, 1996), porém os transportadores EAAT1 (GLAST) e EAAT2 (GLT-1) já foram também identificados (Bianchi et al, 2008). Além disso, o número de passagens

celulares (acima de 100) confere um forte caráter astrocítico às células C6, observado pela reatividade frente a GFAP (dos Santos et al, 2006), um dos principais marcadores astrocíticos. Assim, quando observamos que o resveratrol aumenta a captação de glutamato em células C6, seria provavelmente pela modulação dos três transportadores.

A seguir, estudamos um dos destinos do glutamato após ser captado pelos transportadores glutamatérgicos, a conversão à glutamina, através da atividade da enzima glutamina sintetase. O resveratrol *per se* foi capaz de aumentar a atividade da GS, e apenas após longa exposição ao peróxido de hidrogênio a atividade da GS foi diminuída. A adição conjunta de resveratrol e $H_2O_2$ resultou novamente em um comportamento antioxidante para o modelo I e pró-oxidante para o modelo II. Assim, para o modelo I, mesmo em condições oxidantes o resveratrol é capaz de captar mais glutamato e convertê-lo a glutamina; já no modelo II as condições oxidantes do meio impedem o papel protetor do resveratrol. A enzima GS é bastante sensível ao estresse oxidativo (Banerjee et al, 2008; Halliwell, 2006a; Mates et al, 2002) e provavelmente está inibida frente ao insulto oxidativo ou sofre ação direta da captação de glutamato, que também encontra-se diminuída e dessa forma menos glutamato estaria disponível para conversão a glutamina, via ciclo glutamato-glutamina (Hertz, 2006).

Outro destino do glutamato seria a síntese de GSH, o principal antioxidante do SNC. Observamos que o resveratrol é capaz de aumentar os níveis intracelulares de GSH, o insulto oxidativo reduz estes níveis e a adição de resveratrol e $H_2O_2$ gera novamente comportamento antioxidante para o modelo I, uma vez que resveratrol é capaz de prevenir a queda induzida pelo

agente oxidante e ainda aumentar os níveis de GSH; e pró-oxidante para o modelo II, onde há queda nos níveis de GSH (figuras 3A e 3B – capítulo II).

O efeito antioxidante observado, está de acordo com dados do nosso grupo (de Almeida et al, 2008; de Almeida et al, 2007) e também da literatura científica (Brito et al, 2006; Sharma & Gupta, 2002). Uma vez que a GSH neuronal é dependente da GSH astrocítica, a busca de moléculas antioxidantes pode representar importantes avanços na terapêutica de doenças neurodegenerativas. Sharma & Gupta, 2002 que utilizaram estreptozotocina intracerebroventricular como modelo de demência demonstraram que o resveratrol previne o dano cognitivo e aumenta os níveis de GSH. Em nosso modelo haveria mais substrato para a síntese de GSH e esta por sua vez, poderia evitar a oxidação dos transportadores de glutamato, garantindo assim o efeito antioxidante. Curiosamente, quando falamos em efeito antioxidante do resveratrol no SNC, devemos salientar que ele pode agir tanto como sequestrador (“scavenger”) de espécies reativas, quanto diretamente sobre defesas antioxidantes enzimáticas e/ou não-enzimáticas, como a GSH (de la Lastra & Villegas, 2007).

O efeito pró-oxidante do resveratrol pode ser dependente do trocador cistina/glutamato ($Xc^-$), pois ocorrendo diminuição dos níveis intracelulares de cistina, haveria menor redução deste aminoácido a cisteína e menor quantidade de GSH (Banerjee et al, 2008). Além dessa hipótese, como há menor captação de glutamato, há menos substrato disponível para a síntese de GSH.

A S100B é uma proteína ligante de cálcio que possui atividades intra e extracelular. No meio extracelular a S100B age como uma citocina e seus

efeitos são dependentes de sua concentração, sendo os efeitos tróficos observados em concentrações na ordem nanomolar (Van Eldik & Wainwright, 2003). Aumentos na secreção de S100B foram observados neste trabalho, tanto quando as células astrogliais C6 foram tratadas apenas com resveratrol, como frente ao insulto oxidativo (figura 4 – capítulo II). Demonstramos previamente (Dos Santos et al, 2006), em células C6 aumento na secreção de S100B após 48 horas de exposição ao resveratrol. Aqui observamos aumento após rápida exposição ao resveratrol (1:30 h), no entanto nenhum aumento no modelo II de insulto oxidativo foi observado. Porém, resveratrol e peróxido de hidrogênio podem afetar a secreção de S100B via cinases sensíveis ao estado redox (Leite et al, 2008). No modelo II há predomínio de ações pró-oxidantes e isso poderia afetar a secreção desta proteína. O aumento de S100B pode representar mais um efeito neuroprotetor do resveratrol e este efeito está relacionado ao mecanismo de secreção da S100B, pois já demonstramos que nossos tratamentos não alteram a permeabilidade de membrana celular.

Dados do nosso grupo mostram que a captação de glutamato é estimulada por S100B (Tramontina et al, 2006b), assim onde obtivemos aumento na captação de glutamato também observamos aumento na secreção de S100B. Não podemos determinar qual evento ocorre primeiro, mas dentro de um contexto farmacológico, o resveratrol modula importantes parâmetros gliais, apresentando-se como uma promissora molécula para doenças que afetam sistema glutamatérgico.

O fator nuclear, NFκB, medeia respostas celulares frente aos processos de inflamação, estresse oxidativo, apoptose e proliferação celular (Kundu & Surh, 2004), estando intimamente relacionado ao câncer. Um dos possíveis

mecanismos para o efeito antitumoral do resveratrol é via supressão da ativação do NFκB (Ralhan et al, 2009). Nossos resultados demonstram que o $H_2O_2$, um oxidante clássico, ativa o sistema NFκB, estando de acordo com a literatura que menciona tal ativação pelo estresse oxidativo (Gloire et al, 2006; Nakano et al, 2006). Surpreendentemente, resveratrol *per se* aumenta a ativação deste fator de transcrição em células C6 e frente ao insulto oxidativo aumenta ainda mais a ativação do NFκB. Este resultado merece investigações futuras, porém Gloire et al, 2006, demonstra que níveis intermediários de estresse oxidativo levam a ativação do NFκB, então a oxidação do polifenol no meio de cultivo, com geração de RL e adição de $H_2O_2$ poderia ter formado um ambiente propício a tal evento, independente do perfil observado para os outros resultados.

Nossos modelos de insulto oxidativo levaram à reorganização do citoesqueleto em células astrogliais C6. Esses resultados estão de acordo com outros estudos da literatura (Zhu et al, 2005) e o mecanismo envolvido é dependente de RhoA, uma família de proteínas envolvida na manutenção do citoesqueleto de actina (Cechin et al, 2002; Guasch et al, 2003). Desorganizações no citoesqueleto podem levar a alterações na função das células gliais e isto pode ser induzido por $H_2O_2$ (Zhu et al, 2005). É importante salientar que o resveratrol não altera morfologia celular, não interferindo na via da RhoA.

Conforme discutimos nossos resultados, apresentamos alguns deles com efeito antioxidante e outros com efeito pró-oxidante, sendo este capítulo nominado de “Janus Face”. Para comprovar o efeito anti e pró-oxidante do resveratrol (de la Lastra & Villegas, 2007), avaliamos a produção de radicais

livres através de ensaio de oxidação da diclorofluoresceína (DCFH), um indicador de formação de ERO intracelular (Keller et al, 2004). Concentrações de $H_2O_2$ aumentam a oxidação de DCFH de maneira dose e tempo dependente (figura 8 – capítulo II). Assim, observamos que o insulto oxidativo nos modelos I e II aumenta a formação de RL e o resveratrol frente ao modelo I apresenta-se como antioxidante, diminuindo a produção de RL e para o modelo II potencializa o efeito causado pelo $H_2O_2$, comportando-se como pró-oxidante.

O terceiro capítulo desta tese é uma revisão dos principais resultados do nosso grupo relativos a modulação de parâmetros gliais pelo resveratrol *per se* ou frente a insulto oxidativo. Nele revisamos artigos abordando principalmente as ações do resveratrol sobre a captação de glutamato, a atividade da glutamina sintetase, o conteúdo intracelular de glutationa e a secreção de S100B em condições oxidantes ou não.

Observando o conjunto de nossos resultados (de Almeida et al, 2008; dos Santos et al, 2006; Quincozes-Santos et al, 2009c; Vieira de Almeida et al, 2008) concluímos que os efeitos demonstrados na linhagem astroglial C6 são semelhantes aos relatados em cultura primária de astrócitos, evidenciando que as células C6 apresentam-se como um excelente modelo de estudo das funções astrocíticas.

Também observamos efeitos opostos dependentes de doses de resveratrol. Baixas doses teriam um determinado efeito, enquanto doses maiores um efeito oposto, indicando um fenômeno hormético (Kendig et al). Este fenômeno tem sido observado em estudos clínicos e experimentais, como por exemplo, ensaios dose-resposta. Também os efeitos de restrição calórica e dieta rica em calorias (Kouda & Iki) ou altas e baixas concentrações de

peróxido de hidrogênio sobre proliferação celular podem ser enquadrados como hormese (Day & Suzuki, 2005).

Além disso, a mesma dose de resveratrol (250 µM) não apresentou o mesmo efeito em células C6 e astrócitos primários quando avaliada a captação de glutamato, indicando que apesar das semelhanças, estas células podem apresentar resultados dependentes das condições redox do meio, pois em C6 observamos um aumento na captação com essa dose e em astrócitos efeito oposto.

A proposta desta revisão foi mostrar o papel do resveratrol sobre atividade glial evidenciando assim a ação deste polifenol sobre pontos específicos da sinapse tripartite. Corroborando com essa proposta, discutimos resultados em cultura celular e fatias hipocampais (de Almeida et al, 2008), nos permitindo uma melhor avaliação da interação astrócito-neurônio.

Dessa forma, esta revisão reforça que as células gliais são alvo do resveratrol, demonstrando efeitos deste polifenol sobre a comunicação e sinalização neuroglial. É importante salientar também que as concentrações de resveratrol utilizadas em nossos tratamentos estão de acordo com as doses descritas na literatura como neuroprotetoras em modelos *in vitro* (Wang et al, 2003). Assim, a modulação de parâmetros gliais pelo resveratrol pode trazer importantes avanços na busca de moléculas que melhorem fisiopatologias associadas ao SNC.

Para avaliar o efeito do resveratrol sobre o sistema de defesa antioxidante celular, visto que já havíamos observado um duplo efeito do resveratrol frente ao insulto oxidativo em relação a parâmetros gliais específicos, estudamos no quarto capítulo os níveis de nitritos (NO) e proteínas

reguladoras de sua síntese, mecanismos de defesa antioxidante enzimáticos e a atividade da creatina cinase, uma enzima do metabolismo energético celular.

ERO e ERN podem causar dano a biomoléculas e isto está associado a muitas patologias, incluindo doenças neurodegenerativas (Halliwell, 2006a). O peroxinitrito é um dos mais potentes oxidantes celulares (Halliwell, 2007) e está envolvido na fisiopatologia da doença de Parkinson (Calabrese et al, 2007; Ebadi & Sharma, 2003). O resveratrol *per se* foi capaz de diminuir os níveis de NO em células astrogliais C6 e prevenir o aumento induzido por $H_2O_2$ em ambos os modelos (figura 1 – capítulo IV). Para verificar se o aumento de NO foi dependente da atividade da óxido nítrico sintase (NOS), foi avaliado o efeito do L-NAME ($N^{\omega}$-nitro-L-arginina metil éster), um inibidor da NOS, sobre os níveis de NO. L-NAME apresentou queda nos níveis de NO e a adição conjunta de resveratrol + L-NAME também levou à redução nos níveis de NO, mostrando um efeito sinérgico e também um possível papel inibidor do resveratrol sobre a síntese de NO. Assim, podemos inferir que a prevenção observada em modelos de doença de Parkinson por resveratrol (Jin et al, 2008), possa ser devido em parte pelo efeito inibidor sobre a síntese de NO.

Este efeito inibitório do resveratrol é devido a sua modulação na atividade da iNOS (óxido nítrico sintase induzível) frente ao insulto oxidativo, pois ele previne o aumento observado com $H_2O_2$ (figura 2). Essa resposta está de acordo com outros dados da literatura que demonstram a modulação da atividade de iNOS por resveratrol e outros antioxidantes (Hattori et al, 2002; Lu et al; Youn et al, 2009).

A enzima heme oxigenase (HO) apresenta duas isoformas, HO2 que é constitutivamente expressa e HO1, a forma induzível. A principal função da

heme oxigenase é degradar o grupamento pró-oxidante heme em biliverdina/bilirrubina, ambos conhecidos antioxidantes (Dore et al, 1999a; Dore et al, 1999b). O grupamento heme livre pode ser originado de fontes extracelulares, como pela degradação da hemoglobina; como de fontes intracelulares, a partir do metabolismo de proteínas que contêm heme. Durante situações de hipóxia e excitotoxicidade há aumento na produção do heme, que não pode ser reciclado e então é degradado pela heme oxigenase (Dore et al, 1999a; Sakata et al).

Nós demonstramos que o resveratrol aumenta a HO1 (figura 3), levando à formação dos antioxidantes biliverdina e bilirrubina. No entanto, frente ao insulto oxidativo, o resveratrol apresenta dois comportamentos, no modelo I (1 mM $H_2O_2$/30 minutos) o resveratrol mantém aumentado o imunocontéudo da HO1. Já no modelo II, o resveratrol frente ao oxidante leva a uma diminuição da enzima HO1.

Recentemente, Sakata et al, 2010, demonstraram que a heme oxigenase seria a única candidata pela qual o resveratrol poderia exercer uma sinalização endógena celular que levaria a uma resistência ao estresse oxidativo e, como consequência à neuroproteção. Além disso, existe uma forte associação entre HO1 e iNOS. A HO1 é regulada por um fator de transcrição, o Nrf2 (do inglês “Nuclear related factor 2” ou “Nuclear factor erytroid-2”), que protege células do estresse oxidativo pela ativação de sistemas antioxidantes como a atividade da GPx e aumento dos níveis de GSH (Kode et al, 2008; Wakabayashi et al). O Nrf2 regula a transcrição da HO1, que atua como sequestradora (“scavenger”) de NO e também bloqueadora da atividade da iNOS, prevenindo a produção de NO (Wakabayashi et al). Resveratrol induziu

uma diminuição no imunoconteúdo da iNOS e um aumento na HO1, no modelo I, mostrando um importante efeito antioxidante. A diminuição da HO1 observada no modelo II pode ser mediada pela PKC isoforma delta, de acordo com dados prévios para o antioxidante epigalocatequina (Bastianetto & Quirion; Ogborne et al, 2008) e nós também demonstramos que o resveratrol apresenta efeitos pró-oxidantes dependentes de PKC.

Os níveis de TAR (do inglês “Total Antioxidant Reactivity”) representam a capacidade antioxidante celular (Halliwell & Whiteman, 2004). O resveratrol aumenta os níveis de TAR (figura 4) e $H_2O_2$ reduz tais níveis, porém resveratrol é capaz de proteger essa queda. Esses resultados sugerem que resveratrol exerce efeito protetor contra estresse oxidativo. O efeito antioxidante pode ser ação direta do polifenol ou via modulação de outras vias que culminam na ação antioxidante (Baur & Sinclair, 2006; de la Lastra & Villegas, 2007; Ovesna & Horvathova-Kozics, 2005). Curiosamente, temos discutido comportamentos anti e pró-oxidantes do resveratrol e aqui observamos uma capacidade antioxidante aumentada em ambos os modelos. Isso pode ser explicado metodologicamente porque a técnica de TAR compara as amostras a um padrão de Trolox, um antioxidante clássico, e nosso grupo demonstrou que o resveratrol apresenta maior caráter antioxidante que Trolox e ácido ascórbico em células glias (dados não publicados).

A concentração de RL está relacionada ao balanço entre a produção e a eliminação de espécies reativas por compostos antioxidantes ou enzimas de defesa antioxidante como SOD, CAT e GPx (Droge, 2002). Resveratrol *per se* aumenta a atividade da SOD (tabela 1) e frente ao insulto oxidativo protege células C6 no modelo I, diminuindo os níveis de radical superóxido (Pope et al,

2008; Spanier et al, 2009), mas potencializa a queda no modelo II de insulto oxidativo. A modulação da atividade da SOD é extremamente importante para a manutenção de baixos níveis de RL no SNC (Barbeito et al, 2004). Além disso, a SOD encontra-se principalmente nos astrócitos e quando secretada pode proteger a GSH da oxidação (Pope et al, 2008), sendo assim é um importante mecanismo relacionado à neuroproteção.

A atividade da catalase foi diminuída pelo insulto oxidativo e o resveratrol protegeu as células C6 aumentando tal atividade, mostrando que o peróxido de hidrogênio formado foi decomposto à água (Rodriguez et al, 2004; Smith-Pearson et al, 2008). No modelo II de insulto oxidativo, houve uma queda na atividade da SOD acompanhada por uma queda na atividade da catalase, mostrando acúmulo de superóxido e $H_2O_2$. Além disso, vale salientar que as células C6 apresentam uma elevada atividade da enzima catalase (Funchal et al, 2006a) e por isso, talvez, não sofram modulação direta por antioxidantes, como o resveratrol.

No modelo I, frente ao insulto oxidativo, resveratrol diminui a atividade da GPx, preservando os níveis de GSH, o principal antioxidante do SNC. Nos demais tratamentos, a atividade da GPx não é alterada. Dessa forma, concluímos que nossos modelos de tratamento modulam a atividade das principais defesas antioxidantes enzimáticas contra a ação de radicais livres (Gelain & Moreira, 2008; Kasdallah-Grissa et al, 2007; Khan et al; Meng et al, 2008).

Nós também observamos regulação nos níveis intracelulares de GSH, com proteção pelo resveratrol da queda induzida por peróxido de hidrogênio no modelo I e potencialização do efeito oxidante no modelo II. Além disso,

observamos que o resveratrol *per se* aumenta os níveis extracelulares de GSH. No modelo I de insulto oxidativo, o resveratrol mantém o aumento no conteúdo extracelular de GSH, já no modelo II há uma queda nesses níveis. Como a GSH secretada pelos astrócitos, após ser clivada, poderá sintetizar GSH para os neurônios (Dringen, 2000), o efeito observado tanto para resveratrol *per se* como no modelo I pode sinalizar um importante efeito neuroprotetor, e extrapolando nossos resultados em nível cerebral a ação antioxidante astrocítica do resveratrol poderia beneficiar células neuronais. Também é sabido que $H_2O_2$ pode oxidar GSH a GSSG e assim impediria sua ação tanto intra quanto extracelularmente (Pope et al, 2008).

Outro parâmetro investigado foi a atividade da creatina cinase (CK do inglês “creatine kinase”). Esta enzima exerce um efeito central no metabolismo energético, especialmente em tecidos que consomem muita energia, como cérebro, músculo esquelético e coração, onde atua sobre os níveis de ATP (Andres et al, 2008; Funchal et al, 2006b). A CK também é uma enzima muito sensível ao dano oxidativo (oxidação e nitração). Assim, observamos apenas sobre condições oxidantes modulação na atividade da CK, sendo o resveratrol capaz de proteger o dano induzido por $H_2O_2$ apenas no modelo I.

Como já anteriormente discutido, o resveratrol diminui os níveis de radicais livres observados pela técnica de DCFDA. Frente ao insulto oxidativo, apresenta-se como antioxidante para o modelo I e pró-oxidante para o modelo II. Neste capítulo, verificamos se resveratrol modularia níveis de cálcio e suas implicações no estado redox celular. O aumento do cálcio intracelular é associado ao estresse oxidativo e morte neuronal via receptor glutamatérgico (Banerjee et al, 2008; Coyle, 2006b; Dawson et al, 1992). A ativação do

receptor NMDA poderia gerar NO, via NOS mediada pelo cálcio (Coyle & Puttfarcken, 1993). O EGTA é um quelante de cálcio e o resveratrol exerce efeito antioxidante no modelo I de insulto oxidativo, mostrando-se também como um bloqueador de cálcio capaz de proteger células astroglias contra a excitotoxicidade glutamatérgica e patologias associadas ao estresse oxidativo. Já no modelo II há manutenção do caráter pró-oxidante por resveratrol mais EGTA indicando envolvimento do cálcio intracelular na produção de ERO e consequente toxicidade celular.

Embora, trabalhos mostrem que concentrações de até 100 µM de resveratrol apresentam efeito neuroprotetor *in vitro* (Wang et al, 2002) é importante salientar que a concentração plasmática de resveratrol máxima em humanos, dependendo do consumo, é ao redor de 2 µM e no cérebro 0,4 µM (Gescher & Steward, 2003). Níveis baixos de resveratrol podem ser atribuídos ao rápido metabolismo dessa molécula, com formação de seus metabólitos, resveratrol glicuronídeo e resveratrol sulfato; assim como pelas fontes as quais o resveratrol foi obtido. Assim, nossas concentrações de trabalho estão de acordo com concentrações benéficas *in vitro* do resveratrol, embora ele tenha apresentado diferentes comportamentos dependentes das condições do meio nos ensaios aqui descritos. Cabe ainda destacar que avaliamos um efeito agudo do resveratrol e que talvez a exposição a doses menores de resveratrol, mas por um período prolongado resultem em efeitos semelhantes.

De acordo com suas características de solubilidade, o resveratrol pode ser solubilizado em DMSO (dimetilsulfóxido) ou etanol, sendo que nosso grupo optou por solubilizar em etanol, para aproximar nossos resultados de uma dieta baseada no consumo de vinho, visando estudos *in vivo*, administrando

resveratrol em ratos. É importante mencionar que a presença de álcool não é necessária para a absorção do resveratrol (Goldberg et al, 2003) e nenhum parâmetro analisado nesta tese foi alterado pelo veículo.

Concluindo, a análise de nossos resultados mostra que o resveratrol modula importantes funções das células gliais. Embora o mecanismo de ação do resveratrol ainda não esteja totalmente elucidado, nossos resultados demonstram que a ação protetora do resveratrol envolve a atividade glial, pois entre outros papéis destaca-se sua ação sobre o metabolismo glutamatérgico, como captação de glutamato, atividade da glutamina sintetase e conteúdo de glutationa.

Na proposta de elucidar possíveis vias pelas quais o resveratrol exerça seus efeitos no SNC, demonstramos a modulação da heme oxigenase 1, que recentemente, foi proposta como a principal via pelo qual resveratrol age em situações neuropatológicas (Sakata et al). A grande maioria de nossos resultados mostra dependência das condições oxidantes do meio e como consequência do estado redox celular. Aqui, chamamos a atenção para o consumo de produtos a base de resveratrol que merecem atenção especial na dose a ser consumida, a fim de evitar efeitos pró-oxidantes.

Enfim, este estudo propõe que o resveratrol pode representar um potencial agente terapêutico para o SNC, frente a insultos oxidativos, por modular importantes funções glutamatérgicas que estão associadas a inúmeras patologias do SNC.

## CONCLUSÕES

Os resultados obtidos nesta tese a partir da ação do resveratrol sobre células astrogliais C6 nos permitem concluir que:

- O resveratrol *per se* não induz formação de micronúcleos e frente ao insulto oxidativo previne total ou parcialmente a formação de micronúcleos, apresentando efeitos relacionados à genotoxicidade celular.
- No modelo I o resveratrol protegeu as células da peroxidação lipídica, induzida pelo insulto oxidativo, porém houve potencialização do efeito de lipoperoxidação no modelo II.
- A captação de glutamato foi modulada por resveratrol, apresentando um perfil antioxidante para alta concentração de $H_2O_2$ por curto período de exposição (restaurando e aumentando a captação) e pró-oxidante para baixa concentração de $H_2O_2$ por longo período de exposição, via inibição da PKC, potencializando a queda na captação induzida por $H_2O_2$.
- Resveratrol *per se* aumenta a atividade da enzima glutamina sintetase e frente ao insulto oxidativo, apresenta comportamento antioxidante para o modelo I e pró-oxidante para o modelo II.
- O conteúdo de GSH intracelular é modulado pelo resveratrol, porém frente ao insulto oxidativo há um aumento de GSH após rápida exposição ao $H_2O_2$ e uma redução após longa exposição ao $H_2O_2$.
- A secreção de S100B é aumentada por resveratrol apenas no modelo I de insulto oxidativo.
- O resveratrol induz ativação do fator de transcrição NFκB.
- Ocorre aumento na formação de radicais livres frente ao insulto oxidativo, sendo diminuída por resveratrol no modelo I e potencializada no modelo II.

- Resveratrol diminui os níveis de NO, provavelmente por ação sobre a enzima óxido nítrico sintase.
- Resveratrol diminui a atividade da iNOS nos modelos I e II de insulto oxidativo, assim como da HO1 no modelo II. Porém, há um aumento significativo na atividade da HO1 no modelo I.
- Este polifenol aumenta os níveis de TAR e confere proteção total ou parcial frente ao insulto oxidativo.
- Resveratrol frente aos modelos I e II de insulto oxidativo modula a atividade das principais defesas enzimáticas antioxidantes: SOD, CAT, GPx.
- Os níveis de GSH intra e extracelulares também são modulados por resveratrol. Em condições oxidantes, há um aumento da GSH extracelular no modelo I e uma diminuição no modelo II.
- A atividade da creatina cinase é alterada frente ao insulto oxidativo e apenas após rápida exposição ao $H_2O_2$ ocorre proteção por resveratrol.
- A produção de radicais livres foi medida utilizando-se resveratrol + EGTA e concluímos que a ação anti e pró-oxidante do resveratrol é dependente de cálcio.

**Conclusão Final**

Nossos resultados demonstram que importantes parâmetros bioquímicos astrogliais são modulados por resveratrol, o qual apresenta, geralmente, efeito antioxidante para o modelo I e pró-oxidante para o modelo II, evidenciando que as ações deste polifenol são dependentes do estado redox celular e das condições oxidativas do meio. O artigo de revisão nos permitiu integrar dados obtidos em linhagem celular astroglial, cultura primária de astrócitos e fatias

hipocampais. Também mostramos a modulação de importantes vias de sinalização, como a HO1, que pode justificar a ação do resveratrol em importantes processos neuropatológicos. Assim, fica demonstrado com esta tese que as células gliais são importantes alvos de ação do resveratrol.

## PERSPECTIVAS

- Investigar a ação do resveratrol sobre outros parâmetros gliais, a fim de melhor esclarecer seus efeitos sobre essas células.
- Analisar o efeito do resveratrol em fatias hipocampais, visando o melhor entendimento de sua ação sobre a comunicação neurônio-glia.
- Verificar efeitos *in vivo* do resveratrol com administração de doses diárias (VO e/ou IP) em ratos Wistar para avaliação de parâmetros bioquímicos.

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