UNIVERSIDADE FEDERAL DO RIO GRANDE DO SUL

INSTITUTO DE CIÊNCIAS BÁSICAS DA SAÚDE

PÓS-GRADUAÇÃO EM CIÊNCIAS BIOLÓGICAS: BIOQUÍMICA

EFEITO NEUROPROTETOR DO RESVERATROL EM UM MODELO *IN VITRO* DE ISQUEMIA CEREBRAL: ENVOLVIMENTO DAS VIAS PI3-K e MAPK

Lauren Lúcia Zamin

Orientadora

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Porto Alegre

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Dissertação apresentada ao curso de Pós-Graduação em Ciências Biológicas: Bioquímica da Universidade Federal do Rio Grande do Sul, como requisito parcial à obtenção do grau de Mestre em Bioquímica.

Porto Alegre

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“Transformar o medo em respeito, o respeito em confiança. Descobrir como é bom chegar quando se tem paciência. E para se chegar onde quer que seja, não é preciso dominar a força, mas a razão. É preciso, antes de mais nada, querer”.

Amyr Klink

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RESUMO

O cérebro é altamente dependente de um fluxo sanguíneo contínuo para suprimento de oxigênio e glicose, e por esta razão, eventos isquêmicos resultam em grande degeneração celular. O resveratrol é um antioxidante natural encontrado nas uvas e no vinho tinto que possui efeitos antitumoral e antiinflamatório, bem como propriedades cardioprotetoras. Neste trabalho, nós avaliamos o efeito neuroprotetor do resveratrol em um modelo *in vitro* de isquemia cerebral. Além disso, nós investigamos se este efeito estava correlacionado com as vias de sinalização da fosfoinositol3-quinase (PI3-k) e da proteína quinase ativada por mitógenos (*mitogen-activated protein kinase*-MAPK), ambas envolvidas na regulação da proliferação e sobrevivência celular. Para isto, nós usamos cultura organotípica de hipocampo exposta à privação de oxigênio e glicose (POG) como modelo de isquemia cerebral. A morte celular foi quantificada pela medida da incorporação de iodeto de propídeo (IP), um marcador de células mortas. Nas culturas expostas à POG na presença do veículo, aproximadamente 46% do hipocampo foi marcado com IP, indicando uma alta porcentagem de morte celular. Quando as culturas foram tratadas com resveratrol 10, 25 e 50μM, a morte celular foi reduzida para 22, 20 e 13%, respectivamente. Para elucidar o mecanismo pelo qual o resveratrol exerce seu efeito neuroprotetor nós investigamos a via PI3-k usando o inibidor LY294002 (5μM) e a via MAPK usando o inibidor PD98059 (20μM). A neuroproteção mediada pelo resveratrol (50μM) foi prevenida pelo LY294002, mas não pelo PD98059. A análise por *immunoblotting* revelou que o resveratrol 50μM induziu a fosforilação/ativação da Akt, a fosforilação/inativação da glicogênio sintase quinase-3β (GSK-3β) 1, 6 e 24 h após a POG e a fosforilação/ativação da quinase regulada por sinais extracelulares (*extracellular signal-regulated kinase-1 and -2*-ERK1/2) 6 e 24 h após a POG. Juntos, o aumento da fosforilação da Akt e GSK-3β induzida pelo resveratrol após a POG e o efeito da inibição da PI3-k pelo LY294002 levando a diminuição da neuroproteção mediada pelo resveratrol, sugerem que a via PI3-k/Akt, associada à GSK-3β , estão envolvidas no mecanismo pelo qual o resveratrol protege as culturas organotípicas da morte celular.

ABSTRACT

The brain is highly dependent on continuous blood flow for the supply of oxygen and glucose, for this reason ischemic insults result in severe cell degeneration. Resveratrol is a natural antioxidant occurring in grapes and red wine that has been shown to have anti-cancer and anti-inflammatory effects as well as cardioprotective properties. In this work, we analyzed the neuroprotective effect of resveratrol in an *in vitro* model of cerebral ischemia. We also investigated whether this effect was related with phosphoinositide3-kinase (PI3-k) and mitogen-activated protein kinase (MAPK) signaling pathways, both involved in regulation of cell proliferation and survival. For this purpose, we used organotypic hippocampal slice cultures exposed to oxygen-glucose deprivation (OGD) as a model of ischemia. Cell death was quantified by measuring uptake of propidium iodide (PI), a marker of dead cells. In cultures exposed to OGD in the presence of vehicle, about 46% of the hippocampus was labeled with PI, indicating a robust percentage of cell death. When cultures were treated with resveratrol 10, 25 and 50μM, the cell death was reduced to 22, 20 and 13% respectively. To elucidate a possible mechanism by which resveratrol exerts its neuroprotective effect we investigated the PI3-k pathway using the inhibitor LY294002 (5μM) and MAPK using the inhibitor PD98059 (20μM). The resveratrol (50μM) neuroprotection was prevented by LY294002 but was not by PD98059. Immunoblotting analysis revealed that resveratrol 50μM induced the phosphorylation/activation of Akt and the phosphorylation/inactivation of glycogen synthase kinase-3β (GSK-3β) 1, 6 and 24 h after OGD and phosphorylation/activation of extracellular signal-regulated kinase-1 and -2 (ERK1/2) 6 and 24 h after OGD. Taken together, the increase in phosphorylation of Akt and GSK-3β, induced by resveratrol after OGD, and the effect of the inhibition of PI3-k by LY294002, leading to a decrease in the neuroprotection mediated by resveratrol, suggest that the PI3-k/Akt pathway, together with GSK-3β, are involved in the mechanism by which resveratrol protects organotypic hippocampal slice cultures against cell death.

LISTA DE ABREVIATURAS

a.C. - Antes de Cristo

AKT/PKB - Homóloga Celular ao Oncogene Viral v-AKT/Proteína Quinase B

AP-1 - *Activator Protein*-1

CA1 - Corno de Amonis 1 (*Cornus Ammonis* 1)

COX - Ciclooxigenase

CREB - Proteína Responsiva Ao AMPc (*Cyclic AMP Response Element-Binding Protein*)

d.C - Depois de Cristo

DG - Giro Denteado (*Dentate Gyrus*)

ERK - Quinase Regulada por Sinais Extracelulares (*Extracellular Signal-Regulated Kinase*)

FKHR - Fatores de Transcrição da Forquilha *(Forkhead in Rhabdomyosarcoma)*

GSK-3β - Glicogênio Sintase Quinase 3β - (*Glycogen Synthase Kinase-3β*)

HDL - Lipoproteína de Alta Densidade (*High Density Lipoprotein*)

HPLC - *High Performance Liquid Chromatography*

IGF-1 - Fator de Crescimento tipo-Insulina 1 (*Insulin-like Growth Factor*-1)

JNK - *c-Jun N-terminus Kinase*

LDL - Lipoproteína de Baixa Densidade (*Low Density Lipoprotein*)

MAPK - Proteína Quinase Ativada Por Mitógenos (*Mitogen-Activated Protein Kinase*)

MEK – Quinase Ativada por mitógenos ativadora de ERK (*Mitogen-Activated ERK-activating kinase*)

mTOR - *mammalian Target of Rapamicym*

NFκB - Fator Nuclear Kappa B *(Nuclear Factor Kappa B)*

NGF - Fator de Crescimento do Nervo (*Nerve Growth Factor*)

PDK-1 E 2 - Quinase Dependente de Fosfoinositóis 1 e 2 (P*hophoinositide-Dependent Kinase* 1 e 2)

PH - *Pleckstin Homology*

PKA - Proteína Quinase A

PKC - Proteína Quinase C

POG - Privação de Oxigênio e Glicose

SNC - Sistema Nervoso Central

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1. INTRODUÇÃO

1.1 Isquemia Cerebral

As doenças neurodegenerativas afetam um amplo espectro da população e, na maioria dos casos, levam à incapacidade física e/ou mental, envolvendo as qualidades que fazem a vida dos seres humanos tão especiais, como a memória, a cognição, a fala, a personalidade e os movimentos especializados (Price, 1999). Dentre essas doenças do Sistema Nervoso Central (SNC), podemos destacar as desordens cerebrovasculares, a epilepsia, a doença de Alzheimer, a doença de Parkinson e a esclerose múltipla (Dinargl et al., 1999).

A isquemia cerebral é uma das principais causas de morbidade e mortalidade entre adultos e idosos. Ela está intimamente ligada a uma variedade de fatores de risco como hipertensão, hipercolesterolemia e diabete (Price, 1999). Por possuir uma alta taxa metabólica, estoques de energia limitados e uma alta dependência do metabolismo aeróbico de glicose, o cérebro é mais vulnerável ao dano isquêmico do que os outros tecidos. O tipo mais freqüente de isquemia em humanos é a isquemia focal, que é definida como a interrupção do fluxo sanguíneo para uma parte do cérebro. Outro tipo é a isquemia global, que resulta da interrupção transitória do fluxo sangüíneo para todo o cérebro, como ocorre durante uma parada cardíaca (Lipton, 1999). Além dos danos causados pela falta de oxigênio e metabólitos durante a isquemia, a volta da circulação sangüínea pode aumentar ainda mais a morte neuronal, especificamente nas áreas mais vulneráveis do cérebro (Farooqui et al., 1994; Taylor et al., 1996).

O conhecimento dos eventos moleculares associados à morte celular causada pela isquemia, bem como daqueles envolvidos nas estratégias celulares de sobrevivência e

estímulos nocivos é fundamental para o desenvolvimento de terapias clinicamente efetivas e a consequente diminuição da morte neuronal.

Estudos experimentais identificaram quatro processos dominantes responsáveis pelo dano neuronal pós-isquêmico: aumento de Ca^{2+} intracelular, neurotoxicidade mediada por receptores glutamatérgicos, formação de radicais livres e acidose láctica (Figura 1) (Juurlink e Sweeney, 1997; Dirnagl et al., 1999; Frantseva et al., 2001).

Com a depleção dos estoques energéticos, o potencial de membrana é perdido e os neurônios despolarizam-se. Conseqüentemente, os canais de Ca^{2+} dependentes de voltagem são ativados e aminoácidos excitatórios, particularmente o glutamato, são liberados no espaço extracelular (De Keyser et al., 1999). Ao mesmo tempo, os processos dependentes de energia, como a captação de glutamato, são bloqueados, levando ao acúmulo deste no espaço extracelular. A ativação de receptores glutamatérgicos ocasiona o aumento do Ca^{2+} intracelular pela abertura direta de canais de Ca^{2+} (receptores ionotrópicos), pela ligação aos receptores ligados a proteínas G (receptores metabotrópicos) e favorecendo a saída do Ca^{2+} do retículo endoplasmático. Como resultado da ativação glutamatérgica há aumento do influxo de Na^{+} e Cl^{-} nos neurônios, juntamente com a água, ocasionando edema celular (Dirnagl et al., 1999).

O aumento no Ca^{2+} intracelular desencadeia uma série de eventos citoplasmáticos e nucleares, causando danos no tecido através da ativação de enzimas proteolíticas, endonucleases, lipases, entre outras, levando à morte celular (Mitani et al., 1993). A ativação da fosfolipase A2 e da ciclooxigenase (COX) gera radicais livres acima da capacidade dos mecanismos antioxidantes endógenos, produzindo peroxidação lipídica e dano à membrana (Zhao et al., 1994).

A membrana mitocondrial interna também é afetada pelo distúrbio mediado por radicais livres, formando poros que causam inchamento mitocondrial, suspensão da

produção de ATP e superprodução de radicais livres (Kristian e Siesjo, 1998). Desse modo, o citocromo C pode ser liberado, desencadeando apoptose (Fujimura et al.,1998).

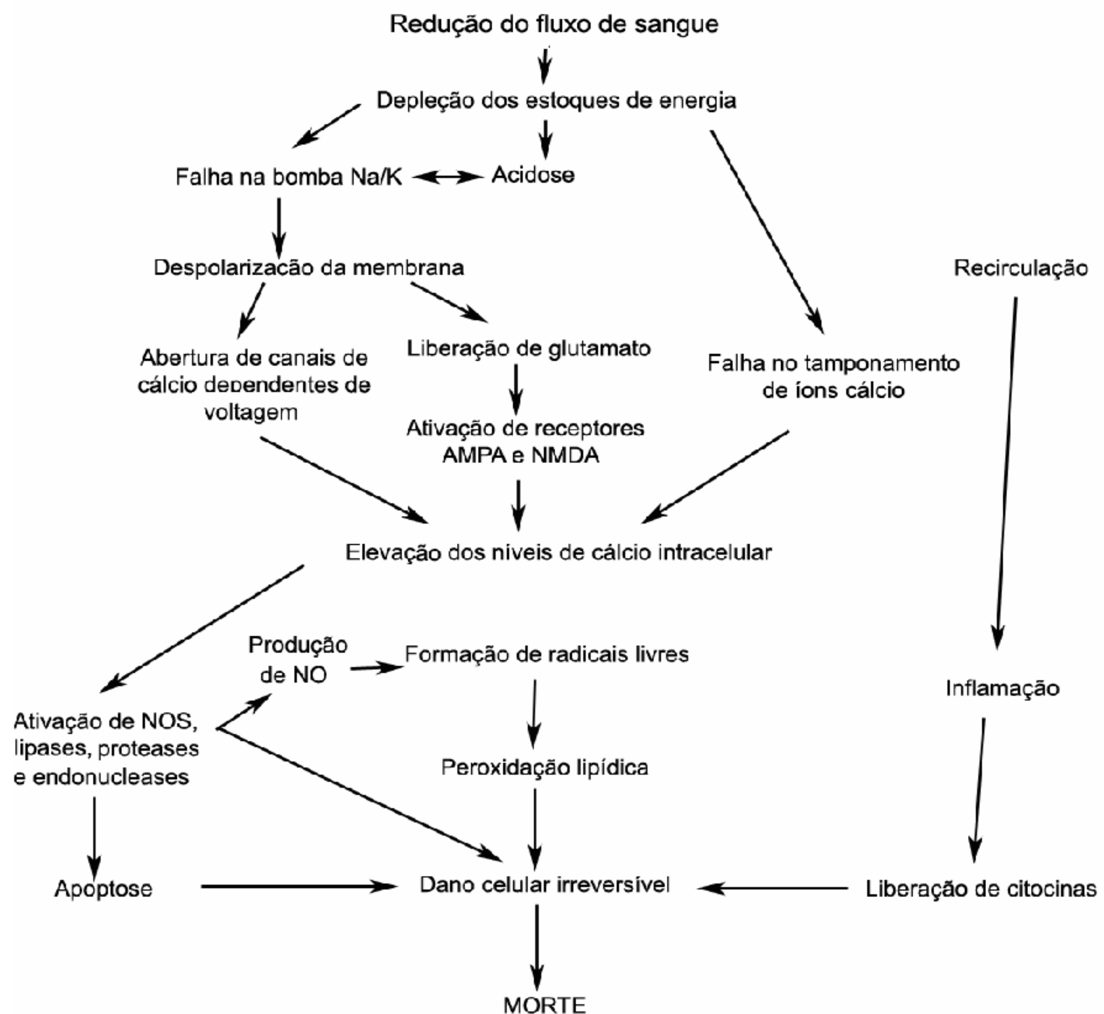

Figura 1 – Visão geral dos mecanismos patofisiológicos ocorridos na isquemia (*Adaptado de De Keyser et al., 1999*).

1.2 Modelos experimentais

Como modelo para o estudo da isquemia cerebral, uma alternativa para a experimentação animal *in vivo* pode ser o uso de sistemas *in vitro* de cultivo de tecidos, como por exemplo, as culturas organotípicas de hipocampo de rato expostas à privação de oxigênio e glicose (Pringle et al., 1997; Cimarosti et al., 2001).

O modelo de cultura organotípica foi desenvolvido em 1981 por Gähwiler e modificado por Stoppini e colaboradores em 1991. Basicamente, trata-se de um método que mantém as fatias de um determinado tecido em cultivo, numa interface entre o ar e o meio de cultivo, podendo permanecer por diversas semanas. Uma das principais características destas culturas é a de manter a organização do tecido tal qual ocorre *in vivo* (Stoppini et al., 1991; Gähwiler et al., 1997). A idade das culturas é denotada em dias pós-natal equivalente, o qual corresponde à idade que o animal tinha quando as fatias foram colocadas em cultivo somado ao número de dias de permanência *in vitro* (Bruce et al., 1995).

Por reproduzirem muitos aspectos da isquemia *in vivo*, tais como a morte neuronal tardia e a vulnerabilidade seletiva (Strasser e Fischer 1995; Laake et al., 1999; Horn et al. 2005), as culturas organotípicas de fatias hipocampais são uma excelente alternativa para o estudo dos mecanismos associados à isquemia cerebral.

1.3 Resveratrol

Escavações em Catal Hüyük (talvez a primeira das cidades da humanidade) na Turquia, em Damasco na Síria, Byblos no Líbano e na Jordânia revelaram a existência de sementes de uvas da Idade da Pedra (Período Neolítico B), cerca de 8.000 a.C.. As mais antigas sementes de uvas cultivadas foram descobertas na Georgia (Rússia) e datam de 7.000 - 5.000 a.C.. A idade dessas coincide com a passagem das culturas avançadas da Europa e do Oriente Próximo de uma vida nômade para uma vida sedentária (Johnson, 1999).

Há inúmeras lendas sobre onde teria começado a produção de vinhos e a primeira delas está no Velho Testamento, onde Noé, após ter desembarcado os animais, plantou um vinhedo do qual fez vinho. Após essa passagem, os egípcios, gregos, sumérios,

romanos, entre outros povos, foram grandes cultivadores da uva e do vinho (Johnson, 1999).

O amor dos gregos pelos vinhos pode ser avaliado pelos "Simpósios", cujo significado literal é "bebendo junto". Eram reuniões (daí o significado atual) onde as pessoas se reuniam para beber vinho em salas especiais, reclinados confortavelmente em divãs, onde conversas se desenrolavam num ambiente de alegre convívio. Todo Simpósio tinha um presidente cuja função era estimular a conversação (Johnson, 1999).

Entre as muitas evidências da sabedoria grega para o uso do vinho são os escritos atribuídos a Eubulus por volta de 375 a.C.: "Eu preparo três taças para o moderado: uma para a saúde, que ele sorverá primeiro, a segunda para o amor e o prazer e a terceira para o sono. Quando essa taça acabou, os convidados sábios vão para casa” (Johnson, 1999).

Existem inúmeros registros do uso medicinal do vinho pelos gregos. Hipócrates fez várias observações sobre as propriedades medicinais do vinho, que são citadas em textos de história da medicina. Galeno (131-201 d.C.), o famoso médico grego dos gladiadores, escreveu um tratado denominado "De antídotos" sobre o uso de preparações à base de vinho e ervas, usadas como antídotos de venenos (Johnson, 1999).

Por volta do ano de 1.300, é publicado o primeiro livro sobre o vinho: "Liber de Vinis". Escrito pelo espanhol Arnaldus de Villanova, médico e professor da Universidade de Montpellier, o livro continha uma visão médica do vinho, provavelmente a primeira desde a escrita por Galeno. O livro cita as propriedades curativas de vinhos em uma infinidade de doenças, tais como: “restabelecer o apetite e as energias, embelezar a face, e manter a pessoa jovem” (Johnson, 1999).

Em 1979 surgiu o primeiro estudo relatando os efeitos benéficos do consumo de vinho sobre a taxa de mortalidade por doenças cardíacas isquêmicas (St Leger et al., 1979), mais tarde confirmadas por Renaud e Lorgeril (1992). Foi também em 1992 que surgiu o primeiro trabalho relacionando este efeito a uma substância em particular presente no vinho: o resveratrol (Siemann e Creasy, 1992).

O estudo feito por Leger e colaboradores, em 1979, suscitou o fato de os franceses possuírem o menor índice de mortalidade por doenças cardíacas isquêmicas. Dentre as inúmeras variáveis testadas, a única que se mostrou significativamente diferente foi o maior consumo de vinho tinto entre os países que demonstraram os índices mais baixos de mortalidade, como a França, Itália e Suíça.

Em 1992, Renaud e Lorgeril destacaram que a alta ingestão de gorduras saturadas esta correlacionada positivamente com a taxa de mortalidade por doenças coronárias cardíacas. Contudo, a situação da França parecia muito paradoxal, visto que nesse país eram encontradas as menores taxas de morte por esta doença. Este paradoxo foi atribuído ao alto consumo de vinho tinto, e a esse fenômeno foi dado o nome de: Paradoxo Francês.

A partir da descoberta do resveratrol (Siemann e Creasy, 1992) inúmeros trabalhos vêm sendo realizados a fim de elucidar os efeitos benéficos desta substância e seus mecanismos de ação. O resveratrol está presente em mais de 70 espécies vegetais, sendo muito abundante em videiras de *Vitis Vinifera* e *Vitis labrusca*, popularmente conhecida como uva Isabel (Jeandet et al., 1991).

O principal significado do resveratrol na biologia da planta consiste em protegê-la de ataques externos, especialmente infecções fúngicas e radiação UV, uma propriedade que inclui o resveratrol na classe dos antibióticos das plantas, conhecido como fitoalexinas (Langeake et al., 1979). Nas plantas encontram-se as formas cis e trans-

resveratrol, sendo a forma trans responsável pelos efeitos biológicos do resveratrol em mamíferos (Soleas et al., 1997).

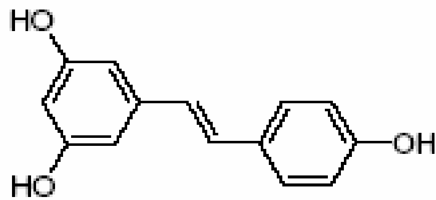

Figura 2 – Estrutura química do *trans*-Resveratrol (*trans*-3,4',5-triidroxiestilbeno)

Em vinhos provenientes de uvas tintas encontram-se as maiores taxas de resveratrol em relação aos vinhos brancos e rosados, pois esta substância é encontrada na casca das uvas tintas. Um estudo realizado por Souto et al. (2001), que analisou 36 tipos diferentes de vinhos brasileiros demonstrou que a quantidade de resveratrol variava entre 0,82 a 5,75 mg/L com um valor médio em torno de 2,57 mg/L, sendo as mais altas concentrações obtidas no vinho da variedade Merlot. Este valor médio é quase o dobro dos vinhos do mesmo tipo oriundos de outros países, como Portugal (1,00 mg/L; Lima et al., 1999), Chile/Argentina (1,21 mg/L; Goldberg et al., 1995), Grécia (0,873 mg/L; Dourtoglou et al., 1999), e Estados Unidos (0,132 mg/L; Lamuela-Raventos et al., 1993).

As concentrações de resveratrol encontradas nos diferentes tipos de vinhos variam em função da infecção por fungos, cultivar da uva, origem geográfica, tipo de vinho e práticas enológicas (Lamuela-Raventos et al., 1999). A hipótese admitida para explicar a maior concentração de resveratrol nos vinhos gaúchos em relação aos vinhos estrangeiros é a alta umidade dos solos da Serra Gaúcha, fato este que favorece a proliferação de fungos (Langkage and Pryce, 1976).

Dentre os diversos efeitos biológicos relacionados ao resveratrol encontram-se a sua alta capacidade antioxidante, diminuindo a lipoperoxidação, atuando como um "*scavenger*" de espécies reativas de oxigênio e diminuindo a oxidação da lipoproteína de baixa densidade (LDL - *low density lipoprotein*) (Miller e Rice-Evans, 1995; Frankel et al., 1993; Chanvitayapongs et al., 1997; Belguendouz et al., 1997). Estas propriedades contribuem para o efeito cardioprotetor do resveratrol, visto que a oxidação da LDL é uma das principais causas do surgimento da aterosclerose (Berliner e Heinecke, 1996). Além disto, o resveratrol diminui a agregação plaquetária, aumenta a lipoproteína de alta densidade (HDL - *high density lipoprotein*), e possui atividade anti-hipertensiva (Bertelli et al, 1995; Araya et al., 2001; Soares de Moura et al., 2004).

A atividade antiinflamatória do resveratrol foi demonstrada através de estudos onde este inibiu a atividade das enzimas COX 1 e 2 e lipooxigenases (Kimura et al., 1985; Subbaramaiah et al., 1998). Além disso, o resveratrol possui uma forte atividade antitumoral, bloqueando o processo carcinogênico nos estágios de iniciação, promoção ou progressão (Jang et al., 1997).

O efeito neuroprotetor do resveratrol foi estudado em vários modelos de lesão cerebral, como o peptídeo β-amilóide em culturas de células PC12 (Jang and Surh, 2003), a oclusão cerebral da artéria média em ratos (Sinha et al., 2002) e a lesão por ácido caínico também em ratos (Virgili e Contestabile, 2000). Nestes trabalhos, o efeito neuroprotetor do resveratrol foi atribuído a sua capacidade antioxidante.

Outros estudos indicam que o resveratrol possui a habilidade de ativar vias de sinalização celular responsável em promover a sobrevivência da célula, como a via Fosfatidilinositol 3-quinase (PI-3k) e a das proteínas quinases ativadas por mitógenos (MAPK - *Mitogen-Activated Protein Kinase*) (Das et al., 2005; Klinge et al., 2005).

1.4 PI3-k e Akt

A PI3-k é uma enzima amplamente expressa que regula vários processos celulares, como proliferação, crescimento e apoptose. Diversos receptores de superfície, especialmente os ligados à tirosina quinase, podem ativar a PI3-k. A PI3-k é formada por duas subunidades: p85 (regulatória) e p110 (catalítica). As subunidades estão dissociadas e juntam-se no momento da ativação, cuja atividade enzimática primária é a fosforilação de fosfatidilinositol (PI-fosfolipídeos de membrana) na posição D3 do grupo inositol. Os PI fosforilados, entre eles fosfatidilinositol trifosfato (PIP3), se ligam a moléculas que possuem domínios homólogos à pleckstrina (PH - *Pleckstrin homology*), recrutando-as para a membrana (Figura 3) (Datta et al., 1999).

Um dos alvos do segundo mensageiro gerado pela ativação da PI3-k é a proteína Akt. A Akt, também conhecida como Proteína Quinase B (PKB) é uma serina/treonina quinase de 60KDa (Datta et al., 1999) envolvida com a regulação da proliferação e sobrevivência celular (Song et al., 2005).

A ativação da Akt é um processo que envolve várias etapas e proteínas adicionais. A ativação da PI3-k por fatores de crescimento resulta em um aumento do PIP3, que interage com o domínio PH da Akt recrutando a quinase para a membrana plasmática e expondo seus resíduos serina e treonina para a fosforilação por quinases associadas à membrana, como a Quinase Dependente de Fosfoionositídeos-1 e -2 (PDK-1 e 2 - *Phosphoinositide-Dependet Kinase-1 e -2*). A PDK-1 fosforila a Akt em um resíduo Thr da alça de ativação (Yano et al, 1998) e a PDK-2 fosforila a Akt na Ser474 da região hidrofóbica C-terminal. A fosforilação em ambos sítios aumenta em 1000 vezes a atividade da Akt (Coffer et al., 1998; Datta et al., 1999).

A Akt é responsável pela fosforilação de vários substratos citosólicos e nucleares que regulam o metabolismo e o crescimento celular. Na sinalização mediada pela

insulina a Akt fosforila a Glicogênio Sintase Quinase-3β (GSK-3β - *Glycogen synthase kinase-3*β), fosfofrutoquinase-2 e *mammalian target of rapamycin* (mTOR) para induzir glicogênese e síntese protéica, enquanto a fosforilação de proteínas que regulam a apoptose como a BAD, caspase-9, fatores de transcrição da forquilha (FKHR - *Forkhead in rhabdomyosarcoma*) e IκB quinase promove proliferação e sobrevivência (Datta et al., 1999). As interações podem ser visualizadas na figura 3.

1.5 GSK-3β

A GSK-3β, encontrada abundantemente no SNC, particularmente em neurônios, é uma serina/treonina quinase inicialmente descrita como uma peça chave no metabolsimo do glicogênio pela sua habilidade de fosforilar e inativar a glicogênio sintase, mas atualmente sabe-se que ela regula diversas funções celulares. A atividade da GSK-3β pode ser reduzida pela fosforilação em resíduo de Ser9 pela Akt, pela Proteína Quinase C (PKC), pela Proteína Quinase A (PKA), MAPK, entre outras.Várias são as funções biológicas mediadas pela GSK-3β, como por exemplo, a regulação de proteínas do metabolismo e sinalização celular (incluindo ciclina D1 e *nerve growth factor* - NGF) proteínas estruturais (incluindo neurofilamentos e tau) e fatores de transcrição (incluindo NFκB - N*uclear Factor-κB* e b-catenina) (Figura 3) (Cohen e Frame, 2001; Grimes e Jope, 2001).

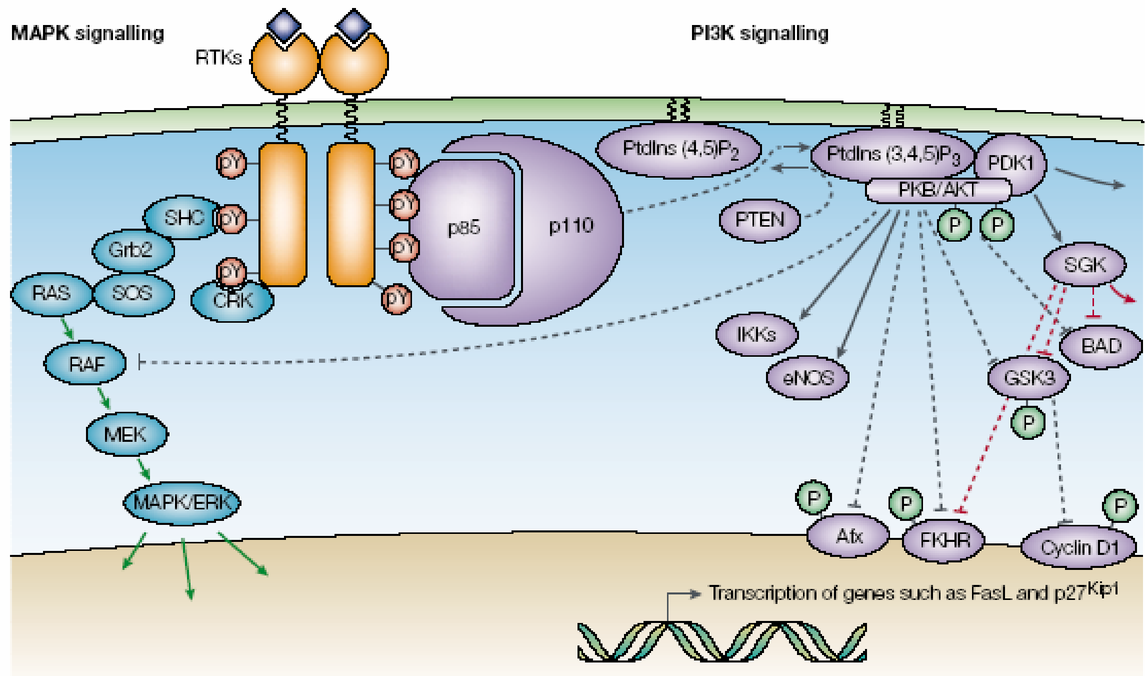

Figura 3 – Vias da PI-3k e MAPK (*Adaptado de Scheid e Woodget, 2001*). Setas inteiras indicam que as proteínas alvo estão sendo ativadas, enquanto setas tracejadas indicam que as proteínas alvo estão sendo inibidas.

1.6 Quinase Regulada por Sinais Extracelulares (ERK - *Extracellular Signal-regulated kinase*)

As MAPK abrangem um grupo de proteínas sinalizadoras utilizadas para diversos acontecimentos celulares, através de eventos de fosforilação. Elas estão presentes em todos os eucariotos e controlam processos como a proliferação, expressão gênica, diferenciação e apoptose (Cano e Mahadevan, 1995; Cohen, 1997). Há três subgrupos principais e distintos de MAPK: ERK, ativada principalmente por estímulos mitogênicos; p38 e JNK (*c-Jun N-terminus Kinase*), ativadas principalmente por estímulos de estresse e citocinas inflamatórias (Davis, 2000; Kyriakis e Avruch, 2001). Cada MAPK opera, basicamente, por uma proteína G ligada à membrana e, no

citoplasma, por uma MAPK quinase quinase (MAPKKK), que fosforila e ativa uma MAPK quinase (MAPKK), que, por sua vez, fosforila e ativa uma MAPK. Os eventos de sinalização mediados pela ERK iniciam através da ativação da Ras, que está envolvida nos passos iniciais de ativação da Raf-1. Após sua ativação, a Raf fosforila e ativa duas MAPK quinases (MAPKKs: MEK1 e MEK2). As MEKs (*Mitogen-activated ERK-activating Kinase)* ativadas funcionam como quinases de dupla especificidade, que fosforilam resíduos treonina e tirosina em duas MAPKs (ERKs) a $p42^{MAPK}$/ERK2 e a $p44^{MAPK}$/ERK1, ativando-as (Rossomando et al., 1989; Gutkind, 2000). Quando ativadas as ERKs se translocam para o núcleo, onde fosforilam e ativam uma variedade de fatores de transcrição, como AP-1 (A*ctivator Protein-1*), CREB (*cyclic AMP-response Element-binding Protein*), NFkB, GSK-3β (Campbell et al., 1998; Weinstein-Oppenheimer et al., 2000).

Considerando que as doenças cerebrais isquêmicas possuem uma alta prevalência, e que o tratamento convencional mostra-se pouco eficaz é importante que se realizem mais estudos a fim de buscar alternativas para o tratamento e a prevenção destas doenças. Sendo o resveratrol um composto que possui um forte potencial terapêutico na prevenção e/ou tratamento de várias doenças, como as doenças cardíacas e cerebrais e que este pode ativar vias de sinalização responsáveis por mediar à sobrevivência celular, como a da PI-3k e das MAPK, este trabalho tem como objetivo central estudar o efeito neuroprotetor do resveratrol em um modelo *in vitro* de isquemia cerebral e seu efeito sobre estas vias.

2. OBJETIVOS

2.1 Objetivo Geral

Investigar o efeito neuroprotetor do resveratrol e os seus possíveis mecanismos de ação.

2.2 Objetivos Específicos

- Avaliar o efeito neuroprotetor do resveratrol em culturas organotípicas de hipocampo de rato submetidas à Privação de Oxigênio e Glicose (POG).

- Investigar se o efeito neuroprotetor do resveratrol está associado a ativação da via PI3-k através do uso do inibidor desta via (LY294002) e a análise por immunoblotting da fosforilação das proteínas Akt e GSK3-β.

- Investigar se o efeito neuroprotetor do resveratrol está associado a ativação da via MAPK através do uso do inibidor desta via (PD98059) e a análise por immunoblotting da fosforilação da proteína ERK1/2.

3. RESULTADO

Os resultados serão apresentados sob a forma de um artigo científico

ARTIGO

Protective Effect Of Resveratrol Against Oxygen-Glucose Deprivation In Organotypic Hippocampal Slice Cultures: Involvement Of Pi3-K Pathway

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PROTECTIVE EFFECT OF RESVERATROL AGAINST OXYGEN-GLUCOSE DEPRIVATION IN ORGANOTYPIC HIPPOCAMPAL SLICE CULTURES: INVOLVEMENT OF PI3-K PATHWAY

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List to abbreviations:

CA1 - *Cornus Ammonis1*

DMSO - dimethylsulphoxide

ERK - extracellular signal-regulated kinase

ERK1/2 - extracellular signal-regulated kinases-1 and -2

GSK-3β - glycogen synthase kinase-3β

HBSS - Hank's balanced salt solution

IGF-1 - insulin-like growth factor-1

MAPK - mitogen-activated protein kinase

MCAO - middle cerebral artery occlusion

MEK - mitogen-activated ERK-activating kinase

OGD - oxygen-glucose deprivation

pAkt - phospho-Akt

pERK1/2 - phospho-ERK1/2

pGSK-3β - phospho-GSK-3β

PI - propidium iodide

PI3-k - phosphoinositide3-kinase

SDS - sodium dodecylsulfate

Abstract - The reduction in the supply of glucose and oxygen to the brain that occurs in cerebral ischemia leads to a complex cascade of cell events that result in neuronal death. Here, we investigated the neuroprotective effect of resveratrol, found in grape seeds and skins, in an *in vitro* model of ischemia. We used organotypic hippocampal slice cultures exposed to oxygen-glucose deprivation (OGD) as a model of ischemia. Cell death was quantified by measuring uptake of propidium iodide (PI), a marker of dead cells. In cultures exposed to OGD in the presence of vehicle, about 46% of the hippocampus was labeled with PI, indicating a robust percentage of cell death. When cultures were treated with resveratrol 10, 25 and 50μM, the cell death was reduced to 22, 20 and 13% respectively. To elucidate a possible mechanism by which resveratrol exerts its neuroprotective effect we investigated the phosphoinositide3-kinase (PI3-k) pathway using LY294002 (5μM) and mitogen-activated protein kinase (MAPK) using PD98059 (20μM). The resveratrol (50μM) neuroprotection was prevented by LY294002 but was not by PD98059. Immunoblotting revealed that resveratrol 50μM induced the phosphorylation/activation of Akt and extracellular signal-regulated kinase-1 and -2 (ERK1/2) and the phosphorylation/inactivation of glycogen synthase kinase-3β (GSK-3β). Taken together, the increase in phosphorylation of Akt and GSK-3β, induced by resveratrol after OGD, and the effect of the inhibition of PI3-k by LY294002, leading to a decrease in the neuroprotection mediated by resveratrol, suggest that the PI3-k/Akt pathway, together with GSK-3β, are involved in the mechanism by which resveratrol protects organotypic hippocampal slice cultures against cell death.

Key Words: Neuroprotection, Akt, GSK-3β, ERK1/2, LY294002, PD98059

Ischemic stroke results from a transient or permanent reduction in cerebral blood flow that leads to a complex cascade of cell events resulting in neuronal death (Dirnagl et al., 1999; Lipton, 1999). Until now, there are no clinically effective therapeutic protocols for amelioration of brain damage by ischemia and reperfusion (White et al., 2000).

Diverse *in vitro* and *in vivo* models have been used to study the mechanisms underlying neuronal degeneration and to evaluate potential neuroprotective effects of pharmacological treatments. Among the *in vitro* systems used to study ischemia-induced injuries, organotypic hippocampal slice cultures, combined with oxygen–glucose deprivation (OGD), offer great advantages because they mimic closely the *in vivo* condition (Pringle et al., 1997; Tavares et al., 2001; Cimarosti et al., 2001; 2005). Cultured slices maintain their cell architecture and interneuronal connections, and neurons survive during the long-term culture and physiologically mature over this period, allowing an extended survival study (Muller et al., 1993; Xiang et al., 2000; Cho et al., 2004).

The notion that red wine may have potential health benefits initially received a great deal of attention following reports that moderate wine consumption was linked to a lower incidence of cardiovascular disease – the so-called “French Paradox” (Renaud and De Lorgevil, 1992). This cardioprotective effect has been attributed to the polyphenol fraction of red wine (Das et al., 1999). A key polyphenol in red wine is resveratrol, trans-3,5,4 trihydroxystilbene, from grape seeds and skins. Resveratrol has been found to act as a strong antioxidant (Belguendouz et al., 1997; Tadolini et al., 2000), and can reduce the oxidation of lipoproteins (Frankel et al., 1993). Resveratrol can also protect the vessels from atherosclerosis (Frankel et al, 1993; Belguendouz et

al., 1997) and inhibit platelet aggregation and cyclo-oxygenase (Bertelli et al., 1995; Subbaramaiah et al., 1998).

In addition to the purported cardioprotective effects of red wine, recent evidences suggest that wine consumption may also protect against certain neurological disorders. Studies have demonstrated the ability of resveratrol to exert protective effects against brain injury due to ischemia/reperfusion in gerbil model (Wang et al., 2002), and epidemiological studies have shown that moderate red wine consumption is significantly correlated with a reduction in the incidence of age-related macular degeneration (Obisesan et al., 1998), Alzheimer's disease (Luchsinger et al., 2004) and stroke (Mukamal et al., 2005).

Although molecular mechanisms underlying the pathogenesis of delayed neuronal death is unclear, histologic and biochemical evidence suggest the involvement of apoptosis in dying cells after ischemia (MacManus et al., 1993; Nitatori et al., 1995). Phosphoinositide3-kinase (PI3-k) pathway is believed to be an important anti-apoptotic signal pathway in neurons (Yuan and Yankner, 2000; Love, 2003). Akt, also known as protein kinase B, is a downstream kinase of PI3-k in growth factor mediated signaling cascades, and the phosphorylation of residues Thr308 and Ser473 is required for its activity (Coffer et al., 1998). One way by which active Akt mediates its anti-apoptotic effects is by phosphorylating and inactivating glycogen synthase kinase-3β (GSK-3β), which plays a key role in apoptosis (Pap and Cooper, 1998).

Another signaling system, which has a role in mediating cell survival, is the extracellular signal-regulated kinase-1 and 2 (ERK1/2), which belongs to the family of mitogen-activated protein kinase (MAPK) pathway (Ahn, 1993). The mitogen-activated ERK-activating kinase/ERK (MEK/ERK) pathway is activated downstream of receptor tyrosine kinases, G protein-coupled receptors and cytokine receptors (Lewis, 1998).

Following neuronal injury, the ERK pathway is activated, suggesting a role in cell death and/or repair (Campos-Gonzales and Kindy, 1992; Hu and Wieloch, 1994; Runden et al., 1998). Recent studies suggest that resveratrol can activate both the PI3-k and the MAPK pathway (Miloso et al., 1999; Das et al., 2005a, 2005b; Klinge et al., 2005).

In order to investigate the neuroprotective effects of resveratrol, we used organotypic cultures of rat hippocampus exposed to an *in vitro* model of cell death using OGD, and we also investigated whether such neuroprotection could be related to PI3-k signaling pathway by Akt activation and GSK-3β inactivation and/or MAPK pathway by activation of ERK1/2.

EXPERIMENTAL PROCEDURE

Organotypic hippocampal slice cultures

Organotypic hippocampal slice cultures were prepared according to the method of Stoppini et al. (1991). Briefly, 400μm thick hippocampal slices were prepared from 6-8-day-old male *Wistar* rats using a McIlwain tissue chopper (all animal use procedures were approved by local Animal Care Committee and were in accordance with the NIH Guide for the Care and Use of Laboratory Animals) and separated in ice-cold Hank's balanced salt solution (HBSS) composed of (mM): glucose 36, $CaCl_2$ 1.26, KCl 5.36, NaCl 136.89, KH_2PO_4 0.44, Na_2HPO_4 0.34, $MgCl_2$ 0.49, $MgSO_4$ 0.44, HEPES 25; fungizone 1% (Gibco, Grand Island, NY, USA) and gentamicine 36μL/100mL (Schering do Brasil, São Paulo, SP, Brazil); pH 7.2. The slices were placed on Millicell culture insert (Millicell®-CM, 0.4μm, Millipore®, Bedford, MA, USA) and the inserts were transferred to a 6-well culture plate (Cell Culture Cluster, Costar®, New York, NY, USA). Each well contained 1mL of tissue culture medium consisting of 50% minimum

essential medium (Gibco), 25% HBSS (Gibco), 25% heat inactivated horse serum (Gibco) supplemented with (mM, final concentration): glucose 36, HEPES 25 and $NaHCO_3$ 4; fungizone 1% (Gibco) and gentamicine 36μL/100mL (Schering do Brasil); pH 7.2. Organotypic cultures were maintained in a humidified incubator gasified with 5% CO_2 atmosphere at 37°C. Culture medium was changed three times/week.

OGD

OGD was achieved by combining hypoxia with aglycemia, according to the method described by Strasser and Fisher (1995), with some modifications (Cimarosti et al., 2001). Figure 1 shows the basic experimental design. After 14 days *in vitro*, the inserts were transferred to a sterilized 6-well plate, and incubated with 1mL of OGD medium consisting of HBSS lacking glucose for 15 min to deplete glucose from intracellular stores and extracellular space. After that, the medium was exchanged for one with the same composition but previously bubbled with N_2 for 30 min and the plate transferred to an anaerobic chamber at 37°C with N_2-enriched atmosphere for 60 min. During this process control slices were maintained in an incubator with 5% CO_2 atmosphere at 37°C. After the deprivation period, slice cultures were incubated in culture medium under normoxic conditions for 24 h, corresponding to the recovery periods.

Drug exposition

Resveratrol was purchased from Calbiochem (San Diego, CA, USA), LY294002 was from Cell Signaling Technology (Beverly, MA, USA) and PD98059 was obtained from New England Biolabs (Beverly, MA, USA). All drugs were dissolved in dimethylsulphoxide (DMSO) (Sigma Chemical, St. Louis, Mo, USA). Resveratrol (10,

25 e 50μM) and DMSO (0.01%) were added to the OGD medium and to the culture medium during the recovery period of 24 h. LY294002 (5μM) and PD98059 (20μM) were administered to the culture medium two hours before OGD period, and maintained during the OGD and the recovery period of 24 h.

Cultures were categorized in four conditions: vehicle-supplemented cultures not exposed to OGD (control DMSO), drug-supplemented group not exposed to OGD (control resveratrol, control LY294002, control resveratrol+LY294002, control PD98059, control resveratrol+PD98059), vehicle-supplemented cultures exposed to OGD (OGD DMSO), and drug-supplemented group exposed to OGD (OGD resveratrol, OGD LY294002, OGD resveratrol+LY294002, OGD PD98059, OGD resveratrol+PD98059).

Quantification of cell death

Cell damage was assessed by fluorescent image analysis of propidium iodide (PI) uptake (Noraberg et al., 1999). After a recovery period of 22 h, 7.5μM PI (Sigma Chemical) was added to the cultures and incubated for 2 h. PI uptake is indicative of significant membrane injury (Macklis and Madison, 1990). Cultures were observed with an inverted microscope (Nikon Eclipse TE 300) using a standard rhodamine filter set. Images were captured and then analysed using Scion Image software (www.scioncorp.com). The area where PI fluorescence was detectable above background was determined using the “density slice” option of Scion Image software and compared to the total hippocampus area to obtain the percentage of damage (Valentim et al., 2003).

Western blotting assay

To investigate the phosphorylation status and immunocontent of Akt, GSK-3β and ERK 1/2 the slices were homogenized in lysis buffer (4% sodium dodecylsulfate (SDS), 2.1mM EDTA, 50mM Tris) 1, 6 and 24 h after OGD. To analyze the effect of the inhibitors we homogenized the slices only 24 h after OGD. Aliquots were taken for protein determination (Peterson, 1983) and β-mercaptoethanol (Sigma Chemical) was added to a final concentration of 5%. Proteins were separated (40μg per lane) on 10% SDS-polyacrylamide gel electrophoresis (Sigma Chemical). After electrophoresis, proteins were electrotransferred to nitrocellulose membranes using a semi-dry apparatus (Bio-Rad Trans-Blot SD, Hercules, CA, USA).

Membranes were incubated for 60 min at 4ºC in blocking solution (Tris-buffered saline containing 5% powdered milk and 0.1% Tween-20), and further incubated with the appropriate primary antibody dissolved in blocking solution overnight at 4ºC. The primary antibodies used were anti-phospho Akt (Ser473) (pAkt, 1:1000; Cell Signaling Technology), anti-Akt (1:1000; Cell Signaling Technology), anti-phospho GSK-3β (Ser9) (pGSK-3β, 1:1000; Cell Signaling Technology), anti-GSK-3β (1:1000; Cell Signaling Technology), anti-phospho ERK1/2 (pERK1/2, 1:1000; Cell Signaling Technology) and anti-ERK1/2 (1:1000; Upstate Biotechnology, New York, NY, USA). The membranes were then incubated with horseradish peroxidase-conjugated anti-rabbit antibody (1:1000; Amersham Pharmacia Biotech, Piscataway, NJ, USA). The chemioluminescence (ECL, Amersham Pharmacia Biotech) was detected using X-ray films (Kodak X-Omat, Rochester, NY, USA). The films were scanned and the percentage of band intensity was analyzed using Optiquant software (Packard Instrument). For each experiment, the test groups were referred to vehicle treated control cultures not exposed to OGD, which were considered 100%, thus assuring the

same signal intensity for control and test groups. The data are expressed as percentage of phosphorylated protein, which was obtained by the ratio of the immunocontent of phospho-protein (pAkt, pGSK-3β or pERK1/2) to the whole amount of the protein (Akt, GSK-3β or ERK1/2) provide by the immunodetection assay with the total antibodies.

Statistical analysis

Data are expressed as mean ± S.E.M. and analyzed for statistical significance by one-way analysis of variance (ANOVA) using a post-hoc Student–Newman–Keuls test for multiple comparisons. Differences between mean values were considered significant when $P < 0.05$.

RESULTS

Resveratrol protects against cell death induced by OGD

The exposure of the cultures to 60 min of OGD and 24 h of recovery caused a marked fluorescence in the hippocampus, indicating a high incorporation of PI, as presented in the photomicrograph in figure 2A (OGD DMSO group). Quantification of PI fluorescence showed that OGD caused about 46% of damage in hippocampus, a significant increase compared to control cultures with a basal hippocampus damage of 4% (figure 2B). Treatment with 10, 25 and 50μM of resveratrol significantly reduced the injury in the hippocampus from 46%, to 22, 20 and 13% respectively (figure 2B). There were no statistical differences among the tested doses, thus we chose the dose of 50μM to investigate a probable mechanism by which resveratrol exerts its neuroprotective effect. Also no difference was detectable between the treatments in the control slices, indicating that resveratrol had no toxic effect in basal conditions (figure

2B). It was not observed cell death after a recovery period of 1 and 6 h (data not shown) as we have previously demonstrated (Horn et al., 2005).

The neuroprotective effect of resveratrol involves PI3-k but not MEK/ERK1/2 pathway

In order to determine whether the PI3-k signaling pathway was involved in the neuroprotective effect of resveratrol, we carried out experiments using LY294002, a specific inhibitor of PI3-k (Vlahos et al., 1994). LY294002 (5µM) abolished the neuroprotection induced by resveratrol (50µM), increasing the PI incorporation from 15% (OGD RSV) to 34% (OGD RSV+LY) (figure 3B). To clarify another probable signaling pathway involved in the neuroprotection by resveratrol we further studied the effect of PD98059, an inhibitor of MEK/ERK pathway (Alessi et al., 1995; Dudley et al., 1995). PD98059 did not prevent the neuroprotection induced by resveratrol that showed a PI uptake of 15% in OGD cultures treated with resveratrol (figure 3B OGD RSV) and 10% in OGD cultures treated with PD98059 and resveratrol (OGD RSV+PD). No neurotoxic effects were observed with inhibitors only (figure 3A).

Resveratrol increase the Akt and GSK3-β phosphorylation in OGD cultures

Considering that LY294002 abolished the neuroprotective effect of resveratrol, we hypothesized that resveratrol would induce Akt activation, a PI3-k-activated protein kinase. The status of Akt phosphorylation was examined after 1, 6 and 24 h of recovery by Western blotting with antibodies against the active form of Akt, phosphorylated at Ser473, as well as its total immunocontent. Resveratrol 50µM significantly increased the percentage of pAkt by 50% in OGD cultures in all recovery periods (figure 4B). There were no differences in the pAkt intensity neither among controls slices or in OGD

DMSO treated group. No alteration in the total amount of Akt was observed (representative Western blotting in figures 4A). To confirm that LY294002 was effective in the blockage of the PI3-k pathway we measured the phosphorylation state of Akt in the presence of LY294002 after 24 h of recovery. Treatment with LY294002 reduced the ratio of pAkt/Akt (figure 5B), confirming the efficacy of this inhibitor.

When Akt is activated (i.e. phosphorylated), it phosphorylates and inactivates GSK-3β. Therefore, GSK-3β phosphorylation was also evaluated after 1, 6 and 24 h after OGD and resveratrol treatment. We found that resveratrol increased the GSK-3β phosphorylation by 65% in OGD cultures in all recovery periods tested (figure 6B). No differences were detected in the percentage of pGSK-3β neither among controls slices or OGD DMSO treated group. It was not detectable alteration in the total amount of GSK-3β (botton line in figures 6A). Figure 7B shows that LY294002 was able to abolish the increase of ratio of pGSK-3β/GSK-3β mediated by resveratrol.

Resveratrol increase ERK1/2 phosphorylation in OGD cultures

Since cell survival and differentiation in several cell types are mediated by ERK1/2 we examined whether the neuroprotection mediated by resveratrol could involve this pathway. Furthermore, the same serine residue in GSK-3β that is targeted by Akt, is now known to be phosphorylated by MAPK cascade (Cohen and Frame, 2001). We measured the levels of the immunocontent of ERK1/2, as well as its phosphorylation status after 1, 6 and 24 h after OGD. Resveratrol (50μM) was also able to increase ERK1/2 phosphorylation in OGD cultures by 80% (figure 8B) after 6 and 24 h of recovery. There were no statistical differences in the phosphorylation status of ERK1/2 in the control cultures and OGD DMSO treated culture, although we could observe a moderate increase on pERK1/2 in OGD DMSO culture. No alteration in the

total amount of ERK1/2 was observed (figure 8A). Figure 3B shows that PD98059 was ineffective in blocking the neuroprotection induced by resveratrol. To determine the efficacy of this inhibitor we analyzed the phophorylation state of ERK1/2 in the presence of PD98059. Western blotting analyses of ERK1/2 confirm that PD98059 was effective in reducing the ERK1/2 phosphorylation (figure 9B).

DISCUSSION

In the present study, we tested whether resveratrol, on active ingredient of red wine, has a neuroprotective effect in ischemic injury and proposed an underlying mechanism by which this neuroprotection occurs. For this purpose, we have used organotypic hippocampal slice cultures to examine its effects in an *in vitro* model of 'ischemic-like insult' using OGD, extensively employed in our research group (Tavares et al., 2001; Valentim et al., 2003; Cimarosti et al., 2005; Horn et al., 2005). Organotypic cultures provide good experimental access to mimic pathophysiological pathways in living tissues and to facilitate design of therapeutic agents (Stoppini et al., 1991). In particular, hippocampal slice cultures combined with OGD could provide a surrogate system to investigate cell loss following ischemic injury to the brain. Using this model, we have shown here that resveratrol at doses of 10, 25 and 50μM protects organotypic hippocampal slice cultures against OGD.

These results go along with findings obtained in other *in vivo* and *in vitro* models of neurodegenerative diseases. Huang et al. (2001) suggest that resveratrol is a potent neuroprotective agent in focal cerebral ischemia caused by middle cerebral artery occlusion (MCAO) in Long-Evans rats. It has been reported that resveratrol attenuated

β-amyloid-induced cytotoxicity, apoptotic features, and intracell reactive oxygen intermediates accumulation in PC12 cells (Jang and Surh, 2003).

The mechanisms of the neuroprotective effects of resveratrol are not fully understood, although some mechanisms have been proposed. In this study we proposed that resveratrol could mediate its neuroprotective effect by activating of two major signaling pathways implicated in supporting neuronal survive: PI3-k and MAPK (Pettmann and Hendersen, 1998). First, we investigated whether resveratrol could activate the PI3-k pathway. For this purpose, we used LY294002. The results presented here show that the neuroprotective effect of resveratrol was prevented by this inhibitor. Second, we investigated whether MEK/ERK1/2 could be involved in the neuroprotective effect of resveratrol. As we showed, PD98055 did not prevent the neuroprotective effect of resveratrol. These results strongly suggest that the PI3-k is involved in the neuroprotection by resveratrol observed in our model of cell death.

It has been shown that the protective effects of PI3-k are mediated primarily by one of its downstream targets – Akt (Franke et al., 1997). Akt has direct effects on the apoptosis pathway, by inhibiting the pro-apoptotic proteins as, among others, Bad, caspase 9, forkhead factors and GSK-3β (Song et al., 1995). Several reports have suggested that apoptosis is underlying the delayed neuronal death in ischemic brain injuries (MacManus et al., 1993; Nitatori et al., 1995). Studies have shown that blocking increased Akt phosphorylation by treatment with LY294002, increases subsequent DNA fragmentation (Noshita et al., 2001). Likewise, reverting the decrease in Akt phosphorylation seen immediately after brain ischemia by treatment with intraventricular administration of insulin-like growth factor-1 (IGF-1), rescued cells from neuronal cell death (Kawano et al., 2001). Taken together, decreased Akt activity accounts for neuronal damage following brain ischemia, and increased Akt activity

observed a few hours later could participate in endogenous neuroprotective responses to ischemia.

Therefore, we hypothesized that resveratrol could protect neurons against cell death induced by OGD by activating PI3-k/Akt proteins. Our results indicated that resveratrol increased Akt phosphorylation after OGD, and this increase was abolished by LY294002. No change in the amount of pAkt or Akt was observed in control slices treated with resveratrol. Furthermore, there was no increase in pAkt or Akt in OGD DMSO group. These results suggest that the neuroprotection mediated by resveratrol could involve an increase of Akt phosphorylation after an insult.

Recently, Das et al. (2005a) showed that resveratrol mediates cardioprotective effect by increasing Akt phosphorylation and this effect was partially abolished by LY294002. Another study has indicated a role of both adenosine A1 and A3 receptors in resveratrol preconditioning, and both use the PI3-k/Akt signaling pathway (Das et al., 2005b). Altogether, these studies pointed to PI3-k as the upstream signaling molecule for resveratrol protection.

Horn et al. (2005) did not observe any significant changes in Akt phosphorylation and immunocontent in CA1 or *Dentate Gyrus* areas of organotypic cultures exposed to OGD after 30 min, 6 and 24 h of reperfusion. However, another group reported a dramatically decrease in Akt phosphorylation at Ser473 immediately following global brain ischemia in the hippocampus of *Wistar* rats. After this transient decrease in Akt phosphorylation, phosphorylation greatly increased within the next 24 h and declined to basal levels within 48 h (Ouyang et al. 1999). Transient upregulation of Akt phosphorylation was also observed in the global ischemic model using 4-vessel occlusion in the rat (Jin et al., 2001). One possible explanation for these apparent controversies results is that the latter used an *in vivo* model of global brain ischemia and

the former used an *in vitro* model of organotypic hippocampal slice cultures exposed to OGD.

Following the PI3-k/Akt pathway, we investigated whether resveratrol had effect on GSK-3β phosphoregulation and whether this effect could involve PI3-k using LY294002. We are the first to demonstrate that resveratrol strongly increase phosphorylation of GSK-3β after OGD, and this increase was abolished by LY294002, suggesting that this could be a probable mechanism by which resveratrol protects against cell death. GSK-3β is an enzyme initially identified as a regulator of glycogen metabolism that also has broader functions, such as regulating signaling proteins (including amyloid precursor protein, cyclin D1, and nerve growth factor receptor), structural proteins (including microtubule-associated proteins-1B and-2, neural cell-adhesion protein, neurofilaments, and tau), and transcription factors (including nuclear factor kappa B, h-catenin, activating protein-1, and heat shock factor-1) as reviewed by Cohen and Frame (2001).

There is growing evidence that GSK-3β is involved in the pathogenesis of central nervous system diseases. There are only a handful of research reports on the expression of GSK-3β in cerebral ischemia. GSK-3β is present in neurons following MCAO (Sasaki et al., 2001). Selective, but not specific GSK-3β inhibition with lithium, has been shown to be protective against a plethora of neurological insults including cerebral ischemia (see reviews Manji et al., 1999; Nonaka and Chuang, 1998), suggesting that a reduction in GSK-3β activity improves brain cell survival. Furthermore, topical application of IGF-1, also a non-specific inhibitor of GSK-3β, significantly reduced infarct size following 60 min MCAO and reduced the number of GSK-3β-immunoreactive neurons (Wang et al., 2000).

Taken together, the increase in phosphorylation of Akt and GSK-3β, induced by resveratrol after OGD, and the effect of the inhibition of PI3-k by LY294002, leading to a decrease in the neuroprotection mediated by resveratrol, suggest that the PI3-k/Akt pathway and GSK-3β could be involved in the mechanism by which resveratrol protects organotypic hippocampal slice cultures against cell death induced by OGD.

Similarly Akt signaling, MAPK signaling pathway is required for the anti-apoptotic effect and neuronal survival in the brain following ischemic insult. Transient decrease in MAPK activity was also evident in the gerbil hippocampus following forebrain ischemia, and such decrease was blocked by intraventricular administration of IGF-1 (Kawano et al., 2001). Recently data have showing that pERK1/2 is upregulated in hippocampal organotypic cultures after OGD (Irving et al., 2001; Namura et al., 2001). Our results showed that resveratrol increased the ERK1/2 phosphorylation after OGD, but the inhibition of this increase by PD98059 did not prevent the neuroprotective effect of resveratrol.

Strikingly, resveratrol only increased the phosphorylation of Akt, GSK-3β and ERK1/2 in the OGD-treated slices, indicating that resveratrol could not be a direct activator of these pathways, but acts as a facilitator of their activation.

While ERK signaling plays a beneficial, neuroprotective role in many systems (Dash et al., 2002; Cavanaugh, 2004; Hetman and Gozdz, 2004), there is growing evidence implicating these kinases in promoting cell death. For example, increased ERK1/2 phosphorylation has been noted in the vulnerable penumbra following acute ischemic stroke in humans (Slevin, 2000). Alessandrini et al. (1999) in model of cerebral ischemia-reperfusion injury provided the first *in vivo* evidence that activation of the MEK/ERK1/2 signaling pathway may contribute to acute brain injuries. In these studies, ERK1/2 activation was blocked using inhibitors of MEK1/2 and led to reduced

neuronal injury and loss of function in mice. More compelling results indicating neuroprotective effects from inhibiting ERK1/2 activation were subsequently obtained in hippocampal slice cultures and hipocampal dissociated neuronal cultures where protein phosphatase inhibition was used to induce cell death (Murray et al., 1998; Runden et al., 1998).

What accounts for the seemingly contradictory effects of MEK1/2 inhibition on neuronal cell survival following acute injury? Differences in outcome resulting from MEK1/2 inhibition may depend not only upon the nature and severity of injury, but also upon drug dosing regimens or the cell type expressing activated ERK1/2. Until cell type-specific inhibition of ERK1/2 activation can be attained, the mechanism responsible for the neuroprotective in vivo effects of MEK1/2 inhibition will remain unresolved (Chu et al., 2004).

Although further work is needed to understand the precise mechanism of neuroprotection provided by resveratrol, the present study adds one evidence that the PI3-k cascade could play a role in resveratrol-induced neuroprotective effect against 'ischemic-like injury'. These findings might be important for understanding beneficial effects of red wine intake to cerebral ischemia and the mechanism of neuroprotection afforded by this beverage.

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Legend to Figures

Figure 1: Experimental design, indicating time-line of culture, drug exposure, OGD and subsequent measurements of cell survival and levels of Akt, GSK-3β and ERK1/2.

Figure 2: Effect of Resveratrol on cell damage induced by OGD for 60 min in organotypic hippocampal cultures. (A): Representative photomicrographs of slices stained with PI after 24 h after exposure to OGD. (B): Quantitative analysis of hippocampus damage 24 h after exposure to OGD. RSV: Resveratrol (10, 25 and 50μM) was added at the moment of lesion induction and maintained during the recovery period. Bars represent the mean ± S.E.M., n=12. * significantly different from control cultures. # significantly different from control and DMSO-treated OGD cultures (one-way ANOVA followed by Student Newman Keuls, $P<0.05$).

Figure 3: Effect of LY294009 and PD98059 on neuroprotective effect of resveratrol on cell damage induced by OGD for 60 min in organotypic hippocampal cultures. A: Representative photomicrographs of cultures showing PI fluorescence 24 h after exposure to OGD. B: Quantitative analysis of hippocampus damage 24 h after exposure to OGD. RSV: Resveratrol (50µM) was added at the moment of OGD induction and maintained in the recovery period. LY: LY294009 (5µM) was added 2 h before OGD, during OGD and maintained in the recovery period. RSV+LY: Resveratrol (50µM) and LY294009 (5µM) were added simultaneously to OGD medium and maintained during the recovery period, LY294009 was also added 2 h before OGD. PD: PD98059 (20µM) was added 2 h before OGD, during OGD medium and maintained during the recovery period. RSV + PD: Resveratrol (50µM) and PD98059 (20µM) were added simultaneously to OGD medium and maintained during the recovery period, PD98059 was also added 2 h before OGD. Bars represent the mean ± S.E.M., n=6. * significantly different from control cultures (indicated by traced line). # significantly different from control cultures and OGD cultures treated with DMSO, LY, RSV+LY, and PD (one-way ANOVA followed by Student Newman Keuls, $P<0.05$).

Figure 4. Effect of treatment with resveratrol on the percentage of phophorylated Akt in organotypic hippocampal cultures. A: Representative Western blottings of pAkt and Akt 1, 6 and 24 h after OGD revealed using specifics antibodies. B: Histograms represent the quantitative Western blotting analysis of Akt phosphorylation state. The densitometric values obtained to phospho- and total-Akt from all treatments were first normalized to their respective vehicle-treated control non-exposed to OGD condition (DMSO bar) (100%). RSV: Resveratrol (50μM) was added at the moment of OGD induction and maintained in the recovery periods of 1, 6 and 24 h. Data are expressed as a ratio of the normalized percentages of pAkt and Akt. Bars represent the mean ± S.E.M., n=6.. * significantly different from controls cultures and OGD cultures treated with DMSO (one-way ANOVA followed by Student Newman Keuls test, $P<0.05$).

Figure 5. Effect of treatment with resveratrol and LY294002 on the percentage of phophorylated Akt in organotypic hippocampal cultures 24 h after OGD. A: Representative Western blottings of pAkt and Akt revealed using specifics antibodies. B: Histograms represent the quantitative Western blotting analysis of Akt phosphorylation state. The densitometric values obtained to phospho- and total-Akt from all treatments were first normalized to their respective vehicle-treated control non-exposed to OGD condition (DMSO bar) (100%). RSV: Resveratrol (50μM) was added at the moment of OGD induction and maintained in the recovery period. LY: LY294009 (5μM) was added to 2 h before OGD, during OGD and maintained in the recovery period. RSV+LY: Resveratrol (50μM) and LY294009 (5μM) were added simultaneously to OGD medium and maintained during the recovery period, LY294009 was also added 2 h before OGD. Data are expressed as a ratio of the normalized percentages of pAkt and Akt. Bars represent the mean ± S.E.M., n=6. # significantly different from controls cultures and OGD cultures treated with DMSO, LY and RSV+LY. * significantly different from controls cultures and OGD cultures treated with DMSO, RSV and RSV+LY (one-way ANOVA followed by Student Newman Keuls test, $P<0.05$).

Figure 6. Effect of treatment with resveratrol on the percentage of phophorylated GSK3-β in organotypic hippocampal cultures. A: Representative Western blottings of pGSK3-β and GSK3-β 1, 6 and 24 h after OGD revealed using specifics antibodies. B: Histograms represent the quantitative Western blotting analysis of GSK3-β phosphorylation state. The densitometric values obtained to phospho- and total- GSK3-β from all treatments were first normalized to their respective vehicle-treated control non-exposed to OGD condition (DMSO bar) (100%). RSV: Resveratrol (50μM) was added at the moment of OGD induction and maintained in the recovery periods of 1, 6 and 24 h. Data are expressed as a ratio of the normalized percentages of pGSK3-β and GSK3-β. Bars represent the mean ± S.E.M., n=6.. * significantly different from controls cultures and OGD cultures treated with DMSO (one-way ANOVA followed by Student Newman Keuls test, $P<0.05$).

Figure 7. Effect of treatment with resveratrol and LY294002 on the percentage of phophorylated GSK-3β in organotypic hippocampal cultures 24 h after OGD. A: Representative Western blottings of pGSK-3β and GSK-3β revealed using specifics antibodies. B: Histograms represent the quantitative Western blotting analysis of GSK-3β phosphorylation state. The densitometric values obtained to phospho- and total-GSK-3β from all treatments were first normalized to their respective vehicle-treated control non-exposed to OGD condition (DMSO bar) (100%). RSV: Resveratrol (50μM) was added at the moment of OGD induction and maintained in the recovery period. LY: LY294009 (5μM) was added to 2 h before OGD, during OGD and maintained in the recovery period. RSV+LY: Resveratrol (50μM) and LY294009 (5μM) were added simultaneously to OGD medium and maintained during the recovery period, LY294009 was also added 2 h before OGD. Data are expressed as a ratio of the normalized percentages of pGSK-3β and GSK-3β. Bars represent the mean ± S.E.M., n=6. * significantly different from controls cultures and OGD cultures treated with DMSO, LY and RSV+LY. (one-way ANOVA followed by Student Newman Keuls test, $P<0.05$).

Figure 8. Effect of treatment with resveratrol on the percentage of phophorylated ERK1/2 in organotypic hippocampal cultures. A: Representative Western blottings of pERK1/2 and ERK1/2 1, 6 and 24 h after OGD revealed using specifics antibodies. B: Histograms represent the quantitative Western blotting analysis of ERK1/2 phosphorylation state. The densitometric values obtained to phospho- and total- ERK1/2 from all treatments were first normalized to their respective vehicle-treated control non-exposed to OGD condition (DMSO bar) (100%). RSV: Resveratrol (50μM) was added at the moment of OGD induction and maintained in the recovery periods of 1, 6 and 24 h. Data are expressed as a ratio of the normalized percentages of pGSK3-β and GSK3-β. Bars represent the mean ± S.E.M., n=6.. * significantly different from controls cultures, OGD cultures treated with DMSO and OGD RSV after 1 h of recovery (one-way ANOVA followed by Student Newman Keuls test, $P<0.05$).

Figure 9. Effect of treatment with resveratrol and PD98059 on the percentage of phophorylated ERK1/2 in organotypic hippocampal cultures 24 h after OGD. A: Representative Western blottings of pERK1/2 and ERK1/2 revealed using specifics antibodies. B: Histograms represent the quantitative Western blotting analysis of ERK1/2 phosphorylation state. The densitometric values obtained to phospho- and total-ERK1/2 from all treatments were first normalized to their respective vehicle-treated control non-exposed to OGD condition (DMSO bar) (100%). RSV: Resveratrol (50μM) was added at the moment of OGD induction and maintained in the recovery period. PD: PD98059 (20μM) was added to 2 h before OGD, during OGD and maintained in the recovery period. RSV+PD: Resveratrol (50μM) and PD98059 (20μM) were added simultaneously to OGD medium and maintained during the recovery period, PD98059 was also added 2 h before OGD. Data are expressed as a ratio of the normalized percentages of pERK1/2 and ERK1/2. Bars represent the mean ± S.E.M., n=6. * significantly different from controls cultures and OGD cultures treated with DMSO, PD and RSV+PD (one-way ANOVA followed by Student Newman Keuls test, $P<0.05$).

Figure 1

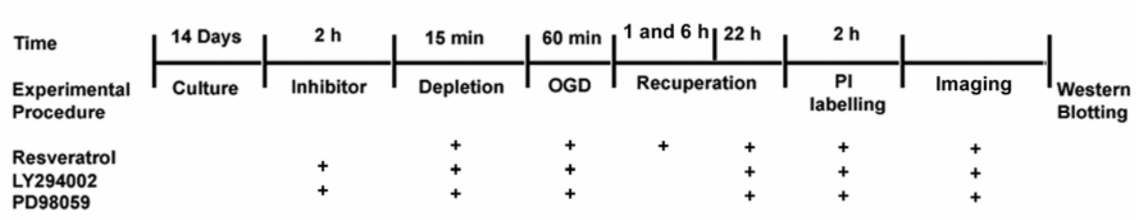


Figure 2

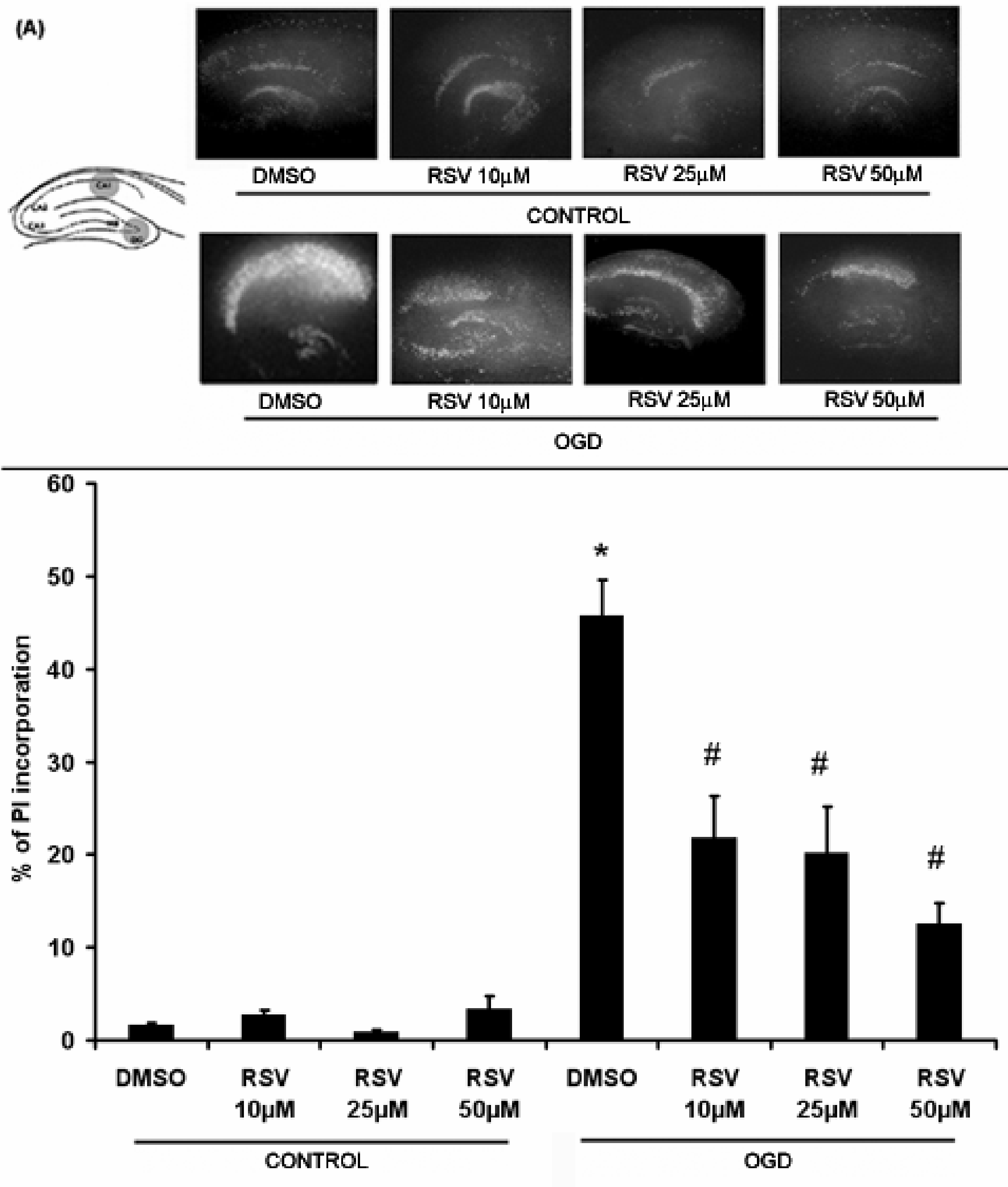


Figure 3

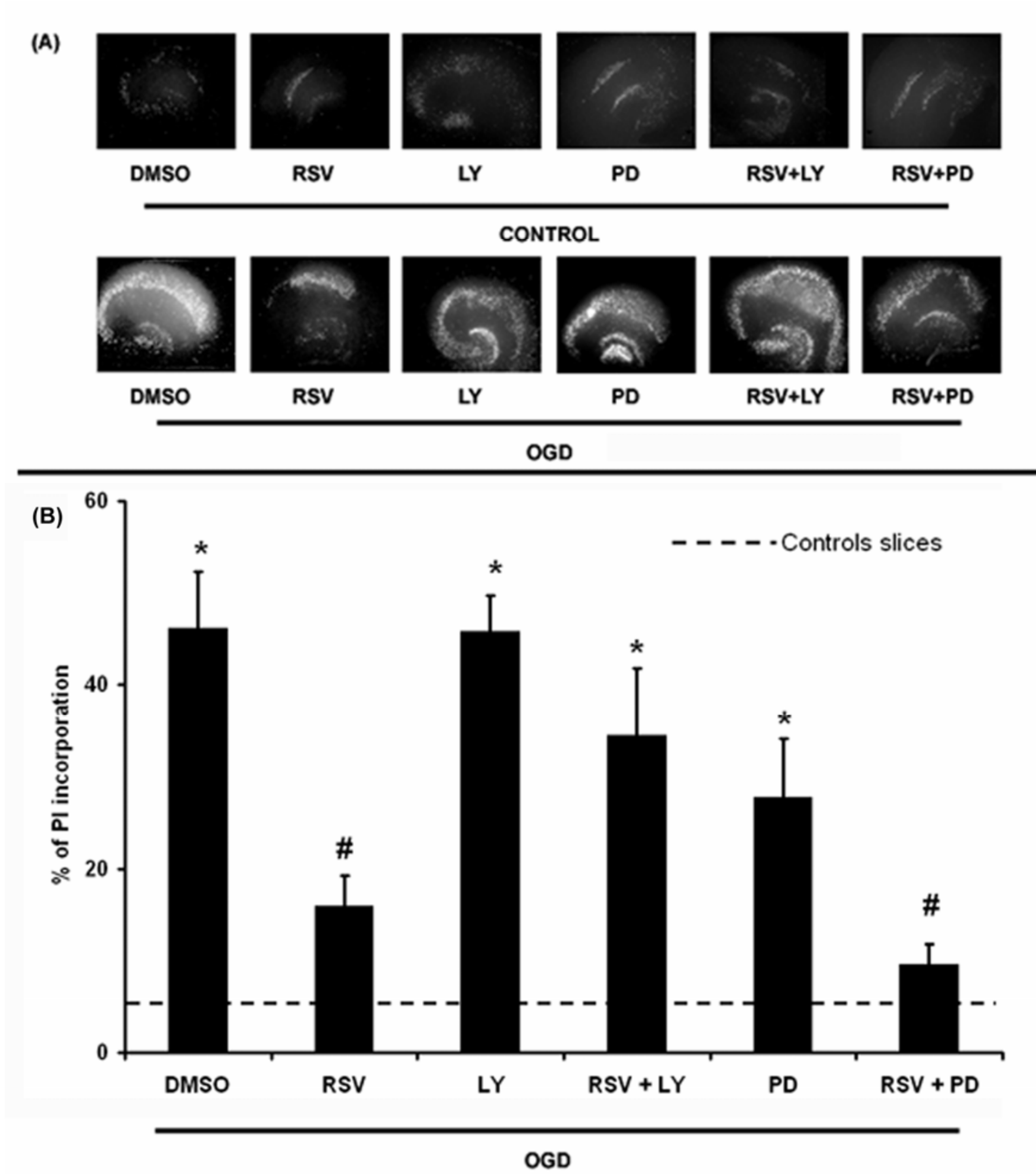


Figure 4

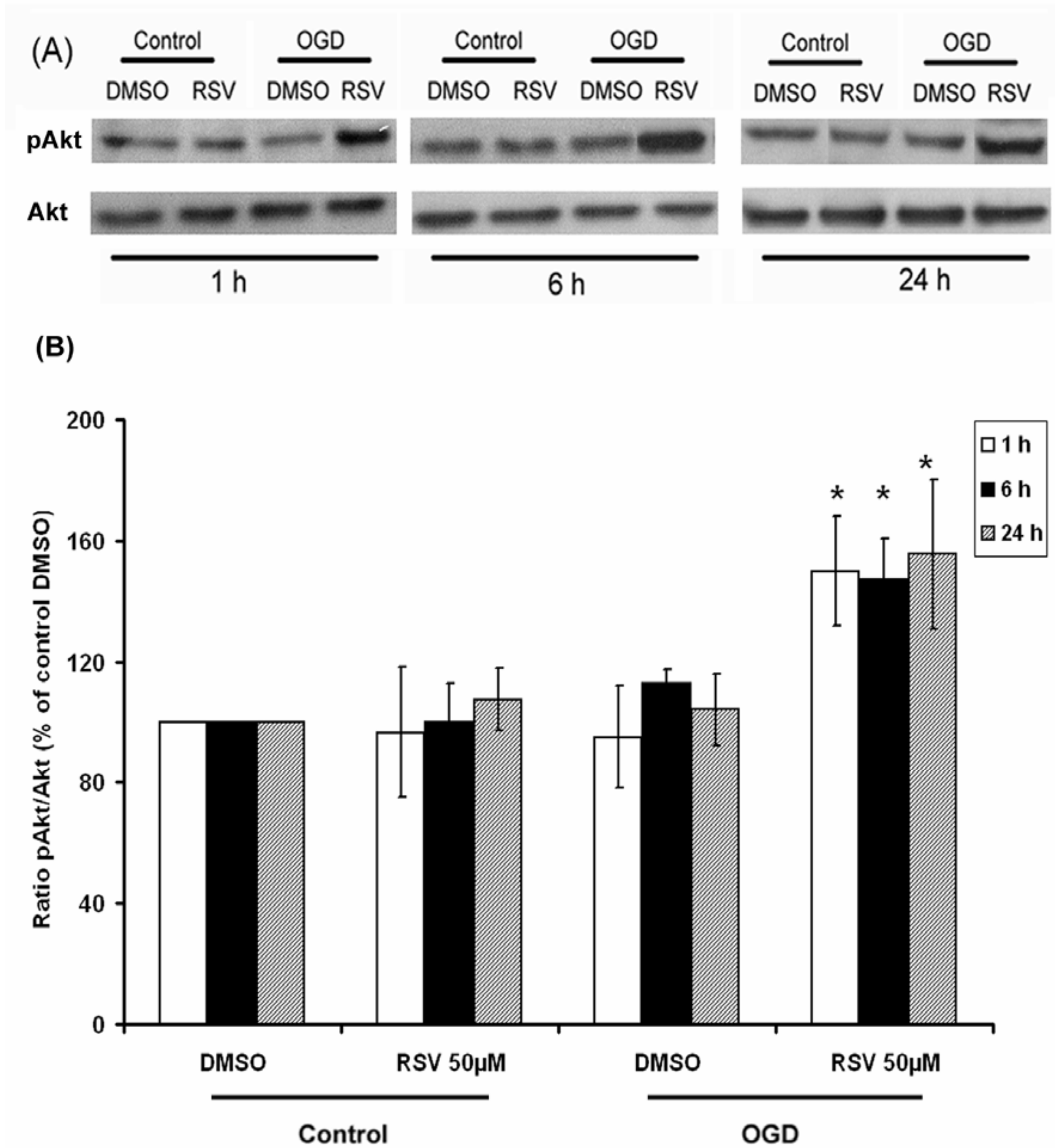


Figure 5

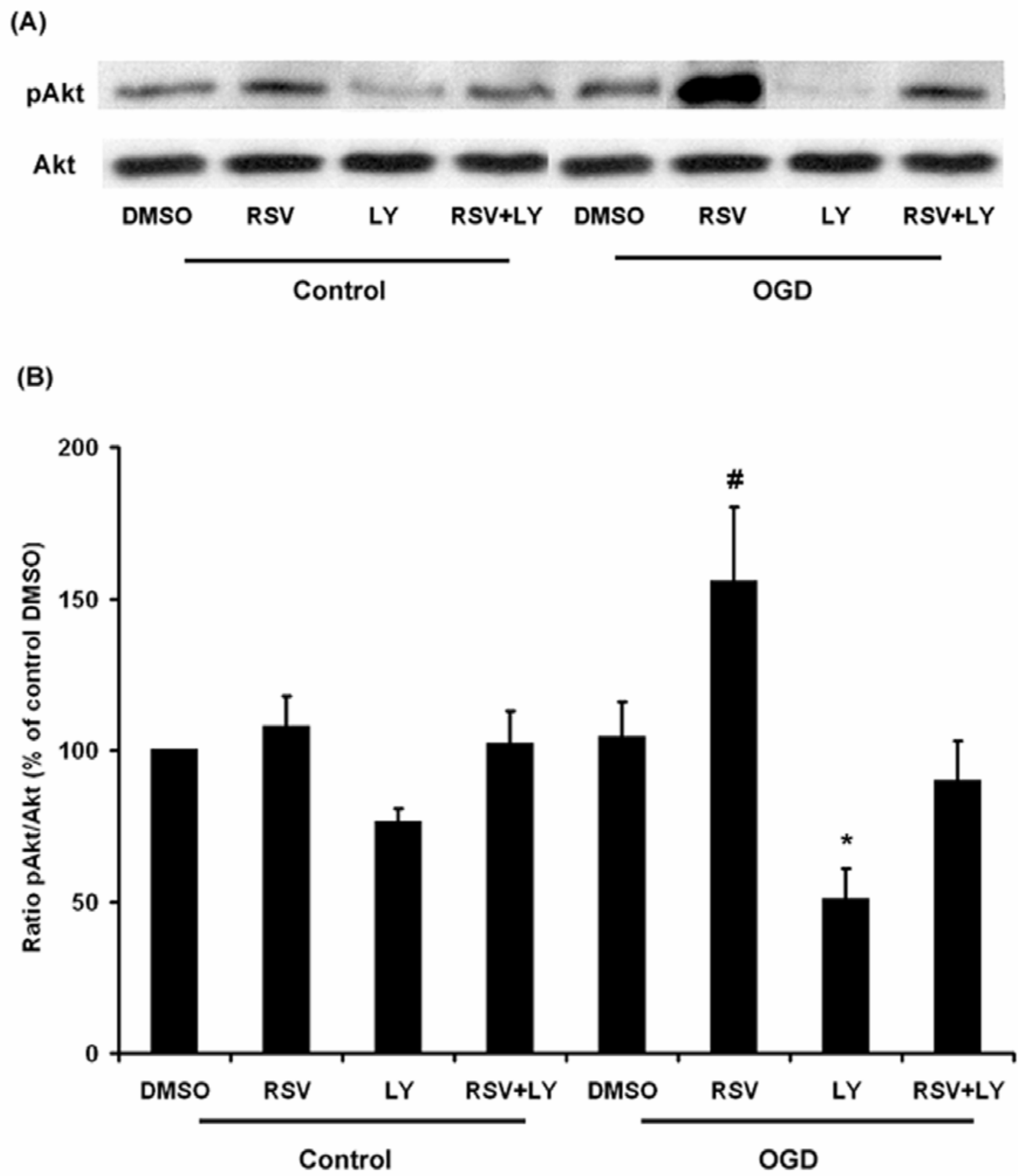


Figure 6

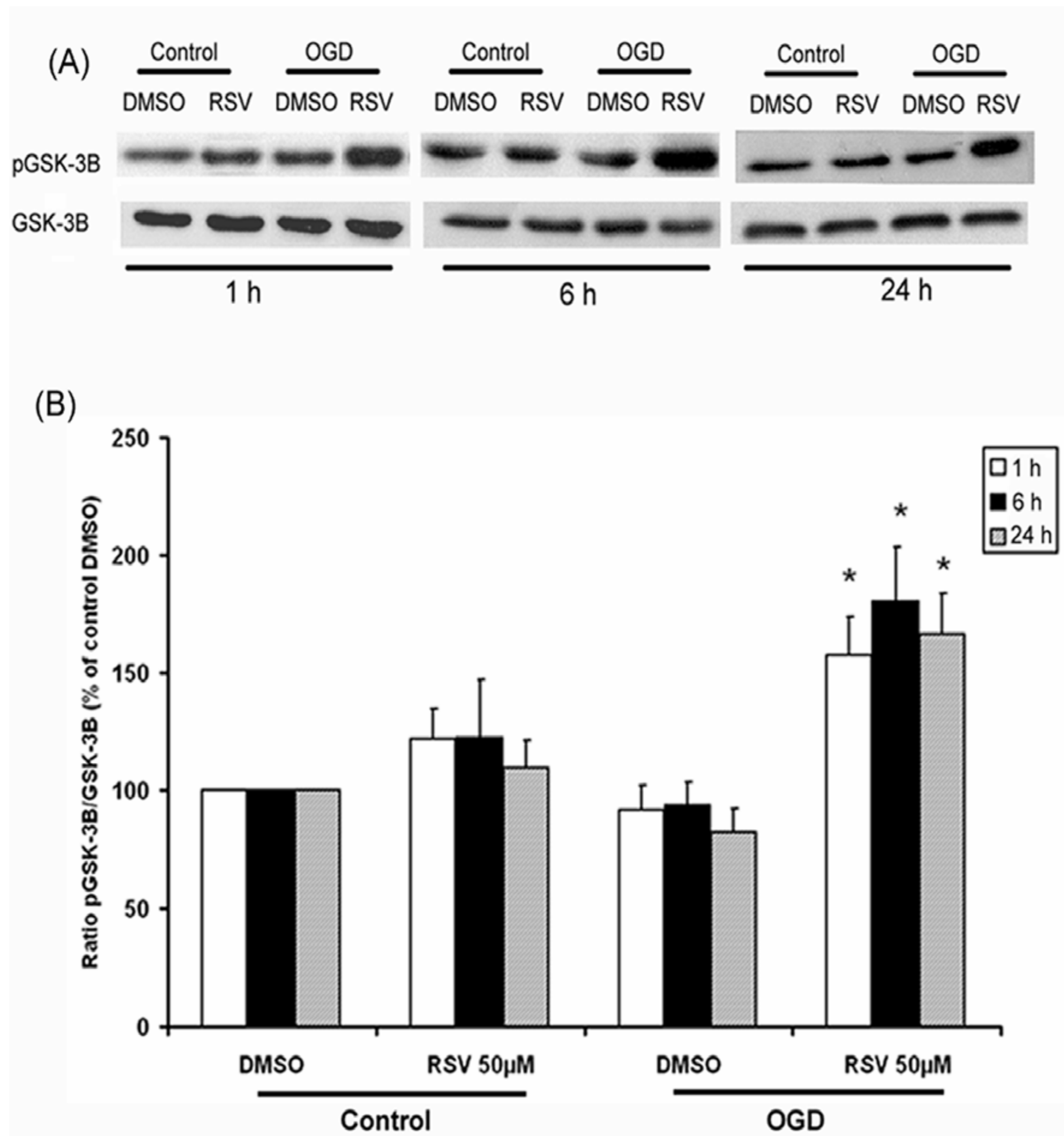


Figure 7

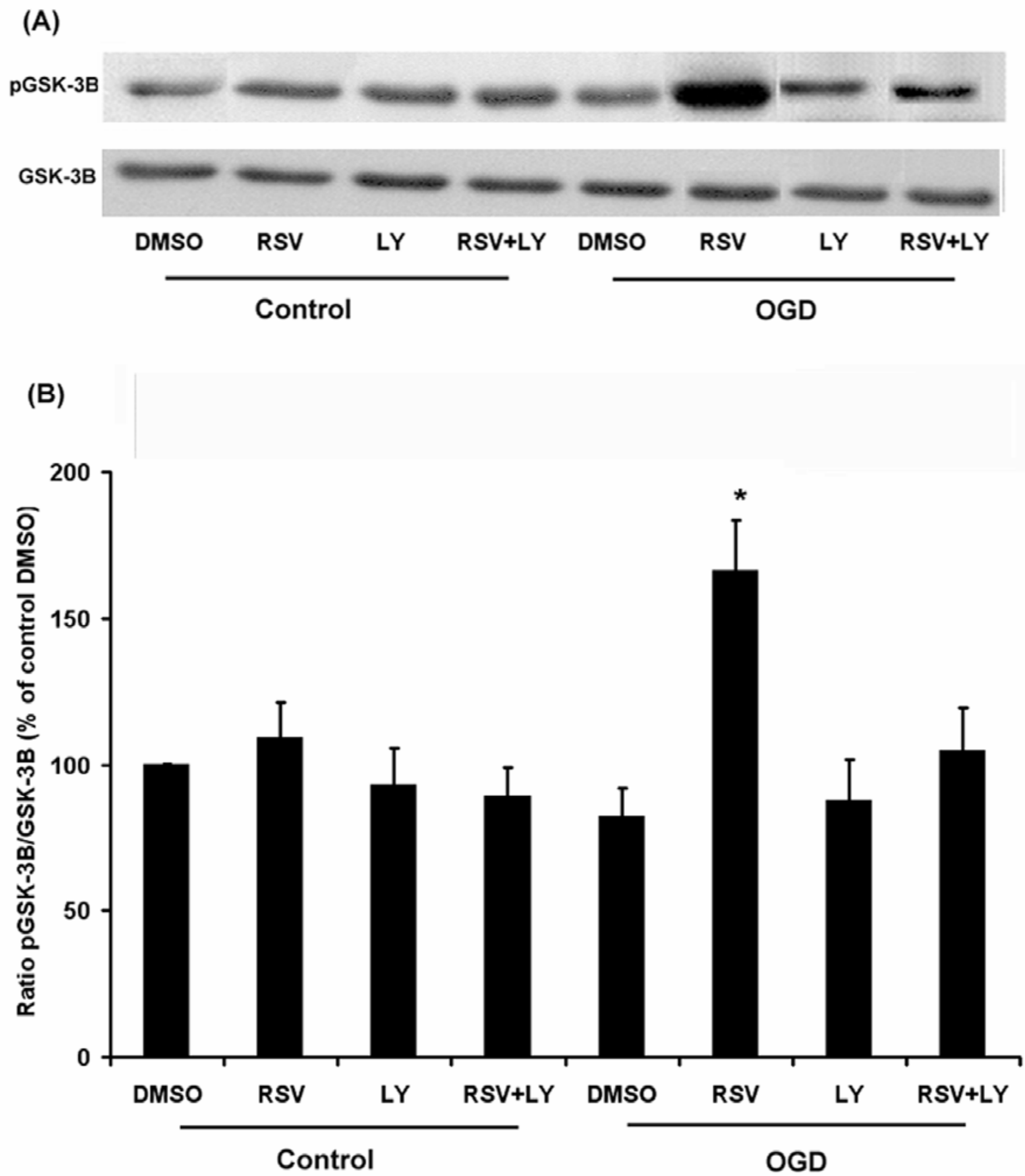


Figure 8

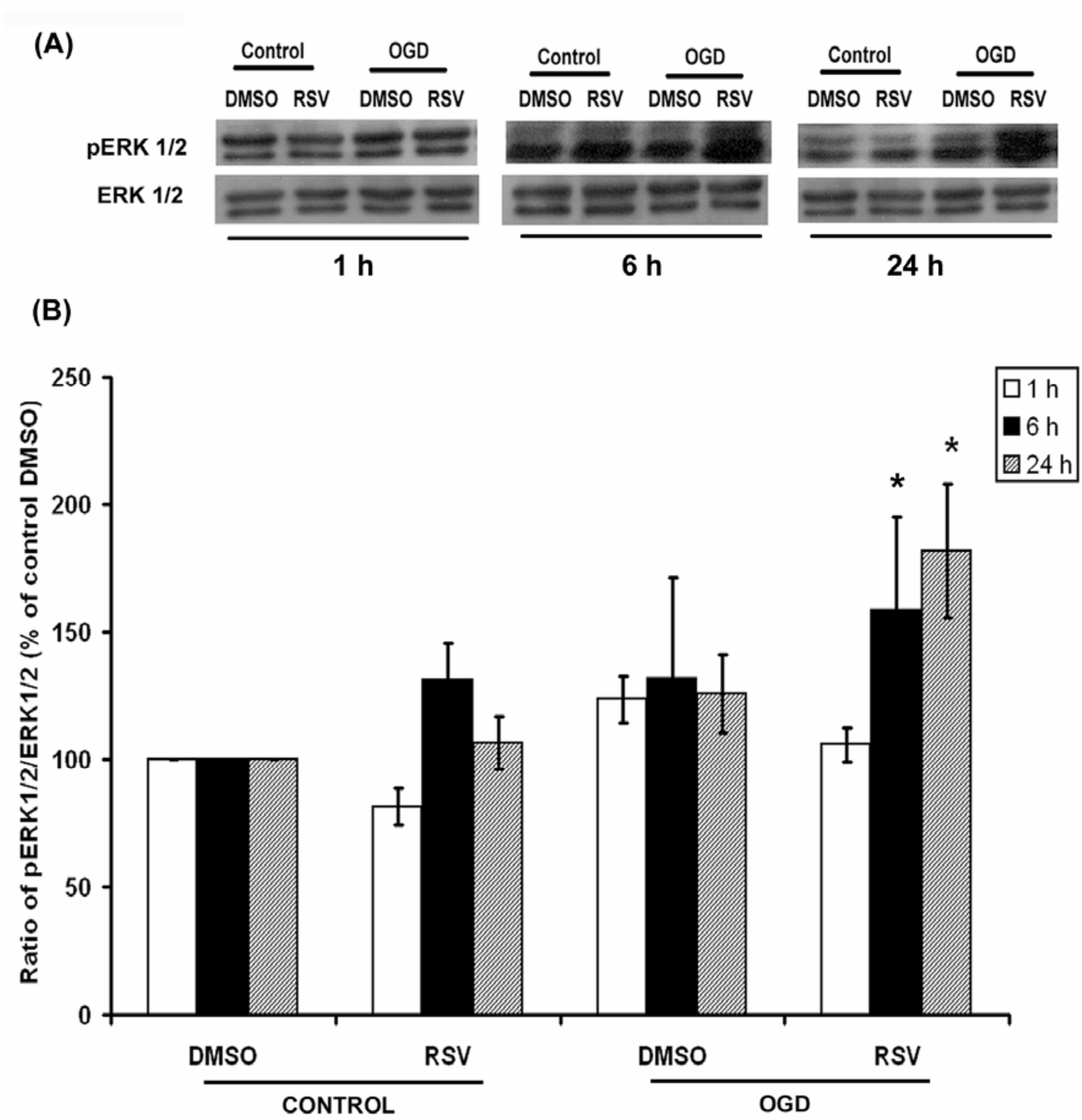
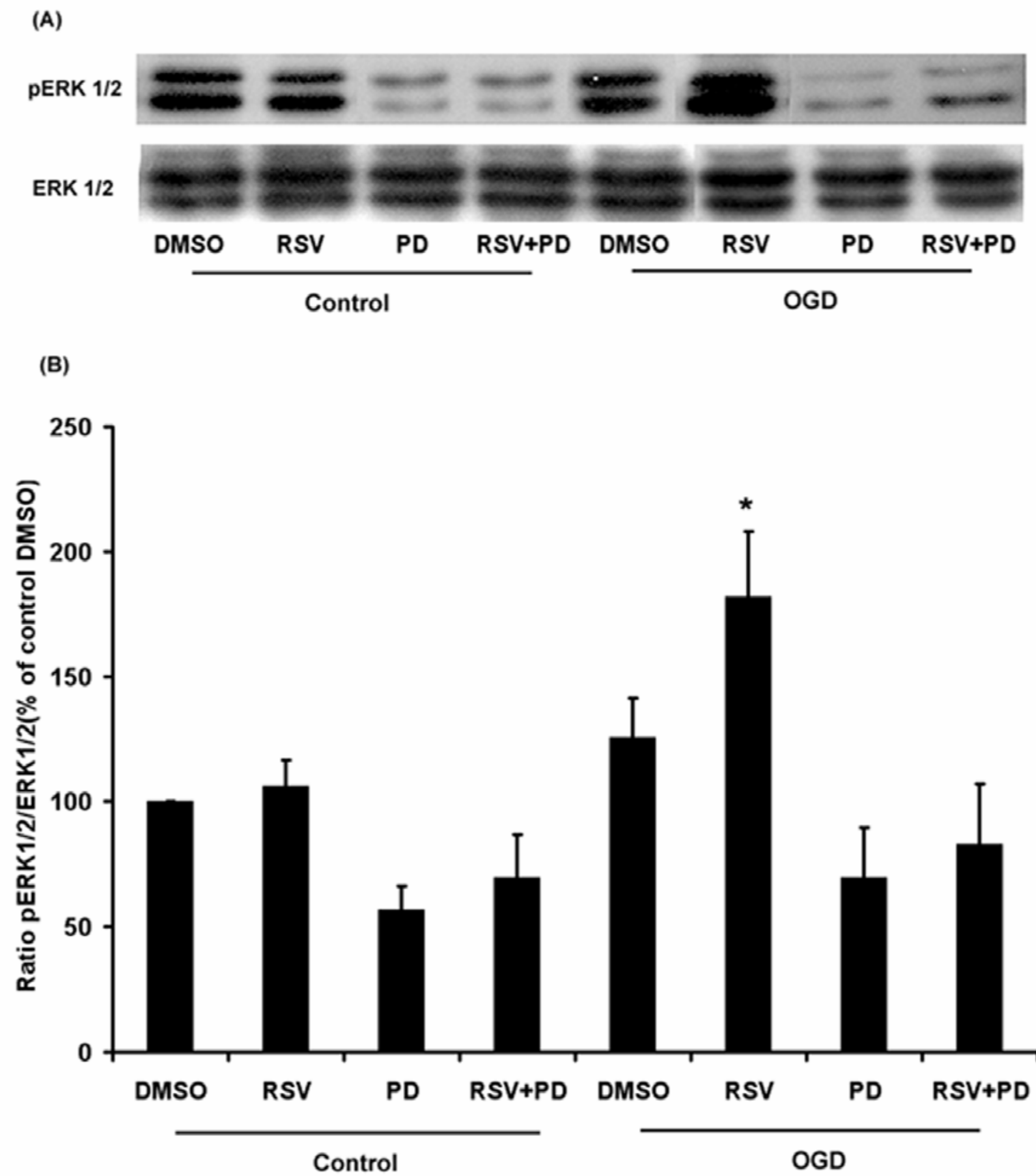


Figure 9



4. DISCUSSÃO

Este trabalho teve por objetivo estudar o efeito neuroprotetor do resveratrol em um modelo *in vitro* de isquemia cerebral e investigar prováveis mecanismos de ação. Para isso, foram utilizadas culturas organotípicas de hipocampo de rato submetidas à POG, um modelo amplamente utilizado pelo nosso grupo de pesquisa para investigar os mecanismos de morte celular, neuroproteção e sinalização celular (Tavares et al., 2001; Valentin et al., 2003; Horn et al., 2005; Cimarosti et al., 2005). Usando este modelo, foi encontrado um grande efeito neuroprotetor do resveratrol nas doses 10, 25 e 50µM.

Dados da literatura demonstraram o efeito neuroprotetor do resveratrol em outros modelos *in vivo* e *in vitro* de lesão cerebral. Wang et al. (2004) demonstraram que o resveratrol protege as células hipocampais da morte excitotóxica mediada pelo ácido caínico em ratos. O resveratrol também atenuou a citotoxicidade do pepetídeo β-amilóide em culturas de células PC12 (Jang e Surh, 2003), protegeu vários tecidos, como coração (Das et al, 2005; Fantinelli et al., 2005), medula espinhal (Kiziltepe et al., 2004) e cérebro (Virgili et al., 2000; Wang et al., 2002; Inoue et al., 2003, Hwang et al., 2004) da morte causada pela isquemia/reperfusão.

Contudo, o mecanismo pelo qual o resveratrol exerce seu efeito neuroprotetor ainda não está bem esclarecido. Alguns destes trabalhos sugerem o forte potencial antioxidante do resveratrol como responsável pelo seu caráter neuroprotetor (Inoue et al., 2003; Jang e Surh, 2003). Neste trabalho, foi proposto que o resveratrol protege as culturas organotípicas da morte induzida pela POG através da ativação de duas vias envolvidas com a sobrevivência neuronal: PI-3k e MAPK (Pettmann e Hendersen, 1998). Alguns estudos indicaram que a apoptose é um dos processos de morte ativados pela isquemia/reperfusão (MacManus et al., 1993; Nitatori et al., 1995). Assim, vias de

sinalização, como as mencionadas acima, que possuem efeito anti-apoptótico, podem ter um grande potencial preventivo a lesões isquêmicas.

No presente estudo, primeiramente foi verificado o efeito do resveratrol sobre a via da PI-3k através do uso do inibidor LY294002, bem como o efeito deste estilbeno sobre as proteínas ativadas por esta via, através da análise da fosforilação/ativação da Akt e fosforilação inativação da GSK-3β. Na seqüência, foi estudado o efeito do resveratrol sobre a via da MAPK através do uso do inibidor PD98055 e também a ativação/fosforilação da proteína ERK1/2 que pertence a esta via.

Os resultados apresentados mostraram que o efeito neuroprotetor do resveratrol foi bloqueado pelo uso do LY294002, mas não pelo uso do PD98055, indicando que a PI-3k é a principal via de sinalização ativada pelo resveratrol após um evento isquêmico. Sendo assim, foi investigado o efeito do resveratrol sobre a Akt, principal proteína efetora da via (Franke et al., 1997). Os resultados mostraram que o resveratrol foi capaz de aumentar a fosforilação desta proteína, e consequentemente a ativação, apenas após a POG, nos diferentes tempos testados, não tendo este efeito sobre as fatias controles.

Recentemente, Das et al., (2005) demonstraram que o resveratrol medeia seus efeitos cardioprotetores através do aumento da fosforilação da Akt e que este efeito foi parcialmente abolido pelo uso do LY294002, resultados estes que vão ao encontro dos mostrados neste trabalho.

Vários estudos mostram a ativação da proteína Akt está presente após um evento isquêmico, resultando assim na prevenção da morte neuronal (Namura et al., 2000; Friguls et al., 2001; Janelidze et al., 2001; Kawano et al., 2002). Porém, estudo prévio do nosso grupo de pesquisa não encontrou alteração na fosforilação e no imunoconteúdo da Akt na região Corno de Amonis 1 (CA1 - *Cornus Ammonis* 1) e Giro Denteado (DG - *Dentate Gyrus*) do hipocampo das culturas organotípicas 30 min, 6 e

24 h após a POG (Horn et al., 2005). No presnete trabalho também não foi encontrado aumento da fosforilação da Akt 1, 6 e 24 h após a POG nas fatias tratadas apenas com o veículo. Uma possível explicação para estes resultados aparentemente controversos é que os trabalhos da literatura citados acima utilizaram modelos *in vivo* de isquemia cerebral, enquanto os nossos trabalhos utilizaram modelos *in vitro*.

Seguindo a via PI-3k/Akt, investigou-se o efeito do resveratrol sobre a proteína pró-apoptótica GSK-3β. Esta proteína está envolvida em várias patologias do SNC, porém existem poucos trabalhos demonstrando a sua relação com os danos causados pela isquemia cerebral. Estudos usando inibidores não específicos da GSK-3β , tais como o lítio e fator de crescimento tipo insulina 1 (IGF-1 - *Insulin-like growth factor* 1), protegeram da morte celular induzida pela isquemia cerebral (Manji et al., 1999; Nonaka e Chuang, 1998; Wang et al., 2000).

Este trabalho foi o primeiro a demonstrar que o resveratrol aumenta a fosforilação/inativação da GSK-3β após a POG, sugerindo que este pode ser um mecanismo pelo qual o resveratrol protege da morte celular. O mesmo perfil de fosforilação encontrado para a Akt foi encontrado para a GSK-3β, onde a fosforilação aumentou apenas após a isquemia, nos três tempos testados, sem ter efeito sobre as culturas controles ou sobre as culturas submetidas à POG tratadas apenas com o veículo.

Este conjunto de resultados, onde o resveratrol aumentou a fosforilação da Akt e da GSK-3β após a POG, e o efeito da inibição da via PI-3k pelo LY294002 abolindo a neuroproteção mediada pelo resveratrol, sugerem que este pode ser o mecanismo pelo qual o resveratrol protege as culturas organotípicas de hipocampo de rato da morte celular induzida pela POG (Figura 4).

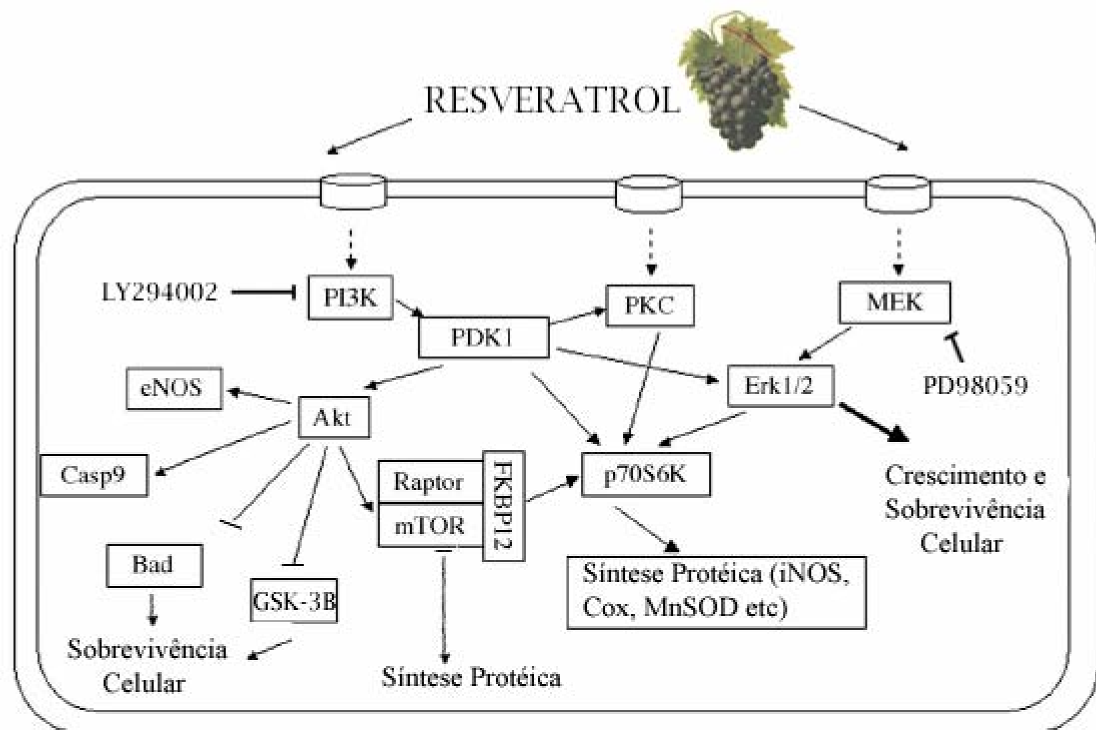


Figura 4 – Resumo esquemático do efeito do resveratrol sobre as vias de sinalização. Flechas (→) indicam ativação da proteína alvo, enquanto setas (⊥) indicam inibição da proteína alvo (*Adaptado de Kis et al., 2003*).

A via da MAPK, assim como a via da PI-3k, possui efeitos anti-apoptóticos e parece estar envolvida com sobrevivência neuronal após um evento isquêmico (Kawano et al., 2001). Além disso, alguns trabalhos demonstraram que o resveratrol pode ativar a ERK1/2 (Miloso et al., 1999; Klinge et al., 2005). Por isso, foi verificado o efeito do resveratrol também sobre esta via. Embora o efeito neuroprotetor do resveratrol não tenha sido abolido pela administração do PD98059, este aumentou a fosforilação da proteína ERK1/2 6 e 24 h após a POG. È curioso notar que mais uma vez o resveratrol foi capaz de aumentar a fosforilação da proteína em estudo apenas após a POG. Este fato pode indicar que o resveratrol não esteja agindo como um ativador direto destas vias, mas como um facilitador da ativação destas, frente a algum estímulo citotóxico.

Embora mais pesquisa seja necessária para entender o preciso mecanismo de neuroproteção mediado pelo resveratrol, este trabalho adiciona mais uma evidência de que a via PI-3k pode ser uma peça chave no efeito neuroprotetor induzido pelo resveratrol. Esses resultados são importantes para entender os efeitos benéficos do consumo de vinho tinto para o SNC e doenças neurodegenerativas como a isquemia cerebral e os mecanismos neuroprotetores proporcionados por esta bebida.

Porém, quanto de resveratrol pode ser absorvido após o consumo de vinho? Sendo o conteúdo de resveratrol no vinho de aproximadamente 5mg/L e assumindo um consumo “moderado” de vinho (250 mL em uma pessoa de 70 kg), o consumo de resveratrol seria de 18µg/kg/dia. Em um recente estudo com indivíduos voluntários sadios, o resveratrol foi administrado em uma dose de 360µg/Kg dissolvido em suco de uva, suco de vegetais ou vinho branco, uma dose 20 vezes maior do que a associada ao consumo “normal” de vinho. Os autores encontraram um pico de 2µM de resveratrol no plasma destes indivíduos 30 minutos após a ingestão, independente da fonte de consumo (Goldberg et al., 2003).

Usando protocolo de *high performance liquid chromatography* (HPLC), Wang et al. (2002) determinaram as concentrações de resveratrol no soro, fígado e cérebro de gerbils em diferentes tempos após a injeção intra peritoneal de resveratrol (30 mg/kg). Eles encontraram que os níveis mais altos de resveratrol no soro deram-se após 1 h de administração, já no cérebro e no fígado após 4 h, mostrando também que o resveratrol possui a capacidade de ultrapassar a barreira hematoencefálica.

Todavia, como qualquer bebida alcoólica, o vinho também causa problemas, quando ingerido além dos limites. Em 100 mL (metade de um copo) existem cerca de 8 a 10 g de etanol. Portanto, quem toma quantidades excessivas de vinho (mais de meio

litro), independente de sua qualidade, está sujeito aos desagradáveis efeitos de intoxicação etanólica no dia seguinte.

Está bem estabelecido que o álcool, em doses mais elevadas, em vez de funcionar como um estimulante, atua como um depressor do SNC, deixando o indivíduo sonolento, letárgico e sem nenhuma disposição para qualquer tipo de atividade, além do risco de se tornar dependente (Tomera, 1999). Nenhum tipo de bebida alcoólica, incluindo o vinho, é indicada para quem sofre de transtornos no aparelho digestivo, úlceras, por exemplo, nem para os que apresentam distúrbios no pâncreas ou no fígado. Aos hipertensos, o consumo de bebida alcoólica também é contra-indicado, porque doses exageradas provocam alterações na pressão arterial.

Não existe uma regra fixa para se dizer qual é o limite de uma pessoa em relação ao álcool. Isso vai depender de alguns fatores, como idade, sexo e estado emocional, além do fato de que cada um tem o seu próprio nível de tolerância ao álcool (German e Walzen, 2000). Portanto, a ordem é moderar. Mas o que é beber moderadamente? O ideal é brindar à sua saúde e ao seu prazer com apenas dois copos de vinho por dia, um no almoço, outro no jantar. Em resumo: o néctar dos deuses é delicioso e faz bem. Quando acompanhado de bom senso, fica melhor ainda (Penna e Hecktheuer, 2004).

Uma fonte de resveratrol alternativa ao consumo de vinho é o suco de uva, que possui doses menores deste estilbeno, mas o fato de não possuir etanol permite que este seja consumido por todos, desde crianças até idosos que ingerem medicamentos incompatíveis os uso de álcool.

Além disso, considerando o exposto acima, os resultados obtidos neste trabalho podem guiar novas linhas de pesquisa para, baseados na estrutura molecular do resveratrol, desenvolver novas drogas que possam minimizar os danos celulares causados pela isquemia cerebral e outras doenças neurodegenerativas.

5. CONCLUSÕES

Os resultados obtidos neste trabalho permitem concluir que:

- O resveratrol protege da morte celular culturas organotípicas de hipocampo de rato submetidas à POG.
- Este efeito neuroprotetor foi abolido pelo uso LY294002, um inibidor da via PI-3k.
- Este efeito não foi prevenido com o uso do PD98059, um inibidor da via das MAPK.
- O resveratrol aumentou a fosforilação/ativação da Akt/PKB nos tempos de 1, 6 e 24 h após a POG, não tendo efeito sobre as culturas controles nem sobre as culturas POG tratadas com o veículo.
- O resveratrol aumentou a fosforilação/inibição da GSK-3β nos tempos de 1, 6 e 24 h após a POG, não tendo efeito sobre as culturas controles nem sobre as culturas POG tratadas com o veículo.
- O resveratrol aumentou a fosforilação/ativação da ERK1/2 6 e 24 h após a POG, não atuando sobre os demais grupos experimentais.

Esses dados nos permitem concluir que o resveratrol tem um forte efeito neuroprotetor contra morte celular induzida pela exposição do hipocampo à POG, e este efeito parece envolver a ativação da via PI-3k/Akt e inibição da GSK-3β.

6. PERSPECTIVAS

- Avaliar o efeito do resveratrol sobre o sistema glutamatérgico avaliando o efeito daquele sobre os transportadores de glutamato bem como estudar a funcionalidade destes através da medida da captação de glutamato em culturas organotípicas de hipocampo de ratos submetidas à POG.

- Avaliar o efeito do resveratrol sobre o sistema purinérgico, usando inibidores de receptores de adenosina, bem como avaliar o efeito daquele sobre a via mediada pela mTOR.

- Estudar o efeito neuroprotetor do resveratrol, do vinho tinto e do suco de uva em um modelo in vivo de isquemia cerebral e investigar os possíveis mecanismos de ação através da análise das vias de sinalização estudadas *in vitro*.

- Verificar o efeito neuroprotetor do resveratrol em culturas organotípicas que receberam o implante de células tumorais e avaliar o efeito antitumoral deste composto em linhagens isoladas de gliomas.

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8. ANEXO

8.1 Normas da Revista à qual o Artigo foi submetido retiradas do site:

http://authors.elsevier.com/GuideForAuthors.html

Guide for Authors

I. GENERAL
II. SUBMISSION OF MANUSCRIPTS **SUBMISSION OF MANUSCRIPTS**
III. PREPARATION OF MANUSCRIPTS **PREPARATION OF MANUSCRIPTS**

I. GENERAL

1. *Neuroscience* publishes original research on any aspect of the scientific study of the nervous system. **Papers most suitable for publication are those that report new observations that directly contribute to our understanding of how the nervous system works.** .

2. *Section Editors*. With the rapid growth of neuroscience into diverse areas ranging from molecular biology to cognitive science, the accurate and fair assessments of papers for publication require the Chief and Associate Editors to seek advice from Section Editors representing all major areas of research. Section Editors suggest appropriate reviewers and also recommend an editorial decision based on the reviews. Section Editors have been appointed in the areas of Behavioral Neuroscience, Cellular, Clinical, Cognitive, Developmental, Molecular, Neuroanatomy, Neuropharmacology, Neuroscience Methods, Systems Neuroscience and Sensory Systems. A special section editor has also been assigned to commission Reviews and Special Issues and another Section Editor is a special statistical consultant.

3. *The Editorial Board*. The international Editorial Board, which is appointed by the Publications Committee of IBRO, consists of specialists in all areas of neuroscience. Each paper is typically evaluated by at least two Editors or ad hoc reviewers. Papers are accepted by the Chief and Associate Editors in consultation with the appropriate Section Editor. **The review and decision process is identical regardless of whether the Chief or Associate Editor oversees the review process.** All manuscripts are subject to any modifications required by the Editorial Assistants to conform to Journal policy.

4. *Types of papers*

(a) **Research papers.** These are full-length papers describing original research. There are no specific page limits although authors are encouraged to be as concise as possible and to use as few, high quality illustrations as necessary to adequately document their findings.

(b) **Rapid Reports**. These are short communications that describe outstanding new discoveries. They will be reviewed by the appropriate Section Editor and either the Chief or Associate Editor and an initial decision will be made as to whether the paper warrants processing as a Rapid Report. The Editors will aim for a two-week time to

decision once a paper is accepted as a Rapid Report. This decision will be based on whether the paper reports a particularly important series of findings that are likely to have high impact on the field. Papers of the type that are typically published in Science or Nature are sought for this category. Papers that are not deemed acceptable for review under this category will be returned to the authors. All accepted Rapid Reports will be first published on the Neuroscience website http://www.neuroscience-ibro.com within 22 working days of acceptance, as a fully-citable article in ScienceDirect between 24-34 working days, and in the print version between 38-48 working days.

(c) **Reviews** (previously know as Commentaries). These are short articles (3000-10,000 words in length), not exhaustive reviews, that are intended to either draw attention to developments in a specific area of research, to bring together observations that seem to point the field in a new direction, to give the author's personal views on a controversial topic, or to direct soundly based criticism at some widely held dogma or widely used technique in neuroscience. Reviews may also provide an historical perspective on an area of neuroscience research. Authors should make their Review understandable to a broad spectrum of neuroscientists. Potential authors are invited to submit a letter of interest to the Section Editor for Reviews and Special Issues or to the Chief or Associate Editors indicating the topic of a potential Review. Proposals for reviews or commentaries should also contain an outline of the contents, including an abstract (<200 words), a list of 10 relevant articles including 5 from the proposer's own research, and a brief statement on why now is a good time to review the topic in question. Reviews will not be accepted for editorial processing unless pre-approved for submission.

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