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

AÇÃO NEUROPROTETORA E MODULADORA DA ANGIOGÊNESE E DA NEUROGÊNESE PROMOVIDA PELO RESVERATROL NA ISQUEMIA CEREBRAL EXPERIMENTAL

FABRÍCIO SIMÃO

Porto Alegre

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FABRÍCIO SIMÃO

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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.

Porto Alegre
2010

Dedicatória

Dedico este trabalho a todos os professores e profissionais que tiveram influência em minha formação e, em especial, aos meus maiores mestres, Marco Simão e Marta Simão, meus pais, não pelos ensinamentos técnicos e científicos, mas sim pelo exemplo de vida, de perseverança, de moral e humildade, e por todo o apoio e amor que me dedicaram. Não haverá trabalho científico ou conquista profissional que se compare ao amor e à união que continuamos construindo durante a nossa vida.

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Sumário

APRESENTAÇÃO

Esta tese está organizada em tópicos: *Introdução, Objetivos, Capítulos* (1 a 6 – referente a artigos científicos), *Discussão, Conclusões, Perspectivas* e *Bibliografia*.

A *Introdução* apresenta o embasamento teórico, que nos levou a formular a proposta de trabalho. O *objetivo geral* e os *objetivos específicos* estão dispostos no corpo da tese, e, especificamente dentro de cada capítulo. Os *capítulos* contêm os artigos científicos, os quais foram organizados como resposta aos objetivos propostos. O quinto e sexto trabalhos foram realizados no laboratório do Prof. Dr. Eng H. Lo (Neuroprotection Research Laboratory, Massachusetts General Hospital, HARVARD University, Boston, USA) durante a realização do doutorado sanduíche, sendo o quinto manuscrito submetido à revista Circulation Research e o sexto manuscrito a ser submetido. Todos os demais trabalhos foram desenvolvidos no departamento de Bioquímica-ICBS- UFRGS.

A seção *Discussão* contém uma interpretação geral dos resultados obtidos nos diferentes trabalhos. Os tópicos seguintes, *Conclusões* e *Perspectivas*, abordam as conclusões gerais da tese, bem como, possibilidades de futuros trabalhos a partir dos resultados descritos.

A seção *Bibliografia* lista as referências citadas na *Introdução* e *Discussão*. As referências utilizadas nos diferentes artigos estão listadas ao final de cada trabalho.

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ACM: Artéria Cerebral Média

Akt/PKB: Proteína Cinase B

AVE: Acidente Vascular Encefálico

ATP: Adenosina Trifosfato

BDNF: Fator de Crescimento Derivado do Encéfalo

Ca^{+2}: Íon Cálcio

CA1: Corno de Ammon

COX: Ciclooxigenase

CREB: Proteína de Ligação ao Elemento de Resposta ao AMP cíclico

DG: Giro denteado

ERK: Cinase Regulada por Sinal Extracelular

EROS: Espécies Reativas de Oxigênio

GFAP: Proteína Glial Fibrilar Ácida

GSK-3β: Glicogênio Sintase Cinase-3β

iNOS: Óxido Nítrico Sintase Induzível

JNK: Jun N-terminal Cinase

MAPK: Proteína Cinase Ativada por Mitógenos

NF-κB: Fator de Transcrição Nuclear

NMDA: N-metil-D-aspartato

NO: Óxido Nítrico

NOS: Óxido Nítrico Sintase

PI3-K: Fosfatidil-Inositol-3-Cinase

PKC: Proteína Cinase C

RSV: Resveratrol

SIRT: Sirtuína

SOD: Superóxido Dismutase

SNC: Sistema Nervoso Central

VEGF: Fator de Crescimento Vascular Endotelial

RESUMO

Estudos com resveratrol (RSV) tiveram um crescimento exponencial nos últimos anos, especialmente focando seu efeito benéfico na saúde humana. Recentemente, foram publicados trabalhos mostrando um forte efeito neuroprotetor, não somente reduzindo lesões cerebrais, mas também promovendo a recuperação funcional após a isquemia cerebral. Entretanto, as bases moleculares para a neuroproteção ainda são desconhecidas. Neste trabalho, avaliamos diversos mecanismos que são modulados pelo resveratrol em modelos experimentais de isquemia cerebral. Inicialmente, demonstramos que o tratamento com RSV, administrado durante 7 dias antes da indução da lesão isquêmica, diminuiu a morte neuronal em hipocampo e córtex cerebral de ratos submetidos a isquemia cerebral global. Em paralelo, reduziu a geração de espécies reativas de oxigênio e nitrogênio. Este efeito foi associado com o aumento de antioxidantes endógenos, além da prevenção do aumento da peroxidação lipídica e da diminuição da atividade da Na^+K^+-ATPase induzidos pela isquemia cerebral global. Tendo em vista que o insulto isquêmico aumentou rapidamente os níveis de espécies reativas de oxigênio, investigamos o efeito do resveratrol sobre o perfil lipídico, e demonstramos que ele foi capaz de prevenir a diminuição de gangliosídeos, fosfolipídios e colesterol observada em hipocampo e córtex de ratos isquêmicos. A análise de vias de sinalização possivelmente envolvidas, mostrou que o efeito neuroprotetor do resveratrol envolve a modulação da via de sobrevivência PI3-K/Akt através da ativação de seus substratos GSK-3β e CREB. Adicionalmente, vias que controlam a neuroinflamação foram moduladas por resveratrol, o qual diminuiu a ativação glial, reduziu a fosforilação da JNK, diminuiu a translocação nuclear de NF-κB, reduzindo sua ativação e seus possíveis substratos iNOS e COX-2. A seguir avaliamos o efeito do resveratrol em promover a angiogênese em células endoteliais cerebrais e demonstramos que ele foi capaz de promover a proliferação, migração e indução da formação de tubo vascular *in vitro*. Além da função da célula, o RSV mostrou mudar o aspecto morfológico de células endoteliais do cérebro associadas com o rearranjo do citoesqueleto e relocalização de β-catenina e VE-caderina. A avaliação de vias de sinalização associadas com a angiogênese promovida pelo resveratrol, mostrou o envolvimento das vias PI3-K/Akt e MAPK/ERK, que regularam eNOS modulando os níveis de VEGF e metaloproteinases. Considerando que angiogênese e neurogênese são processos acoplados, investigamos o efeito do RSV no aumento de fatores tróficos e na plasticidade neuronal. Demonstramos que o RSV promoveu a recuperação funcional e está intimamente relacionada com o aumento da angiogênese e neurogênese após a isquemia cerebral focal. Em conjunto, nossos resultados abrem a perspectiva para o desenvolvimento de uma nova estratégia terapêutica para o tratamento da isquemia cerebral.

ABSTRACT

Recent studies with resveratrol (RSV) have been growing exponentially throughout the years, especially focusing on its beneficial effect on human health. Recently published studies, showing strong neuroprotective effects of RSV, not only reduce brain damage, but also promotes functional recovery after stroke. However, the molecular basis for neuroprotection is still unknown. In this study, we evaluated various mechanisms that are modulated by RSV in experimental models of cerebral ischemia. Initially, we demonstrated that by administering RSV, for 7 days prior to global cerebral ischemia, decreased neuronal cell death in the cerebral cortex and hippocampus of rats as well as reducing the generation of oxygen and nitrogen reactive species. The observed neuroprotection was associated with increased endogenous antioxidants, a decrease of Na^+ K^+ ATPase, and the prevention of lipid peroxidation. With an ischemic insult, levels of ROS rapidly increase which may lead to a decrease in lipids. However, we investigated the effects of RSV on lipid profiles and it showed that it was able to prevent a decrease in the amount of gangliosides, phospholipids, and cholesterol observed in the hippocampus and cortex of ischemic rats. There are several possible signaling pathways of RSV. Analysis of these pathways reveals that the neuroprotective effects of RSV involve the modulation of PI3-K/Akt survival pathway by activating its substrates GSK-3β and CREB. Furthermore, RSV modulates pathways involved in neuroinflammation, decreasing glial activation, phosphorylation of JNK, nuclear translocation and activation of NF-κB and its possible substrates of iNOS and COX-2. Additionally, we also investigated the effects of RSV on angiogenesis in brain endothelial cells. Results showed that RSV was able to promote proliferation, migration and vascular tube formation *in vitro*. Signaling pathways associated with angiogenesis by RSV, involves PI3-K/Akt and MAPK/ERK pathways which both regulate eNOS in the modulation of VEGF and metalloproteinase levels. Furthermore, RSV showed that the morphological change of brain endothelial cells was associated with cytoskeletal rearrangement and relocation of β-catenin and VE-cadherin. Considering that angiogenesis and neurogenesis are coupled processes, we investigated the effects of RSV on the increase of trophic factors and neuronal plasticity. We demonstrate that RSV increased functional recovery as well as intimately related to the increase of angiogenesis and neurogenesis after focal cerebral ischemia. Therefore, our results open the perspective for the development of a new therapeutics for the treatment of stroke.

1. INTRODUÇÃO

1.1 ISQUEMIA CEREBRAL

No Brasil, a doença cerebrovascular é uma das principais causas de óbito e dependência, observando-se uma incidência anual de 156 casos a cada 100.000 habitantes (RADANOVIC, 2000). O Acidente Vascular Encefálico (AVE) pode ser definido como uma perturbação focal ou global da função cerebral, de rápido desenvolvimento, supostamente de origem vascular, e que resulta em sinais clínicos duradouros ou morte sem outra causa aparente, a não ser de origem vascular (WHO, 1988). Pode tratar-se de uma interrupção do suprimento sangüíneo ou de uma hemorragia encefálica, que, normalmente, envolve prejuízo das vias sensoriais e motoras. Aproximadamente 80% dos AVEs são decorrência da oclusão vascular, sendo que a artéria mais comumente ocluída é a artéria cerebral média (ACM) ou suas ramificações profundas (STOKES, 2004).

O encéfalo é altamente dependente de fluxo sangüíneo contínuo para o suprimento de oxigênio e glicose, sendo mais vulnerável ao dano isquêmico do que os outros tecidos. Isto porque a bioenergética cerebral normal tem algumas características especiais, que incluem uma taxa metabólica alta, estoques de energia limitados e uma grande dependência do metabolismo aeróbico da glicose. A redução da taxa de fluxo sangüíneo e/ou conteúdo arterial de oxigênio pode afetar gravemente a função cerebral, ocasionar alterações bioquímicas e moleculares, e manifestar-se como seqüela neurológica (LIPTON, 1999; RODRIGO *et al.*, 2005).

Embora diversos estudos venham melhorando o conhecimento sobre o complexo funcionamento fisiológico e patológico do cérebro, inclusive sobre os mecanismos associados à morte celular no cérebro isquêmico, as opções farmacológicos para prevenir e/ou tratar a isquemia cerebral são ainda muito limitadas. O conhecimento dos eventos moleculares associados à morte celular

causada pela hipóxia-isquemia, bem como daqueles envolvidos nas estratégias celulares de sobrevivência a estímulos nocivos é fundamental para o desenvolvimento de terapias clinicamente efetivas e a conseqüente diminuição da morte celular.

1.2 MECANISMOS BÁSICOS DA MORTE CELULAR NA ISQUEMIA

O tecido encefálico, submetido à isquemia, passa por uma série de eventos complexos e intrincados, os quais são denominados, em conjunto, de "cascata isquêmica". Em poucos minutos de oclusão vascular, uma seqüência complexa de eventos fisiopatológicos espaciais e temporais acontece em certa ordem, apresentando importantes inter-relações entre si, e perdurando por várias horas ou dias (DURUKAN & TATLISUMAK, 2007). Decorrente da falha energética, ocorre despolarização neuronal, excessiva liberação e falha na recaptação do neurotransmissor glutamato, aumento dos níveis intracelulares de Ca^{2+}, produção excessiva de espécies reativas de oxigênio (EROS), depleção dos níveis de enzimas anti-oxidantes, produção de ácido araquidônico e mediadores inflamatórios, além da ativação de segundos-mensageiros envolvidos na sinalização da morte celular programada (LO *et al.*, 2003). Em função de todas essas modificações e da ativação de enzimas que danificam a estrutura das membranas celulares, ocorre perda da compartimentalização, abalo da homeostase celular e, finalmente, morte neuronal (Figura 1) (LO *et al.*, 2003).

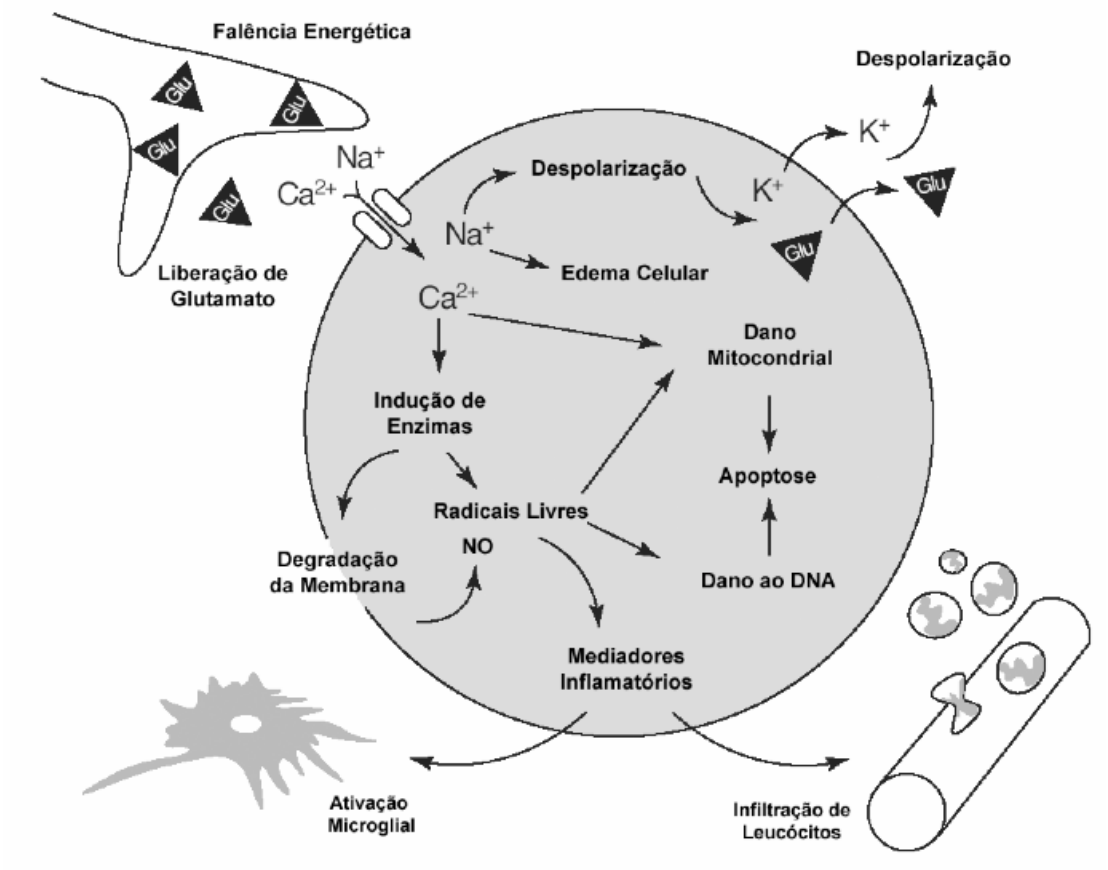

Figura 1. Visão geral simplificada dos mecanismos patofisiológicos ocorrendo no cérebro isquêmico (Modificado de DIRNAGL et al., 1999).

1.3 MODELOS EXPERIMENTAIS DE LESÃO ISQUÊMICA

1.3.1 Isquemia Cerebral Global (ICG)

A isquemia cerebral global ocorre geralmente em pacientes que sofrem de uma variedade de condições clínicas, incluindo a parada cardíaca, choque e asfixia, além de pacientes submetidos à cirurgia cardíaca complexa (SALAZAR *et al.*, 2001; BERNARD *et al.*, 2002; NUSSMEIER *et al.*, 2002). Além de prejuízo aos outros órgãos da hipoperfusão sistêmica, as seqüelas neurológicas da lesão cerebral são variadas e constituem um amplo espectro que inclui coma, convulsões e disfunção

cognitiva (MURKIN *et al.*, 1999; LLINAS *et al.*, 2000). A ICG é definida como uma diminuição do fluxo sanguíneo cerebral (FSC) a um patamar crítico que propaga o dano cerebral para todo o cérebro. Os episódios isquêmicos globais são mais comumente produzidos por oclusões de vasos e menos comumente por parada circulatória cerebral completa.

Os modelos animais em ratos de ICG incluem: (1) oclusão dos quatro vasos (4-VO) por eletrocoagulação de ambas artérias vertebrais, com oclusão transitória de ambas as artérias carótidas 24 horas mais tarde como descrito por PULSINELLI & BRIERLEY (1979). A isquemia é induzida quando o rato, já recuperado da anestesia, tem as artérias carótidas ocluídas. Verifica-se a falta de resposta do animal, com perda do reflexo do endireitamento, sinal de isquemia efetiva. A reperfusão é obtida pela restauração do fluxo carotídeo; (2) oclusão dos dois vasos, a isquemia é induzida pela oclusão bilateral das artérias carótidas combinada com hipotensão sistêmica (50 mmHg) (SMITH *et al.*, 1984).

Nestes modelos animais, populações específicas de neurônios no cérebro, incluindo neurônios piramidais de CA1 do hipocampo, neurônios do córtex e estriado, além de células de Purkinje do cerebelo (FONNUM *et al.*, 1984), são suscetíveis a lesões. A sensibilidade destas populações neuronais é variada e depende da duração e gravidade da isquemia, onde um perfil típico temporal é observado após a ICG. Neurônios da área de CA1 são os mais sensíveis à isquemia (3-5 minutos), enquanto os neurônios da área do estriado são mais resistentes (15-20 minutos) (KOEHLER *et al.*, 1996). Após a reanimação e recirculação cerebral, a progressão da lesão neuronal, que é irreversível, também difere nestas populações neuronais de forma seletiva. Por exemplo, a lesão neuronal é observada dentro de 3 horas de recirculação no estriado, em neurônios de tamanho médio, mas com atraso de 72-168 horas nos neurônios do

hipocampo, um fenômeno conhecido como “morte neuronal tardia” (KOEHLER *et al.*, 1996).

1.3.2 Isquemia Cerebral Focal (ICF)

Os modelos de isquemia cerebral focal envolvem principalmente a oclusão da artéria cerebral média (ACM). Esses modelos tem sido amplamente utilizados pela sua relevância para as condições clínicas do AVE. Um grande número de modelos de oclusão da ACM vem sendo utilizado, tanto de oclusão de natureza permanente quanto temporária (GINSBERG & BUSTO, 1998).

A ICF é caracterizada pela presença de um centro de tecido densamente isquêmico, e um tecido mais periférico com menor densidade isquêmica, parcialmente suprido pelos vasos colaterais. Em 1981, TAMURA e colaboradores descreveram o modelo de oclusão proximal permanente da ACM através de craniotomia. Outro modelo de oclusão foi o descrito por KOIZUMI e col. (1986) e modificado por LONGA e col. (1989), no qual a isquemia é produzida por um filamento intraluminal introduzido em sentido retrógrado através da artéria carótida externa, progredindo anterogradamente pela artéria carótida interna até encontrar resistência (Figura 2). Isso obstrui a ACM e interrompe o fluxo colateral proveniente da artéria comunicante anterior. A principal diferença entre o modelo descrito por TAMURA (1981) e o descrito por KOIZUMI e col. (1986) está no fato de que no segundo permite-se a reperfusão da área isquêmica, além de não ser necessária a craniotomia.

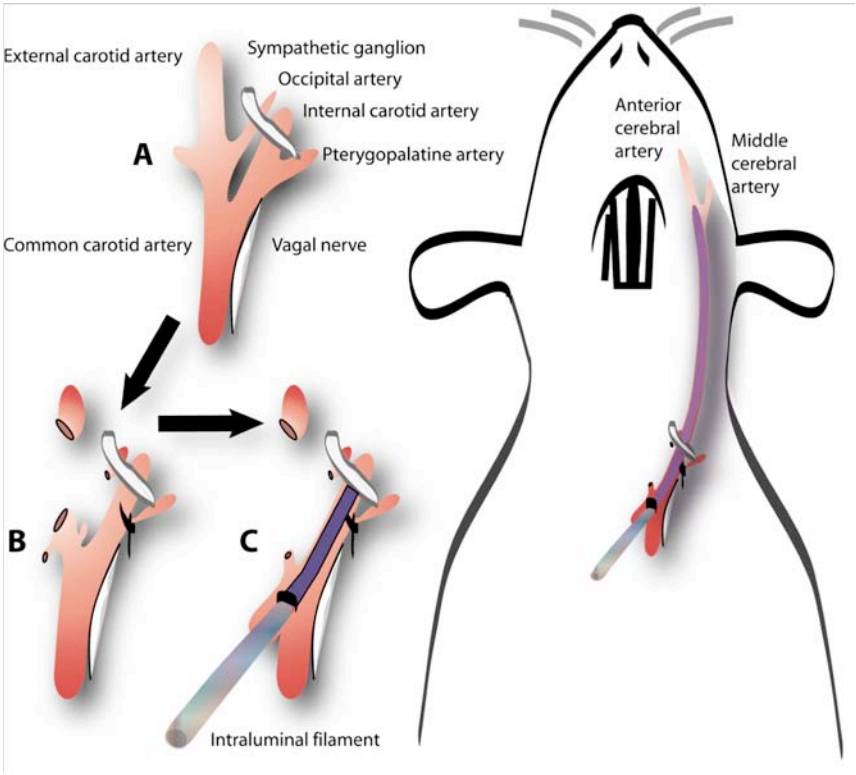

Figura 2. Desenho esquemático do modelo de obstrução da artéria cerebral média. (A) Orientação anatômica, (B) divisão da artéria carótida externa, divisão dos pequenos ramos e ligação da artéria pteriogopalatina, (C) Inserção do filamento.

Nos modelos de ICF da ACM o centro do tecido densamente isquêmico geralmente engloba a parte lateral da área do caudado putâmen e o neocórtex adjacente, pois esses são tecidos cuja perfusão depende totalmente desse vaso sangüíneo. As áreas periféricas ao núcleo isquêmico são perfundidas pela artéria cerebral anterior e artéria cerebral posterior, além de outros vasos colaterais, a maioria dos quais leptomeningeais. A vitalidade do tecido presente no núcleo isquêmico encontra-se usualmente comprometida quando a isquemia se prolonga para além dos 15 a 30 minutos iniciais de isquemia sob temperatura normal.

Durante a isquemia, ocorre redução gradativa do dano tecidual do centro para a periferia, de forma que o dano máximo ocorre na área central do infarto. A região em torno do foco da lesão é denominada de “penumbra isquêmica” e normalmente é

suprida por vasos colaterais à artéria inicialmente ocluída (MEHTA *et al.*, 2007). A penumbra é uma zona caracterizada pelo silêncio elétrico sem perda da polarização da membrana (ASTRUP *et al.*, 1981; HAKIM, 1992), podendo permanecer viável por muitas horas e reter um considerável potencial para recuperação da função neural. Se o dano neuronal puder ser freado nesta região, uma recuperação neurológica substancial pode ser possível mesmo em um indivíduo que se apresenta inicialmente com um quadro clínico correspondente a um AVE severo (ALBERS, 1997). Funcionalmente, podemos definir a penumbra como a parte da região isquêmica que é potencialmente recuperável (SIESJO, 1992).

1.4 ANGIOGÊNESE

A angiogênese é um processo altamente dinâmico e complexo, podendo ser ativado pela hipóxia, que consiste no crescimento de novos vasos sanguíneos a partir de estruturas vasculares pré-existentes. Estudos em animais e humanos demonstraram que a angiogênese endógena pode desempenhar um papel importante no AVE isquêmico, melhorando a recuperação funcional (KRUPINSKI *et al.*, 1994; WEI *et al.*, 2001). A angiogênese poderia ser interpretada como um mecanismo de defesa natural ajudando a restaurar a oferta de oxigênio e nutrientes para o tecido cerebral. Estas observações suportam a consideração de angiogênese como um potencial alvo terapêutico no curso da isquemia.

Após a interrupção do fluxo sangüíneo, a condição hipóxico-isquêmica em torno de uma lesão rapidamente aciona a transcrição de uma variedade de genes que podem estar envolvidos no processo de angiogênese (BECK *et al.*, 2009). Por exemplo, a produção do fator de crescimento vascular endotelial (VEGF) e citocinas pró-inflamatórias criam um ambiente permissivo para o surgimento e proliferação de

células endoteliais (ZHANG *et al.*, 2002; SLEVIN *et al.*, 2006; BECK *et al.*, 2009). Estudos utilizando camundongos e/ou ratos com oclusão temporária ou permanente da artéria cerebral média demonstraram que as células endoteliais que cercam a área do infarto cerebral começam a proliferar mais rapidamente 12-24 h após a isquemia (MARTI *et al.*, 2000; HAYASHI *et al.*, 2003), o que leva a um aumento da vascularização na região peri-infarto 3 dias após a lesão isquêmica. Estudos com cérebro humano demonstraram que a ativação da angiogênese ocorre em 3-4 dias após o episódio isquêmico (KRUPINSKI *et al.*, 1993, 1994). Esta fase do início da angiogênese está associada com a quebra da barreira hemato-encefálica (BHE) (SANDOVAL *et al.*, 2008) e em uma semana após o AVE, foi observado um aumento significativo da densidade microvascular (SLEVIN *et al.*, 2006). Com o tempo de sobrevivência, os conglomerados de micro-vasos aumentam de tamanho, podendo dar origem a um aumento do volume e fluxo sangüíneo cerebral (LIN *et al.*, 2002). Além disso, uma diminuição da permeabilidade do vaso pode ser observada ao longo do tempo (Figura 3), que é sugestivo para o remodelamento de pericitos, astrócitos e outras células que estão envolvidos na integridade da BHE.

A formação de novos vasos após o AVE pode (1) contribuir para a recuperação do tecido danificado por restaurar o metabolismo em neurônios sobreviventes, (2) facilitar a remoção de restos necróticos e/ou (3) aumentar a oferta de compostos neurotróficos para remodelação neuronal (por exemplo, para sinaptogênese) (MANOONKITIWONGSA *et al.*, 2001; ZHANG *et al.*, 2002; HAYASHI *et al.*, 2003; SLEVIN *et al.*, 2006; ARAI *et al.*, 2009; BECK *et al.*, 2009). No entanto, se a angiogênese de fato dá origem ao restabelecimento funcional de redes vasculares ao longo do curso de uma lesão que ainda não está clara, continua a ser um tópico importante para futuras investigações.

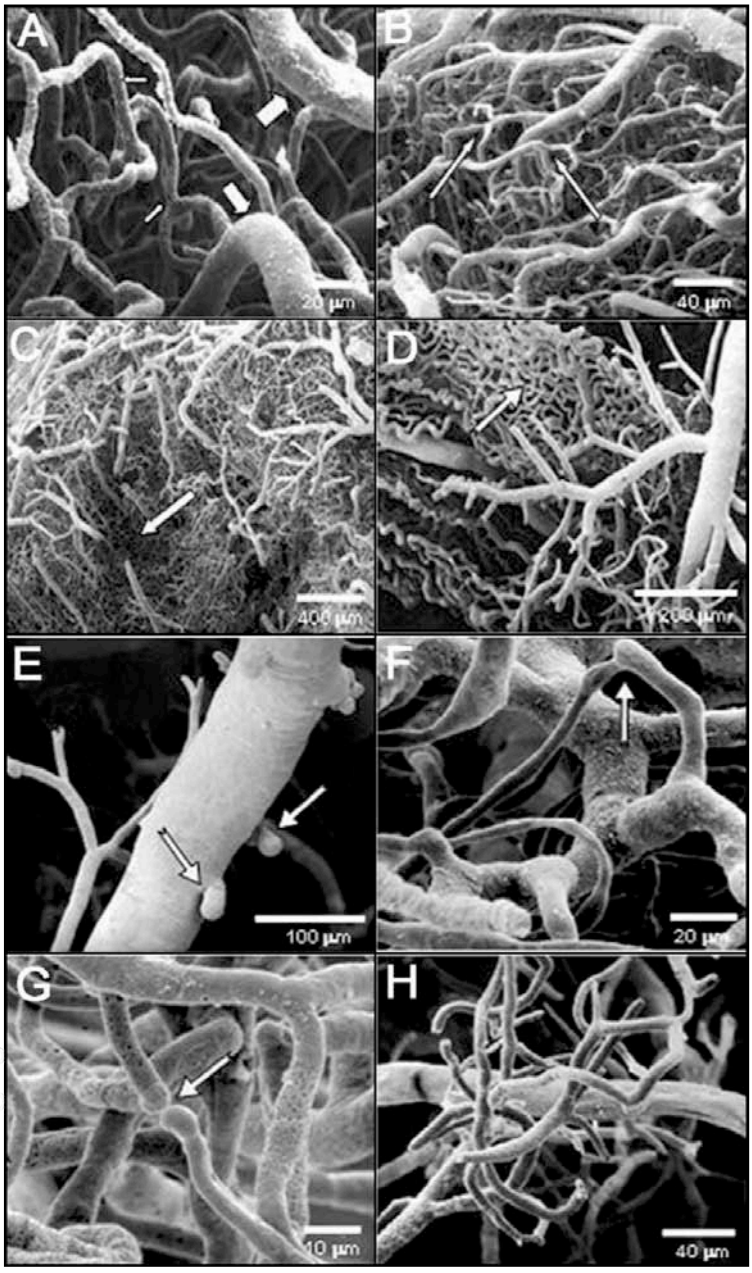

Figura 3. Angiogênese endógena ocorre na penumbra pós-isquemia. Imagens por microscopia eletrônica de leptomeninges (setas grandes) e pequenas arteríolas (setas pequenas) no cérebro normal (A e B). (C) Áreas de infarto onde os vasos sanguíneos são visíveis (seta), e (D) microvassos estressados 24h após a oclusão da ACM. (E e F) Projeções vasculares são visíveis 3 dias após a oclusão da ACM (setas), e (G e H) conexões de pequenos microvassos associados com vasos ao redor (seta). Adaptado de SLEVIN et al., 2006.

Para explorar a angiogênese com o objetivo de minimizar as conseqüências desencadeadas por uma lesão em curso, deve-se focar na avaliação das funções de moléculas específicas as quais atuarão dentro de uma janela de tempo definida. A

partir de evidências experimentais e com uma massa crítica de tais informações poderemos projetar terapias angiogênicas pertinentes e personalizadas para tratar a evolução heterogênea da lesão. O pensamento atual suporta a idéia que a promoção da angiogênese e da neurogênese (ZHANG *et al.*, 2005), através do recrescimento da estrutura vascular, pode fornecer a recuperação molecular necessária e como consequência o suporte para a recuperação anatômica da rede neural (OHAB *et al.*, 2008). De uma forma geral, acredita-se que a angiogênese é terapeuticamente vantajosa, porém deve-se reconhecer que existe uma variedade clínica muito grande em pacientes com AVE.

Muitos fatores de crescimento podem estar envolvidos na angiogênese, mas o mediador protótipo é o VEGF. Em teoria, as terapias de VEGF podem promover diretamente a neurorestauração, como agente neuroprotetor, ou indiretamente induzindo angiogênese (FERRARA *et al.*, 2001; CARMELIT & STORKEBAUM, 2002; SUN *et al.*, 2003). VEGF induz as células endoteliais a proliferação, migração e sobrevivência, e também é conhecido por promover a neurogênese, melhorar o aprendizado e memória e inibir a apoptose (CAO *et al.*, 2004; GREENBERG & JIN, 2004). Em modelos animais, uma injeção intracerebroventricular de VEGF, começando 24 horas após o início da isquemia cerebral focal, promoveu a estimulação da angiogênese coincidindo com a redução do volume do infarto (SUN *et al.*, 2003). Um aumento na angiogênese por VEGF em ratos foi associada com a redução do déficit neurológico, após a oclusão ACM (ZHANG *et al.*, 2000). Estes dados demonstram que o VEGF promove revascularização após o AVE.

Angiogênese e neurogênese são processos acoplados (PALMER *et al.*, 1988). Co-cultura de células endoteliais com células progenitoras neurais aumentam a proliferação e diferenciação de células progenitoras neurais (TENG *et al.*, 2008). No

cérebro de ratos adultos, células progenitoras neurais são localizadas junto a células endoteliais na zona subventricular e no giro denteado (PALMER *et al.*, 2000). Além disso, um aumento do suporte neurotrófico angiogênico para neurônios recém-gerados e neuroblastos foram encontrados se concentrarem em torno dos vasos sanguíneos após o AVE (THORED *et al.*, 2007). A Neurogênese ocorre dentro de um nicho angiogênico, podendo assim, ser mecanicamente ligados.

1.5 NEUROGÊNESE

A neurogênese no sistema nervoso central de mamíferos adultos ocorre por toda vida (ALTMAN & DAS, 1965). A neurogênese é primariamente um processo do desenvolvimento que envolve a proliferação, migração e diferenciação de célula tronco neural ou célula progenitora. Ocorre em regiões discretas do cérebro adulto, incluindo a zona subventricular (ZSV) dos ventrículos laterais (LUSKIN, 1993) e a zona subgranular (ZSG) do giro dentado (GD) (KAPLAN & HINDS, 1977). Os neurônios que surgem na ZSV migram em direção ao bulbo olfatório através de uma corrente migratória rostral (CMR) (LOIS *et al.*, 1996), e novos neurônios deixando a ZSG migram para a camada de célula granulares do GD (Figura 4). A neurogênese nestas regiões está sujeita a regulação fisiológica por glicocorticóides, fatores de crescimento, neurotransmissão excitatória, aprendizado, estresse e podem ser modificados farmacologicamente (CAMERON & MCKAY, 1999; CHEN *et al.*, 2000). Eventos patológicos podem também estimular a neurogênese no cérebro adulto.

A lesão cerebral isquêmica desencadeia mecanismos de reparo molecular e celular que contribuem para a recuperação e podem incluir ativação da neurogênese no cérebro adulto (CRAMER & CHOPP, 2000). Após a oclusão da ACM em

roedores, a população de células positivas à bromodeoxyuridina (BrdU) aumenta na ZSV e giro denteado, com pico em torno de 7-14 dias após a isquemia (TAKAGI *et al.*, 1999; JIN *et al.*, 2001; KEE *et al.*, 2001; ZHANG *et al.*, 2001; ARVIDSSON *et al.*, 2002; PARENT *et al.*, 2002; TUREYEN *et al.*, 2004). Este aumento é devido principalmente ao aumento da produção de células positivas para marcadores de neuroblastos (nestina, doublecortina, PSA-NCAM, β-tubulina) após o AVE (CHEN *et al.*, 2004). A ZSV expande com neuroblastos positivos a doublecortina (DCX) que migram para fora da ZSV em cadeias ao lado de astrócitos, semelhante à sua migração normal na CMR (LOIS *et al.*, 1996; PERETTO *et al.*, 1997), e para dentro do estriado lesionado alguns dias após a MCAO.

A resposta da ZSV após a lesão não é limitada à produção de novos neurônios. Células-tronco neurais (NSCs) da ZSV podem se diferenciar em neurônios, astrócitos e oligodendrócitos. Células da glia radial funcionam como progenitores gliais e fornecem um suporte para a neurogênese durante o desenvolvimento, mas também podem atuar como células-tronco neurais na ZSV de adulto pela divisão e produção de astrócitos e neurônios (DOETSCH *et al.*, 1999; GREGG & WEISS 2003; WEISSMAN *et al.*, 2003; MERKLE *et al.*, 2004). Após a oclusão da ACM, há um aumento de células da glia radial na ZSV e essas células parecem apoiar a migração dos neurônios após lesão (ZHANG *et al.*, 2007). A astrogliose induzida pela isquemia pode ocorre dentro da região do estriado isquêmico e pode guiar a migração de neuroblastos da ZSV para o núcleo isquêmico (ZHANG *et al.*, 2005; OHAB & CARMICHAEL, 2008).

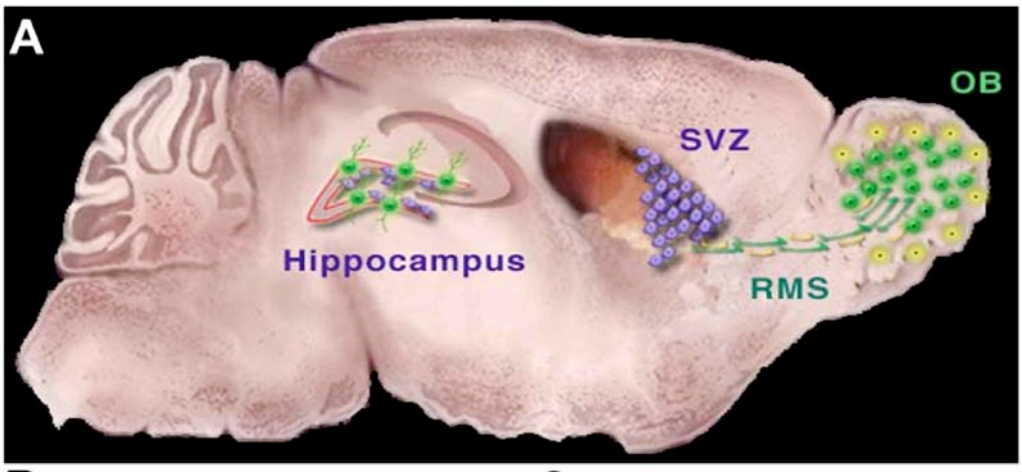

Figura 4. Neurogênese no encéfalo adulto. SVZ, zona subventricular; RMS, corrente migratória rostral. Adaptado de Lichtenwalner & Parent JCBFM, 2006.

Os estudos mencionados apóiam a hipótese de que a neurogênese estaria envolvida na fisiopatologia da isquemia cerebral. Estes resultados foram interpretados como evidências para migração direcionada de precursores neuronais em direção a lesão, possivelmente em resposta a um sinal químico. Dessa forma, manipulações na neurogênese no sentido de estimulá-la poderiam se tornar uma alternativa terapêutica para a isquemia cerebral.

1.6 RESVERATROL

1.6.1 Aspectos Gerais

O resveratrol (3,4',5-trihidroxiestilbeno, RSV) é um polifenol presente em mais de 70 espécies de plantas com diversas atividades biológicas que incluem propriedades antioxidantes, antiinflamatórias e antitumorais. O resveratrol foi identificado pela primeira vez em 1940 nas raízes de *Verartum grandiflorum O. Loes* (TAKAOKA, 1940), cuja função biológica é a proteção contra agentes potencialmente lesivos à estrutura da planta e a estresses ambientais. A raiz de

Polygnum cuspidatum, erva usada na medicina popular chinesa e japonesa, apresenta os níveis mais elevados de resveratrol (SOLEAS *et al.*, 1997).

Quimicamente, trata-se de um composto polifenólico derivado da fenilalanina, que contém dois anéis aromáticos com hidroxilas reativas em sua estrutura e que pode se apresentar sob duas formas isoméricas: *cis* e *trans*-resveratrol. O processo de isomerização ocorre pela ação da luz e o isômero *trans* é o principal responsável pelos efeitos biológicos do resveratrol em mamíferos (figura 5) (SOLEAS *et al.*, 1997). Por ser uma fitoalexina, antibiótico das plantas, o conteúdo de RSV presente nas diferentes fontes pode variar amplamente, dependendo de fatores que podem gerar mais ou menos estresse, tais como: ambiente de cultivo, clima, exposições a patógenos e, no caso dos vinhos, método de produção (SIGNORELLI & GHIDONI 2005).

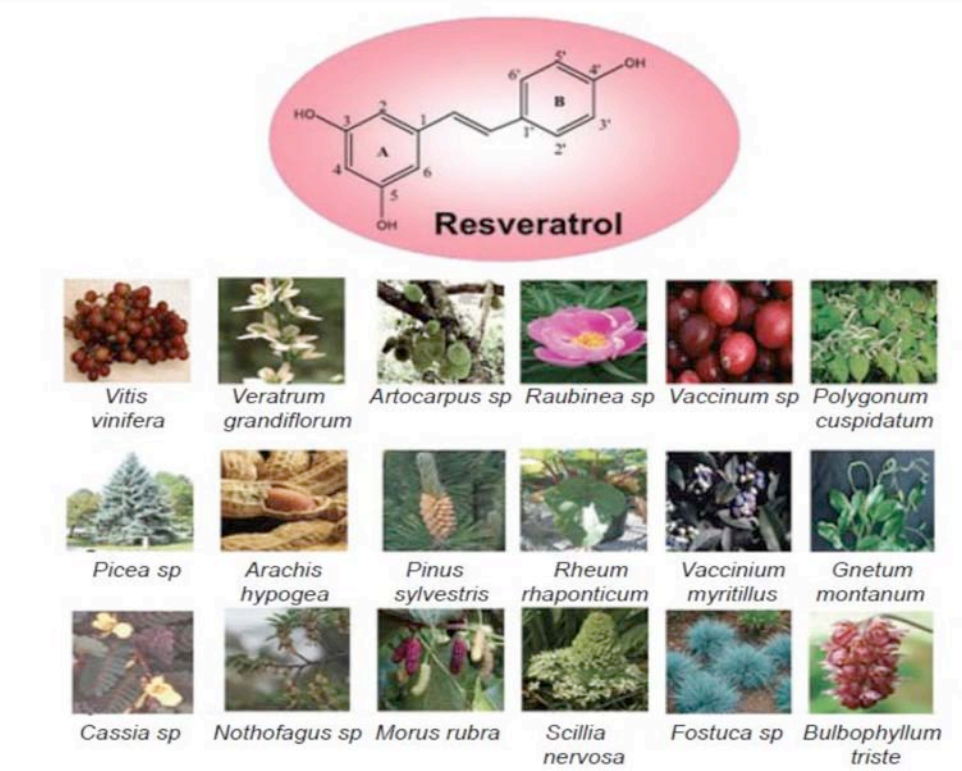

Figura 5. Estrutura e fontes de resveratrol. Adaptado de HARIKUMAR E AGGARWAL, 2008.

Atualmente, muitos estudos sobre resveratrol são direcionados aos vinhos (em especial, ao vinho tinto) e apontam a presença da molécula nessa bebida, sob suas duas formas isoméricas (PERVAIZ, 2003). O efeito do resveratrol já foi testado e se mostrou benéfico na prevenção e tratamento de uma série de doenças, dentre elas câncer, diabetes, distúrbios cardiovasculares, doenças neurodegenerativas, auto-imunes e metabólicas (BAUR & SINCLAIR, 2006a).

1.6.2 Alvos Moleculares

1.6.2.1 Propriedades Gerais

A diversidade dos efeitos biológicos do resveratrol deriva de sua capacidade de afetar diversos alvos moleculares. Estes alvos podem ser divididos em duas categorias: aqueles modulados por interação física direta com o resveratrol (figura 6) e aqueles modulados indiretamente, através da mudança nos níveis de expressão de diversos genes (figura 7).

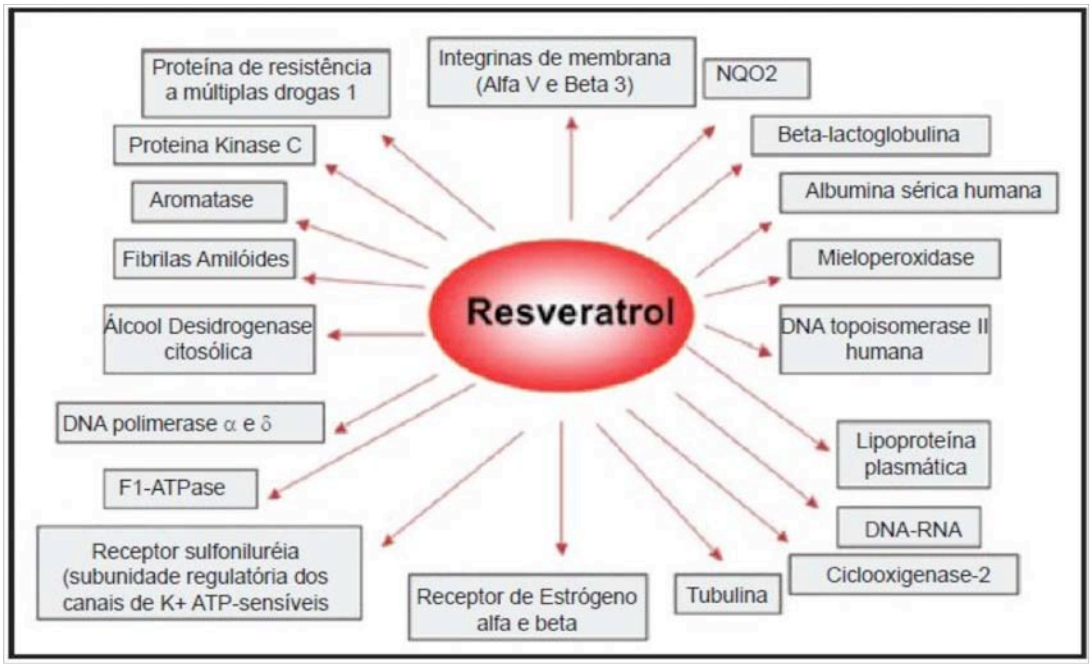

Figura 6. Moléculas com as quais o resveratrol se liga diretamente. NQO2 – NAD(P)H:quinone oxidoreductase 2. Adaptado de HARIKUMAR E AGGARWAL, 2008.

Uma das mais conhecidas propriedades do resveratrol é a capacidade antioxidante, atenuando a acumulação de espécies reativas de oxigênio. Dados da literatura mostram que resveratrol é capaz de impedir aumento das espécies reativas de oxigênio e a morte por apoptose causada por um insulto oxidativo (JANG & SURH, 2001; DE SALVIA *et al.*, 2002; LOSA, 2003; OVESNA *et al.*, 2006). Sabe-se que o resveratrol pode agir como um "scavanger" contra o radical hidroxil e o ânion superóxido (LEONARD *et al.*, 2003; SOARES *et al.*, 2003). Além disso, o resveratrol é capaz de aumentar a atividade das enzimas envolvidas no estresse oxidativo como SOD, catalase e glutationa peroxidade de forma dose-dependente (MOKNI *et al.*, 2007), assim como o conteúdo de glutationa (SHARMA & GUPTA, 2002; ATES *et al.*, 2007). O efeito neuroprotetor do resveratrol já foi demonstrado em modelos de lesão cerebral com o peptídeo β- amilóide em culturas de células PC12, atribuindo esse resultado à sua capacidade antioxidante (JANG & SURH, 2003), à inibição de receptores glutamatérgicos pós- sinápticos, diminuindo a excitotoxicidade glutamatérgica (GAO *et al*., 2006b).

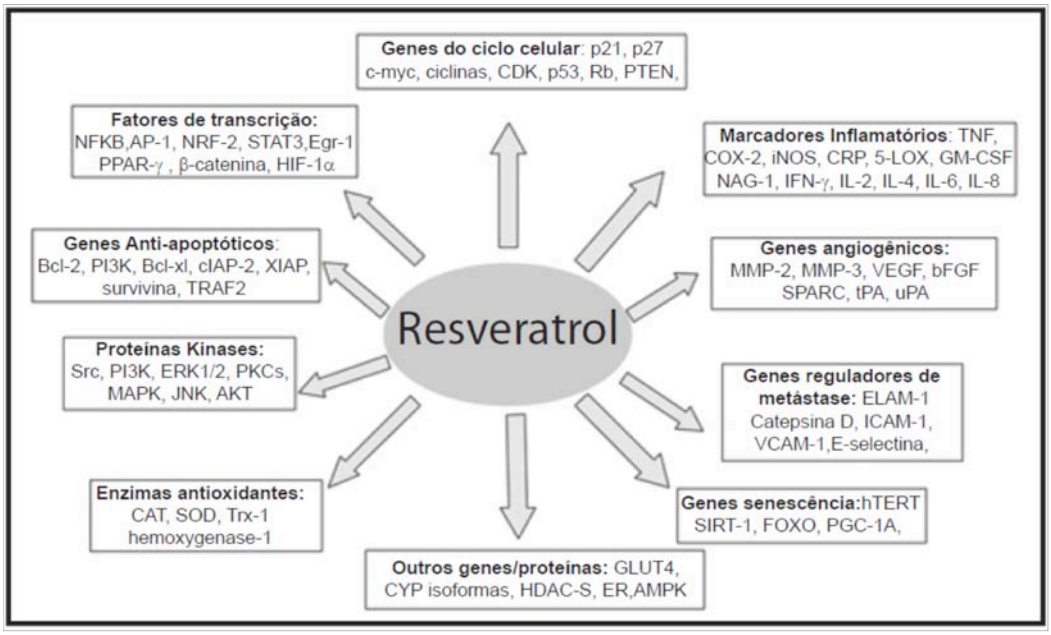

Figura 7. Produtos gênicos modulados pelo resveratrol. Adaptado de HARIKUMAR E AGGARWAL, 2008.

Os efeitos antiinflamatórios são mediados através da regulação negativa de vários marcadores como do fator de necrose tumoral (TNF), ciclooxigenase-2 (COX-2), óxido nítrico sintase induzível (iNOS), proteína C-reativa, interferon-γ, e várias interleucinas (Revisado em HARIKUMAR & AGGARWAL, 2008). O resveratrol reduz as inflamações agudas e crônicas, evitando todas as características do processo inflamatório (CHEN *et al.*, 2005).

Estudos recentes demonstram que a modulação da via de sinalização das sirtuinas com resveratrol é uma poderosa ferramenta contra uma série de lesões. Além disso, o resveratrol seria capaz de mimetizar os efeitos da restrição calórica, fenômeno caracterizado por ativar as sirtuinas e aumentar a longevidade. As sirtuinas são histonas diacetilases dependentes de NAD (dinucleotídeo de nicotinamida e adenina) que participam na fisiopatologia de uma série de modificações relacionadas à idade e que, de acordo com estudos experimentais, estariam associadas à longevidade (ANEKONDA, 2006). Camundongos submetidos a uma dieta hipercalórica, mas com concomitante administração de resveratrol apresentaram um aumento significativo na sobrevida (BAUR *et al.*, 2006b). Anteriormente, já havia sido relatado o efeito do resveratrol sobre o aumento da longevidade em diversas espécies como *Saccharomyces cerevisiae*, *Caenorhabditis elegans* e *Drosophila melanogaster* (WOOD *et al.*, 2004). Em lesões do sistema nervoso, o tratamento com RSV seria capaz de induzir a expressão do NAD e de sirtuinas, os quais seriam responsáveis pela ativação de genes relacionados à proteção neuronal (ARAKI *et al.*, 2004; BEDALOV & SIMON, 2005; RAVAL *et al.*, 2008).

1.6.1.2 Resveratrol e Isquemia Cerebral

É crescente o número de estudos sugerindo que o resveratrol possa exercer um papel significativo regulando funções importantes no SNC, especialmente sob condições patológicas (DORE, 2005; ANEKONDA, 2006). Por exemplo, o tratamento *in vivo* mostra que o resveratrol atravessa a barreira hemato-encefálica e é capaz de proteger contra isquemia cerebral (SINHA *et al.*, 2002; WANG *et al.*, 2002). Os efeitos neuroprotetores do resveratrol têm sido demonstrados em modelos de isquemia focal e global, em diversas doses, vias e tempos de administração. O pré-condicionamento pelo resveratrol resultou na redução do volume isquêmico e diminuição do conteúdo de água (BAUR & SINCLAIR, 2006). A compreensão do mecanismo molecular do pré-condicionamento desencadeado pelo resveratrol, que protege contra lesão cerebral isquêmica e no período de reperfusão, é fundamental. Têm sido apontados diferentes mecanismos moleculares envolvidos nos efeitos neuroprotetores do resveratrol. Em um estudo, HUANG e col. (2001a) mostraram que o resveratrol eficientemente reduziu o volume do infarto em ratos Long-Evans submetidos a isquemia cerebral focal numa dose baixa sem qualquer efeito colateral e sugeriu que os efeitos benéficos do resveratrol estejam relacionados a sua atividade na agregação plaquetária, na vasodilatação, na ação antioxidante ou por todos estes mecanismos juntos. Efeito semelhante foi observado em ratos Wistar, onde SINHA e col. (2002) demonstraram que o pré-condicionamento pelo resveratrol preveniu o dano motor, diminuiu os níveis de malondialdeído, glutationa reduzida e diminuiu o volume do infarto depois da isquemia focal usando uma dose de 20 mg/kg intraperitoneal por 21 dias. Por outro lado, WANG e col. (2001) observaram que resveratrol suprimiu a indução da expressão do RNAm da interleucina-6 em células corticais constituídas de 85–90% de astrócitos (positivo a GFAP), 5–10% de

microglia (positivo a Mac-1) e 2–3% de neurônios (positivo a MAP-2) expostas a privação de oxigênio-glicose *in vitro* seguida de reoxigenação. GAO e col. (2006a) demonstraram que o resveratrol teve efeito protetor em lesão isquêmica aguda através da inibição da MMP-9. Em modelo de anóxia-reoxigenação de isquemia usando mitocôndria cerebral de rato Wistar, resveratrol inibiu a liberação de citocromo c, diminuiu a produção do ânion superóxido e o consumo de O_2 (ZINI *et al.*, 2002). Em neurônios hipocampais de ratos, resveratrol inibiu correntes de K^+ ativados por voltagem, sugerindo que pode ser útil para tratar a lesão cerebral isquêmica (GAO *et al.*, 2006b).

1.6.1.3 Vias de Sinalização Envolvida na Proteção

O resveratrol tem a capacidade de modular a função de proteínas regulatórias envolvidas na transdução de sinal, e como conseqüência modulando uma variedade de atividades celulares incluindo crescimento celular, sobrevivência e morte (DAS *et al*., 2005a, b, c; ZAMIN *et al*., 2006). Alguns alvos de ação do resveratrol são: o óxido nítrico (NO) (HATTORI *et al.*, 2002; IMAMURA *et al.*, 2002; HUNG *et al*., 2004), receptores de adenosina (DAS *et al.*, 2005a, b), diferentes vias de sinalização celular como a da PI3-K (DAS *et al.*, 2006a, b), da MAPK (DAS *et al.*, 2006a, b) e estas têm sido apontadas como mediadoras dos efeitos farmacológicos do resveratrol. Nosso grupo demonstrou que o resveratrol diminuiu a morte celular pela ativação das vias PI3-K/Akt e Erk em cultura organotípica hipocampal exposta a privação de oxigênio-glicose (ZAMIN *et al.*, 2006). Também foi demonstrada a indução de SOD2 mitocondrial por resveratrol através da ativação das vias de sinalização PI3-K/Akt e GSK-3β/β-catenina (FUKUI *et al.*, 2010). A ação do resveratrol nas vias de

sinalização tem permitido uma maior compreensão dos mecanismos farmacológicos e certamente contribuirá para o desenvolvimento de estratégias terapêuticas.

1.6.1.4 Ação Pro-Angiogênese

Crescem as evidências que sugerem o envolvimento de resveratrol na regulação da formação vascular, como um promotor da angiogênese (WANG *et al.*, 2007a, 2010). Até agora, o mecanismo responsável pela ação vasodilatadora do resveratrol não foi completamente determinada, mas pode incluir a estimulação do óxido nítrico, uma vez que estudos fisiológicos indicam maior produção de NO *in vitro* e *in vivo*, em resposta ao tratamento agudo com resveratrol (WALLERATH *et al.*, 2002). Além disso, estudos em cultura de células endoteliais de cordão umbilical de humanos (HUVEC) têm revelado que o resveratrol aumenta tanto a expressão quanto a atividade da eNOS, sugerindo a possibilidade de que, além do efeito vasodilatador na fase aguda, a exposição crônica ao resveratrol poderia afetar a função vasomotora. Adicionalmente, o resveratrol aumentou a proliferação e migração de células progenitoras endoteliais de humanos e aumentou a expressão de VEGF, sugerindo que o resveratrol tem propriedades angiogênicas (WANG *et al.*, 2007a). Concomitantemente, o resveratrol foi capaz de promover vasculogênese em HUVEC através da expressão de VEGF e formação de tubo vascular em cultura. A modulação de VEGF foi dependente da acumulação nuclear e atividade de β-catenina, sendo que seu modulador negativo, GSK-3β, estava menos ativo nas células tratadas com resveratrol (WANG *et al.*, 2010). Em outro estudo, polifenóis do vinho tinto promoveram angiogênese em animais submetidos à ligadura da artéria femural através da via de sinalização PI3-K/-Akt-eNOS e aumento da produção de VEGF

(BARON-MENGUY *et al.*, 2007). Por outro lado, GAN e col. (2009) demonstraram que o resveratrol foi capaz de aumentar a capacidade de neovascularização de células mononucleares da medula óssea de camundongos diabéticos. Neste estudo, resveratrol aumentou a capacidade angiogênica, que foi correlacionada com um aumento da expressão de eNOS e VEGF, bem como aumento da atividade da SOD e que foi inversamente correlacionada com a produção de ROS. Mais estudos focando o mecanismo de vias que modulam a angiogênese são necessárias para validar o uso do resveratrol como terapia neurovascular em lesões cerebrais.

2. OBJETIVOS

2.1 Objetivo Geral

O objetivo geral deste trabalho foi estudar os mecanismos moleculares associados à neuroproteção mediada por resveratrol, focando na sua propriedade antioxidante, sua ação em vias de sinalização celular e seu efeito sobre a modulação da angiogênese e neurogênese contra o dano celular induzido pela isquemia cerebral.

2.2 Objetivos Específicos

1. Investigar os efeitos neuroprotetores do resveratrol em um modelo *in vivo* de lesão isquêmica, utilizando o modelo de isquemia cerebral global. Verificar se a neuroproteção mediada por resveratrol estaria relacionada:

A- A atividade da enzima Na^+,K^+-ATPase e parâmetros do estresse oxidativo em córtex cerebral e no hipocampo, assim como, analisar a neuroproteção através da avaliação da morte celular.

B- Com a composição de lipídios, para isto investigamos o efeito do resveratrol sobre o conteúdo e distribuição de gangliosideos, sobre as concentrações de fosfolipídios e colesterol em córtex cerebral e hipocampo

C- Analisar o efeito do resveratrol sobre vias de sinalização celular focando na ativação da via PI3-K/Akt a qual pode induzir um aumento endógeno de CREB e diminuir a ativação da GSK-3β e assim promover a neuroproteção.

D- Avaliar a modulação de parâmetros associados a inflamação através da ativação do NF-κB e da JNK, além da regulação de iNOS e COX-2.

2. Verificar o efeito do resveratrol na promoção da angiogênese em células microvasculares cerebrais de endotélio humano através da via de sinalização do NO/VEGF/MMPs .

3. Investigar o efeito do resveratrol na modulação da neurogênese, angiogênese e na recuperação funcional em animais submetidos à isquemia cerebral focal .

3. CAPÍTULO 1

Artigo: Resveratrol prevents oxidative stress and inhibition of $Na^{+}K^{+}$-ATPase activity induced by transient global cerebral ischemia in rats – Publicado no *Journal of Nutritional Biochemistry*

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Resveratrol prevents oxidative stress and inhibition of Na^+K^+-ATPase activity induced by transient global cerebral ischemia in rats

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Abstract

Increased oxidative stress and energy metabolism deficit have been regarded as an important underlying cause for neuronal damage induced by cerebral ischemia/reperfusion (I/R) injury. In this study, we investigated the oxidative mechanisms underlying the neuroprotective effects of resveratrol, a potent polyphenol antioxidant found in grapes, on structural and biochemical abnormalities in rats subjected to global cerebral ischemia. Experimental model of transient global cerebral ischemia was induced in Wistar rats by the four vessel occlusion method for 10 min and followed by different periods of reperfusion. Nissl and fluoro jade C stained indicated extensive neuronal death at 7 days after I/R. These findings were preceded by a rapid increase in the generation of reactive oxygen species (ROS), nitric oxide (NO), lipid peroxidation, as well as by a decrease in Na^+K^+-ATPase activity and disrupted antioxidant defenses (enzymatic and non-enzymatic) in hippocampus and cortex. Administrating resveratrol 7 days prior to ischemia by intraperitoneal injections (30 mg/kg) significantly attenuated neuronal death in both studied structures, as well as decreased the generation of ROS, lipid peroxidation and NO content. Furthermore, resveratrol brought antioxidant and Na^+K^+-ATPase activity in cortex and hippocampus back to normal levels. These results support that resveratrol could be used as a preventive, or therapeutic, agent in global cerebral ischemia and suggest that scavenging of ROS contributes, at least in part, to resveratrol-induced neuroprotection.

Keywords: Oxidative stress; Free radicals; Cerebral ischemia; Resveratrol; Neuroprotection; Na^+K^+-ATPase

1. Introduction

Cerebral ischemia is caused by a deficiency in blood supply which triggers various pathophysiological changes. Brief periods of global cerebral ischemia result in delayed neuronal death (DND), which occurs 3–7 days after the initial ischemic insult in selective vulnerable brain regions, especially in the cortex and the CA1 subfield of hippocampus [1]. During the reperfusion period after ischemia, increased oxygen supply results in overproduction of reactive oxygen species (ROS), which are involved in the process of cell death. ROS, such as superoxide anions, hydroxyl free radicals, hydrogen peroxide and nitric oxide are produced as a consequence of metabolic reactions and central nervous system activity [2]. These reactive species are directly involved in oxidative damage of cellular macromolecules such as nucleic acids, proteins, and lipids in ischemic tissues, which can lead to cell death [3]. In fact, the release of ROS and increased lipid peroxidation can be detected early after ischemia/reperfusion (I/R), e.g., 1–3 h, an interval in which there was no sign of neuronal death [4,5]. Superoxide and hydroxyl radical cause severe cell membrane damage by inducing lipid peroxidation [6]. Inducible nitric oxide (NO) synthase is up-regulated after I/R, which results in excessive NO production. This excess NO reacts with superoxide to form peroxynitrite, a powerful radical that can induce neuronal death after cerebral ischemia [7]. The enzymatic antioxidant activity of the tissue affected by I/R is particularly important for the primary endogenous defense against the ROS induced injury. Superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GSH-Px) are endogenous antioxidants which play an important role in the prevention of oxidative damage [8]. In the presence of excess oxygen species, inactivation of detoxification systems and degradation of antioxidants, endogenous antioxidative defences are highly effective [3]. In light of the oxidative hypothesis of ischemia-induced cell death, there is an increasing interest focusing on natural products that may ameliorate DND due to cerebral ischemia. Numerous antioxidants and scavengers of ROS have been tested, and many have shown neuroprotective effects [9-13].

Na^+K^+-ATPase is the enzyme, located to the cytoplasmatic membrane responsible for the active transport of sodium and potassium ions in the nervous system, maintaining the ionic gradient necessary for neuronal excitability and regulation of neuronal cell volume. It has been demonstrated that this enzyme is susceptible to

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free radical attack [14,15], and there are some reports showing that Na^+K^+-ATPase activity is decreased after cerebral ischemia [16] and in various chronic neurodegenerative disorders [14].

Resveratrol (RSV: 3,5,4′-trihydroxy-*trans*-stilbene) is found in many plant species such as grapes, berries and peanuts and exhibits pleiotropic health beneficial effects, including anti-oxidant, anti-inflammatory, cardioprotective, neuroprotective and anti-tumor activities [17-21]. Wang and colleagues [22] showed that resveratrol decrease delayed neuronal cell death, reducing the reactive astrogliosis and microglial activation in gerbils. It was demonstrated that resveratrol is the main responsible for cardiovascular benefits associated with moderate wine consumption [23], and this beneficial effect has been attributed to its strong antioxidant activity [17,19]. Although direct neuroprotective effects of resveratrol against oxidative stress have been studied in the PC12 cells and animals [17,24], there is no such investigation in models of global cerebral ischemia in rats. Therefore, considering that an enhancement of antioxidant activities in brain tissue may be potentially beneficial for neuronal recovery from I/R injury, we investigated the effectiveness of resveratrol pretreatment against brain injury caused by transient global cerebral ischemia in rats. In the present study we analyzed cell damage, Na^+K^+-ATPase activity and oxidative stress parameters both in the CA1 subfield of hippocampus and cerebral cortex.

2. Methods

2.1. Experimental procedures

Adult male Wistar rats (290–330 g) were obtained from the Central Animal House of the Department of Biochemistry, Institute for Basic Health Sciences, Federal University of Rio Grande do Sul, Porto Alegre, RS, Brazil. Animals were maintained under a 12/12-h light/dark cycle, in an ambient temperature (24±1°C) colony room. Animals were provided with a constant supply of food (ad libitum) and water. Animal care followed the official governmental guidelines in compliance with the Federation of Brazilian Societies for Experimental Biology and experimental protocols were conducted with the approval of the Ethics Committee of the Federal University of Rio Grande do Sul, Brazil.

2.2. Transient global cerebral ischemia

Transient global cerebral ischemia was induced by the four-vessel occlusion method described by [1], with minor modifications [25]. Briefly, rats were anesthetized with a mixture of 70% nitrous oxide, 30% oxygen and 1.5% halothane during surgical operation. Vertebral arteries were permanently electrocoagulated through the alar foramina at the first cervical vertebrae and the common carotid arteries were then exposed, carefully separated from the vagus nerve and isolated. Twenty-four hours later, transient global ischemia was accomplished by bilateral occlusion of the common carotid arteries with aneurysm clips for 10 min. Reperfusion started when aneurysm clips were removed and patency of arteries was confirmed by inspection. The rectal temperature was monitored and maintained at 37±0.5°C with a feedback-controlled heating-pad. Animals which did not lose the righting reflex or convulsed during the ischemic episode were excluded. Sham-operated group underwent the same procedures except for the occlusion of carotids [25].

2.3. Experimental protocols

Resveratrol (courtesy of Pharmascience, Montreal, Canada) was freshly prepared in 50% ethanol [17]. It was administered at the dose of 30 mg/kg, intraperitoneally everyday for 7 days before ischemia, volume not exceeding 0.1 ml/100 g rat weight. The dose of RSV was selected on the basis of earlier reports, which have demonstrated its antioxidant property in different experimental models in doses ranging from 8–40 mg/kg [17,22].

Rats were randomly divided into four groups: Sham + vehicle, Sham + RSV, ischemia + vehicle and ischemia + RSV. After the animals were sacrificed, cortex and hippocampal tissues were isolated for biochemical studies in 1 and 24 h. Another group of animals were prepared for histopathological examination after 7 days of injury. The vehicle-treated group received 0.1 ml of 50% ethanol v/v per 100 g for 7 days and subjected to global cerebral ischemia. The vehicle-treated sham group received 0.1 ml of 50% ethanol v/v /100 g for 7 days. Preliminary experiments confirmed that, in our biochemical and histopathological examinations, the vehicle-treated group was comparable to control.

2.4. Histological analysis

2.4.1. Nissl staining

Neuronal cell loss was assessed by histological examination of Nissl-stained brain sections at the level of the dorsal hippocampus from sacrificed animals 7 days after ischemia. Animals were deeply anesthetized with pentobarbital (50 mg/kg, i.p.) and then transcardially perfused with cold saline followed by 4% paraformaldehyde in phosphate-buffered saline (0.1 M; pH 7.4). Brains were removed and immersed in fixative (4°C overnight). Coronal sections (20 μm) were cut at the level of the dorsal hippocampus (3.3–4.0 mm posterior from bregma) with a cryostat (Leica Microsystems, Germany). Every fourth section was collected and either stained with Cresyl violet or with Fluoro Jade C. The number of surviving pyramidal neurons per 250-μm length of the medial CA1 pyramidal cell layer was counted bilaterally in four sections per animal under a light microscope at 40× magnification. Cell counts from the right and left hippocampus on each of the four sections were averaged to provide a single value (number of neurons per 250 μm length) for each animal.

2.5. Fluoro-Jade C staining

Fluoro-Jade C is a polyanionic fluorescein derivative which sensitively and specifically binds to degenerating neurons. Its high affinity to degenerating neurons with green fluorescence has made it an excellent marker for detecting degenerating neurons. The sections were air dried for 1 h and afterward were immersed in a basic alcohol solution consisting of 1% sodium hydroxide in 80% ethanol for 5 min. They were then rinsed for 2 min in 70% ethanol, following 2 min in distilled water, and then incubated in 0.06% potassium permanganate solution for 10 min. Slides were rinsed with distilled water for 1-2 min and then incubated to a 0.0001% solution of Fluoro-Jade C dissolved in 0.1% acetic acid for 15 min. The slides were then rinsed three times with distilled water for 1 min each. Excess water was drained onto a paper towel and the slides were then air dried. Air-dried slides were cleared in xylene for at least 1 min and a coverslipped was placed with DPX (Sigma) mounting media.

2.6. Measurement of ROS

To assess the ROS formation, 2′,7′-dichlorofluorescin (DCFH-DA, Sigma) was used as a probe [26]. Within the cell, esterases cleave the acetate groups on DCFH-DA, thus trapping the reduced probe (DCFH) intracellularly. ROS in the cells leads to the oxidation of DCFH, yielding the fluorescent product DCF. An aliquot of the sample was incubated with DCFH-DA (100 μM) at 37°C for 30 min, chilling the reaction mixture in ice terminated the reaction. The formation of the oxidized fluorescent derivative (DCF) was monitored at excitation and emission wavelengths of 488 and 525 nm, respectively, using a fluorescence spectrophotometer. The free radical content was quantified using a DCF standard curve and results were expressed as nanomoles of DCF formed per milligram of protein.

2.7. Determination of NO metabolites

Nitrites (NO_2) were determined using the Griess reagent, in which a chromophore with a strong absorbance at 540 nm is formed by reaction of nitrite with a mixture of naphthyl-etilenediamine (0.1%) and sulphanilamide (1%). $NaNO_2$ was used as standard to calculate NO_2 concentrations. Results were expressed as nanomoles per milligram of protein of nitrates plus nitrites [27].

2.8. Na^+,K^+-ATPase activity assay

The reaction mixture for Na^+,K^+-ATPase activity assay contained 5.0 mM $MgCl_2$, 80.0 mM NaCl, 20.0 mM KCl and 40.0 mM Tris–HCl with pH 7.4, in final volume of 200 μl. The reaction was initiated by ATP addition. Controls were carried out under the same conditions with the addition of 1.0 mM ouabain. Na^+,K^+-ATPase activity was calculated by the difference between the two assays as described by Wyse et al. [28]. Released inorganic phosphate (Pi) was measured by the method of Chan et al. [29]. Specific enzyme activity was expressed as nanomoles of Pi released per minute per milligram of protein. All samples were run in duplicate.

2.9. Thiobarbituric acid-reactive substances (TBARS)

TBARS measures malondialdehyde (MDA), a product of lipid peroxidation caused mainly by hydroxyl free radicals. A mixture of 50 μl of 8.1% sodium dodecyl sulfate and 1.5 ml of 20% acetic acid solution was adjusted to a pH of 3.5. Afterward, the mixture was combined with 1.5 ml of 0.8% aqueous solution of thiobarbituric acid and was added to 500 μl of tissue homogenate in a Pyrex tube and heated in a boiling water bath for 60 min [30]. After cooling with tap water, the mixture was centrifuged at 1000×*g* for 10 min. The organic layer was taken and the resulting pink color was measured in a spectrophotometer at a wavelength of 535 nm. TBARS was calculated as nanomoles of malondialdehyde formed per milligram of protein. The results were reported as nanomoles of TBARS per milligram of protein.

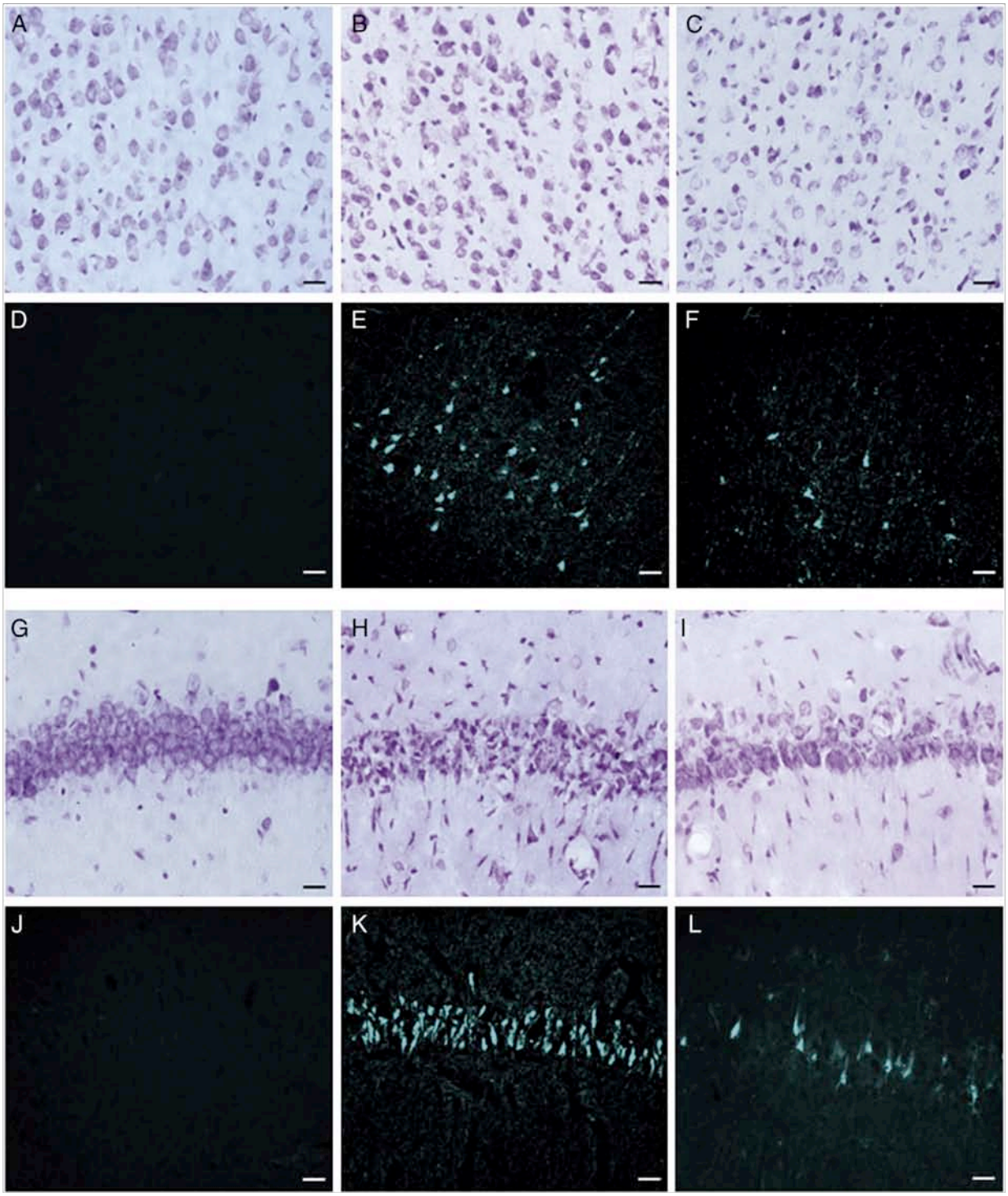

Fig. 1. Representative photomicrographs of Nissl and Fluoro Jade C-stained cells in the cortex and hippocampus (CA1). Vehicle-treated sham group (A and D); vehicle-treated ischemic group (B and E); and RSV-treated ischemic group (C and F) at 7 days of reperfusion in cortex. Vehicle-treated sham group (G and J); vehicle-treated ischemic group (H and K); and RSV-treated ischemic group (I and L) at 7 days of reperfusion in hippocampus. RSV was administered i.p (30 mg/Kg) for 7 days before ischemia (original magnification ×400; scale bar=50μm).

2.10. Total radical-trapping antioxidant potential (TRAP)

TRAP, which represents the quantity of non-enzymatic antioxidant defenses in tissue was determined by measuring the chemiluminescence intensity of luminol induced by 2,2′-azo-bis-(2-amidinopropane) (ABAP) thermolysis [31] in a Wallac 1409 Scintillation Counter. The initial chemiluminescence value was obtained by the addition of 3 ml of 10 mM ABAP, dissolved in 50 mM sodium phosphate buffer pH 7.4, and 10 μl of 5.6 mM luminol into a glass scintillation vial. Ten microliters of 160 μM

Trolox (water-soluble α-tocopherol analogue, used as standard) or tissue supernatant were then added to the vial, producing a decrease in the chemiluminescence value until the antioxidants present were depleted and chemiluminescence reaches its initial values. The time taken by the sample to keep the chemiluminescence low is called induction time and is directly proportional to the antioxidant capacity of the tissue. The induction time of the tissue was compared to that presented by Trolox. Results were reported as nanomoles of Trolox per milligram of protein.

2.11. Antioxidant enzymes

2.11.1. Superoxide dismutase assay

SOD activity is based on the capacity of pyrogallol to autoxidize, a process highly dependent on its substrate, superoxide. [32]. The inhibition of autoxidation of the compound occurs in the presence of SOD, whose activity can be then indirectly assayed spectrophotometrically at 420 nm. A calibration curve was performed with purified SOD as standard, to calculate the activity of SOD present in the samples. The results were reported as units per milligram of protein.

2.11.2. Catalase assay

CAT activity was assayed according to Aebi [33] by measuring the decrease in absorbance at 240 nm in a reaction medium containing 20 mM H_2O_2, 0.1% Triton X-100 and 10 mM potassium phosphate buffer, pH 7.0. One CAT unit is defined as 1 μmol of hydrogen peroxide consumed per minute and the specific activity is reported as units per milligram of protein.

2.11.3. Glutathione peroxidase assay

GSH-Px activity was measured according to the method described by Wendel [34] using *tert*-butyl hydroperoxide as substrate. Nicotinamide adenine dinucleotide phosphate (NADPH) disappearance was monitoredspectrophotometrically at 340 nm in a medium containing 2 mM glutathione, 0.15 U/ml glutathione reductase, 0.4 mM azide, 0.5 mM *tert*-butyl hydroperoxide and 0.1 mM NADPH. One GSH-Px unit is defined as 1 μmol of NADPH consumed per minute and the specific activity is represented as units per milligram of protein.

2.12. Statistical analysis

All assays were performed in duplicate and the mean was used for statistical analysis. Data were analyzed by one-way analysis of variance (ANOVA) followed by the Duncan multiple test. All analyses were performed using the SPSS software. Values of $P<.05$ were considered to be significant.

3. Results

3.1. RSV is neuroprotective against I/R-induced DND

Microphotographies of the cortex and hippocampal CA1 subfield for each group are shown in Fig. 1. Histological observation shows that neurons in the cortical layer (Fig. 1A) and CA1 pyramidal cell (Fig. 1G) are clear and moderate-sized with normal ultrastructure in vehicle-treated sham rats. In the vehicle-treated ischemic rats, pyramidal neurons exhibited either significant shrinkage, dark staining appearance with minimal cytoplasm or outright loss of neurons and widespread damage was evident after 7 days (Fig. 1B and H). These neuropathological signs were not present in RSV treated animals, which reduced neuronal loss and moderate morphologic changes (Fig. 1C and I). Fluoro-Jade C histofluorescence stains are remarkably helpful in identifying neurodegeneration, which is indicated by a bright fluorescence. A representative Fluoro-Jade C labeling is presented in the cortex (Fig. 1D–F) and hippocampus (Fig. 1J–L). No Fluoro-Jade C positive fluorescence staining was noted in the cortex (Fig. 1D) and hippocampal (Fig. 1J) regions of sham-operated group. On the other hand, hippocampal and cortex sections from vehicle-treated ischemic rats showed a dramatic increase in Fluoro-Jade C positive cells in the cortical (Fig. 1E) and CA1 pyramidal (Fig. 1K) cell layer, whereas very few positive cells were seen in similar sections of the RSV-treated animals (Fig. 1F and L). Global ischemia induced extensive death of pyramidal cells in the cerebral cortex ($P<.001$ vs. vehicle-treated sham rats) and hippocampal CA1 subfield ($P<.001$ vs. vehicle-treated sham rats) at 7 days post ischemia (Fig. 2). RSV greatly reduced the ischemia-induced neuronal loss in hippocampus ($P<.01$ vs. vehicle-treated ischemia and vehicle-treated sham rats) and cerebral cortex ($P<.01$ vs. vehicle-treated ischemia and vehicle-treated sham rats), as shown in Fig. 2.

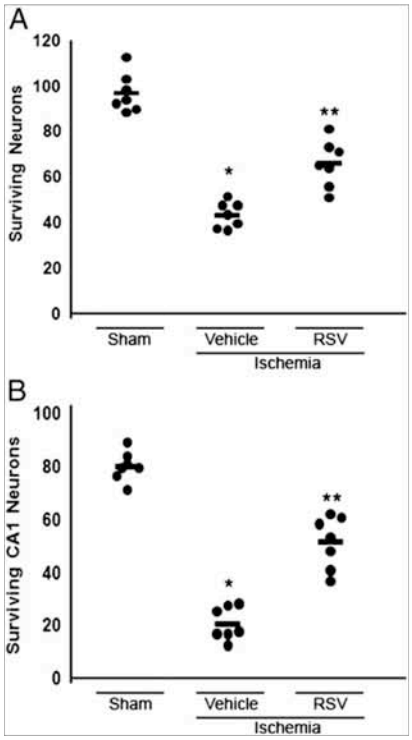

Fig. 2. Influence of RSV on numbers of survival neurons in cortex (A) and hippocampus (CA1) (B) in rats after 7 days of reperfusion. Data are mean±S.D. ($n=7$ each). One-way ANOVA revealed significant interactions between ischemia and RSV. *$P<.001$ compared with vehicle-treated sham group; **$P<.01$ compared with vehicle-treated ischemic and vehicle-treated sham group.

3.2. Biochemical analysis

3.2.1. ROS and NO content

The fluorogenic compound DCFH-DA is one of the most prominent markers to reflect the overall oxidative status in cells. As shown in Fig. 3A–B, rats that had undergone transient global cerebral ischemia for 10 min followed by 1 h of reperfusion exhibited 100% and 99% increase in ROS production in the cortex ($P<.01$) and hippocampus ($P<.01$), respectively, as compared with vehicle-treated sham animals. Decreases in basal levels were recorded at 24 h of reperfusion in cortex and hippocampus. The I/R induced ROS overproduction at 1 h of reperfusion was significantly decreased by RSV in cortex ($P<.01$) and hippocampus ($P<.01$) (Fig. 3A-B). No alterations in DCFH levels were observed in sham animals treated with or without RSV.

Nitrite is the major end-product of nitric oxide, which was undertaken to monitor NO production during ischemia. NO content significantly increased 1 h after transient global cerebral ischemia in cortex ($P<.01$) and hippocampus ($P<.001$) when compared to vehicle-treated sham rats (Fig. 3C-D). After 24 h of cerebral injury, NO content returned to basal level. Administration of RSV significantly inhibited the increase of NO content in cortex ($P<.05$) and hippocampus ($P<.01$) when compared to vehicle-ischemic group (Fig. C-D). No alterations in NO content was observed in sham animals, treated with or without RSV.

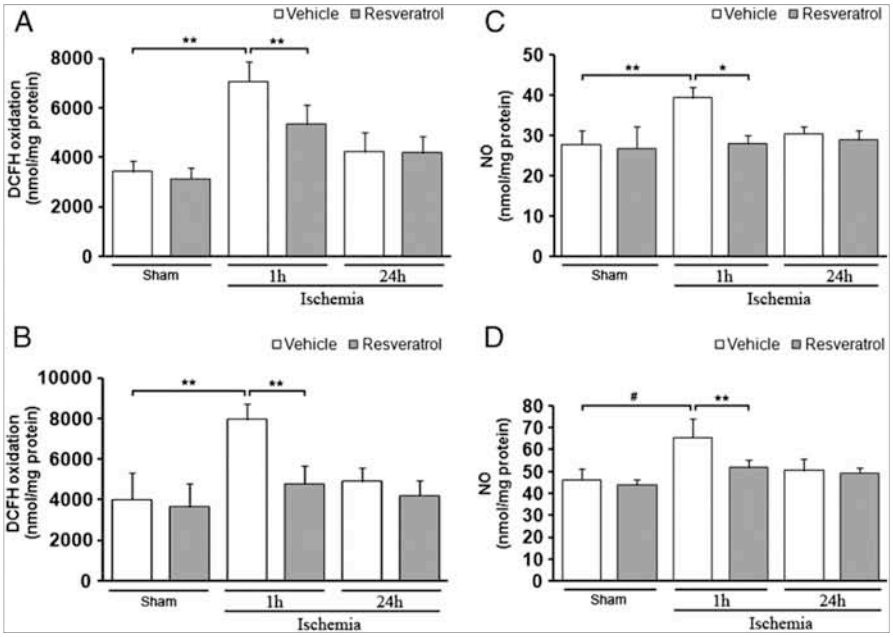

Fig. 3. Effect of RSV on ROS generation (A and B) and NO content (C and D) from cortex (A and C) and hippocampus (B and D) of rats at 1h and 24h after global cerebral ischemia. ROS levels were assessed by dichloroflurescein (DCF) assay. NO content was assessed by Griess reagent. Data are mean±S.D. (n=6 each). *P<.05; **P<.01; #P<.001.

3.2.2. Lipid peroxidation

When subjected to global cerebral ischemia, animals showed a significant increase in MDA in the cortex (P<.001) and hippocampus (P<.05) after 1 h (Fig. 4A–B). After 24 h of cerebral injury, MDA levels remained increased in the cortex (P<.01) and hippocampus (P<.05). As shown in Fig. 4A, ischemia-mediated lipid peroxidation decreased significantly in RSV-treated rats in cortex 1 (P<.05) and 24 h (P<.05) after ischemia. Hippocampus showed the same pattern and reversed the alterations in MDA levels in 1 (P<.05) and 24 h (P<.05) after ischemia (Fig. 4B). No alterations in MDA levels were observed in sham operated animals treated with or without RSV.

3.2.3. Na^+K^+-ATPase

As shown in Fig. 5(A–B), ischemia significantly reduces Na^+,K^+-ATPase activity in the cortex (P<.01) and hippocampus (P<.05) after 1 h when compared to vehicle-treated sham groups. After 24 h of reperfusion, maximal decrease of Na^+,K^+-ATPase activity was observed in the cortex (P<.01) and hippocampus (P<.05). In RSV-treated ischemia, the decrease in Na^+,K^+-ATPase was significantly smaller in the cortex (1 h, P<.05 and 24 h, P<.05) (Fig. 5A) as well as in the hippocampus (1 h, P<.05 and 24 h, P<.05) after ischemia (Fig. 5B). The administration of RSV and vehicle in the sham animals did not alter Na^+,K^+-ATPase activity.

3.2.4. The activities of antioxidant enzymes

The effect of RSV on the antioxidant capacity of hippocampus and cortex homogenates was studied by determining TRAP, which represents non-enzymatic antioxidant quantity. It can be observed in Fig. 5C that global cerebral ischemia decreased TRAP in the cortex in 1 h (P<.05) and 24 h (P<.05) when compared to vehicle-treated sham group. Furthermore, ischemia significantly decreased TRAP in the hippocampus in 1 h (P<.05) and 24 h (P<.05) when compared to vehicle-treated sham groups (Fig. 5D). RSV-treated rats significantly prevented the alterations on the antioxidant capacity in the cortex 24 h (P<.05) after reperfusion when compared to vehicle-treated ischemic group (Fig. 5C). Antioxidant capacity in hippocampus was significantly prevented by RSV-treatment in 1 h (P<.05) and 24 h (P<.05) after reperfusion when compared to ischemia-group (Fig. 5D).

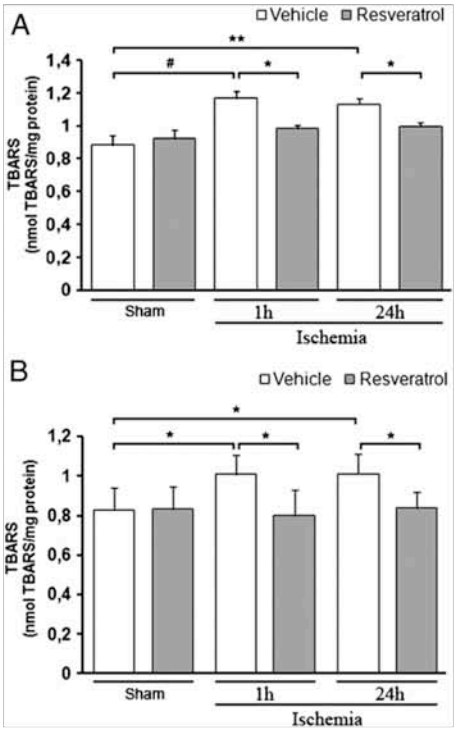

Fig. 4. Effect of RSV on lipid peroxidation level in rats cortex (A) and hippocampus (B) at 1 and 24 h following 10 min of global cerebral ischemia. TBARS is expressed as nanomoles of malondialdehyde per mg protein. Data are mean±S.D. (n=6 each). *P<.05; **P<.01; #P<.001.

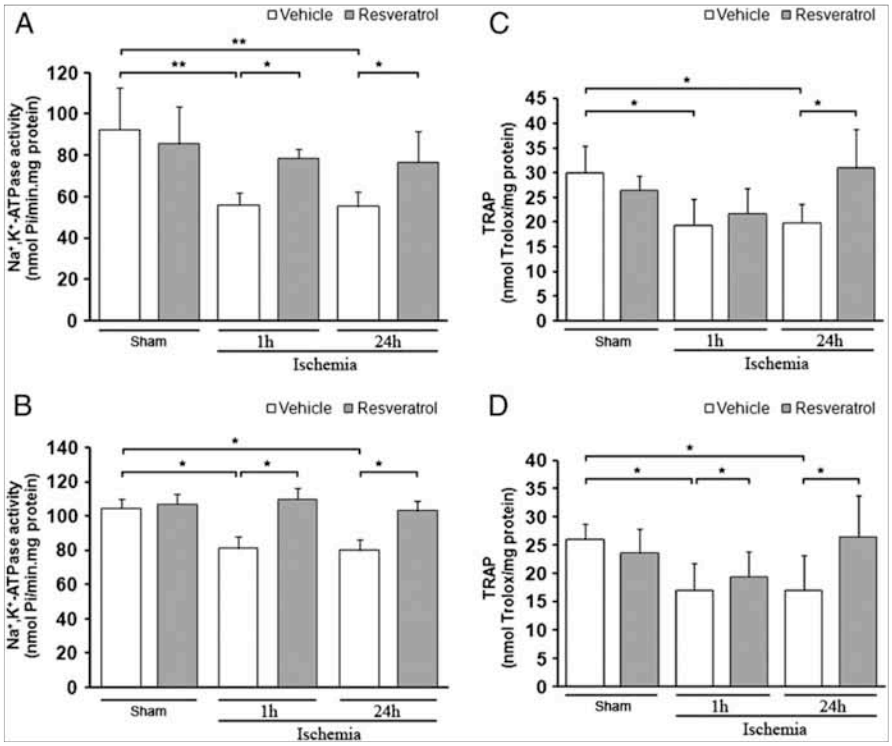

Fig. 5. Effect of global cerebral ischemia after 1 and 24 h and RSV treatment on Na^+,K^+-ATPase activity and antioxidant capacity (TRAP) in cortex (A and C) and hippocampus (B and D). Na^+,K^+-ATPase is expressed in nanomoles of Pi per milligram of protein and TRAP in nanomoles of Trolox per milligram of protein. Data are mean±S.D. (n=6 each). *P<.05; **P<.01.

After 1 h and 24 h of reperfusion, the hippocampus of ischemic animals showed a significant increase in SOD and GPx, whereas there was a significant decrease in catalase, as compared with the vehicle-treated sham animals (Table 1). Cortex showed a significant increase in SOD and a significant decrease in catalase, as compared with the vehicle-treated sham animals (Table 1). Treatment with RSV markedly reversed the alterations in enzymatic parameters brought about by I/R. The values were almost restored to near-normal levels with no significant differences versus the vehicle-treated sham group.

Table 1
Effect of RSV on hippocampus and cortex oxidative stress parameters (SOD, catalase and GPx levels) after global cerebral ischemia

Groups (n=6)	Time of reperfusion (h)	SOD (U/mg protein)	CAT (U/mg protein)	GPx (U/mg protein)
Hippocampus				
Control	–	6.57±0.21	4.21±0.19	21.03±2.20
Control+RSV	–	7.46±0.71	4.02±0.32	23.31±2.85
Isch.+vehicle	1	7.78±0.74a*	2.98±0.41a#	25.91±0.59a#
Isch.+RSV	1	6.41±0.25b#	4.16±0.51b#	22.71±1.01b*
Isch.+vehicle	24	7.93±1.15a*	3.26±0.45a*	26.01±3.04a#
Isch.+RSV	24	6.36±1.04c#	4.24±0.92c*	21.50±2.71c#
Cortex				
Control	–	4.69±0.18	4.68±0.63	23.74±2.73
Control+RSV	–	4.94±0.19	4.47±0.69	26.11±2.97
Isch.+vehicle	1	5.29±0.38a*	3.00±0.8a*	24.48±1.74
Isch.+RSV	1	4.97±0.32b*	4.48±1.03b*	21.97±1.65
Isch.+vehicle	24	5.26±0.29a*	3.10±0.91a*	24.11±0.81
Isch.+RSV	24	4.76±0.36c*	4.41±1.20c*	21.93±2.68

RSV (30 mg/kg) was administered intraperitoneally 7 days before occlusion. Values are expressed as mean±S.D. with n=6 per group.
*P<.05 and #P<.01: a, vs. control animals; b, vs. ischemic animals 1 h after reperfusion and c, vs. ischemic animals 24 h after reperfusion (tested using one-way ANOVA followed by Duncan multiple comparison test).

4. Discussion

The removal of pathologically generated free radicals during I/R seems to be a viable approach to neuroprotection. The present study demonstrates that pretreatment with RSV prevented both structural and biochemical abnormalities in rats subjected to global cerebral ischemia. RSV pretreatment protected the brain tissue against the increase of lipid peroxidation, NO levels and ROS formation as well as the decrease of Na^+K^+-ATPase activity and disrupted antioxidant defenses (enzymatic and non-enzymatic). As oxidative stress is closely associated with ischemic neuronal death after global cerebral ischemia, the preservation of the neuronal integrity in our model was provided presumably via the direct and indirect antioxidant properties of RSV [17,18]. Supporting this assumption, RSV prevented the loss of pyramidal cells in the hippocampal CA1 layer and cerebral cortex, a finding consistent with a previously published studies reporting that RSV ameliorates neuronal damage in CA1 following I/R [22,35].

Our results have shown a sustained increase of lipid peroxidation markers following transient global ischemia, which was at 1 h and 24 h of recirculation. These findings are consistent with other studies, which have found that the early increased level of lipid peroxidation persists for several days (1 h–4 days) after brief global cerebral ischemia in the hippocampus and 1 h to 24 h in cortex [4,36]. ROS production in the cortex and hippocampus returned to basal levels after being increased at 1 h and at 24 h after transient global cerebral

ischemia. These results are in agreement with other studies, which have found an early increase in ROS generation and a decrease in the hippocampus 24 h after brief global cerebral ischemia. In addition, high levels of LPO were observed in the hippocampus and cortex 24 h after ischemia. This may possibly involve mechanisms that account for the late increase in oxidative damage, delaying the induction of ROS-generating enzymes like cyclooxygenase-2 [37] and nitric oxide synthase [38]. RSV pretreatment decreased LPO levels, an effect that could be attributed to its capacity to scavenge ROS, indicating that the neuroprotection conferred by RSV is due to its anti-oxidative effect of attenuating ROS formation and lipid peroxidation. RSV was showed to restore decrease lipid peroxidation in different tissues exposed to ischemia [17,39,40]. A number of studies have demonstrated the antioxidant properties of RSV, for example, its ability to protect against oxidative DNA damage in stroke-prone hypertensive rats [41], to suppress lipid peroxidation [42] and to inhibit cerebral mitochondrial ROS production [43].

One of the ROS that elevates in cerebral ischemia is NO nevertheless; its precise role in this neuropathology remains controversial [7,44]. NO is beneficial as a messenger or modulator, but in conditions such as oxidative stress, it is potentially toxic. The toxic effects of NO may be attributed to peroxynitrite ($ONOO^{\cdot -}$), which is a reaction product of NO with superoxide ($O_2^{\cdot -}$) [45]. It may be suggested that diminishing the formation of $ONOO^{\cdot -}$ might reduce the potential for damage to neurons and supporting elements in the brain observed with ischemia and reperfusion. NO synthase activity and NO release are greatly increased in the acutely ischemic brain [46]. We showed that measurement of NO in the hippocampus and cortex homogenate revealed that all were significantly increased response to I/R. The data is consistent with increased oxidative stress. The level of NO in the brain was diminished in response to treatment with RSV. RSV was shown to increase NO levels up to basal levels in normal conditions [47-49]. Under physiological conditions, NO is an important endogenous vasodilator that regulates cerebral blood flow, and mediate vascular response to a diverse group of stimulations. Recently, Kiziltepe et al., [39] showed that RSV pretreatment increased NO levels in plasma during ischemia but not during the reperfusion period. Therefore, we suggest that our findings are consistent with this report and decreasing NO in the reperfusion period could reduce the delayed neuronal death induced by oxidative stress.

Neuronal damage caused by global cerebral ischemia is associated with an imbalance in ionic homeostasis. It has been shown that inhibition of Na^+,K^+-ATPase activity by ouabain induces the release of neurotransmitters including glutamate, which is proposed to play a major role in neuronal death after excitotoxic and ischemic insults [39]. A possible mechanism to explain ischemia-induced inhibition of Na^+,K^+-ATPase is the well-documented increase in free fatty acids and ROS following ischemia [50]. It has been demonstrated that the structural properties [51] and lipid composition [52] of synaptosomal membrane are essential for enzyme activity and that even low concentrations of free fatty acids and free radicals inhibit Na^+,K^+-ATPase activity of rat brain. Therefore, the period of reperfusion is highly suitable for lipid peroxidation. Previous findings suggest that reperfusion produces a decrease of Na^+,K^+-ATPase activity in early periods that is maintained during 24 h, corroborating findings from Wyse and colleagues [16]. The present data shows that RSV pretreatment prevents the inhibition of Na^+,K^+-ATPase activity in the hippocampus and cortex, suggesting that during cerebral ischemia, RSV decreases ROS formation and impairment in membrane permeability due to lipid peroxidation causing a decrease in Na^+,K^+-ATPase enzyme activity. In regards to the possible consequences of the inhibition of Na^+,K^+-ATPase activity to neural cellular metabolism and function, it should be stressed that there is increasing evidence suggesting that alterations in Na^+,K^+-ATPase activity may be a link between many common neurotoxic mechanisms in neurons [14]. In this context, blockage of the Na^+,K^+-ATPase activity secondary to energy depletion and free radical attack may be a common event in the apoptotic cascade [53].

Oxidative stress is the result of a redox imbalance between the generation of ROS and the compensatory response from the endogenous antioxidant network. In the present study, SOD and GPx activities increased in ischemic rats. The increase in GPx activity may be a compensatory response for an increase in endogenous H_2O_2 production in ischemic brain because ROS formation promotes the oxidation of fatty acids with resulting H_2O_2 formation [54]. The increase in SOD activity could be due to its induction by increased production of superoxide and H_2O_2 was reported to act as an inducer of tissue SOD. Increases in both SOD and GPx activities may thus be an adaptive response for increased oxidative stress in the brain tissue. RSV, by scavenging ROS, prevents the elevation of those antioxidant enzyme activities in ischemic rat brain. The presence of molecules and macromolecules capable of transforming active radicals into inactive species (e.g., chain-breaking antioxidants) constitutes one of these defenses. This type of defense includes a variety of compounds bearing different reactive centers (e.g., phenols, thiols) with widely different hydrophobicities that allow the trapping of both hydrophobic and hydrophilic radicals. In this regard, there is great interest in the evaluation of the total reactive antioxidant potential (TRAP), and several procedures have been developed to measure it in biological fluids and animals [55]. The interest in this type of determination resides in that they can provide information regarding the system's capacity to withstand oxidative stress disbalances. In this view, decreasing TRAP to energy depletion and free radical attack may be an event in the global cerebral ischemia-induced delayed neuronal death. RSV pretreatment brought TRAP measurements back to normal levels in the cortex and hippocampus and contributed to the neuroprotective effect after transient global cerebral ischemia.

In conclusion, this study suggests that RSV pretreatment attenuates ischemia/reperfusion-induced cerebral injury decreasing oxidative stress and maintaining Na^+K^+-ATPase activity. These observations suggest that RSV may be a clinically viable protective agent against a variety of conditions where cellular damage is a consequence of oxidative stress. In addition, RSV may have the potential to be used in the prevention of neurodegenerative diseases such as forebrain ischemia.

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4. CAPÍTULO 2

Artigo: Resveratrol prevents global cerebral ischemia-induced decrease of lipid content in rat hippocampus and cerebral cortex – Submetido a *Neuroscience Research*

Resveratrol prevents global cerebral ischemia-induced decrease of lipid content in rat hippocampus and cerebral cortex.

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SHORT COMMUNICATION

ABSTRACT

Changes in brain membrane lipid composition are one of the important mechanisms proposed to be involved on the neuronal damage pathway triggered by brain ischemia and reperfusion. It has been shown that resveratrol, a polyphenol found in grapes, modulates oxidative stress and lipid peroxidation. Here we investigated gangliosides, phopholipids and cholesterol contents in hippocampus and cerebral cortex from rats subjected to global cerebral ischemia treated or not with resveratrol. Our experiments indicate that lipid metabolism is important for development of ischemic damage and suggest that resveratrol treatment 7 days prior to ischemia may prevent membrane lipid loss.

Keywords: cerebral ischemia, resveratrol, gangliosides, phospholipids, Cholesterol.

Neuronal damage subsequent to transient cerebral ischemia is a multifactorial process involving several overlapping mechanisms. It has been shown that brain contains many complex lipids, such as gangliosides, phospholipids and cholesterol, presenting a variety of important physiological functions in neurons (Agranoff & Hajra, 1994). Among them, gangliosides are a family of sialic acid-containing glycosphingolipids present in a high concentration on neuronal membranes. They play important biochemical roles in cell biology, taking part of processes like cell differentiation and maturation, synaptogenesis, intercellular communication and neuronal plasticity (Yu et al., 2009). Phospholipids perform structural functions in the membrane, as well as cellular signaling functions. Cholesterol is an essential lipid component of plasma membranes modulating its physicochemical properties and contributing to raft formation, so consequently affecting membrane protein function (Ohvo-Rekilä et al., 2002). Changes on lipid membranes were observed in different experimental CNS damages such as neonatal hypoxia-ischemia (Ramirez et al., 2003), malnutrition (Trindade et al., 1992), organic acid and amino acid inborn errors (Stefanello et al. 2007; Wajner et al., 1988).Resveratrol (RSV; *3,5,4´-trihydroxy-trans-stilbene*) is found in many plant species such as grapes, berries and peanuts and exhibits pleiotropic health beneficial effects, including anti-oxidant, anti-inflammatory, cardioprotective and anti-tumor activities (de la Lastra & Villegas, 2005; Delmas et al., 2005). In a previous study we demonstrated that resveratrol has neuroprotective effect against global ischemia injury by modulating oxidative stress, lipid peroxidation and Na^+,K^+,ATPase activity, as well as decreasing delayed neuronal death observed after ischemia/reperfusion (I/R)

(Simão et al., in press). Since these parameters are related with neuronal membrane lipid composition, our proposal aimed to investigate the effect of resveratrol pre-treatment on the content and distribution of gangliosides, as well as phospholipid and cholesterol concentrations in cerebral cortex and rat hippocampus after transient global cerebral ischemia.

Adult male Wistar rats (290–330 g) were obtained from the Department of Biochemistry, Federal University of Rio Grande do Sul, Porto Alegre, Brazil. Animal care followed the official governmental guidelines in compliance with the Federation of Brazilian Societies for Experimental Biology. Transient global cerebral ischemia was induced by the four-vessel occlusion method. Briefly, rats were anesthetized with a mixture of 70% nitrous oxide, 30% oxygen and 1.5% halothane during surgical operation. On day 1, both cervical vertebras were exposed, and the visible vertebral arteries were permanently electrocauterized. On day 2, both common carotid arteries were exposed and occluded with aneurysm clips for 10 min. Sham-operated animals received exactly the same surgical procedure but without arterial occlusion (Simão et al., 2010). Resveratrol (courtesy Pharmascience, Montreal, Canada) was intraperitoneally administered at the dose of 30 mg/kg for 7 days prior ischemia. Ethanol (50%, 0.1 mL v/v/100 g) was treated into rats as vehicle according to the same volume and time schedule of resveratrol. After the reperfusion periods of 1, 24 h and 7 days, animals were sacrificed, cerebral cortex and hippocampus were isolated for biochemical studies.

After remotion, hippocampi and cortex were weighed and homogenized in a 2:1 mixture of chloroform:methanol (C:M, 2:1, v/v) to a 20-fold dilution of tissue mass and centrifuged at 800 x g for 10 min. The sediment was re-

homogenized in C:M (1:2) to a 10-fold dilution of original sample mass (Folch et al., 1959). The C:M extracts were combined and the this pool was used for the following determinations. Aliquots corresponding to the tenth part from this pool (total lipid extract) were used for total ganglioside determination by the N-acetyl-neuramic acid (NeuAc) quantification with the thiobarbituric acid method described by Skoza e Mohos (Skoza et al., 1976). Ganglioside species were analyzed by thin layer chromatography (TLC) on 10 X 10 cm Merck plates of silica gel 60 using a developing tank described by Nores et al. (1994). Aliquots of the total lipid extracts containing 4 nmol of NeuAc suspended in 10 μL C:M (1:1) were spotted on 8 mm lanes. TLC was developed, sequentially, with two mixtures of solvents, firstly C:M (4:1, v/v) and secondly C:M: 0.25 % CaCl2 (60:36:8, v/v). Ganglioside profile was visualized with resorcinol reagent. The chromatographic bands were quantified by scanning densitometry at 580 nm with Geliance 600 Image System (PerkinElmer, USA). The terminology used herein for gangliosides is that recommended by Svennerholm (Svennerholm et al., 1963). Total phospholipid evaluations were done using aliquots corresponding to the fourth part of the total lipid extract original volume were evaporated and quantified according to the method of Bartlett (1959). The absolute total quantity of phospholipids-inorganic phosphorus was expressed as nmol Pi/mg tissue weight. Cholesterol determinations were performed using aliquots of 100 μL of total lipid extract. These volumes were evaporated, the residue suspended in isopropanol and used for cholesterol evaluations according to the Trinder enzymatic method (Bergmeyer et al., 1974). Data are expressed as mean + S.D. Analysis of variance (ANOVA) was applied to the means to determine

statistical differences between experimental groups. *Post hoc* comparisons were performed with Tukey test for multiple comparisons. Values of $p < 0.05$ were considered to be significant.

Global cerebral ischemia caused a significant decrease in the gangliosides, phospholipids and cholesterol contents in the hippocampus observed at 24 h of reperfusion ($p<0.05$) (Fig. 1A-C), which remained decreased after 7 days of reperfusion ($p<0.05$). However 1 h after injury, this effect was not detected. Resveratrol-treated group prevented the lipid concentration decreased significantly in hippocampus 24 h ($p<0.05$) and 7 days ($p<0.05$) (figure 1A-C) when compared with ischemia vehicle group. The same pattern of results was observed in cerebral cortex at 24 h ($p<0.05$) and 7 days ($p<0.05$) after ischemia (Fig. 2A-C) when compared with sham group. In a similar fashion, resveratrol treated-group in cerebral cortex prevented the lipid concentration decreased in 24 h ($p<0.05$) and 7 days ($p<0.05$) (figure 2A-C) when compared with ischemia vehicle group. No changes on lipid levels were observed in sham-operated animals treated or not with resveratrol. The profile of the ganglioside species from vehicle and resveratrol treated rat hippocampus and cerebral cortex, and from different experimental groups was apparently unaffected (TLC not shown). The four main gangliosides present in the brain (GM1, GD1a, GD1b and GT1b) were detected in both rat cerebral regions studied. Table 1 shows that the percentage distribution of these ganglioside species was also apparently unaltered. Therefore all gangliosides have their absolute quantity variations similar to the total ganglioside content changes observed in this study.

In the present study, we evaluated the effect of resveratrol treatment on total lipid content in hippocampus and cerebral cortex of rats subjected to global cerebral ischemia. Results showed that resveratrol significantly prevented the reduction on the total content of gangliosides, phospholipids and cholesterol in hippocampus and cerebral cortex induced by global cerebral ischemia. Brain damage following cerebral ischemia results from mechanisms directly related to the ischemic insult *per se* (energy failure and acute cellular necrosis) and from secondary mechanisms that occur over an extended time. These mechanisms might include: disruption of ionic homeostasis, activation of degrading enzymes, reduction in Na^+,K^+-ATPase activity, increase on cellular oxidative stress that could damage DNA, cell proteins and lipids, by this way leading to cellular death (Northington et al., 2001). One or more of these processes lead to a structural membrane destabilization and failure of its function. In global ischemia, cerebral phospholipid changes are thought to result from increased phospholipases (Edgar et al., 1982), and consequently alter the plasma membrane structure. Since membrane phospholipids are directly involved in regulating Na^+,K^+-ATPase activity (Harris et al., 1985), it is hypothesized that lipid changes associated with ischemia, decrease the order of membrane components resulting in decreased Na^+,K^+-ATPase activity, therefore leading to an energy-dependent ionic imbalance.

In this context, we have previously demonstrated that RSV pretreatment protected the brain tissue against the increase of lipid peroxidation, NO levels and ROS formation as well as the decrease of Na^+K^+-ATPase activity and disrupted antioxidant defenses (enzymatic and non-

enzymatic) induced by global cerebral ischemia (Simão et al., 2010). We propose that the alterations caused by global ischemia on lipid content could be, at least in part, due to oxidative damage of the membrane lipids, since we showed high levels of lipid peroxidation and reactive oxygen species. This fact could induce changes in lateral assembly of glycosphingolipids, unsaturated glycerophospho- lipids and cholesterol and alter Na^+,K^+-ATPase activity as suggested by other investigators (Welker et al., 2007). Cholesterol is an important component of myelin (Agranoff et al., 1994), our result showed that after 7 days of global cerebral ischemia the content of cholesterol was reduced suggesting an impairment of axon myelination. The profile of different ganglioside species from control and global ischemia groups were apparently unaffected, however, absolute quantity decreased in hippocampus and cerebral cortex. Resveratrol brought gangliosides content in hippocampus and cortex back to control levels. Since gangliosides can prevent glutamate and kainite induced neurotoxicity, these molecules could be considered a class of negative modulators of specific enzyme activation caused by the persistent stimulation of excitatory amino acid receptors. It has been reported that gangliosides inhibit the intensification of free radicals reactions induced by glutamate (Avrova et al., 1998) and also protect polyenoic fatty acids from oxidative damage in synaptosomes of rat brain (Tyurina et al., 1993). In addition, gangliosides could prevent excitatory aminoacid-induced neuronal death following brain ischemia and might reflect in the neuronal stabilization and consequently in the neuronal network (Skaper et al., 1989). In conclusion, the present study demonstrates a deleterious effect on important structural

lipid components in consequence of global cerebral ischemia, which can be prevented by resveratrol.

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Legends of figures

Figure 1. Concentration of gangliosides (A), phospholipids (B) and cholesterol (C) in the hippocampus of rats submitted to global cerebral ischemia followed by different reperfusion periods. Bars represent mean ± S.D. of 8 determinations. Ganglioside concentrations are expressed in nmol NeuAc/mg protein, phospholipid concentrations are expressed in nmol Pi/mg protein and cholesterol concentrations are expressed in µg cholesterol/mg protein. * $p<0.05$ vs. vehicle-treated sham group, # $p<0.05$ vs. vehicle-treated ischemia group.

Figure 2. Concentration of gangliosides (A), phospholipids (B) and cholesterol (C) in the cerebral cortex of rats submitted to a global cerebral ischemia episode followed by different reperfusion periods. Bars represent mean ± S.D. of 8 determinations. Ganglioside concentrations are expressed in nmol NeuAc/mg protein, phospholipid concentrations are expressed in nmol Pi/mg protein and cholesterol concentrations are expressed in µg cholesterol/mg protein. * $p<0.05$ vs. vehicle-treated sham group, # $p<0.05$ vs. vehicle-treated ischemia group.

Figura 1

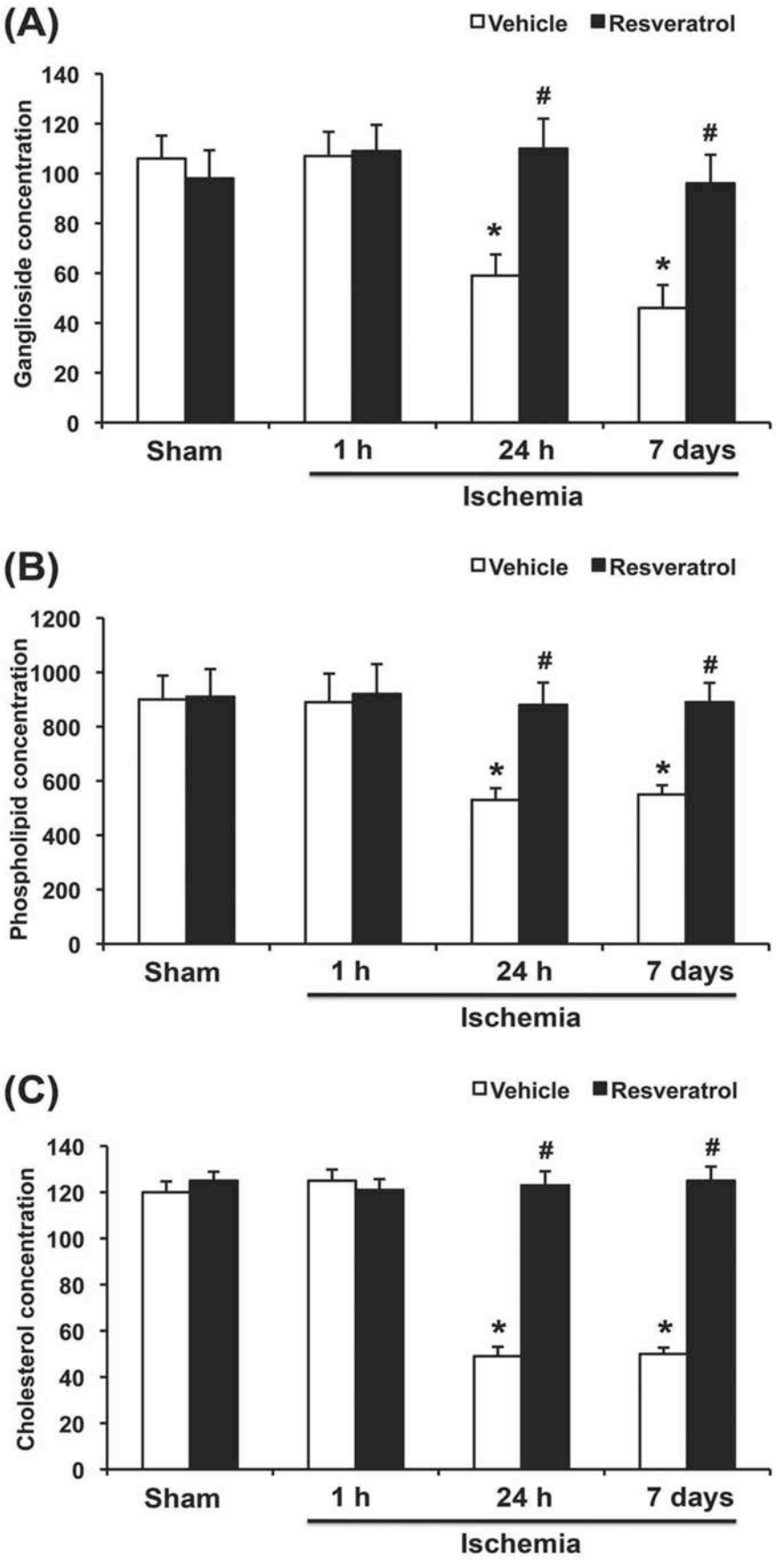
(A)
Vehicle
Resveratrol
Ganglioside concentration
140
120
100
80
60
40
20
0
*
#
Sham
1 h
24 h
7 days
Ischemia
(B)
Vehicle
Resveratrol
Phospholipid concentration
1200
1000
800
600
400
200
0
*
#
Sham
1 h
24 h
7 days
Ischemia
(C)
Vehicle
Resveratrol
Cholesterol concentration
140
120
100
80
60
40
20
0
*
#
Sham
1 h
24 h
7 days
Ischemia

Figura 2

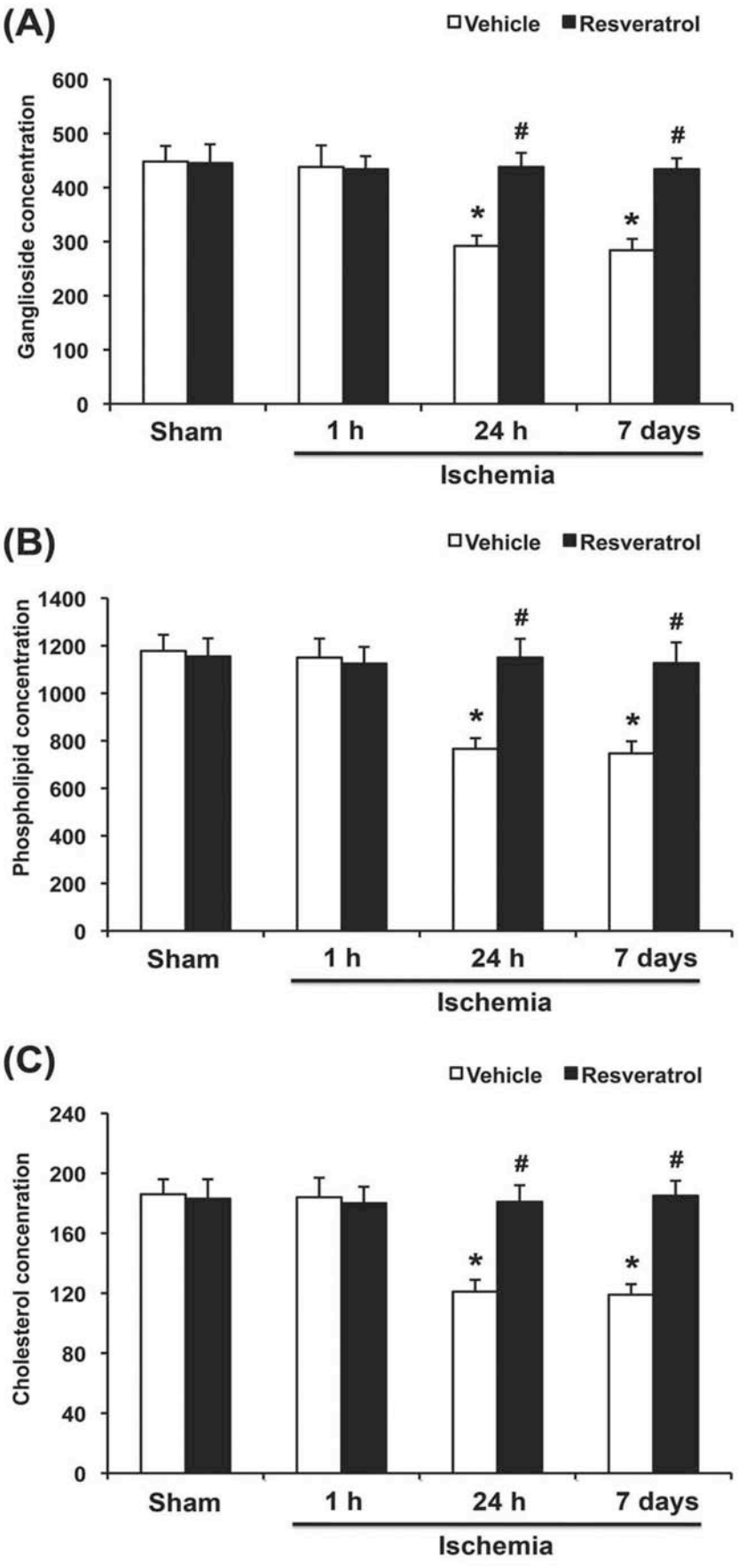
(A)
Vehicle
Resveratrol
Ganglioside concentration
600
500
400
300
200
100
0
*
#
Sham
1 h
24 h
7 days
Ischemia
(B)
Vehicle
Resveratrol
Phospholipid concentration
1400
1200
1000
800
600
400
200
0
*
#
Sham
1 h
24 h
7 days
Ischemia
(C)
Vehicle
Resveratrol
Cholesterol concenration
240
200
160
120
80
40
0
*
#
Sham
1 h
24 h
7 days
Ischemia

Table 1. Ganglioside distribution in hippocampus and cerebral cortex of rats at different times of reperfusion after global cerebral ischemia.

	Hippocampus				Cortex			
		Ischemia				ischemia		
Group	Sham	1 h	24 h	7 days	Sham	1 h	24 h	7 days
Vehicle								
GM1	15.7±0.9	14.4±2.5	14.4±1.3	17.7±2.0	19.5±1.8	18.7±1.9	17.4±1.4	18.8±1.7
GD1a	33.6±1.8	34.7±2.4	36.8±3.6	34.2±2.5	34.5±2.9	34.2±3.1	35.3±3.4	36.2±3.5
GD1b	19.2±1.6	18.7±1.5	17.3±4.6	16.8±3.5	18.7±1.9	19.7±1.8	22.9±2.7	21.7±2.5
GT1b	31.5±1.8	32.2±3.7	31.5±3.4	31.3±3.7	27.3±2.3	27.4±2.1	24.4±2.2	23.3±2.4
Resveratrol								
GM1	14.8±1.0	13.3±4.3	12.3±1.8	13.4±1.7	19.3±1.4	18.6±1.5	17.1±1.3	18.6±1.7
GD1a	35.8±2.1	29.7±2.6	30.9±3.1	29.8±1.9	33.6±3.4	35.3±3.3	34.8±3.2	33.4±3.7
GD1b	18.5±2.3	17.5±2.9	18.4±1.9	16.2±1.7	18.5±1.7	17.9±1.8	21.8±1.9	22.3±2.1
GD1b	30.9±1.9	39.5±3.9	39.4±3.7	40.6±4.4	28.6±2.5	28.2±2.7	26.3±2.4	25.7±2.3

Resveratrol (30 mg/kg) was administered intraperitoneally 7 days before artery occlusion. Results correspond to densitometric analysis of 8 TLC. Every ganglioside is expressed as a percentage of total gangliosides content. Data are mean ± SD.

5. CAPÍTULO 3

Artigo: Resveratrol-mediated neuroprotection in global cerebral ischemia correlates with CREB and GSK-3β activation-dependent of PI3-K/Akt – Submetido a *Experimental Neurology*

Resveratrol-mediated neuroprotection in global cerebral ischemia correlates with CREB and GSK-3β phosphorylation-dependent of PI3-K/Akt

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Abstract

Accumulating evidence indicates that the polyphenol resveratrol potently protects against cerebral ischemia damage due to its oxygen free radicals scavenging and antioxidant properties. However, the role and contribution of PI3-K/Akt/GSK-3β and cyclic-AMP response element binding protein (CREB) is unclear and was the subject of the current study. The purpose of this study was to determine whether resveratrol protects against delayed neuronal cell death in hippocampal CA1 following transient global cerebral ischemia in rats and to study the cell signaling mechanism responsible for the neuroprotective effects of resveratrol. Experimental model of transient global cerebral ischemia was induced in Wistar rats by the four vessel occlusion method for 10 min and followed by different periods of reperfusion. Nissl stained indicated extensive neuronal death at 7 days after I/R. Administration of resveratrol by i.p. injections (30 mg/kg) for 7 days before ischemia significantly attenuated neuronal death. Both the Akt/GSK-3β and CREB signaling pathways appear to play a critical role in RSV neuroprotection, as RSV pre-treatment increased the phosphorylation of Akt/GSK-3β and CREB in 1h and 4h in CA1 hippocampus after global ischemia. Furthermore, administration of LY294002, an inhibitor of PI3-K, compromised the neuroprotective effect of RSV and decreased the level of p-Akt, p-Gsk-3β and p-CREB after injury. Taken together, the results suggest that resveratrol protects against delayed ischemic neuronal death in the hippocampal CA1 by maintaining the pro-survival states of Akt/GSK-3β and CREB signaling pathways pointing to that

resveratrol's neuroprotection is mediated in part by induction of CREB and GSK-3β through PI3-K/Akt-dependent.

Keywords: Cerebral ischemia, resveratrol, neuroprotection, CREB, GSK-3β, PI3-K/Akt.

1. INTRODUCTION

Resveratrol (3, 4, 5-trihydroxy-trans-stilbene) occurs naturally in grapes and a variety of medicinal plants, which possesses multiple biological activities (Delmas et al., 2005; Lastra and Villegas, 2005). In particular, recent experimental evidences have demonstrated that resveratrol exhibits neuroprotective effect in a variety of model for cerebral ischemic stroke (Huang et al., 2001; Gao et al., 2006). The resveratrol neuroprotective effects have been associated with its antioxidant (Sinha et al., 2002; Wang et al., 2002; Kiziltepe et al., 2004; Lu et al., 2006), anti-platelet aggregation and vasodilatory effect (Shigematsu et al., 2003), anti-inflammation (Birrel et al., 2005) and anti-ageing properties (Wood et al., 2004).

Resveratrol has been shown to modulate functions of many regulatory proteins involved in signal transduction, and to affect a variety of cellular activities including cell growth, survival and death (Das, 2005; Zamin et al., 2006). Nitric oxide (NO) (Hattori et al., 2002; Imamura et al., 2002; Hung et al., 2004), adenosine receptors (Das et al., 2005a,b), PI3-kinase (Das et al., 2006a,b), and mitogen-activated protein kinase (MAPK) (Das et al., 2006a,b) have been proposed to mediate the cardioprotective effect of resveratrol-induced pharmacological preconditioning. Furthermore, inhibition of these signaling pathways can attenuate the protective effects of resveratrol. On the other hand, the relationship between neuroprotective effect of resveratrol and signal molecules in the ischemic brain has not been well elucidated. Our previous study (Zamin et al., 2006) reveals that resveratrol could exert neuroprotective effect against ischemic injury by modulating PI3-K and MAPK signaling pathway in organotypic culture submitted to oxygen glucose

deprivation. In addition, the induction of mitochondrial SOD2 by resveratrol was mediated through the activation of the PI3-K/Akt and GSK-3β/β-catenin signaling pathways in HT22 cells (Fukui et al., 2010). Moreover, Chen and colleagues (2005) showed that resveratrol augments cellular antioxidant defense capacity by induction of HO-1 via Nrf2-ARE signaling through the transient activation of Akt and Erk1/2.

In the present work, we tested the hypothesis that resveratrol can induce endogenous increase of CREB and decrease the activation of GSK-3β in the brain to promote neuroprotection by activating the PI3-K/Akt pathway in rat hippocampi after global cerebral ischemia.

2. Methods

2.1. Transient global cerebral ischemia and Resveratrol treatment

Adult male Wistar rats (290–330 g) were obtained from the Central Animal House of the Department of Biochemistry, Institute for Basic Health Sciences, Federal University of Rio Grande do Sul, Porto Alegre, RS, Brazil. Animals were maintained under a 12/12 h light/dark cycle, in an ambient temperature (24 ± 1 °C) colony room. Animals were provided with a constant supply of food (*ad libitum*) and water. Animal care followed the official governmental guidelines in compliance with the Federation of Brazilian Societies for Experimental Biology and experimental protocols were conducted with the approval of the Ethics Committee of the Federal University of Rio Grande do Sul, Brazil. Transient global cerebral ischemia was induced using a previously described rat model or four-vessel occlusion (Simao et al., in press). In briefly, rats were anesthetized with a mixture of 70% nitrous oxide, 30% oxygen and 1.5% halothane during surgical operation. Global ischemia was induced by cauterization and cutting of both vertebral arteries and temporarily occluding both common carotid arteries for 10 min. Reperfusion started when aneurysm clips were removed and patency of arteries was confirmed by inspection. The rectal temperature was monitored and maintained at 37 ± 0.5 °C with a feedback-controlled heating-pad. Sham operations were performed in additional animals using identical surgical procedures, except that the common carotid arteries were not occluded.

Resveratrol (RSV, courtesy Pharmascience, Montreal, Canada) was freshly prepared in 40% propylene glycol. It was administered at the dose

of 30 mg/kg, intraperitoneally every day for 7 days before ischemia. The vehicle-treated group received the same amount of propylene glycol for 7 days. In selected experiments, LY294002 (5 μL of 5 mM/L in dimethylsulfoxide/PBS) or vehicle were infused into the right i.c.v. within 10 min before ischemia with a Hamilton syringe, using the following coordinates from bregma: anteroposterior, 0.8 mm; lateral 1.5 mm; depth, 3.5 mm.

2.2 Cell fractionation and Western Blot Analisys

The rats were sacrificed under anesthesia at 1 h, 4 h and 7 day after ischemia. The hippocampal CA1 region was micro-dissected from both sides of the hippocampal fissure at 0 ^{o}C and quickly frozen in liquid nitrogen. The hippocampal samples were homogenized in buffer (10 mM HEPES, 10 mM KCl, 100 mM EDTA, 1.5 mM MgCl2, 0.05% Triton X-100, 1 mM PMSF, proteinase inhibitor cocktail, 20mM DTT, 0.5% NP40). The homogenate was then centrifuged at 4 °C at 13,000 g for 5 min. The resulting (1) pellet and (2) supernatant were treated separately to acquire the nuclear and cytosolic fractions, respectively. (1) The pellet was washed twice, resuspended in nuclear lysis buffer (20 mM Hepes, 400 mM NaCl, 0.25 mM EDTA, 25% glycerol, 1.5 mM MgCl2, 25 mM DTT, proteinase inhibitor cocktail) and centrifuged at 4 °C at 13,000 g for 20 min. The resulting supernatant was the nuclear fraction. (2) The resulting supernatant was recentrifuged at 17,000 g for 20 min, to isolate the cytosolic fraction; the pellet was discarded. The cytosolic or nuclear fractions were analyzed for protein content. 60 mg protein of each sample was heated at 100 °C for 5min with loading buffer containing

0.125 M Tris- HCL (PH 6.8), 20% glycerol, 4% SDS and 0.002% bromophenol blue, then separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) using 10-12% acrylamide gels. The proteins were transferred onto nitrocellulose membranes (pore size, 0.45 mm). Blotting membranes were incubated with 3% BSA in TBST (10 mmol/L Tris (PH 7.5), 150 mmol/L NaCl, 0.05% Tween-20) and probed with corresponding primary antibodies at 4 °C overnight. Blots were probed with antibodies recognizing Akt, phosphorylated-Akt (p-Akt) at Ser-473; GSK-3β, phosphorylated-GSK-3β (p-GSK-3β) at Ser9; CREB; and phosphorylated CREB (p-CREB) at Ser-133 (Cell Signaling Technology, Beverly, MA, USA). The bands on the membranes were scanned and analyzed with an image analyzer (Labworks Software, UVP Upland, CA, USA). Phospho-protein signals were expressed as a ratio to the corresponding total protein. Normalized means were then expressed relative to the ratio for sham-treated animals.

2.4. Nissl Staining

Neuronal cell loss was assessed by histological examination of nissl-stained brain sections at the level of the dorsal hippocampus from sacrificed animals 7 d after ischemia. Animals were deeply anesthetized with pentobarbital (50 mg/kg, i.p) and then transcardially perfused with cold saline followed by 4% paraformaldehyde in phosphate-buffered saline (0.1 M; pH 7.4). Brains were removed and immersed in fixative (4 °C overnight). Coronal sections (20 μm) were cut at the level of the dorsal hippocampus (3.3–4.0 mm posterior from bregma) with a cryostat (Leica Microsystems, Germany). Every

fourth section was collected and stained with Cresyl violet. The number of surviving pyramidal neurons per 250 μm length of the medial CA1 pyramidal cell layer was counted bilaterally in five sections per animal under a light microscope at 40X magnification. Cell counts from the right and left hippocampus on each of the four sections were averaged to provide a single value (number of neurons per 250 μm length) for each animal.

2.5 Statistical analysis

Data were analyzed by one way ANOVA followed by the Tukey multiple test. All analyses were performed using the Statistical Package for the Social Sciences (SPSS) software. Values of $p<0.05$ were considered to be significant.

3. Results

Akt signaling pathway plays a critical role in resveratrol-mediated neuroprotection

Akt is a master protein kinase that promotes neuronal survival after brain ischemia (Zhang et al. 2004), we therefore hypothesize that the Akt pathway may be important in resveratrol-mediated neuroprotection against CA1 neuronal injury induced by global cerebral ischemia in rats.

To prove our hypothesis, we first detected the phosphorylation of Akt in hippocampal CA1 tissues at 1, 4 and 24 h after global ischemia. At 1 h after ischemia, there was an increase in the phosphorylation of Akt in vehicle-treated groups; this effect was further enhanced by resveratrol and maintained after 4 h after ischemia (Fig. 1A and B). To confirm the protective role of Akt, we performed Akt inhibition experiment by injecting LY294002, a PI3-K inhibitor, into ICV 10 min before ischemia, and it was analyzed its effects on the phosphorylation of Akt and on the resveratrol mediated neuroprotection against ischemia. As shown in Fig. 1C and D, LY294002 significantly reduced the phosphorylation of Akt, which consequently compromised the neuroprotective effect of resveratrol (Fig. 4 and 5), indicating that Akt plays a critical role in the resveratrol-mediated neuroprotection against CA1 neuronal injury induced by transient global cerebral ischemia in rats.

Figure 1 and 2 near here

Phosphorylation of GSK-3β signaling pathway is involved in resveratrol-mediated neuroprotection

We previously reported that the GSK-3β signaling pathway plays a key role in the neuroprotective effect of resveratrol against injuries induced by oxygen-glucose deprivation (Zamin et al., 2006). To detect the role of GSK-3β phosphorylation in CA1 regions after global ischemia in rats, we performed western blots. As shown in Fig. 2A and B, an increase in the phosphorylation of GSK-3β was noticed at 1 h in vehicle treated rats, and this was significantly increased by resveratrol and remained high at 4h.

To confirm the role of GSK-3β as a substrate of PI3-K/Akt, we subsequently performed PI3-K inhibition experiment by injecting LY294002, a PI3-K inhibitor, into ICV before ischemia. As shown in Fig. 2C and D, LY294002 significantly reduced the phosphorylation of GSK-3β, indicating that GSK-3β inhibition is modulated by PI3-K signaling pathway.

Resveratrol increases CREB phosphorylation in the CA1 region after global ischemia

Resveratrol increases CREB phosphorylation in the CA1 region after global ischemia. It has been previously demonstrated that an increase in CREB phosphorylation is essential to the protective role of resveratrol against cardio injury induced by ischemia (Das et al., 2006). To determine whether CREB is also involved in resveratrol-mediated neuroprotection in the rat

global cerebral ischemia model, we performed western blots using the same protein samples as previously described. As shown in Fig. 3A and B, there was a significant increase in p-CREB in the resveratrol-treated group at 1 h post-ischemia, maintained at 4 h, when compared to the sham-operated group. Also the level of p-CREB increase in the vehicle-treated group at 1 h post-ischemia.

To confirm the role of CREB as a substrate of PI3-K/Akt, we subsequently performed PI3-K inhibition experiment by injecting LY294002. As shown in Fig. 3C and D, LY294002 significantly reduced the phosphorylation of CREB, indicating that CREB activation is modulated by PI3-K signaling pathway.

Figure 3 and 4 near here

3.1. Resveratrol is neuroprotective against brain ischemia through PI3-K pathway

Microphotographies of the hippocampal CA1 subfield for each group are shown in Fig. 4. Histological observation shows that neurons in the CA1 pyramidal cell layer (Fig. 4A and B) are clear and moderate-sized with normal ultrastructure in vehicle-treated sham rats. In the vehicle-treated ischemic rats, pyramidal neurons exhibited either significant shrinkage, dark staining appearance with minimal cytoplasm or outright loss of neurons and widespread damage was evident after 7 days (Fig. 4C and D). These neuropathological signs were decreased in resveratrol treated animals, which

reduced neuronal loss and moderate morphologic changes (Fig. 4G and H), while pretreatment with LY294002 and resveratrol aggravated cell death (Fig. 4I and J) compared with only resveratrol pretreatment. Global ischemia induced extensive death of pyramidal cells in the hippocampal CA1 subfield ($P < 0.001$ vs. vehicle-treated sham rats) at 7 days post-ischemia (Fig. 5). Resveratrol greatly prevented the ischemia-induced neuronal loss in hippocampus ($P < 0.05$ vs. vehicle-treated ischemia), as shown in Fig. 5. The LY294002-treated group decreased the neuroprotection induced by resveratrol in hippocampal CA1 regions (Fig. 5).

Figure 5 near here

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4. Discussion

The results of the current study provides evidence that activation of PI3-K signaling pathway by resveratrol rapidly induces activation of a Akt/GSK-3β/CREB signaling pathway and results in neuroprotective effects in the hippocampal CA1 region following global cerebral ischemia. Moreover, the inhibition of PI3-K signaling pathways plays critical roles in resveratrol-mediated neuroprotection. The data presented here confirm the observed neuroprotective effects of resveratrol to the prevention of hippocampal CA1 neuronal death induced by transient global cerebral ischemia (Wang et al., 2002, Della-Morte et al., 2009; Simão et al., in press).

The cell protective mechanisms of resveratrol involve Akt, Erk, Nitric oxide, CREB (Hattori et al., 2002; Imamura et al., 2002, Das et al., 2005A,B; Das et al., 2006A,B; Zamin et al., 2006) which are all downstream signaling events induced by resveratrol. Inhibition of individual routes of downstream signaling pathways compromises the neuroprotective effects of resveratrol, suggesting that these pathways are essential to the neuroprotective action of resveratrol. Akt has been recognized as one of the main proteins kinases that promote neuronal survival (Yano et al. 2001; Zhang et al. 2004). Previous work using different ischemic models has shown that the protective effect of resveratrol is dependent on Akt activity. For example, cell death induced by oxygen and glucose deprivation in an *in vitro* model of brain slice ischemia (Zamin et al., 2006) and cardiac ischemia (Das et al., 2003) was diminished by resveratrol-enhanced Akt activity. We demonstrate here that resveratrol enhances the Akt phosphorylation in hippocampal CA1 area, and that the PI3K/Akt inhibitor LY294002 abolishes the neuroprotective effects of resveratrol. Therefore, Akt plays an essential role in the neuroprotective effects of resveratrol against ischemic CA1 neuronal death.

Several downstream targets of Akt have been recognized to be apoptosis regulatory molecules, including bcl-2-family member BAD (Yang et al., 2003), GSK-3β (Grimes and Jope, 2001), CREB (Shaywitz and Greenberg, 1999). We examined two downstream targets of Akt. Our data demonstrated an enhanced phosphorylation of GSK-3β at Ser9 in resveratrol-treated groups, and it is well known that its phosphorylation negatively regulates its proapoptotic activity (Grimes and Jope, 2001; Jope and Johnson, 2004). This ubiquitous and constitutively active kinase regulates neuronal

differentiation and survival via phosphorylation of nuclear factor of activated T cells, cyclin D1, and b-catenin, among others (Doble and Woodgett, 2003). A role for GSK-3β in ischemia has been demonstrated by the use of a GSK-3β inhibitor to decrease infarct size in rodents (Kelly et al., 2004). In agreement with previous report showing that GSK-3β activity is decreased by resveratrol in organotypic culture (Zamin et al., 2006; Somervaille et al., 2001), our findings demonstrated that resveratrol was able to increase the level of the inactive form of GSK- 3β 1 hr and 4 hr after reperfusion. Inactivation of GSK-3β by PI3-K/Akt pathway was likely to be important in resveratrol-mediated neuroprotection, given the abundance of GSK-3β in the central nervous system and its role in ischemia.

CREB, a major nuclear transcription factor that transduces cAMP activation of gene transcription, is another regulatory downstream target molecule of Akt (Shaywitz and Greenberg, 1999). CREB has been recognized as an important nuclear factor for cell survival. Overexpresson of a dominant negative CREB transgene induced apoptosis in T cells (Barton et al., 1996). A recent study showed that CREB contributed to cell survival in response to growth factor stimulation (Du and Montminy, 1998). Our results showed induction of CREB in response to resveratrol treatment. Like nuclear factor-κB, CREB is also a target for several signaling pathways mediated by a variety of stimulation. For example, insulin-like growth factor-1-stimulated CREB phosphorylation was decreased by wortmannin, an inhibitor of PI3-K, suggesting a role of Akt in CREB activation. Considering that, it is most likely that the role of CREB in cognitive preservation in our study is limited to its

neuroprotective action that reduce ischemic neuronal cell death in the hippocampal CA1 region following.

In conclusion, the current study provides important new evidence that activation of PI3-K/Akt signaling pathway involve CREB activation and GSK-3β inhibition in neuroprotection by resveratrol following global cerebral ischemia. Given that prophylactic resveratrol administration initiates biochemical events that are known to increase brain tolerance to harmful factors, we suggest that the drug may induce activation of survival pathways after the ischemic insult. The activation of these complex and interrelated pathways may rescue neuronal cells.

Acknowledgements

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Legends of figures

Fig. 1. Resveratrol enhances the phosphorylation of Akt in hippocampal CA1 after ischemia. (a) Representative western blots showing the levels of p-Akt and Akt in the CA1 region at serial time points following ischemia. (b) The average levels of p-Akt in the CA1 region were increased in the Resveratrol-treated ischemia group at 1 h and 4 h following global ischemia. Data are presented as means ± SD. *$p < 0.05$ vs. vehicle- treated ischemia group at the same time point; $^{\#}p < 0.05$ vs. vehicle- treated sham group. (c) Representative western blots showing p-Akt and Akt levels in the CA1 region at 1 h following ischemia. S: sham-operated; S+R: Resveratrol-treated sham; LY: LY294002-treated ischemia group. (d) The average levels of p-Akt in the CA1 region were quantified and showed an increase in the resveratrol-treated group at1 h following global ischemia, which was decreased by the co-administration of LY294002. S: sham-operated; S+R: Resveratrol-treated sham; LY: LY294002-treated ischemia group. Data are means ± SD. *$p < 0.05$ vs. vehicle-treated ischemia group, $^{\#}p < 0.05$ vs. resveratrol-treated ischemia group.

Fig. 2. Resveratrol enhances the phosphorylation of GSK-3β in hippocampal CA1 after ischemia. (a) Representative western blots showing the levels of p-GSK-3β and GSK-3β in the CA1 region at serial time points following ischemia. (b) The average levels of p-GSK-3β in the CA1 region were increased in the Resveratrol-treated ischemia group at 1 h and 4 h following global ischemia. Data are presented as means ± SD. *$p < 0.05$ vs. vehicle-

treated ischemia group at the same time point; $^{\#}p < 0.05$ vs. vehicle- treated sham group (c) Representative western blots showing p-GSK-3β and GSK-3β levels in the CA1 region at 1 h following ischemia. S: sham-operated; S+R: Resveratrol-treated sham; LY: LY294002-treated ischemia group. (d) The average levels of p-GSK-3β in the CA1 region were quantified and showed an increase in the resveratrol-treated group at1 h following global ischemia, which was decreased by the co-administration of LY294002. S: sham-operated; S+R: Resveratrol-treated sham; LY: LY294002-treated ischemia group. Data are means ± SD. *p < 0.05 vs. vehicle-treated ischemia group, $^{\#}p < 0.05$ vs. resveratrol-treated ischemia group.

Fig. 3. Resveratrol enhances the phosphorylation of CREB in hippocampal CA1 after ischemia. (a) Representative western blots showing the levels of p-CREB and CREB in the CA1 region at serial time points following ischemia. (b) The average levels of p-CREB in the CA1 region were increased in the Resveratrol-treated ischemia group at 1 h and 4 h following global ischemia. Data are presented as means ± SD. *p < 0.05 vs. vehicle- treated ischemia group at the same time point; $^{\#}p < 0.05$ vs. vehicle- treated sham group (c) Representative western blots showing p-CREB and CREB levels in the CA1 region at 1 h following ischemia. S: sham-operated; S+R: Resveratrol-treated sham; LY: LY294002-treated ischemia group. (d) The average levels of p-CREB in the CA1 region were quantified and showed an increase in the resveratrol-treated group at1 h following global ischemia, which was decreased by the co-administration of LY294002. S: sham-operated; S+R: Resveratrol-treated sham; LY: LY294002-treated ischemia group. Data are means ± SD. *p < 0.05 vs. vehicle-treated ischemia group, $^{\#}p < 0.05$ vs.

resveratrol-treated ischemia group.

Fig. 4. Representative photomicrographs of cresyl violet-stained sections of the hippocampi. Rats were sham operated (A, B), at 10 min of ischemia followed by 7 d of reperfusion with the administration of DMSO (C, D). The rats subjected to 10 min of ischemia followed by 7 d of reperfusion with administration of LY294002 (E, F). The rats subjected to 10 min of ischemia followed by 7 d of reperfusion with the administration of resveratrol (30 mg/kg) for 7 d before ischemia (G, H). Rats were pretreated with resveratrol and LY294002 (I, J). Data were obtained from 6 independent animals (n=6 rats), and the results of a typical experiment are presented. Boxed areas in left column are shown at higher magnification in right column. A, C, E, G, I: ×40; B, D, F, H, J: ×400. Scale bar in I=100 μm; Scale bar in J=20 μm.

Fig. 5. Influence of resveratrol on numbers of survival neurons in hippocampus (CA1) in rats after 7 days of reperfusion. Data are mean±SD (n=6 rats). One-way ANOVA revealed significant interactions between ischemia and resveratrol. *P < 0.001 compared with vehicle-treated sham group; $^{\#}P$ < 0.05 compared with vehicle-treated sham group, $^{\&}P$ < 0.05 compared with resveratrol-treated ischemia group.

Figura 1

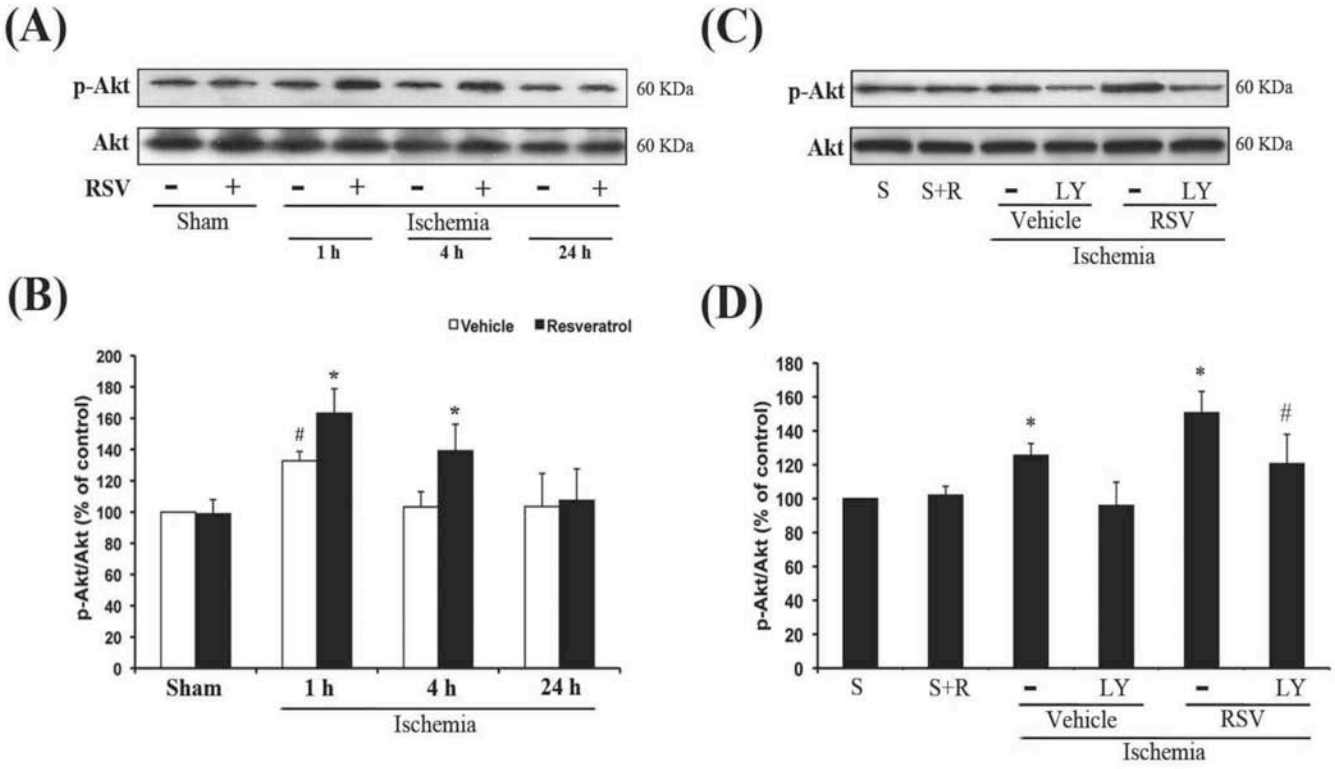
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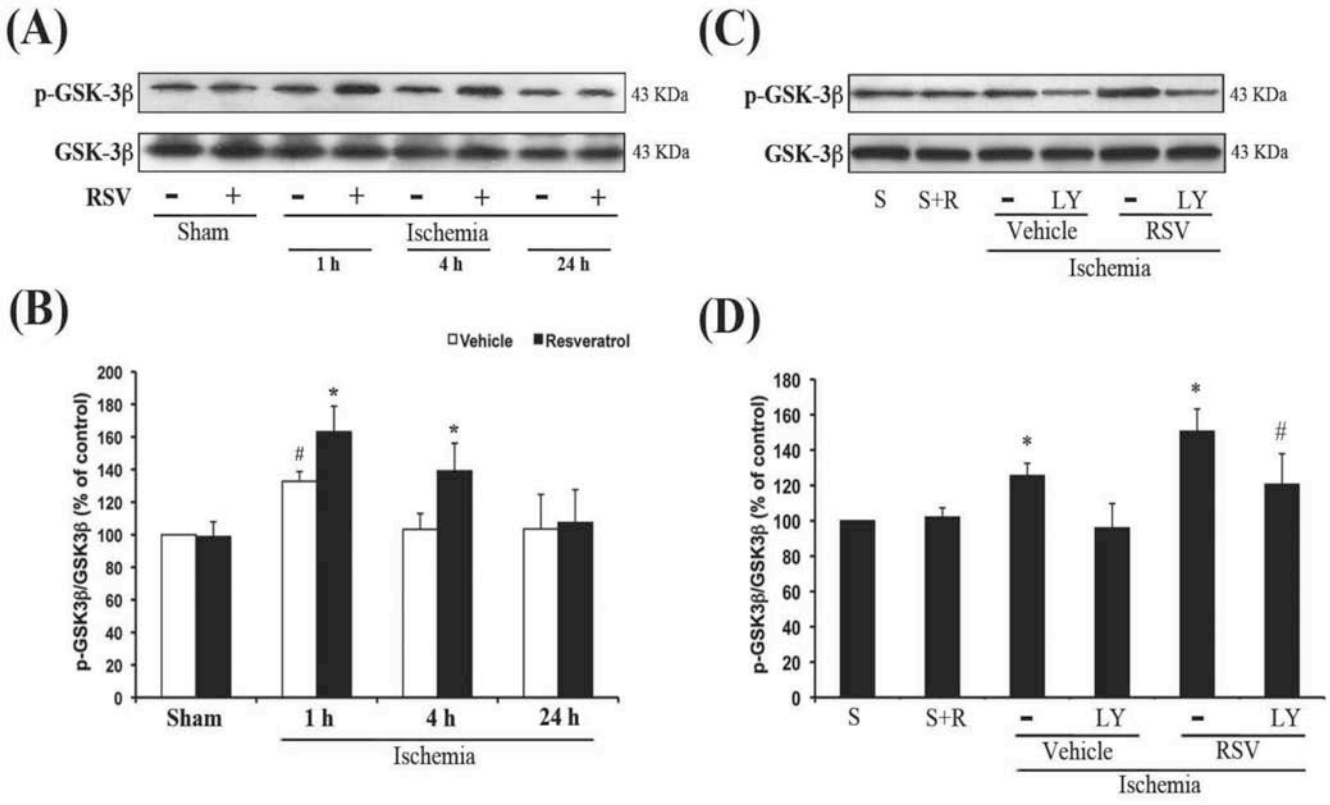
Figura 2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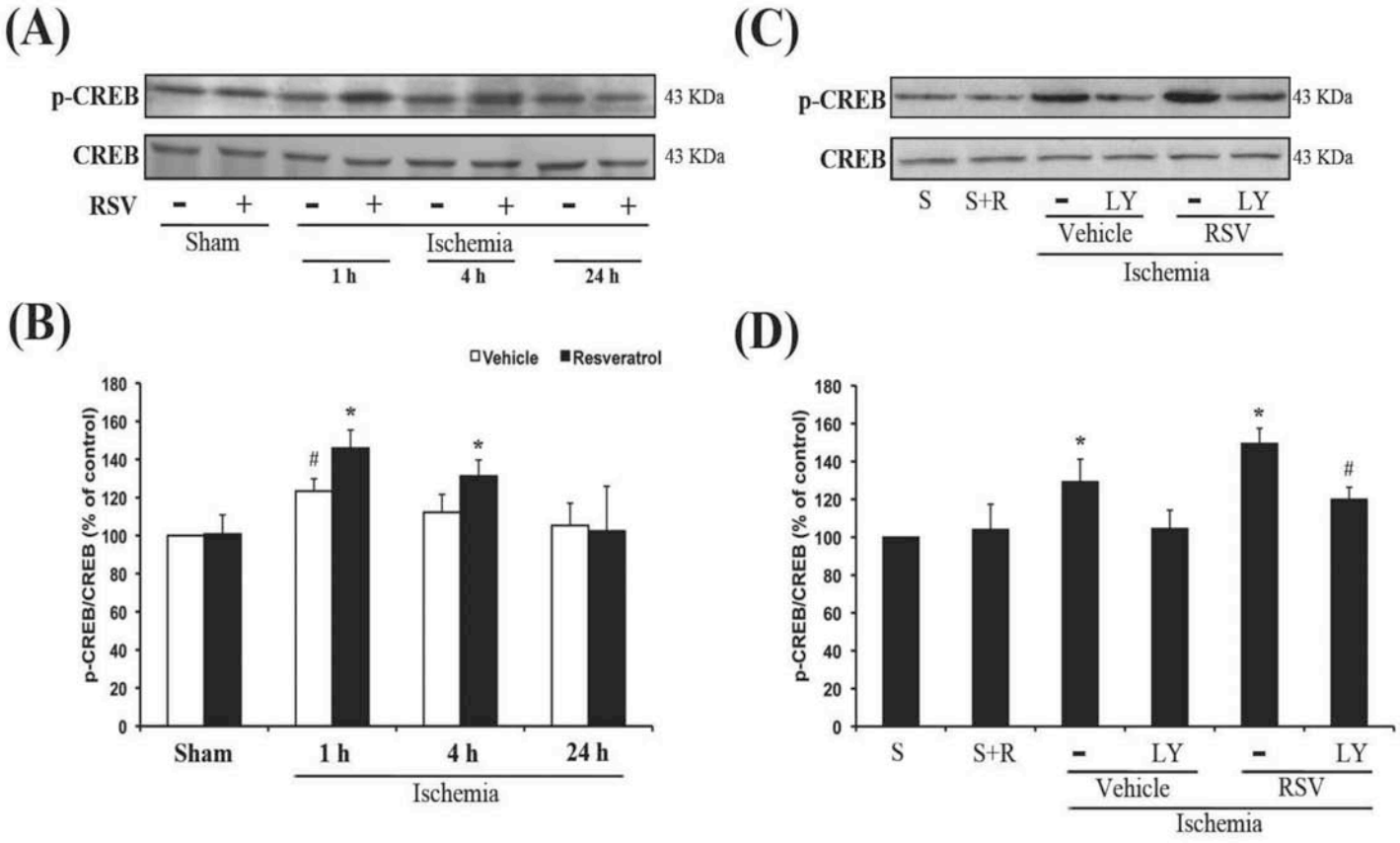
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Figura 4

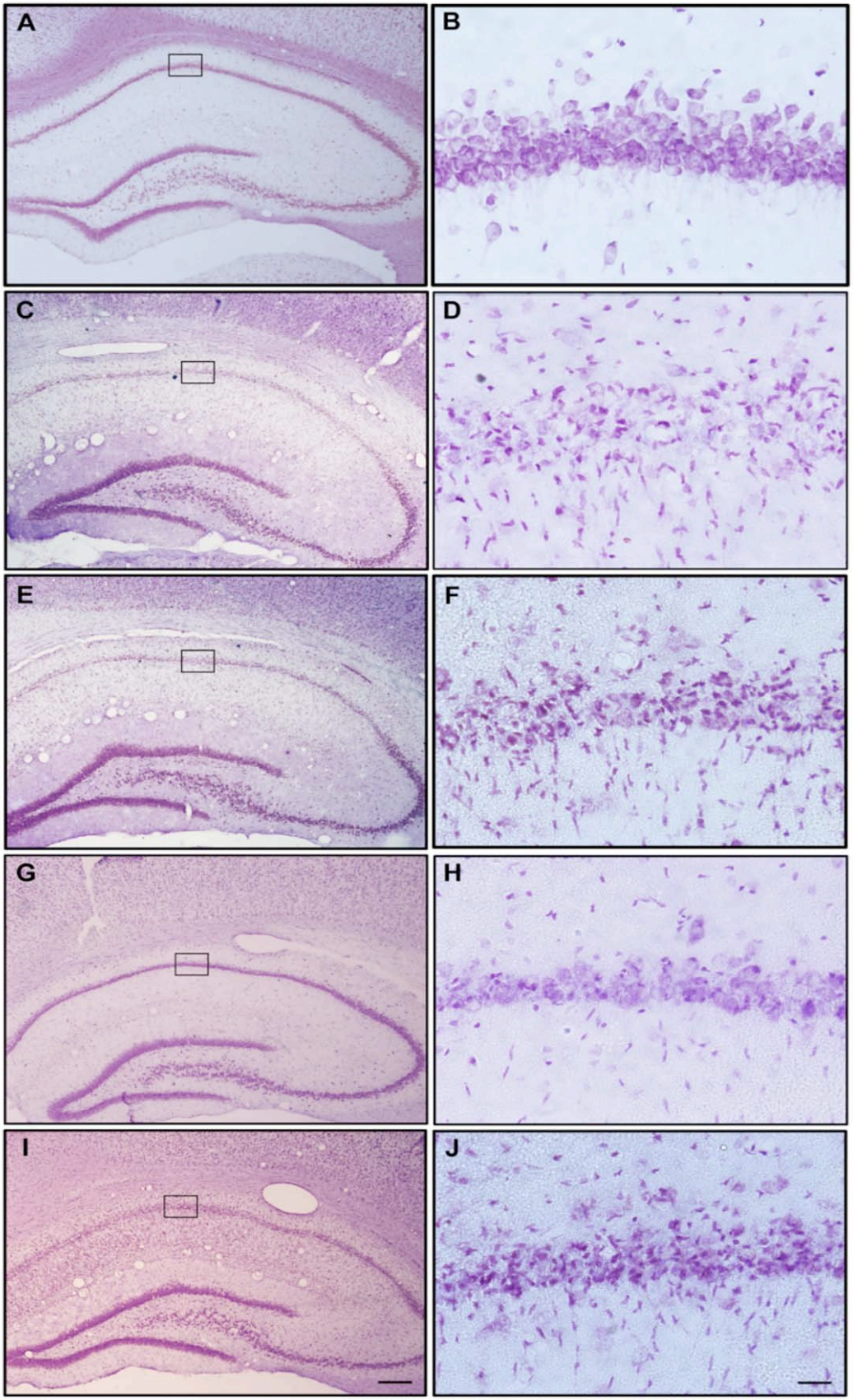
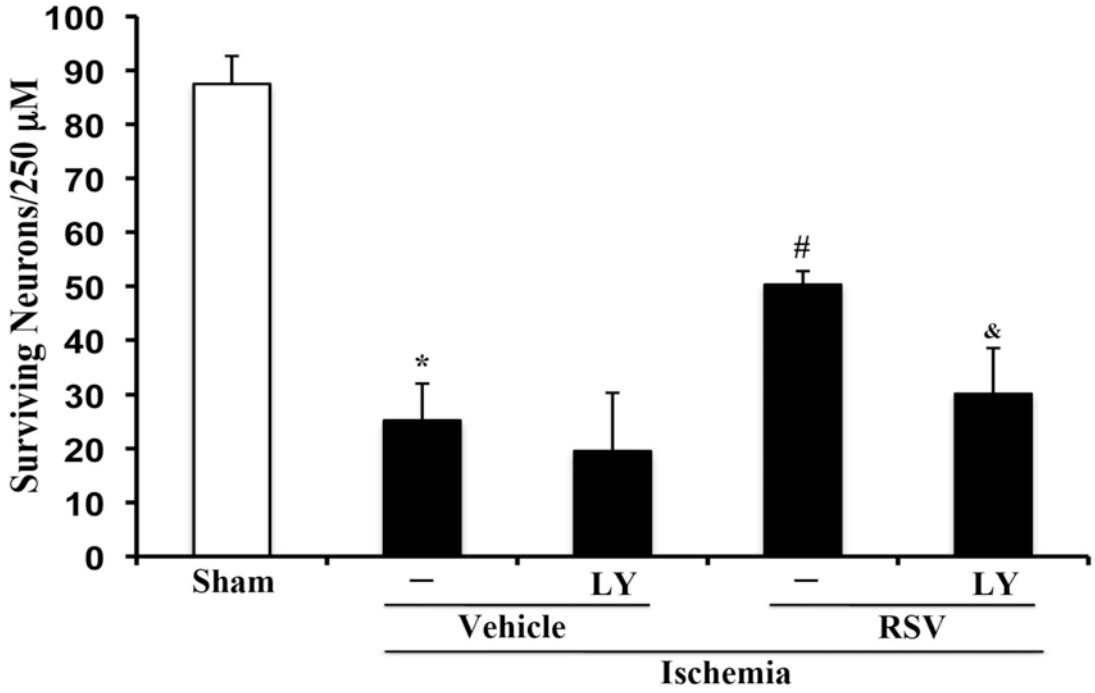


Figura 5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6. CAPÍTULO 4

Artigo: NF-κB activation following transient global cerebral ischemia is modulated by resveratrol – Submetido a *Journal of Neuroscience Research*

NF-κB activation following transient global cerebral ischemia is modulated by resveratrol

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Abstract

We investigated the effects of resveratrol treatment on the expression of JNK, the activity of NF-κB, and the signaling pathways related to NF-κB activation through iNOS and COX-2 in a rat model of global cerebral ischemia (GCI). JNK phosphorylation was enhanced by GCI; this effect was inhibited by the administration of resveratrol (30 mg/Kg, i.p) 7 days before ischemia. Pre-treatment with resveratrol prevented the ischemia-induced NF-κB activation and translocation to the nucleous observed in vehicle-treated animals. In addition, levels of inducible nitric oxide synthase (iNOS) and cyclooxygenase-2 (COX-2) in the hippocampal CA1 region at 6 h and 24h after GCI were attenuated by resveratrol. Furthermore, astroglial and microglial activation increased at 7 days after GCI and were markedly attenuated by resveratrol. These results suggest that the neuroprotective effects of resveratrol are due to its anti-inflammatory effects and to its upregulation of NF-κB and JNK phosphorylation.

Keywords: Cerebral ischemia, resveratrol, NF-κB, JNK, iNOS, COX-2.

INTRODUCTION

Resveratrol has been suggested as a potential agent to treat ischemic stroke, because of its anti-oxidative and anti-inflammatory actions that are important in reducing ischemic brain damage (Sun et al., 2002; de La Lastra and Villegas, 2005). The anti-inflammatory response of resveratrol was realized from its ability to downregulate pro-inflammatory cytokines (Pervaiz, 2003). Recently, it has been postulated that resveratrol attenuate NF-κB activation (Das and Das, 2007) by enhancing SIRT1 activity (Kubota et al., 2009; Singh et al., 2010), which in turn lead to significant suppression of the inflammation-related molecules. Furhermore, based on the results of *in vitro* studies that demonstrated that resveratrol reduces neuronal death induced by microglial activation (Chen et al., 2005; Okawara et al., 2007; Bureau et al., 2008), it can be postulated that resveratrol may exert anti-inflammatory activity by suppressing cytokine expression and microglial activation in the ischemic brain. In addtion, Wang et al., 2002 showed that resveratrol decreases microglia activation and astrogliose reaction after global cerebral ischemia.

Inflammation is an orchestrated response involving the rapid upregulation and activation of a variety of genes. NF-κB is the most important transcription factor in inflammatory responses that can be activated by reactive oxygen species (ROS), cytokines, or viruses. When activated, it induces a number of target genes including those encoding cytokines, cell adhesion molecules, oxidative-stress related enzymes, cell surface receptors,

and acute-phase proteins. (Hayden and Ghosh, 2008). There is ample evidence that NF-κB is activated in global cerebral ischemia. Most investigators found NF-κB to be activated in neurons (Clemens et al., 1997; Schneider et al., 1999; Stephenson et al., 2000; Huang et al., 2001; Nurmi et al., 2004). NF-κB can modulate several genes as ciclooxygenases (COX-2), which is upregulated in neurons, glial cells and infiltrating leukocytes in cerebral ischemia (Nakayama et al., 1998; Koistinaho et al., 1999). Induction of inducible nitric oxide syntase (iNOS) expression has been shown in models of focal and global ischemia. In a previous study, mice that were deficient in iNOS showed smaller infarcts than their wild-type counterparts after experimental ischemia (Ferriero et al., 1996; Iadecola et al., 1997).

The c-Jun N-terminal kinase (JNK) group of MAP kinases is activated in response to pro-inflammatory cytokines, different environmental stressful stimuli and ischemia (Rosette et al., 1996). JNK activity is necessary for the expression of inflammatory mediators, such as cytokines, metalloproteinases, and adhesion molecules (Raman et al., 1998; Han et al., 2001). Activation of JNK has been reported in global cerebral ischemia, which leads to cell death of hippocampal CA1 neurons (Guan et al., 2005). It has been proved that the expression of several inflammatory mediators requires ROS (Krunkosky et al., 2003; Zou et al., 2004). Antioxidants can block most of the known stimuli that activate NF-κB and JNK (Schreck et al., 1992). Our previous paper Simao et al., (in press) showed an antioxidant effects of resveratrol through decreasing ROS and NO after global cerebral ischemia. Because it seems reasonable that transcriptional activation may be necessary for the process of cell death and the close association of stress oxidative with activation of NF-κB and

JNK, our hypothesis was determine if resveratrol could modulate NF-κB and JNK and regulates iNOS and COX-2 after cerebral global ischemia.

2. Methods

2.1. Transient global cerebral ischemia and Resveratrol treatment

Adult male Wistar rats (290–330 g) were obtained from the Central Animal House of the Department of Biochemistry, Institute for Basic Health Sciences, Federal University of Rio Grande do Sul, Porto Alegre, RS, Brazil. Animals were maintained under a 12/12 h light/dark cycle, in an ambient temperature (24 ± 1 °C) colony room. Animals were provided with a constant supply of food (*ad libitum*) and water. Animal care followed the official governmental guidelines in compliance with the Federation of Brazilian Societies for Experimental Biology and experimental protocols were conducted with the approval of the Ethics Committee of the Federal University of Rio Grande do Sul, Brazil. Transient global cerebral ischemia was induced using a previously described rat model or four-vessel occlusion (Simao et al., in press). In briefly, rats were anesthetized with a mixture of 70% nitrous oxide, 30% oxygen and 1.5% halothane during surgical procedure. Global ischemia was induced by cauterization and cutting of both vertebral arteries and temporarily occluding both common carotid arteries for 10 min. Reperfusion started when aneurysm clips were removed and patency of arteries was confirmed by inspection. The rectal temperature was monitored and maintained at 37 ± 0.5 °C with a feedback-controlled heating-pad. Sham operations were performed in additional animals using identical surgical

procedures, except that the common carotid arteries were not occluded.

Resveratrol (RSV, courtesy Pharmascience, Montreal, Canada) was freshly prepared in 40% propilenoglicol. It was administered at the dose of 30 mg/kg, intraperitoneally every day for 7 days before ischemia. The vehicle-treated group received the same amount of propilenoglicol for 7 days.

2.2 Cell fractionation and Western Blot Analisys

The rats were sacrificed under anesthesia at 1 h, 4 h and 7 day after ischemia. The hippocampal CA1 region was micro-dissected from both sides of the hippocampal fissure at 0 ^{o}C and quickly frozen in liquid nitrogen. The hippocampal samples were homogenized in buffer (10 mM HEPES, 10 mM KCl, 100 mM EDTA, 1.5 mM MgCl2, 0.05% Triton X-100, 1 mM PMSF, proteinase inhibitor cocktail, 20mM DTT, 0.5% NP40). The homogenate was then centrifuged at 4 °C at 13,000 g for 5 min. The resulting (1) pellet and (2) supernatant were treated separately to acquire the nuclear and cytosolic fractions, respectively. (1) The pellet was washed twice, resuspended in nuclear lysis buffer (20 mM Hepes, 400 mM NaCl, 0.25 mM EDTA, 25% glycerol, 1.5 mM MgCl2, 25 mM DTT, proteinase inhibitor cocktail) and centrifuged at 4 °C at 13,000 g for 20 min. The resulting supernatant was the nuclear fraction. (2) The resulting supernatant was recentrifuged at 17,000 g for 20 min, to isolate the cytosolic fraction; the pellet was discarded. The cytosolic or nuclear fractions were analyzed for protein content. 60 mg protein of each sample was heated at 100 °C for 5min with loading buffer containing 0.125 M Tris- HCL (PH 6.8), 20% glycerol, 4% SDS and 0.002% bromophenol blue, then separated by sodium dodecyl sulfate-polyacrylamide gel

electrophoresis (SDS-PAGE) using 10-12% acrylamide gels. The proteins were transferred onto nitrocellulose membranes (pore size, 0.45 mm). Blotting membranes were incubated with 3% BSA in TBST (10 mmol/L Tris (PH 7.5), 150 mmol/L NaCl, 0.05% Tween-20) and probed with corresponding primary antibodies at 4 °C overnight. Blots were probed with antibodies recognizing JNK, phosphorylated-JNK (p-JNK); NF-κB, phosphorylated- NF-κB (p- NF-κB); iNOS; COX-2; GFAP and Isolectin B4 (Cell Signaling Technology, Beverly, MA, USA). The bands on the membranes were scanned and analyzed with an image analyzer (Labworks Software, UVP Upland, CA, USA). Phospho-protein signals were expressed as a ratio to the corresponding total protein and expressed relative to β-actin in the same sample. Normalized means were then expressed relative to the ratio for sham-treated animals.

2.3 Immunohystochemistry

Brains were post-fixed by immersion for 24 h in fixative and cryoprotected in 30% sucrose. Tissues were rapidly frozen in isopentane chilled using dry ice and serially sectioned in the coronal plane throughout the rostrocaudal extent of the hippocampus. Twenty micron sections were thaw-mounted onto gelatin-coated slides and stored at -70 ^{o}C. Immunocytochemisty was performed using the avidin-biotin peroxidase system ABC kit (Vector Labs, Burlingame, CA). Briefly, tissue sections were incubated with serum to block non-specific staining followed by treatment overnight with primary cd11b (Serotec). Sections were stained with the ABC immunoperoxidase system according to the recommendations of the

manufacturer. The reaction product was visualized by development with 3,3X-diaminobenzidine DAB and H_2O_2. GFAP was used in the same way but secondary antibody (Alexa 488, rabbit) was conjugated to fluorescence. Stained sections were dehydrated, mounted in DCX and examined using a Nikon Microscope.

2.4 Statistical analysis

Data were analyzed by one way ANOVA followed by the Tukey multiple test. All analyses were performed using the Statistical Package for the Social Sciences (SPSS) software. Values of $p<0.05$ were considered to be significant.

3. Results

3.2. Effect of resveratrol on glial cell changes induced by global cerebral ischemia in hippocampal CA1 area

Immunoblot analyses for microglial activation obtained from rats subjected to 10 min of ischemia was increased at 7 days after global cerebral ischemia (GCI) (Fig 1 A and B). The results of the immunostained for CD11b after 7 days of ischemia were consistent with the immunoblot for microglia (Fig 1 C). Resveratrol significantly reduced activated microglia. Similarly, activated astrocytes (immunoblot with GFAP antibody) were significantly increased in CA1 at 7 days after GCI, and resveratrol significantly decreased these activated astrocyte immunoreactivity (Fig. 1 A and B). The results of the immunostained for GFAP after 7 days of ischemia were consistent with the immunoblot (Fig 1 C).

Figure 1 and 2 near here

3.2 Resveratrol decreases the expression of NF-κB p65

The expression of NF-κB p65 in GCI was examined. The level of phospho- NF-κB p65 on nucleus was significantly increased in brain tissue subjected to GCI. As shown in Fig. 2A, high level of NF-κB p65 was observed in GCI rats on nucleus and decreased in cytoplasm. The levels of NF-κB p65 were markedly decreased on nucleus after resveratrol treatment (Fig. 2A).

3.3. Effect of resveratrol on iNOS and COX-2 changes induced by GCI in the hippocampal CA1 region

Immunoblot analyses with antibodies against COX-2 and iNOS exhibited a single band (about 72 kDa and 110 kDa, respectively) in protein extracts from microdissected CA1 area of the hippocampus of control and experimental rats. COX-2 and iNOS expression in the CA1 regions obtained from rats subjected 10 min of ischemia were increased in 6 h and 24 hs after insult (Fig. 3A, B and C). Resveratrol pre-treatment for 7 days before GCI decreased COX-2 and iNOS expression in both time periods (Fig. 3A, B and C).

3.4 Resveratrol modulates JNK activation

We next examined Resveratrol upon activation of JNK, a pro inflammatory factor. Fig. 4A–B shows that p-JNK levels are increased at 1 h and 24 hs after reperfusion as compared to sham. Total JNK levels did not change regardless of time or treatment. Resveratrol significantly inhibited p-JNK levels at both time points following GCI.

Figure 3 and 4 near here

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4. Discussion

In the present study, resveratrol displayed neuroprotective effect against global cerebral ischemia-reperfusion (I/R) damage through decreased microglial and astrocyte activation in CA1 area. In addition, resveratrol attenuated I/R-induced NF-κB translocation to nucleus and JNK, iNOS and COX-2 regulation. These activities of resveratrol against neuroinflammation appear to target activated microglia, resulting in the reduction of pro-inflammatory factors through the modulation of signal transduction pathways. Upon activation, microglia is capable of secreting a range of pro-inflammatory factors including prostaglandins, chemokines, cytokines, proteinases, ROS and NO. Our data corroborate with Wang et al., 2002, where resveratrol decreased reactive astrogliose and microglia activation in global cerebral ischemia. In addition, our previous study showed that resveratrol decresed ROS and NO formation (Simao et al., in press), both factor that contribute to promote inflammation.

Microglia act as sensors of pathological events in the brain, and it has been demonstrated that they were rapidly activated after brain damage (Kreutzberg, 1996). Astrocytes are also dynamically involved in a wide range of brain pathologies, including trauma, ischemia, and neurodegeneration (Schipke and Kettenmann, 2004). Furthermore, transient cerebral ischemia induces the activations and proliferations of microglia and astrocytes (Kato et al., 1994; Denes et al., 2007), which after activation release many cytotoxic agents that probably cause neuronal injury (Giulian and Vaca, 1993; Tichauer et al., 2007). Recently, several studies have suggested that inhibiting microglial activation attenuates ischemic injury (Suk, 2004; Fox et al., 2005).

In the present study, the expression of activated microglia and astrocytes in CA1 area at 7 days after global ischemia were significantly higher in ischemic than in sham control, which is consistent with previous reports (Wang et al., 2002). Thus, it appears reasonable that the activations of microglia and astrocytes play important causative roles in neuronal damage resulting from I/R. Moreover, these increases in activated microglia and astrocytes were found to be dramatically reduced by administering resveratrol for 7 days before ischemia. However, the mechanism whereby resveratrol reduces the activations of microglia and astrocytes after global ischemia requires further study.

Many studies suggest that NF-κB might be one of the key regulators of inflammation after brain damage (Ridder and Schwaninger, 2009). Activation of the transcription factor NF-κB is required for the expression of cytokines or adhesion molecules. In the cytoplasm, NF-κB is bound to inhibitor κB (IκB) when inactive; however, when cells are stimulated, NF-κB translocates to the nucleus by separating from IκB, functioning as a transcription activity factor for the target gene. The localization of p65 in the nuclei in the present study bears similarities to previous findings in the model of global ischemia where NF-κB was localized to nuclei in the hippocampal CA1 area after four-vessel occlusion (Clemens et al., 1997). We demonstrate here that resveratrol decreases the phosphorylation of NF-κB in nucleous of hippocampal CA1 area.

Several targets genes of NF-κB have been recognized to be inflammation-associated that encode proinflammatory mediators such as

iNOS and COX-2 (Ridder and Schwaniger, 2009). Induction of iNOS expression has been shown in models of focal and global ischemia. It has been reported that mice lacking iNOS gene and iNOS inhibition showed protection in cerebral ischemia (Iadecola et al., 1997). Besides iNOS, COX2 influence the brain function through production of oxygen free radicals and prostanoids. Furthermore, several reports have suggested that the induction of COX-2 contributes to ischemic brain damage (Ohtsuki et al., 1996; Nogawa et al., 1997; Nakayama et al., 1998), and others reported that COX-2 still remains upregulated in CA1 hippocampal cells even at 3 days after ischemia (Nakayama et al., 1998; Koistinaho et al., 1999). Therefore, both iNOS and COX-2 seems to offer important targets, since their blockade even at 6-24 h after ischemia appears neuroprotective (Sugimoto and Iadecola, 2003). In the present study, COX-2 and iNOS expression were found to be dramatically increased in CA1 region at 6 and 24 h after ischemia and could contribute to brain injury through their production such as NO and toxic prostanoids. In the present study, resveratrol reduced COX-2 and iNOS upregulations trigged by global ischemia. Resveratrol inhibits NF-κB activation, which may explain its effect on the expression of iNOS and COX-2.

It has been reported that JNK is an essential mediator of relevant proinflammatory functions in microglia and the intervention on JNK pathway may be a therapeutic approach for treating inflammatory neurological diseases (Jang et al., 2008). The stress-activated JNK/MAPKs mainly function as mediators of cellular stress including focal and global cerebral ischemia (Alessandrini et al., 1999; Irving and Bamford, 2002) by phosphorylating intracellular enzymes, transcription factors and cytosolic proteins involved in

cell survival, inflammatory cytokine production and apoptosis (Lee and Young, 1996; Harada and Sugimoto, 1999). We found that JNK increased after ischemia in the hippocampus and resveratrol attenuate JNK activation after GCI likely contributes significantly to its neuroinflammatory actions.

In summary, resveratrol treatment effectively decreased NF-κB and JNK activation and decreased the global cerebral ischemia-induced glial activation. In addtion, resveratrol decreased iNOS and COX-2 regulation. These results provide evidence that the resveratrol induces its effect in the protection against ischemic brain damage with a more complex mechanism that also involves anti-inflammatory properties.

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Legends of figures

Figure 1. Resveratrol reduced astrocyte and microglia activation in the microdissected CA1 region 7 days following global cerebral ischemia (GCI). (A) Representative bands of GFAP and Isolectina B4 (IB4) in CA1 hipocampi at 7 days after GCI. (B) The average levels of GFAP and Isolectina B4 in the CA1 region were decreased in the Resveratrol-treated ischemia group at 7 days of GCI. (C) Representative photomicrographs of CD11b (upper panel, A-C) and GFAP (under panel, D-F) positive cells in the hippocampal CA1 region at 7 day after GCI. A and D, sham group; B and E, ischemic group; C and F, Resveratrol-treated ischemic group. Data are presented as means ± SEM of 4 animals. *P < 0.05, compared with the sham control. $^{\#}$P < 0.05, compared with the vehicle-ischemic group. Magnification: A-C, 40x; D-F, 100x. Bar = 100 μm.

Figure 2. Effects of resveratrol on the expressions of NF-κB at 6 h following global cerebral ischemia (GCI). (A) Representative western blot of nuclear and cytoplasmatic NF-κB (p65) in the microdissected CA1 region after GCI. Resveratrol decreased nuclear translocation induced by GCI.

Figure 3. Resveratrol decreased iNOS and COX-2 activation following global cerebral ischemia. (A) Representative western blot data of iNOS and COX-2 in the microdissected CA1 region at 6 h and 24 h following global cerebral ischemia. (B) Bar graph represent iNOS level expressed as the average percentage increase (mean ± SD) over basal levels (n=5). *P < 0.05 vs. sham; $^{\#}$P < 0.05 vs. ischemia. (C) Bar graph represent COX-2 level expressed

as the average percentage increase (mean ± SD) over basal levels (n=5). *P < 0.05 vs. sham; $^{\#}$P < 0.05 vs. ischemia.

Figure 4. Resveratrol decreased JNK activation following global cerebral ischemia (GCI). (A) Representative western blot data of JNK in the microdissected CA1 region at 1 h and 24 h following GCI. (B) Bar graph represent p-JNK/JNK levels expressed as the average percentage increase (mean ± SD) over basal levels (n=5). *P < 0.05 vs. sham; $^{\#}$P < 0.05 vs. ischemia.

Figure 1

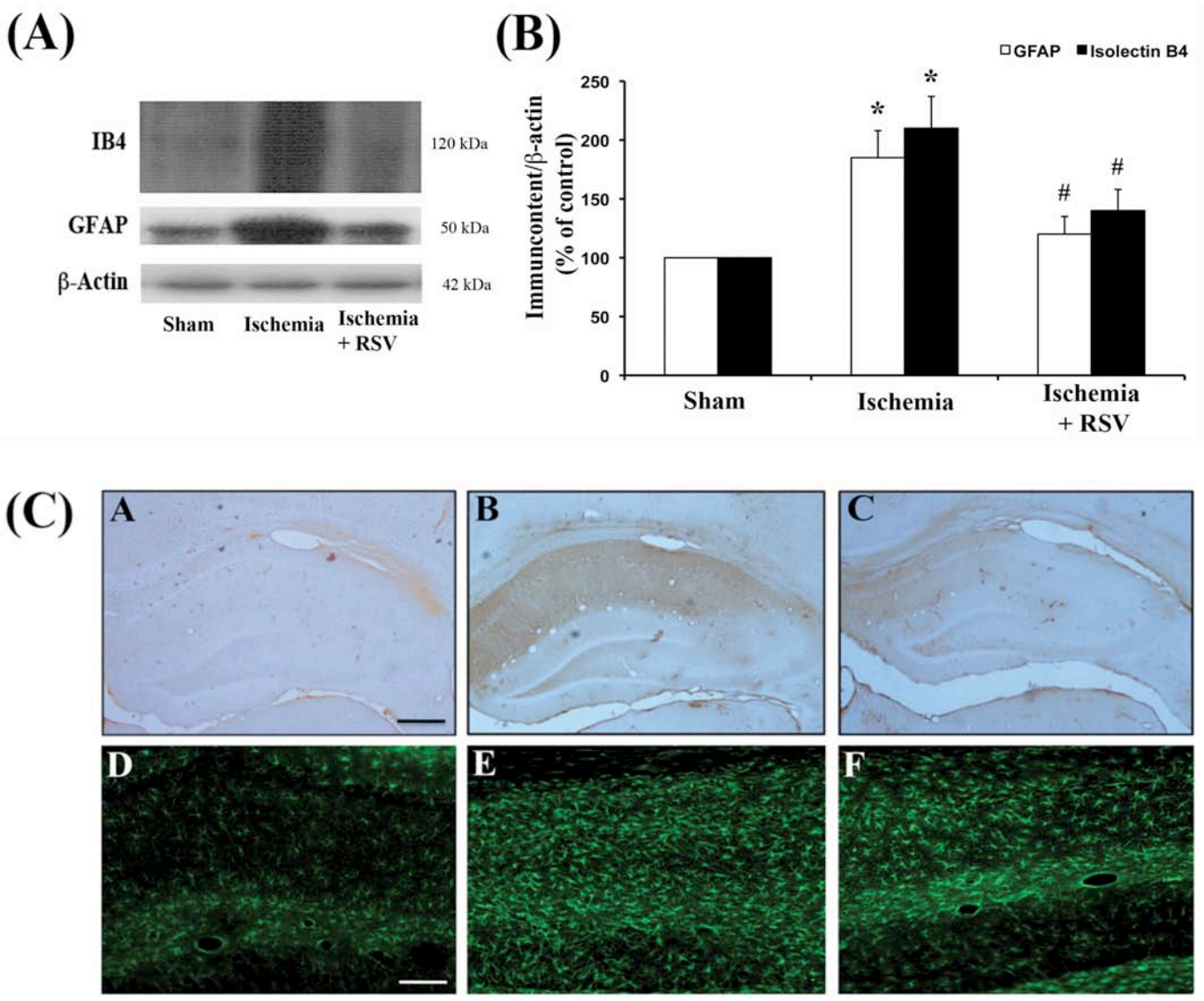

(A)
IB4
GFAP
β-Actin
120 kDa
50 kDa
42 kDa
Sham
Ischemia
Ischemia + RSV
(B)
GFAP
Isolectin B4
Immuncontent/β-actin (% of control)
250
200
150
100
50
0
*
*
#
#
Sham
Ischemia
Ischemia + RSV
(C)
A
B
C
D
E
F

Figure 2

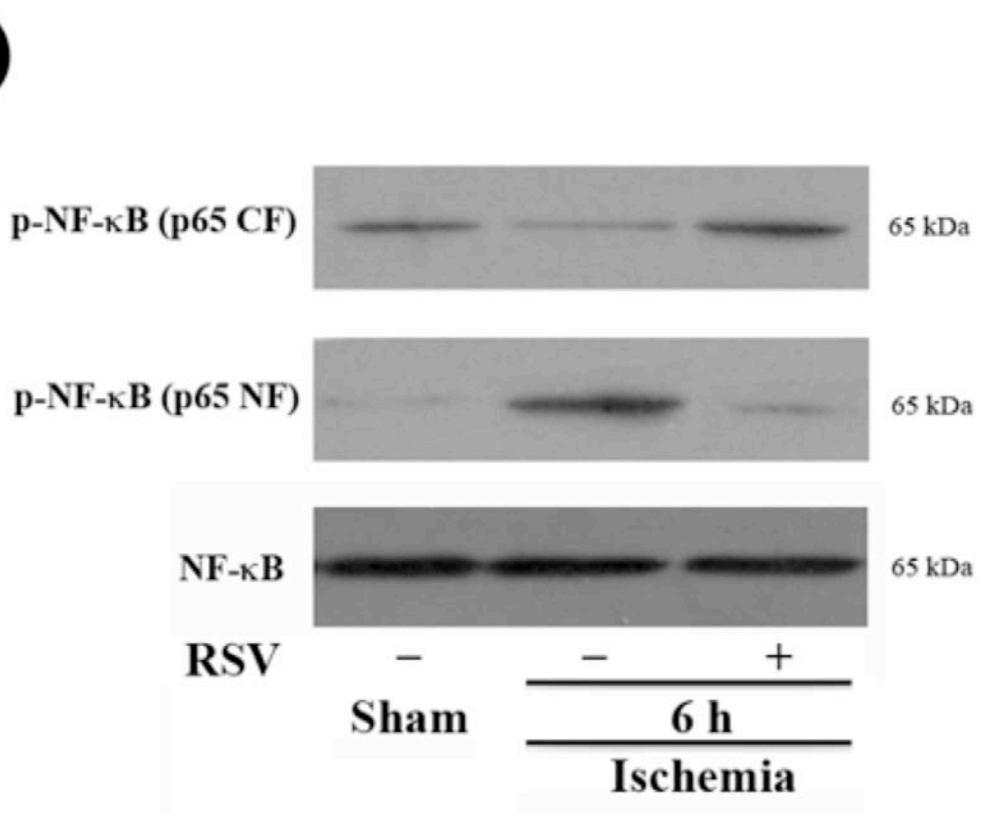
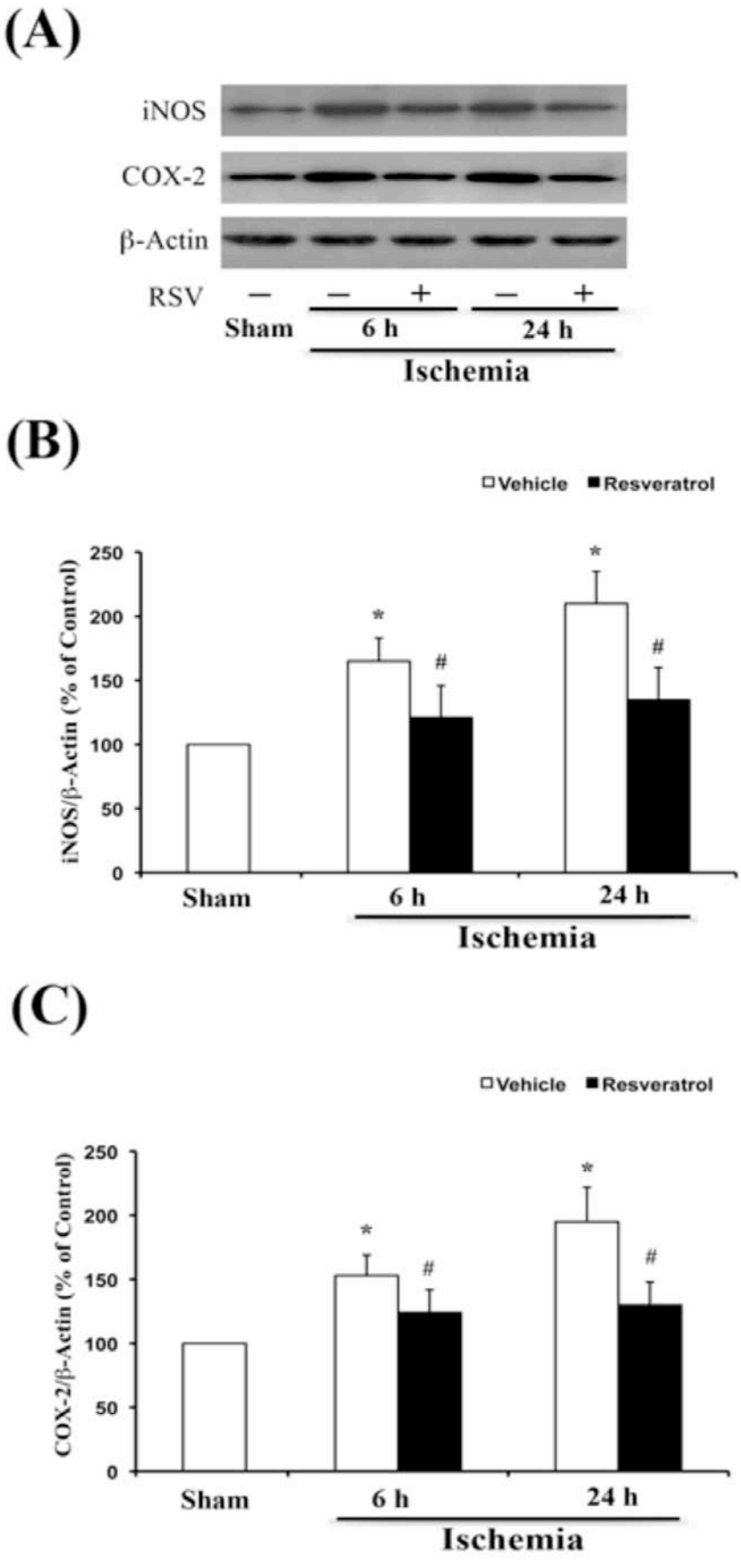

(A)
p-NF-κB (p65 CF)
65 kDa
p-NF-κB (p65 NF)
65 kDa
NF-κB
65 kDa
RSV
−
−
+
Sham
6 h
Ischemia

Figure 3


(A)
iNOS
COX-2
β-Actin
RSV
−
−
+
−
+
Sham
6 h
24 h
Ischemia
(B)
Vehicle
Resveratrol
iNOS/β-Actin (% of Control)
250
200
150
100
50
0
*
#
*
#
Sham
6 h
24 h
Ischemia
(C)
Vehicle
Resveratrol
COX-2/β-Actin (% of Control)
250
200
150
100
50
0
*
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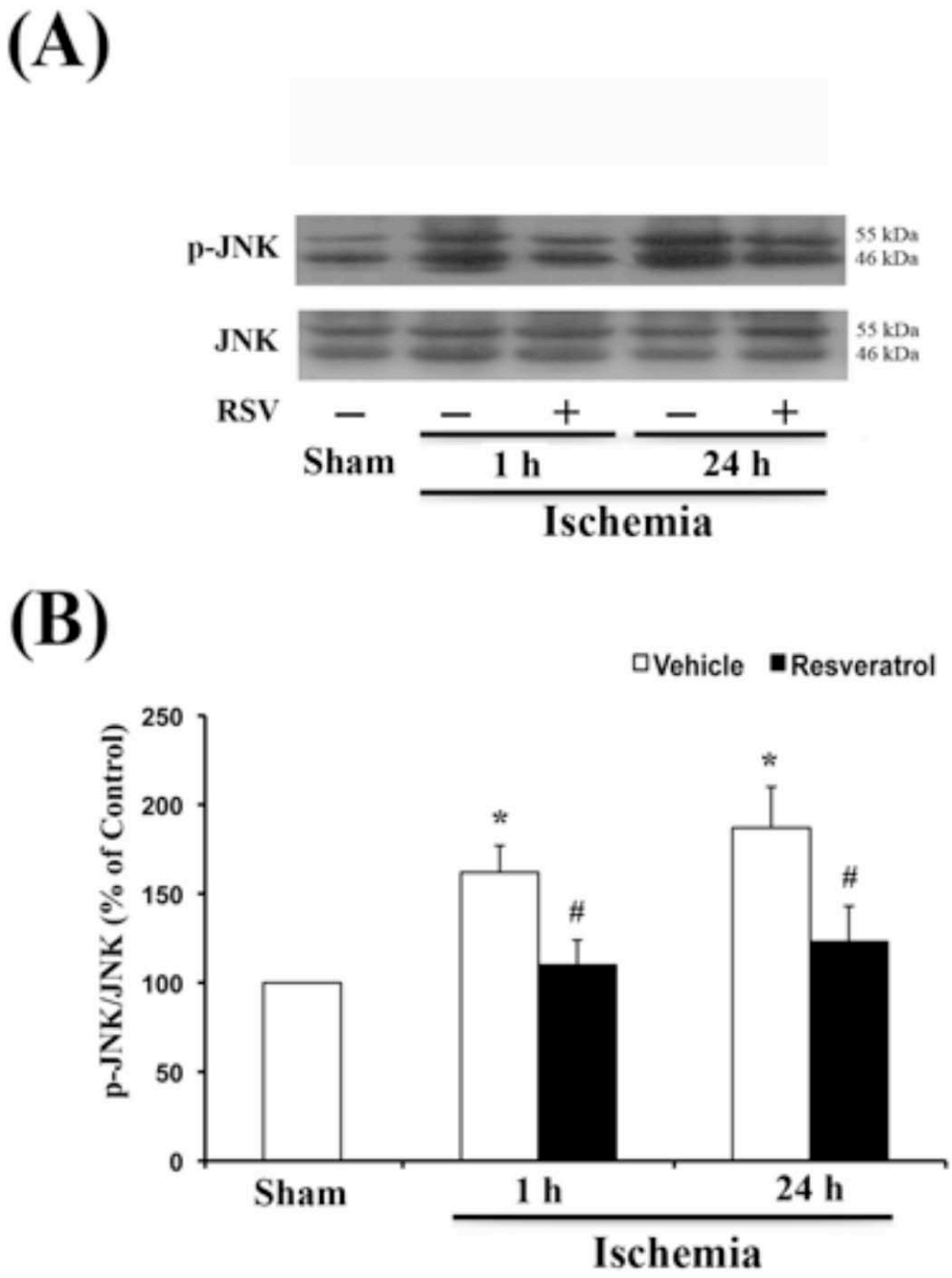
*
#
Sham
6 h
24 h
Ischemia

Figure 4


(A)
p-JNK
55 kDa
46 kDa
JNK
55 kDa
46 kDa
RSV
Sham
1 h
24 h
Ischemia
(B)
Vehicle
Resveratrol
p-JNK/JNK (% of Control)
250
200
150
100
50
0
*
#
*
#
Sham
1 h
24 h
Ischemia

7. CAPÍTULO 5

Artigo: Pro-angiogenic effects of resveratrol in brain endothelial cells: nitric oxide-mediated regulation of vascular endothelial growth factor and metalloproteinases – Submetido a *Circulation Research*

Pro-angiogenic effects of resveratrol in brain endothelial cells: nitric oxide-mediated regulation of vascular endothelial growth factor and metalloproteinases

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ABSTRACT

Modulating the sirtuin signaling pathway with resveratrol (RSV) is a powerful way of protecting the brain against a wide variety of stress and injury. Recently, it has been proposed that RSV not only reduces brain injury but also promotes recovery after stroke. But the underlying mechanisms are unknown. Here, we tested the hypothesis that RSV promotes angiogenesis in cerebral endothelial cells and dissected the signaling pathways involved. Treatment of cerebral endothelial cells with RSV promoted proliferation, migration and capillary formation in Matrigel assays. Consistent with these pro-angiogenic responses, RSV altered endothelial morphology resulting in cytoskeletal rearrangements of β-catenin and VE-cadherin. These effects of RSV were accompanied by activation of PI3-kinase/Akt and ERK MAP kinase signaling pathways that led to eNOS upregulation and increased NO levels. Subsequently, elevated NO signaling increased VEGF and MMP levels. Sequential blockade of these signaling steps prevented RSV-induced angiogenesis in cerebral endothelial cells. These findings provide a mechanistic basis for the potential use of RSV as a candidate therapy to promote angiogenesis and neurovascular recovery after stroke.

Keywords: resveratrol, eNOS, VEGF, MMPs, angiogenesis, endothelium

INTRODUCTION

It is increasingly recognized that neurovascular responses play a critical role in the ability of the brain to recover after stroke. Signals and substrates of neurogenesis and neuroplasticity are tightly co-regulated with angiogenesis and vascular remodeling.[1] This may not be surprising since the fundamental mechanisms of neurogenesis and angiogenesis are evolutionarily conserved so that similar mediators are involved in both phenomena.[2] After stroke, newborn neuroblasts migrate along perivascular routes,[3] and promotion of angiogenesis enhances neurogenesis and functional recovery.[4,5] Hence, therapies that enhance neurovascular remodeling should provide new opportunities for repairing damaged brain tissue after stroke.[6]

Accumulating data suggest that resveratrol (RSV) is a potent neuroprotectant against stroke.[7-10] The underlying mechanisms of these effects involve modulation of sirtuin signaling pathways that upregulate pro-survival mediators such as FOXO and MnSOD while dampening cell death signals such as bax and caspases.[11,12] More recently, however, it has been proposed that besides acute neuroprotection, RSV may also have beneficial effects on stroke recovery.[7] But the mechanisms that might mediate these phenomenon remain unknown.

In the present study, we test the hypothesis that RSV can promote angiogenesis in cerebral endothelial cells. Our findings suggest that RSV activates PI3-kinase/AKt and ERK MAP kinase signaling pathways that upregulate eNOS and increases extracellular levels of VEGF and MMPs. These data suggest that RSV may be a candidate therapy for stroke recovery by promoting angiogenesis via a programmed NO/VEGF/MMP signaling axis.

MATERIALS AND METHODS

Reagents and Cells

Resveratrol (>99%) was purchased from Sigma-Aldrich (St. Louis, MO). A stock solution of resveratrol was made in dimethyl sulfoxide at a concentration of 10 mM. MEK inhibitor - U0126 (Calbiochem), a PI3-K inhibitor - LY294002 (Calbiochem), a VEGF antagonist (sFlt1) (Calbiochem), a VEGF receptor blocker - SU1498 (Calbiochem). A human cerebral microvascular endothelial cell line (THBMEC)[13] was cultured in RPMI 1640 containing 10% fetal bovine serum, 10% Nu-Serum, 2 mM L-glutamine, 1 mM pyruvate, essential amino acids, and vitamins.

Proliferation Assay

In vitro endothelial cell proliferation THBMEC cells was seeded into 96-well ($1x10^5$ cells/well) flat bottom plates in triplicate with medium alone (control) or medium containing different concentrations of recombinant RSV (0.1-10 μM). Cell proliferation was determined with WST proliferation Assay kit (Dojindo, Japan) according to the manufacturer's instructions. Briefly, serum-starved cells were dose dependently treated with RSV (0.1- 10 μM) for 24hs. Following RSV treatment, 10 μl WST reagent was added to 100 μl of fresh culture medium in each well. Absorbance was recorded at 490 nm (EL800, Bio-Tek Instruments, Inc., Winooski, VT, USA), and calculated as a ratio against untreated cells.

SDS-PAGE Zymography

Conditioned media were collected and concentrated using a centricon (Millipore, Bedford, MA). Equal amounts of cell for each sample were mixed with 2x sample buffer and loaded on a 10% polyacrylamide gel incorporated with 0.1% gelatin for electrophoresis. MMP2 and MMP9 zymographic standards were used as positive controls (Chemicon). After electrophoresis, gels were renaturated and then incubated with developing buffer at 37°C for 72 hr, and stained for 1 h with 0.1% Coomassie brilliant blue. Gelatinolytic activity was visualized 1h after destained as a transparent band against a blue background.

Matrigel Tube Formation Assay for Angiogenesis

The standard Matrigel assay was used to assess the spontaneous formation of capillary-like structures. Cells ($8x10^4$ cells/well) were seeded in 24-well plates in serum-free media previously coated with Growth Factor-Reduced Matrigel Matrix (BD Biosciences), and incubated at 37 ^oC for 18 hr. The degree of tube formation was determined by counting the number of tubes in four random fields from each well. Data were analyzed as a percentage of the tube numbers in untreated control wells.

Endothelial cell migration assay

Cells ($1x10^5$) in RPMI containing 0.5% FBS were plated onto the upper transwell chamber (6.5 mm, 8 μM pore Corning Costar, Cambridge, MA) coated with 0.1% collagen Type I (BD Bioscience). The bottom chamber contained 700 μL of medium (serum free) alone or medium containing different concentrations of RSV. The cells were incubated for 18h at 37 °C and cells

that did not pass through the membrane pores were removed. Migrated cells were stained with crystal violet and counted in five random fields (200×).

NO and VEGF Assays

NO concentrations in conditioned media were determined using QuantiChrom Nitric Oxide Assay Kit (BioAssay Systems, Hayward, CA) according to the manufacturer's instructions.

VEGF levels in supernatants were determined using a human VEGF ELISA kit according to the manufacturer's instructions (R&D Systems). Known concentrations of VEGF (0 to 500 pg/mL) were used to generate standard curves for each assay.

Immunoblotting

Cells were lysed in lysis buffer (Cell Signaling Technology) and clarified, and protein concentration was determined with the Bradford assay (Bio-Rad). Total lysates of cells (50-100 μg per lane) were separated in precast 4-12% NuPage gels (Invitrogen), and proteins were transferred to nitrocellulose membrane (Invitrogen). After blocking with 0.2% I-block (Tropix), membranes were incubated overnight at 4°C with indicated primary antibodies, and 1 h at room temperature with horseradish peroxidase-conjugated secondary antibodies (Amersham). The immune complexes were visualized by enhanced chemiluminescence (Amersham). All immunoblots were repeated for at least three independent experiments.

Statistical Analysis

All of experiments were done in duplicate or triplicate, repeated three to four times independently. Quantitative data were expressed as mean$\pm$SD and analyzed with ANOVA followed by Tukey multiple comparisons. Differences of $P<0.05$ were considered significant.

RESULTS

Resveratrol augments proliferation, migration and tube formation

Cerebral endothelial cells were incubated with different concentrations of RSV (0.1 to 10 μM) over 24 hrs. Proliferation, migration and matrigel tube formation assays were examined as in vitro markers of angiogenesis. RSV induced a dose-dependent increase in endothelial proliferation (Fig 1A). Flow cytometry analysis confirmed that RSV induced a significant increase in the proliferative S phase while decreasing the resting G^0/G^1 phase of the cell cycle (Fig 1B). Along with increased proliferation, RSV also enhanced endothelial cell migration as quantified with a transwell assay (Fig 1C-D). Matrigel assays showed that RSV induced tube formation in a dose-dependent manner (Fig 1E-F). In all proliferation, migration and tube formation assays, the most effective concentrations of RSV appeared to peak around 5 μM.

Immunostaining confirmed that RSV induced morphological responses that were consistent with a pro-angiogenic phenotype in cerebral endothelial cells. In control untreated cells, normal resting state profiles were observed with F-actin mostly found in unorganized fibers and membrane structures, β-catenin localized to the cytoplasm, and VE-cadherin localized at intercellular junctions (Fig 1G). After RSV treatments, cytoskeletal rearrangements were observed with edge ruffling and F-actin stress fiber formation, relocation of β-catenin to the nucleus, and movement of VE-cadherin from membrane junctions into the cytoplasm (Fig 1G).

RSV upregulates NO via PI3-kinase/Akt and MAPK/ERK signaling

RSV robustly activated PI3-kinase signaling in cerebral endothelial cells, leading to a rapid increase in phsopho-Akt levels (Fig 2A-B). Activation of this pathway was significantly blocked by the specific PI3-kinase inhibitor LY294002 (Fig 2C-D). Additionally, the ERK MAP kinase pathway was also triggered by RSV, with clear increases in phospho-ERK levels (Fig 2E-F). ERK signaling was pharmacologically confirmed with the MEK/ERK inhibitor U0126, which significantly lowered RSV-induced phospho-ERK levels (Fig 2G-H).

Since Akt and ERK pathways are known to be related to NO signaling, we next examined the role of eNOS in RSV-induced angiogenesis. Incubation of cerebral endothelial cells with RSV rapidly increased phosphorylation of eNOS (Fig 3A-B). Activation of eNOS occurs downstream of PI3-K/Akt and MAPK/ERK signaling since blocking these pathways with LY294002 or U0126 significantly decreased RSV-induced phospho-eNOS levels (Fig 3C-D). Consistent with the increased phosphorylation of eNOS, RSV amplified NO levels in endothelial-conditioned media (Fig 3E). Co-treatment with the NOS inhibitor L-NAME suppressed the RSV-induced NO response (Fig 3F). The sequential activation of this PI3-kinase/Akt-ERK-eNOS cascade was confirmed with further inhibitor experiments. Co-treatments with either the PI3-K/Akt inhibitor (LY294002) or the MEK/ERK inhibitor (U0126) significantly decreased RSV-induced NO levels (Fig 3F). Finally, matrigel assays demonstrated that these pathways were required for RSV-induced

angiogenesis in cerebral endothelial cells. Blockade of any of these steps in the signaling cascade (PI3-K/Akt, ERK or eNOS) significantly suppressed RSV-induced tube formation (Fig 4A-B).

RSV increases VEGF and MMPs via NO-dependent signaling

To directly link RSV-induced signaling with angiogenesis, we assessed the well-established pro-angiogenic mediator VEGF. Immunostaining (Fig 5A) and western blots (Fig 5B-C) showed that VEGF expression in cerebral endothelial cells was strongly upregulated by RSV. This effect was dependent on NO. Inhibition of NOS with L-NAME significantly decreased the ability of RSV to upregulate VEGF (Fig 5D-E).

Cellular upregulation of VEGF was accompanied by a release of protein into extracellular space. VEGF levels in endothelial-conditioned media were clearly increased by RSV (Fig 5F). Extracellular VEGF release was also NO-dependent since L-NAME significantly decreased RSV-induced NO in conditioned media (Fig 5G).

Consistent with elevated protein levels, activation of VEGF signaling was detected in RSV-treated endothelial cells. Levels of the phosphorylated receptor phospho-VEGFR2 was increased by RSV, indicating that active signaling was indeed taking place (Fig 5H-I). These pathways involved autocrine signaling since blockade of the VEGF with s-Flt1 dampened the ability of RSV to activate the VEGF pathway and phosphorylate VEGFR2 (Fig 5H-I).

Besides VEGF, another major class of pro-agiogenic mediators comprise MMPs. Exposure of cerebral endothelial cells to RSV upregulated both MMP-2 and MMP-9 (Fig 6A). Induction of MMPs was also dependent on NO since the inhibitor L-NAME prevented RSV from upregulating both MMP-2 and MMP-9 (Fig 6B-C).

Crosstalk between VEGF and MMP signaling may exist. Exposing cerebral endothelial cells to VEGF directly upregulated MMP levels (Fig 6B-C). And the ability of RSV to induce MMPs can be prevented by either antagonizing VEGF with s-Flt1 or a blocking the VEGFR2 receptor with SU1498 (Fig 6B-C). Taken together, these data demonstrated that RSV strongly activates both VEGF and MMP pathways.

RSV-induced angiogenesis is dependent on VEGF and MMPs

Next, we asked whether RSV-induced angiogenesis in cerebral endothelial cells was indeed dependent on the control of VEGF and MMP mechanisms. As expected, RSV increased tube formation in matrigel assays. Blocking either VEGF or MMP signaling potently suppressed these RSV-induced effects. Co-treatments with the VEGF antagonist sFlt-1, or the VEGFR2 blocker SU1498, or the global MMP inhibitor GM6001, all significantly decreased RSV-induced tube formation (Fig 7A-B).

DISCUSSION

RSV is a potent neuroprotectant that has been shown to reduce neuronal death in many experimental models of stroke and brain injury.[7-10] But emerging data now suggest that RSV may also be used to promote recovery after stroke.[7] The major finding of the present study is that RSV can promote angiogenesis in cerebral endothelial cells. The mechanisms of this phenomenon appear to involve upstream control of NO via PI3-kinase/Akt and ERK signaling, and downstream induction of VEGF and MMPs (Fig. 8). These data provide a mechanistic basis for the potential application of RSV as a candidate therapy for neurovascular repair.

Although several groups have previously reported generation of NO by RSV;[14-18] the precise signaling pathways are not well established. RSV upregulated eNOS expression and NO generation in HUVECs.[16] But in our study, the major effects of RSV appeared to take place via phosphorylation rather than absolute alterations of protein levels. eNOS was constitutively expressed in cerebral endothelial cells but the basal level of phosphorylation was minimal. RSV increased ser1177 phosphorylation of eNOS within minutes. Ser1177 phosphorylation is critical for eNOS activity.[19] In our experiments, RSV-induced eNOS phosphorylation led to prompt generation of NO.

To dissect how RSV regulates eNOS phosphorylation, we assessed two major kinase pathways, PI3-kinase and ERK MAP kinase. Incubation of endothelial cells with the PI3-kinase inhibitor LY294002 or the MEK/ERK inhibitor U0126, both abrogated eNOS phosphorylation induced by RSV. It is interesting to note that activation of PI3-kinase and ERK are required for angiogenesis, since either PI3-kinase or ERK inhibitors reduced RSV-induced tube formation. Functionally, the balance between these two signals may allow fine-tuning of eNOS activation, and the redundancy of the two signaling pathways may ensure robust NO generation. Collectively, our observations highlight the importance of PI3-kinase/Akt and ERK pathways in RSV-induced NO production, and both appear to be necessary for cerebral endothelial angiogenesis.

It is well known that many important mediators execute the angiogenic program downstream of NO. Acting as a messenger molecule, NO mediates the majority of endothelium-dependent responses in the brain.[20] Treatment of cells with NO donors increases VEGF, [21] and inhibitors of NO synthase such as L-NAME can block VEGF generation.[22] eNOS deficient mice have impaired angiogenesis as well as impaired neurogenesis and recovery of neuronal functional following experimental stroke.[23] Limbs from eNOS knockout mice exhibit significant impairment in angiogenesis response, suggesting that NO can induce angiogenesis through VEGF.[24] Hence, VEGF may be an important mechanism by which RSV induces angiogenesis. Sen and colleagues[25] demonstrated upregulation of VEGF expression at dermal wound site with a combination of resveratrol and grape seed proanthocyanidins. In addition, Wang et al.[26] reported that resveratrol increased VEGF production and further

induced vasculogenesis in endothelial progenitor cells. Consistent with these previous reports, our results demonstrate the induction of NO and VEGF by RSV. The importance of these mechanisms is confirmed by the fact that blockade of any of these NO or VEGF signaling steps potently suppressed RSV-induced angiogenesis in our cerebral endothelial models. Furthermore, positive feedback loops may also be involved. The receptor VEGFR2 can also be upregulated by VEGF stimulation, leading to enhanced VEGF signaling and angiogenesis.[27] Our data confirm that RSV upregulates both VEGF and VEGFR2, and inhibition of upstream NO signals can dampen all components of the VEGF response and angiogenesis.

Besides VEGF, neurovascular proteolysis via MMPs represent another major mechanism for angiogenesis.[28-30] To form new blood vessels, activated endothelial cells must degrade the basement membrane and remodel the extracellular matrix around neovascular sites. Our findings reveal that RSV directly upregulated MMP-2 and MMP-9 production in cerebral endothelial cells, whereas inhibition of MMPs significantly reduced RSV-mediated angiogenesis. Taken together, these findings support a causal link between RSV-induced MMP responses and angiogenesis in cerebral endothelial cells. Furthermore, MMPs are known to be regulated by a number of cytokines and growth factors.[30] In our cerebral endothelial system, crosstalk between MMPs and VEGF was apparent. Inhibition of VEGF signaling potently suppressed RSV-induced MMPs, and prevented angiogenesis.

Taken together, our findings suggest that RSV may be a novel way to promote angiogenesis in cerebral endothelial cells. But there are also several caveats to keep in mind. First, although our data provide cellular and pharmacologic proof-of-principle for RSV in cerebral angiogenesis, in vivo validation of these mechanisms remain to be obtained. The pro-angiogenic utility of RSV as a potential stroke recovery therapy should be explored in future experiments. Second, our findings are only focused on endothelium. But it is now recognized that multi-cellular interactions between endothelial cells and astrocytes and pericytes are required for neurovascular homeostasis.[31] Further dissection of how RSV affects cell-cell signaling in the entire neurovascular unit is warranted. Finally, our studies are only focused on VEGF and MMPs. Our data suggest that VEGF and MMP pathways are critical because blocking them significantly impair RSV-mediated angiogenesis. But angiogenesis is known to recruit complex networks of genes and proteins.[32] Future investigations utilizing proteomics approaches may be required to fully catalog the effects of RSV on cerebral endothelium.

In summary, the present study provides mechanistic evidence that RSV promotes angiogenesis in cerebral endothelial cells via NO, VEGF and MMP signaling. Further in vivo and clinical exploration of these pathways is warranted to validate these experimental findings and develop RSV as a potential neurovascular repair therapy for stroke and brain injury.

Acknowledgments

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Legends of figures

Figure 1. Resveratrol stimulates brain endothelial cell proliferation, migration and tube formation. (A) Serum-starved THBMEC were treated with RSV (0.1–10 μM) for 24 h and cell growth was assessed for proliferation assay by WST. Bar graph shows that the proliferation of THBMEC incubated with indicated concentration of RSV increased. Results were expressed as percentage of cells in relation to basal (untreated) and represent the mean of triplicates. *p<0.05 vs. control, n=4. (B) Effect of RSV on cell cycle distribution. THBMEC cells were treated with RSV for 24 h. Flow cytometry analysis showed that RSV significantly increased the proportion of cells in S phase (gray bar) and decreased the proportion of cells in the G_0/G_1 phase (black bar). Data are presented as the mean±SD. *p<0.05, **p<0.01 vs. control. (C) Migration of serum-starved THBMEC in response to RSV (0.1–10 μM) were examined using transwell chamber assay for 10 h. Cells undergoing migration in response do RSV were captured using a light microscope at ×200 magnification. (D) Increased migration of THBMEC cells treated with RSV. The values represent the average number of cells migrated (mean ± SD) of three independent experiments in duplicate. *p>0.01 vs. control. (E) RSV promoted matrigel angiogenesis**.** THBMEC (8×10^4) were plated on Matrigel-coated, 24-well plates and were incubated for 20 hours in medium alone or with RSV. Photomicrographs represent the matrigel tube formation after 20-hour incubation detected by phase-contrast microscopy (magnification, ×200). (F) Bar graph shows that RSV promoted THBMEC tube formation. *p<0.05, **p<0.01 vs. control. Each experiment was performed four times in duplicate. The results are expressed as mean ± SD. (G) Cytoskeleton rearrangement in brain endothelial cells following treatment with RSV. F-actin rearrangement in THBMEC cells in response to RSV (5 μM) was examined using immunofluorescence analysis by phailoidin staining. THBMEC with media alone showed stress fibers aligned across the cell axis. Stress fibers disappeared forming membrane ruffles in THBMEC treated with 5 μM of RSV in media for 20 min. Immunocytochemistry to show localization of β-catenin and VE-cadherin in THBMEC treated with RSV was performed. β-catenin was localized on the membrane of THBMEC cells treated with media alone, whereas it was found more in the cytoplasm and nucleus of cells treated with 5 μM of RSV. VE-cadherin was localized at the intercellular junctions of THBMEC cells treated with media alone, whereas it was found more in the cytoplasm of cells treated with 5 μM of RSV.

Figure 2. Regulation of PI3-K and MAPK signaling pathway by RSV. (A) Exposure to 5 μM RSV triggered PI3-K/Akt signaling as indicated by elevated levels of phospho-Akt for indicated time periods. Representative images from four individual experiments are shown. (B) Bar graph represent p-Akt/ total Akt levels expressed as the average percentage increase (mean ± SD) over basal levels (n=4). *p<0.05, **p<0.01 vs. control. (C) Serum-starved THBMEC were pretreated with a selective PI3-K inhibitor (LY294002, 10 μM) for one hour before incubation with 5 μM RSV for 10 minutes. Representative blot from four individual experiments are shown. (D) Bar graph represent p-Akt/ total Akt levels expressed as the average percentage increase (mean ± SD) over basal levels (n=4). *p<0.05, **p<0.01 vs. control. (E) Serum starved THBMEC were treated with 5 μM RSV for up 30 minutes and Erk phosphorylation was measured by western blot analysis. Representative images from four individual experiments are shown. (F) Bar graph represent p-Erk/ total Erk levels expressed as the average percentage increase (mean ± SD) over basal levels (n=4). *p<0.05, **p<0.01 vs. control. (G) Serum-starved THBMEC were pretreated with a selective MEK inhibitor (U0126, 10 μM) for one hour before incubation with 5 μM RSV for 10 minutes. Representative images from four individual experiments are

shown. (H) Bar graph represent p-Erk/ total Erk levels expressed as the average percentage increase (mean ± SD) over basal levels (n=4). *p<0.05, **p<0.01 vs. control. The cell lysates were subjected to Western blotting with the indicated antibodies. The results are represented as a ratio of phosphorylated to total protein and expressed as percentage of control.

Figure 3. eNOS is regulated by RSV and downstream of PI3-K and MAPK signaling pathway. (A) Serum-starved THBMEC were treated with 5 μM RSV for indicated time period. Representative images from four individual experiments are shown. (B) Bar graph represent p-eNOS/total eNOS levels expressed as the average percentage increase (mean ± SD) over basal levels (n=4). *p<0.05 vs. control. (C) Serum-starved THBMEC were pretreated with a selective PI3-K (LY294002, 10 μM) and MEK (U0126, 10 μM) inhibitor for one hour before incubation with 5 μM RSV for 20 minutes. Representative images from four individual experiments are shown. (D) Bar graph represent p-eNOS/total eNOS levels expressed as the average percentage increase (mean ± SD) over basal levels (n=4). *p<0.05, **p<0.01 vs. control. eNOS phosphorylation on Ser1177 was measured by in-cell western blot analysis using a specific antibody as described in the Methods section. The results are represented as a ratio of phosphorylated to total protein and expressed as percentage of control. (E) Serum starved THBMEC were treated with 5 μM RSV for up 30 minutes. The stable end product of nitric oxide, nitrite, was measured in the medium using the Griess reaction. Medium total nitrite content corrected for protein content of the cell layer was estimated according with company product. Composite data from four experiments are shown relative to control (mean $\pm$ SD). *p<0.05 vs. control. (F) Serum-starved THBMEC were pretreated with a selective PI3-K (LY294002, 10 μM), MEK (U0126, 10 μM) and NOS (L-NAME, 3 mM) inhibitor for one hour before incubation with 5 μM RSV for 20 minutes. Composite data from four experiments are shown relative to control (mean $\pm$ SD). *p<0.05 vs. control, $^{\#}$p<0.05 vs. RSV group.

Figure 4. Involvement of PI3-K, MAPK and NOS signaling pathway in RSV-induced capillary tube formation. (A) Serum-starved THBMEC were pretreated with a selective PI3-K (LY294002, 10 μM), MEK (U0126, 10 μM) and NOS (L-NAME, 3 mM) inhibitors for one hour before incubation with 5 μM RSV. Photomicrographs represent the matrigel tube formation after 20-hour incubation detected by phase-contrast microscopy (magnification, ×200). (B) RSV-induced brain endothelial cell capillary tube formation was significantly inhibited when THBMEC were pre-incubated with inhibitors. Results are means $\pm$ SD of four experiments in duplicate. *p<0.05 vs. control; $^{\#}$p<0.05 vs. RSV group.

Figure 5. Vascular endothelial growth factor (VEGF) induced by RSV is nitric oxide synthase (NOS)-dependent. **(**A) Serum-starved THBMEC were treated with 5 μM RSV for 24h. Immunocytochemistry for VEGF demonstrated a strong stained for RSV-brain endothelial cells. (B) Serum-starved THBMEC were treated with 5 μM RSV for indicated time periods and performed western blotting. Representative blots from four individual experiments are shown. Deferoxamine (DFX) was used as a positive control for VEGF generation. (C) Bar graph represent VEGF/β-actin levels expressed as the average percentage increase (mean ± SD) over basal levels (n=4). *p<0.05, **p<0.01 vs control group. The cell lysate were collected and Western blotting was performed. (D) VEGF induced by RSV is NOS-dependent. Serum-starved THBMEC were pretreated with a selective NOS inhibitor (L-NAME, 3 mM) for one hour before incubation with 5 μM RSV for 24h. Representative blots from four individual experiments are shown. (E) Bar graph represent VEGF/β-actin levels expressed as the average percentage increase (mean ± SD) over basal levels (n=4).

*p<0.05 vs control group; #p<0.05 vs. RSV group. (F) VEGF levels in the conditioned media, as confirmed by ELISA, were also higher in RSV and DFX-treated THBMEC. The VEGF concentrations were calculated from the standard curve. *p<0.05, **p<0.01 vs. control. (G) VEGF levels in the conditioned media, as confirmed by ELISA, were higher in RSV and decreased when cells were pretreated with a selective NOS inhibitor (L-NAME, 3 mM). *p<0.05 vs. control; #p<0.05 vs. RSV group. (H) Serum-starved THBMEC were pretreated with a selective VEGF antagonist (sFLT-1, 10 μM) for one hour before incubation with 5 μM RSV for 24h. Representative blots for p-VEGFR2/total VEGFR2 from four individual experiments are shown. (I) Bar graph represent p-VEGFR2/total VEGFR2 levels expressed as the average percentage increase (mean ± SD) over basal levels (n=4). *p<0.05 vs control group; #p<0.05 vs. RSV group.

Figure 6. Effect of RSV on MMP-2 and MMP-9 secretion. Gelatin zymograph analysis (A) shows MMP-2 and MMP-9 activities in the conditioned medium for 24h harvested from control and different concentrations of RSV (0.1-10 μM). (B) Serum-starved THBMEC were pretreated with a selective VEGF (sFLT-1, 10 μM), VEGFR2 (SU1498, 5 μM) and NOS (L-NAME, 3 mM) inhibitors for one hour before incubation with 5 μM RSV for 24h. VEGF was used as a positive control. Representative zymograms from four individual experiments are shown. (C) Bar graph represent MMP-2 and MMP-9 levels expressed as the average percentage increase over basal levels (n=4). *p<0.05 vs. control group; #p<0.05 vs. RSV group. Data shown are means ± SD. The values represented are relative to basal.

Figure 7. Involvement of VEGF, VEGFR2 and MMPs signaling pathway in RSV-induced capillary tube formation. (A) Serum-starved THBMEC were pretreated with a selective VEGF (sFLT-1, 10 μM), VEGFR2 (SU1498, 5 μM) and MMPs (GM6001, 10 μM) inhibitors for one hour before incubation with 5 μM RSV. Photomicrographs represent the matrigel tube formation after 20-hour incubation detected by phase-contrast microscopy (magnification, ×200). (B) RSV-induced brain endothelial cell capillary tube formation was significantly inhibited when THBMEC were pre-incubated with inhibitors. Results are means ± SD of four experiments in duplicate and expressed as percentage of control. * p<0.05 vs. control; #p<0.05 vs. RSV group.

Figure 8. A schematic diagram showing the proposed mechanism by which intracellular signaling pathway involved in RSV-induced brain endothelial cell angiogenesis.

Figura 1

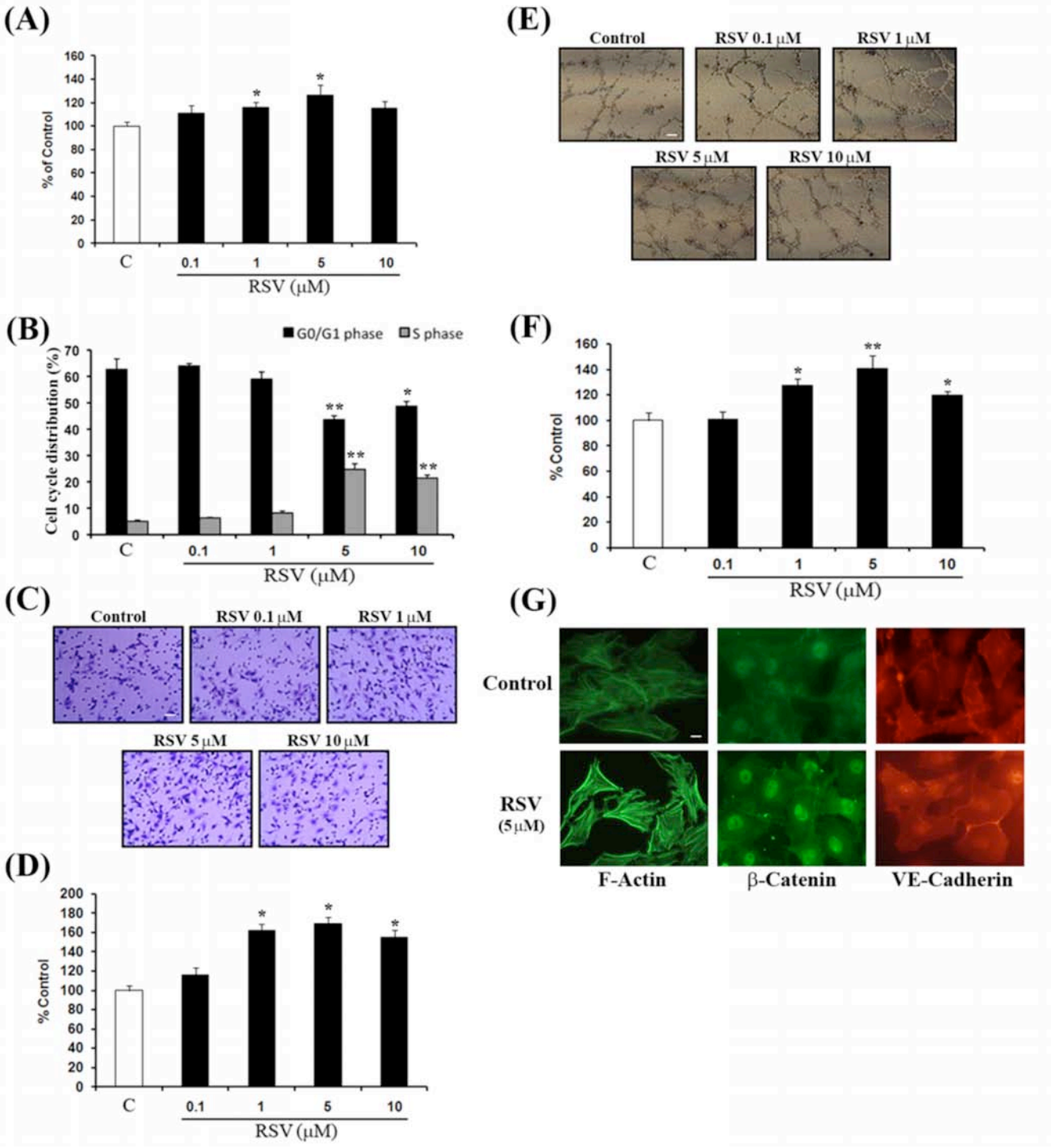
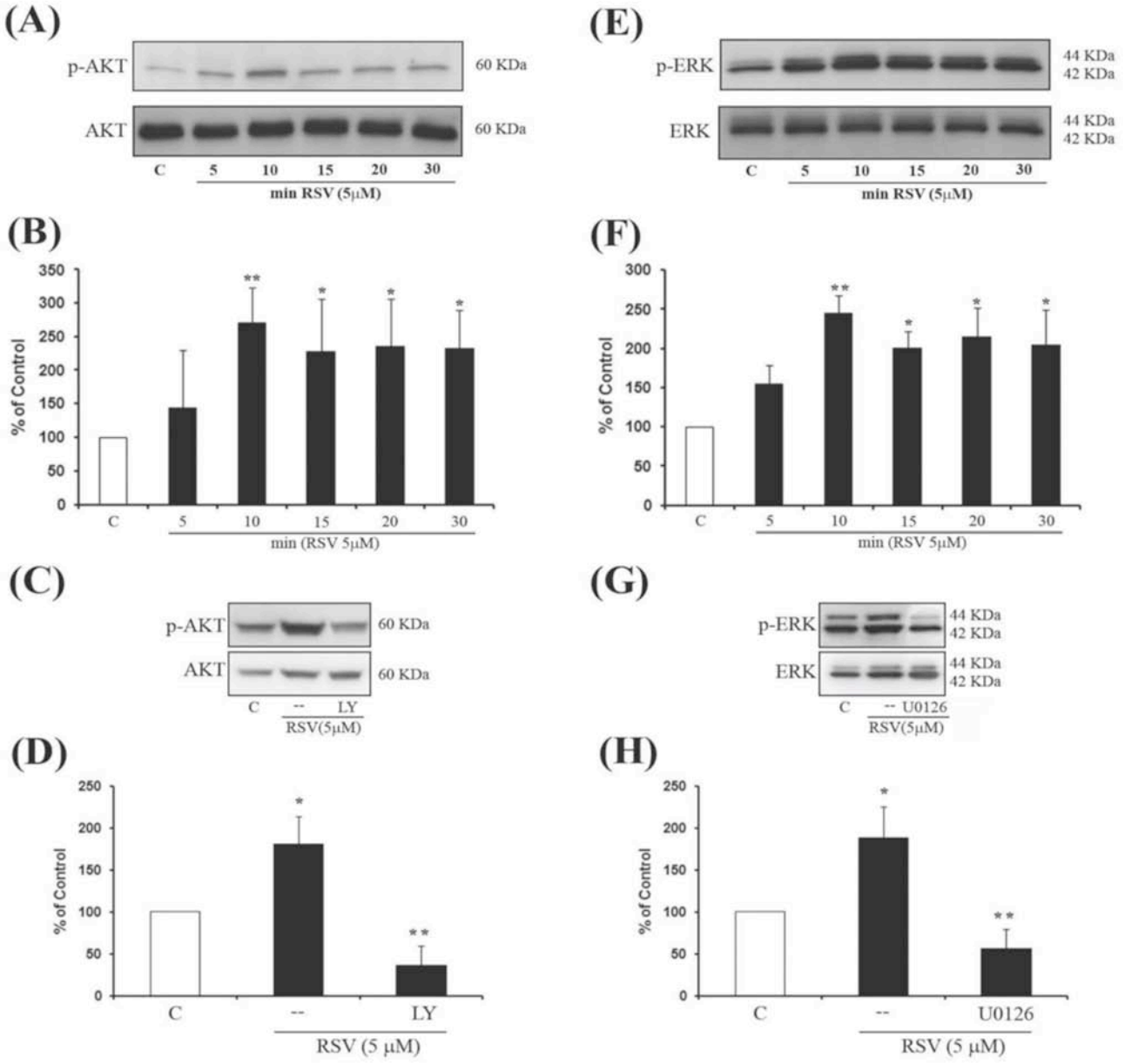

(A)
% of Control
C
0.1
1
5
10
RSV (μM)
(B)
G0/G1 phase
S phase
Cell cycle distribution (%)
C
0.1
1
5
10
RSV (μM)
(C)
Control
RSV 0.1 μM
RSV 1 μM
RSV 5 μM
RSV 10 μM
(D)
% Control
C
0.1
1
5
10
RSV (μM)
(E)
Control
RSV 0.1 μM
RSV 1 μM
RSV 5 μM
RSV 10 μM
(F)
% Control
C
0.1
1
5
10
RSV (μM)
(G)
Control
RSV
(5 μM)
F-Actin
β-Catenin
VE-Cadherin

Figura 2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AKT
60 KDa
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min RSV (5μM)
(E)
p-ERK
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42 KDa
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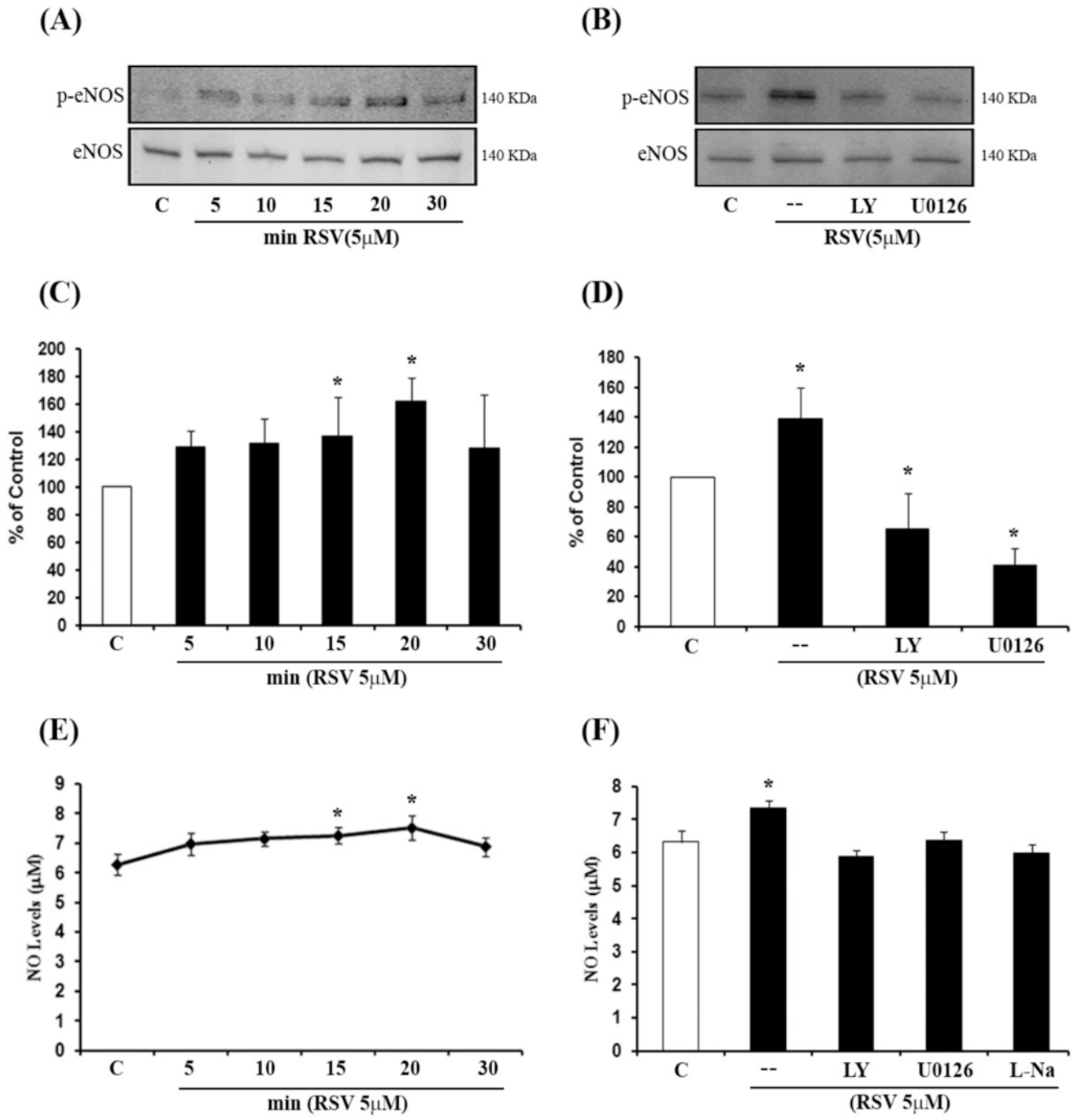
44 KDa
42 KDa
C 5 10 15 20 30
min RSV (5μM)
(B)
% of Control
C 5 10 15 20 30
min (RSV 5μM)
(F)
% of Control
C 5 10 15 20 30
min (RSV 5μM)
(C)
p-AKT
60 KDa
AKT
60 KDa
C -- LY
RSV(5μM)
(G)
p-ERK
44 KDa
42 KDa
ERK
44 KDa
42 KDa
C -- U0126
RSV(5μM)
(D)
% of Control
C -- LY
RSV (5 μM)
(H)
% of Control
C -- U0126
RSV (5 μM)

Figura 3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p-eNOS
eNOS
140 KDa
140 KDa
C 5 10 15 20 30
min RSV(5μM)
(B)
p-eNOS
eNOS
140 KDa
140 KDa
C -- LY U0126
RSV(5μM)
(C)
% of Control
C 5 10 15 20 30
min (RSV 5μM)
(D)
% of Control
C -- LY U0126
(RSV 5μM)
(E)
NO Levels (μM)
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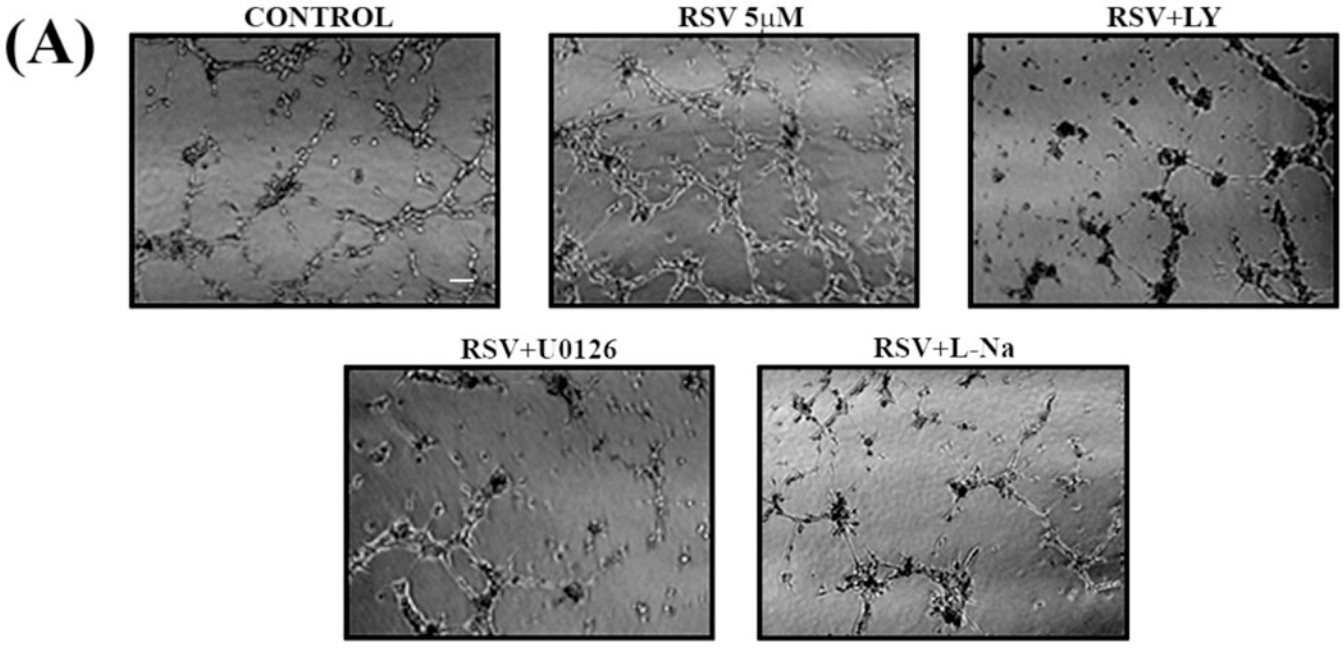
min (RSV 5μM)
(F)
NO Levels (μM)
C -- LY U0126 L-Na
(RSV 5μM)

Figura 4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CONTROL
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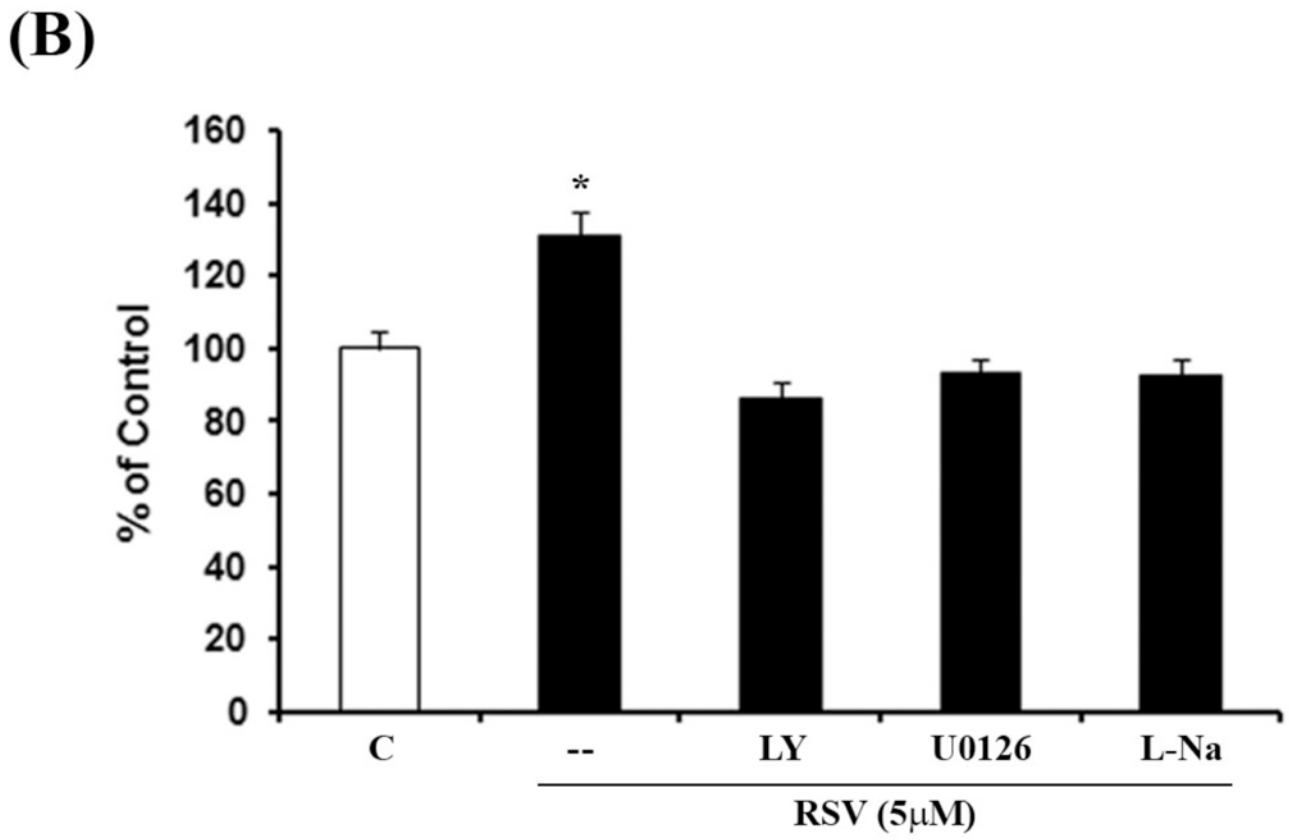
RSV+LY
RSV+U0126
RSV+L-Na


(B)
% of Control
160
140
120
100
80
60
40
20
0
*
C
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LY
U0126
L-Na
RSV (5μM)

Figura 5

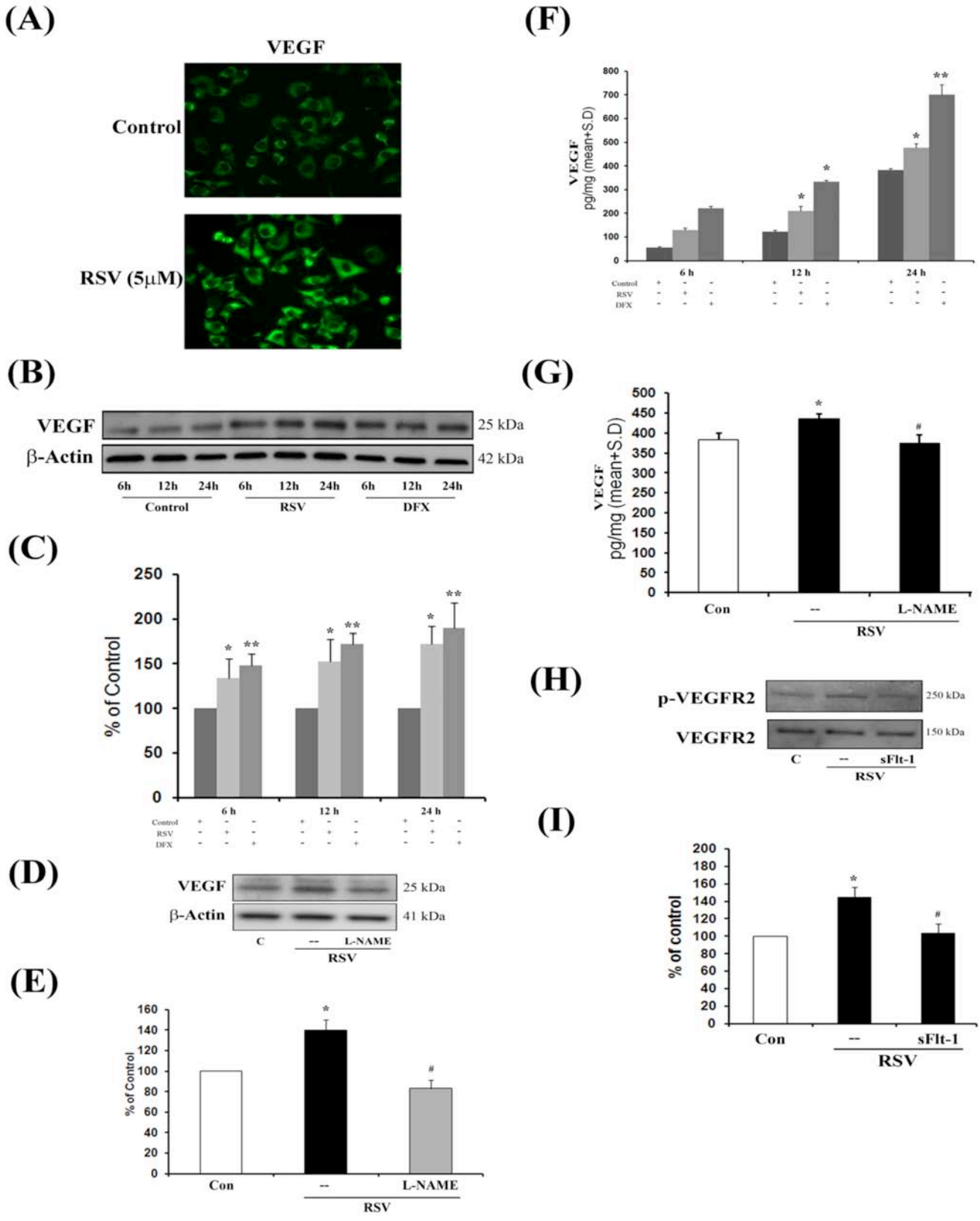

(A)
VEGF
Control
RSV (5μM)
(B)
VEGF
β-Actin
25 kDa
42 kDa
6h 12h 24h 6h 12h 24h 6h 12h 24h
Control
RSV
DFX
(C)
% of Control
6 h
12 h
24 h
Control
RSV
DFX
(D)
VEGF
β-Actin
25 kDa
41 kDa
C -- L-NAME
RSV
(E)
% of Control
Con -- L-NAME
RSV
(F)
VEGF pg/mg (mean+S.D)
6 h
12 h
24 h
Control
RSV
DFX
(G)
VEGF pg/mg (mean+S.D)
Con -- L-NAME
RSV
(H)
p-VEGFR2
VEGFR2
250 kDa
150 kDa
C -- sFlt-1
RSV
(I)
% of control
Con -- sFlt-1
RSV

Figura 6

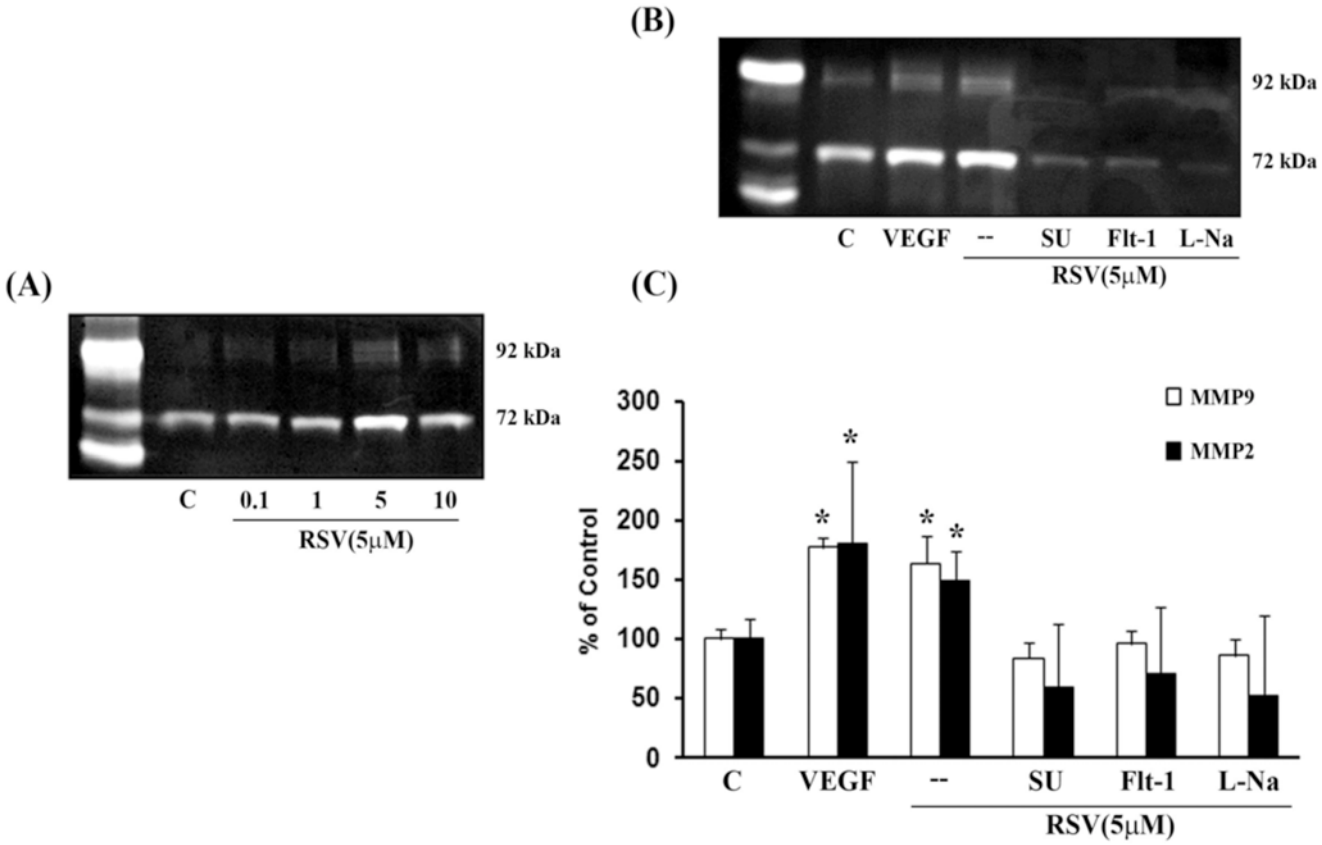
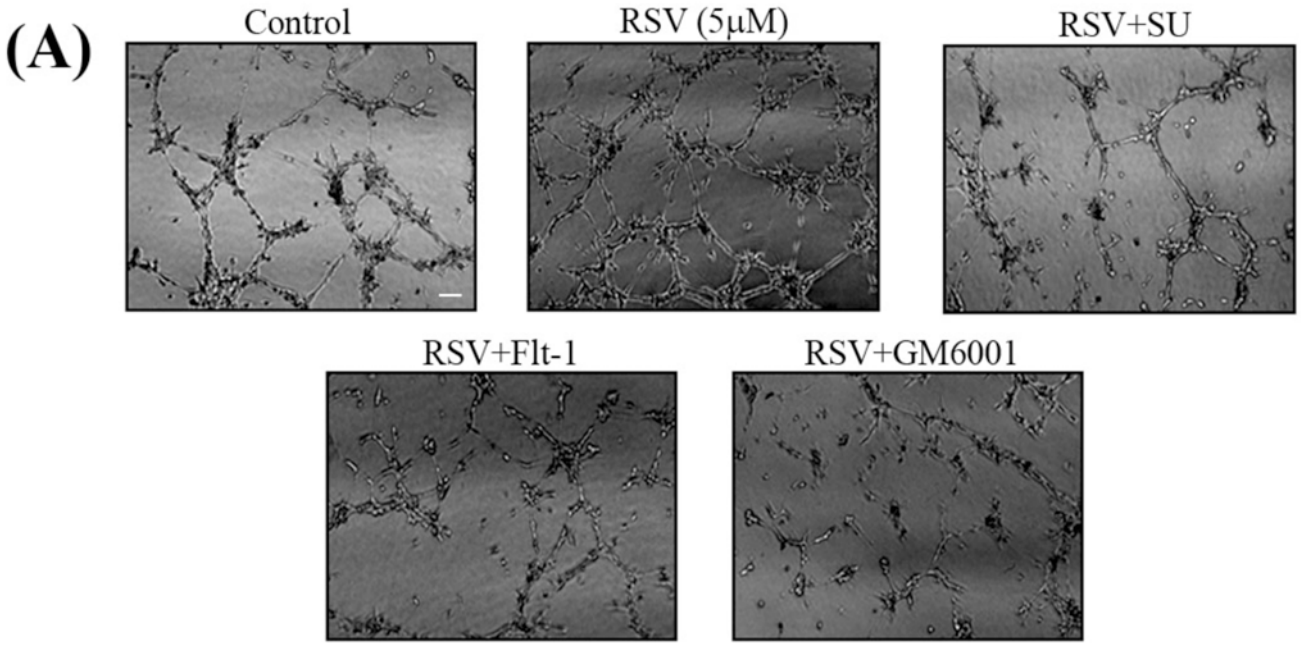

(A)
92 kDa
72 kDa
C
0.1
1
5
10
RSV(5μM)
(B)
92 kDa
72 kDa
C
VEGF
--
SU
Flt-1
L-Na
RSV(5μM)
(C)
MMP9
MMP2
% of Control
300
250
200
150
100
50
0
C
VEGF
--
SU
Flt-1
L-Na
RSV(5μM)

Figura 7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RSV+Flt-1
RSV+GM6001

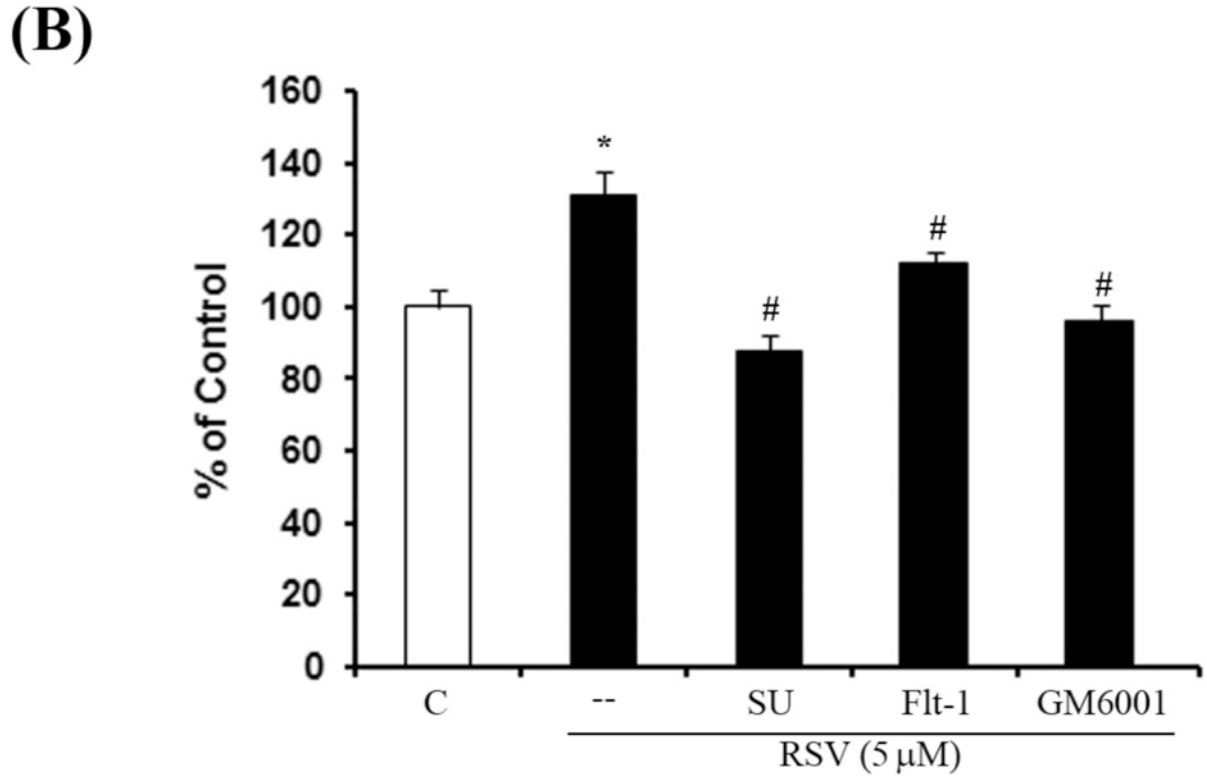
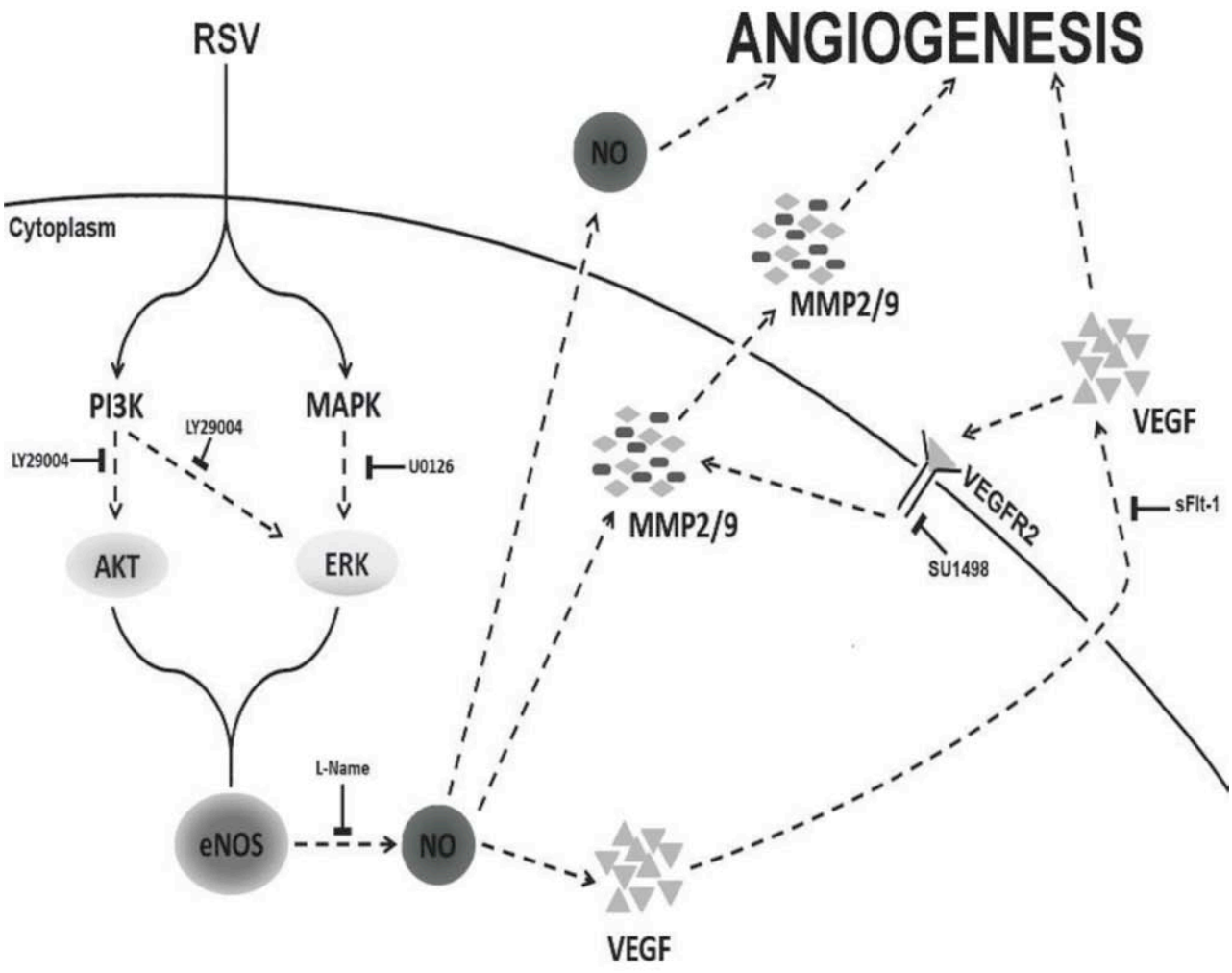

(B)
% of Control
160
140
120
100
80
60
40
20
0
*
#
#
#
C
--
SU
Flt-1
GM6001
RSV (5 μM)

Figura 8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VEGFR2
sFlt-1
AKT
ERK
SU1498
L-Name
eNOS
NO
VEGF

8. CAPÍTULO 6

Artigo: Resveratrol promotes angiogenesis, neurogenesis and functional recovery after stroke – Manuscrito a ser submetido

Resveratrol promotes angiogenesis, neurogenesis and functional recovery after stroke

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Abstract

Background and Purpose— We tested the hypothesis that resveratrol promotes functional recovery, angiogenesis and neurogenesis after stroke. ***Methods***—Mice were treated with resveratrol at 72 hours after the onset of MCAO. An array of behavior tests was performed. Mice were euthanized 14 days after stroke for measurements of angiogenesis and neurogenesis. In vitro, neurospheres derived from the subventricular zone (SVZ) from the embryos mice were treated with resveratrol to measure cell proliferation and differentiation. ***Results***—Treatment with resveratrol significantly improved functional recovery, along with increases in density of cerebral microvessels and BrdU/lectin positive cells at the stroke boundary. In addition, resveratrol treatment significantly increased the number of BrdU-positive cells and doublecortin immunoreactivity in the ipsilateral SVZ in 14 days after stroke. Resveratrol treatment significantly increased brain levels of vascular endothelial growth factor (VEGF) and brain-derived neurotrophic factor (BDNF). In vitro, incubation of neurospheres derived from embryos mice treated with resveratrol enhanced proliferation and differentiation in neurons. In addition, resveratrol significantly increased SVZ explant cell migration in vitro. Anti-BDNF and anti-VEGF antibody significantly inhibited resveratrol-induced SVZ explant cell migration, indicating a prominent role for BDNF and VEGF in progenitor cell migration. ***Conclusion***— These data indicate that resveratrol promotes angiogenesis, brain plasticity and enhances functional recovery after stroke. In addition, VEGF and BDNF likely contribute to these restorative processes.

Keywords: resveratrol, stroke, neurogenesis, angiogenesis, VEGF, BDNF

Introduction

Functional recovery after stroke and brain injury may be related to neurogenesis and compensatory responses within the injured brain (Lo et al., 2010). How to induce neurogenesis and promote functional recovery after neuronal injury is an area of intense interest, with obvious clinical implications. Angiogenesis is coupled with neurogenesis in the brain, and neurogenesis occurs within an angiogenic niche (Palmer et al., 2000). The neurovascular niches provide microenvironments for neural stem cells (NSCs) in the adult brain; newly generated, immature neurons are closely associated with the remodeling vasculature. The generation of new vasculature facilitates coupled neurorestorative processes including neurogenesis and synaptogenesis, which improve functional recovery (Li and Chopp, 2009; Zhang and Chopp, 2009). Angiogenesis and neurogenesis may play a significant role in mediating functional recovery following experimental stroke (Chopp et al., 2008; Li and Chopp, 2009; Lu et al., 2005; Wu et al., 2008). Neurorestorative agents that increase angiogenesis and neurogenesis have been shown to improve functional outcome following brain injury (Zhang and Chopp, 2009).

Preclinical studies have shown that acute treatment with resveratrol reduces cerebral infarction in rats after transient ischemia, and delayed administration of resveratrol promotes neurological functional recovery (Dong et al., 2008; Shin et al., 2010). RSV is a potent neuroprotectant that has been shown to reduce neuronal death in many experimental models of stroke and brain injury (Dong et al., 2008; Sakata et al., 2010; Shin et al., 2010; Sun et al., 2010). These data suggest that, in addition to the neuroprotective effect, resveratrol has a capacity to promote brain repair after stroke (Dong et al.,

2008). However, the effect of resveratrol on neurogenesis in ischemic brain has not been investigated.

Despite strong evidence for the neuroprotective benefits of resveratrol in the treatment of acute stroke, the effects of resveratrol on angiogenesis, neurogenesis and neurological function during stroke recovery have not been investigated.

Methods

MCAO Model and Resveratrol Administration

Adult male CD-1 mice (age 2 to 3 months, weight 35-40g) were purchased from Charles River (Wilmington, MA, USA). Mice were anesthetized with isoflurane. Transient right MCAO was induced by advancing a 7-0 surgical nylon suture coated with silicon from the external carotid artery into the lumen of the internal carotid artery to block the origin of the MCA (Mao et al, 1999). Resveratrol was dissolved in propileneglicol 40% and injected intraperitoneally (i.p.) starting day 3 after MCAO.

After MCAO, mice were randomly divided into the following groups: Group 1, control; Group 2, MCAO+ Vehicle (0.1 mL 40% propileneglicol) daily from day 3 to day 14; Group 3, resveratrol (10 mg/kg) daily from day 3 to day 14. To identify newly formed DNA in ischemic brain, mice received injections of bromodeoxyuridine (BrdU, Sigma Chemical, 50 mg/kg in saline), intraperitoneally (i.p.) starting from day 3 after MCAO and daily thereafter until the mice were killed. Functional tests were performed on Groups 1, 2 and 3.

Behavioral Tests

To test whether resveratrol promotes neurologic recovery after stroke, an array of behavioral tests were performed after MCAO, and at 1, 3, 7, 10 and 14 days after MCAO by an investigator who was masked to the experimental groups.

Modified neurologic severity scores (mNSS): Neurologic function was graded on a scale of 0 to 14 (normal score 0; maximal deficit score 14, Li et al, 2000). mNSS is a composite of motor, reflex and balance tests. In the severity scores of injury, one score point is awarded for the inability to perform the test or for the lack of a tested reflex; thus, the higher the score, the more severe is the injury.

Foot-fault test: For locomotor assessment, mice were tested for placement dysfunction of forelimbs with the modified foot-fault test (Hernandez and Schallert, 1988). The total number of steps (movement of each forelimb) that the mouse used to cross the grid was counted, and the total number of foot-faults for left forelimb was recorded. The percentage of foot-fault of the left paw to the total number of steps is presented.

Corner test: Mice were placed between two boards with dimensions 30x30x2.5 cm^3 for each in the home cage (Zhang et al, 2002). The non-ischemic animals turn back from either left or right, randomly. The ischemic animals preferentially turn toward the impaired side. The number of turns taken on each side was recorded from 10 trials for each test.

Neurosphere Culture

SVZ cells were dissociated from embryos on day 13th. The cells were plated at a density of 2x10^5 cells per 10 mL in medium DMEM/F12 containing 20 ng/mL of epidermal growth factor (EGF; sigma), basic fibroblast growth factor (bFGF; sigma), 2μg/mL Heparin (sigma) and 5% N2 suplement (Invitrogen). Passage 2-5 cells were used in the study. To examine the effects of resveratrol on neurosphere differentiation, neurospheres were plated directly onto poly-ornithine/laminin- coated glass coverslips in 2% serum medium containing resveratrol (0.1, 0.5 and 1 μM). Incubation was terminated at 7 days after plating and immunostaining for neuronal (TUJ-1) and astrocyte (GFAP) markers were performed for evaluation of differentiation. Next, we examine the effects of resveratrol on neurosphere secondary formation,

Immunohistochemical Assessment

Mice subjected to MCAO followed by treatment with vehicle or resveratrol daily for 14 days were killed at day 14 after MCAO. Brains were fixed by transcardial perfusion with PBS and store on -70 ^oC.
Immunohistochemical staining: To measure whether resveratrol treatment promotes trophic factor expression, angiogenesis and neurogenesis, BrdU, doublecortin (DCX), tubulin isotype III (TUJ-1) and Lectin immunostaining were performed. For immunohistochemical analysis a rat anti-BrdU (1:1000; abcam), mouse anti-β-tubulin III (TuJ-1, 1:1000; sigma), goat anti-doublecortin (1:200; Santa Cruz Biotechnology Inc) and Lectin from were used. Control experiments consisted of staining brain coronal tissue sections as outlined above, but omitted the primary antibodies.

Quantification: For semiquantitative measurements of TUJ1, DCX, BrdU and Lectin densities, five slides from each block, with each slide containing 8 fields consisting of cortex and stratum from the ischemic border area and SVZ area were digitized under a x20 objective (Olympus BX40) using a 3-CCD color video camera (Sony DXC-970MD) interfaced with an MCID image analysis system (Imaging Research, St. Catharines, Canada). The ischemic border zone is defined as the area surrounding the lesion, which morphologically differs from the surrounding normal tissue (Nedergaard et al, 1987). The digitalized images were then contrast-enhanced to clearly differentiate positivity from background, and a thresholding procedure was established to determine the proportion of immunoreactive area within each fixed field of view. Data are presented as a percentage of area, in which the BrdU, DCX, TUJ-1 and Lectin immunopositive areas in each field were divided by the total areas in the field.

Western Blotting

Tissue was lysed in lysis buffer (Cell Signaling Technology) and clarified, and protein concentration was determined with the Bradford assay (Bio-Rad). Total lysates of cells (50-100 μg per lane) were separated in precast 4-12% NuPage gels (Invitrogen), and proteins were transferred to nitrocellulose membrane (Invitrogen). After blocking with 0.2% I-block (Tropix), membranes were incubated overnight at 4°C with indicated primary antibodies (VEGF, BDNF and β-actin), and 1 h at room temperature with horseradish peroxidase-conjugated secondary antibodies (Amersham). The immune complexes were

visualized by enhanced chemiluminescence (Amersham). All immunoblots were repeated for at least three independent experiments.

Subventricular Zone (SVZ) Explant Cultures and Cell Migration Measurement In Vitro

To measure whether resveratrol induces neural cell migration, SVZ explant cultures were prepared from adult male mice subjected to MCAO for 7 days (Dutton and Bartlett, 2000; Lois and Alvarez-Buylla, 1993). The methods for extracting and employing SVZ explants from rats have been widely used and are well developed (Katakowski et al, 2003). The SVZ was dissected from the ipsilateral SVZ of the rat brain. Tissue was minced with scalpels into pieces of 0.1 mm in each dimension. Explants were cultured within Matrigel (BD Biosciences) in wells with 500 mL of Neuralbasal-A Medium containing 2% B27 supplement (Invitrogen). The cultured SVZ explants were treated in the absence or presence of: (a) control; (b) anti-BDNF neutralizing antibody (100 ng/mL, Promega); (c) anti-VEGF neutralizing antibody (100 ng/mL, R&D system); (d) resveratrol (1 μM); (e) resveratrol (1 μM) and anti-BDNF neutralizing antibody (100 ng/mL); (f) resveratrol (1 μM) and anti-VEGF neutralizing antibody (100 ng/mL) for 7 days. Cell migration from the SVZ explant was measured using a phase contrast microscope and photographed at x100 magnification with a digital camera. The average linear distance of cell migration from the edge of the SVZ explant was captured and measured at day 7 using the MCID software. This average distance was assessed in each explant culture.

Statistical Analysis

One-way analysis of variance (ANOVA) followed by Student- Newman–Keuls test was used. The data were presented as means±SE. A value of $p<0.05$ was taken as significant.

Results

Treatment With resveratrol Improves Neurological Outcome

To determine whether resveratrol plays a role in neurological functional impairment and recovery after stroke, mice were subjected to MCAo, and a battery of functional tests were performed. Neurological functional recovery was significantly attenuated in resveratrol group on NSS (Fig 1 A), foot-fault (Fig. 1 B) and corner (fig. 1 C) tests at 14 d after stroke compared with vehicle-group in mice subjected to MCAo (Fig. 1).

Resveratrol Enhances Angiogenesis

Treatment with resveratrol significantly increased BrdU-reactive endothelial cell numbers (Fig 2 G) and vascular density (Fig 2 H) in the ischemic border, as compared with the vehicle-treated animals. BrdU with lectin double immunostaining shows that BrdU-positive endothelial cells localize in the vessel (Fig 2 C, F). These data indicate that resveratrol enhances angiogenesis in the ischemic brain.

Resveratrol Enhances Neurogenesis

To test whether resveratrol promotes neuronal migration and proliferation, BrdU, DCX and TUJ1 immunostaining were performed in the brain coronal sections. Figure 3 shows that resveratrol after stroke significantly increased BrdU in SVZ area (C and D). In addition, DCX (Fig 4 B and C) and TUJ-1 (Fig 4 E) positive cells in the SVZ and ischemic border, respectively, showed increase in animals treted with resveratrol as compared with vehicle-treated mice. These findings suggest that resveratrol promotes cell migration and differentiation into neurons.

Resveratrol modulates neural stem cell in vitro

Complementing the *in vivo* indication of neurogenesis, neurospheres derived from embryous 13^{th} day mice exhibited a significant ($P<0.05$) increase in the number of TuJ1 (neuronal marker)-positive cells (Figure 5 B-E) when treated with resveratrol dose-depende compared with the control (Figure 5 A and E). Figure 5 (F and G) shows that resveratrol significantly increases the secondary neurosphere formation (Fig 5 F) and proliferation measured using MTT assay (Fig 5 G). These data indicate that resveratrol directly affects SVZ neurosphere formation and promoted neuronal progenitor cell proliferation.

Effects of Resveratrol on VEGF and BDNF expression

In the ischemic peri-infarct region, expression of VEGF and BDNF, were analyzed using Western blot. In stroke plus resveratrol animals, the protein level of VEGF was increased at 14 days after stroke (Fig 6 A and B). In the case of BDNF expression, there was also significant increase in

resveratrol group compared with stroke-only and sham- operated groups (Fig 6 A and C).

Effect of Resveratrol on Cell Migration in SVZ Explant Cultures

Figure 7 shows that resveratrol promote SVZ explant cell migration (E), compared with control (B). Anti-BDNF and Anti-VEGF antibody significantly inhibited cells migration compared with control (Fig 7 C, D and H). Coculture anti-BDNF and anti-VEGF with resveratrol significantly inhibited resveratrol-induced SVZ neural cell migration, compared with resveratrol alone (Fig 7 F, G and H).

Discussion

In the current study, we have demonstrated that resveratrol when administered to mice starting 3 days after MCAO, evokes significant improvement in functional neurologic recovery. In addition, we have also shown that resveratrol promotes: (1) concomitant with this neurological benefit is a significant induction of angiogenesis in the ischemic brain; (2) neurogenesis and neuronal plasticity as well as increases the expression of VEGF and BDNF after stroke in mice; (3) neural cell migration in SVZ explants mediated by BDNF and VEGF; (4) resveratrol increase neurogenesis *in vitro* and differentiation in neurons. Collectively, these *in vivo* and *in vitro* data strongly support a role for resveratrol in promoting brain plasticity and recovery from stroke.

Stroke stimulates neurogenesis in the SVZ and newly generated neuroblasts in the SVZ migrate to the ischemic boundary region (Jin et al., 2003; Zhang et al., 2001; Arvidsson et al., 2002). The present study indicates that resveratrol augments neurogenesis in the ischemic SVZ region. Neurogenesis enhanced by resveratrol is likely involved in multiple mechanisms. In addition, our *in vitro* data indicate that, in the presence of the bFGF and EGF, resveratrol promotes neural progenitor cell proliferation and 2^{nd} neurosphere formation. Furthermore, resveratrol enhances the ischemic neural progenitor cell differentiation into neurons. Thus, resveratrol can promote neurogenesis by directly acting on enhancement of proliferation and differentiation of SVZ neural progenitor cells.

The present *in vivo* data show that resveratrol substantially increased DCX-positive cells in the SVZ area. DCX-positive cells are migrating neuroblasts and are generated in the SVZ (Alvarez- Buylla et al., 2000). Under physiological condition, these neuroblasts migrate to the olfactory bulb, where they differentiate into neurons (Alvarez-Buylla et al., 2000). After focal cerebral ischemia, neuroblasts in the SVZ migrate to the ischemic boundary region to replace damaged neurons (Jin et al., 2001; Zhang et al., 2001; Arvidsson et al., 2002). Our *in vitro* data indicate that resveratrol has a direct effect on promoting neural progenitor cell migration and it is modulated by BDNF and VEGF. Together with in vivo results, the present study suggests that, in addition to an increase in the number of neuroblasts, resveratrol may promote SVZ neuroblast migration to the ischemic boundary.

Our data show that treatment with resveratrol initiated 3 days after stroke significantly improved functional outcome. Augmentation of proliferation, migration, and survival of neuroblasts may contribute to the observed improvement of functional outcome. The 10mg/kg dose of resveratrol used in this study is consistent with the neuroprotective dose of resveratrol used in pretreatment of animals after stroke (Dong et al, 2008). Wang and collegues (2007) have shown that *in vitro*, resveratrol has a dose–response curve on inducing prolifration and migration in progenitor endothelial cells.

In addition to its role in inducing angiogenesis, VEGF also stimulates neurogenesis and axonal outgrowth, and improves the survival of mouse superior cervical, dorsal root ganglion neurons, and mesencephalic neurons (Hess et al, 2002; Sondell et al, 1999). VEGF is mitogenic for astrocytes and

promotes growth/ survival of neurons (Silverman et al, 1999). Our previous data have shown that resveratrol promotes angiogenesis as well as increases VEGF expression. We propose that the resveratrol-induced increase of VEGF may not only cause angiogenesis but also provide a supportive microenvironment, which can enhance the neuronal and synaptic plasticity. Neurogenesis occurs in close proximity to blood vessels, where VEGF expression is high and angiogenesis is ongoing (Palmer et al, 2000). Furthermore, resveratrol has shown increase BDNF expression in 14 days after stroke. The newly activated and expanded vasculature substantially increases the production and release of BDNF, whose induction is both spatially and temporally associated with recruitment of new neurons (Leventhal et al, 1999).

Resveratrol induces neuronal migration in the SVZ explant culture. Inhibition of BDNF and VEGF decreases neuronal migration in the SVZ explant. These data suggest that neuronal plasticity is closely linked with BDNF/VEGF may facilitate resveratrol-induced neuronal plasticity after stroke. Resveratrol-mediated functional benefit might be derived from the upregulation of trophic factors such as VEGF/BDNF and induction of angiogenesis, neurogenesis, and synaptic plasticity.

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Legends to Figure

Figure 1. Resveratrol promotes functional recovery. Mice were treated with or without resveratrol (10mg/kg) starting at 72 hours after stroke daily for 14 days (n=12 group). Functional tests were performed before MCAO and 1, 3, 7, 10 and 14 days after MCAO. *P<0.05 vs. stroke control. (A) Neurological severity score test (NSS); (B) Foot-fault test; (C) Corner test.

Figure 2. Resvertrol enhances angiogenesis. A-C, stroke group; D-F, resveratrol-treated stroke group. A and D, cerebral vessels immunostained with Lectin at 14 days after stroke. B and E, BrdU immunoreactive cells. C and F , BrdU immunoreactive endothelial cells at the ischemic boundary region of a representative mice treated with resveratrol at 10 mg/Kg (F), BrdU-positive endothelial cells of a representative mouse from the stroke-only group (C). (G) Quantitative data of BrdU/Lectin positive cells at the ischemic boundary regions. (H) Quantitative data of vascular density at the ischemic boundary regions. Numbers of cerebral vessels increased in the ischemic region in mice treated with resveratrol at 10 mg/Kg (D) compared with the stroke-only mice (A). *p<0.05 vs. stroke group.

Figure 3. The effect of resveratrol on cell proliferation in the SVZ. Proliferation of neuroblast in the SVZ was evaluated by BrdU-positive cells 14 days after stroke in sham-operated mice (A), stroke-only mice (B), and resveratrol mice (C). (D) Ischemia significantly increased the number of BrdU-positive cells in the ipsilateral SVZ, measured 14 days post-ischemia, in both ischemic stroke-

only mice and resveratrol mice compared with sham-operated mice. N=6 animals in each group. Data are expressed as mean ± SEM. *p < 0.05 vs. sham group; #p < 0.05 vs stroke group.

Figure 4. Resveratrol enhanced migration and neurogenesis. The effects of resveratrol on neuroblasts migration were examined by immunostaining of BrdU (green) and DCX (red) 14 days after the onset of ischemia on SVZ area. (A) Double-labeling of DCX/BrdU-positive cells in the SVZ area of ischemic stroke-only and stroke plus resveratrol mice. (B) Quantitative data of DCX/BrdU positive cells at the SVZ region. (C) Quantitative data of DCX positive cells at the SVZ region. (D) Neurogenesis in the ischemic border region was examined by the colocalization of the neuronal marker TUJ-1 (red) and the proliferation marker BrdU (green) 14 days after stroke. Arrows show double-labeling of TUJ-1/BrdU positive cells in the ischemic peri-infarct region of ischemic stroke-only and stroke plus resveratrol mice. There were more TUJ-1/BrdU-positive cells in resveratrol group compared with stroke-only group (E). Cell count was performed in four randomly chosen fields in the penumbra region per section. The total number of cells in four sections was summarized for each animal. Cell counts show increased numbers of TUJ-1/BrdU-positive cells in resveratrol group compared with stroke-only group. N=5 animals in each group. Data are expressed as mean ± S.D. *p < 0.05 vs. sham group. #p < 0.05 vs. ischemic stroke group.

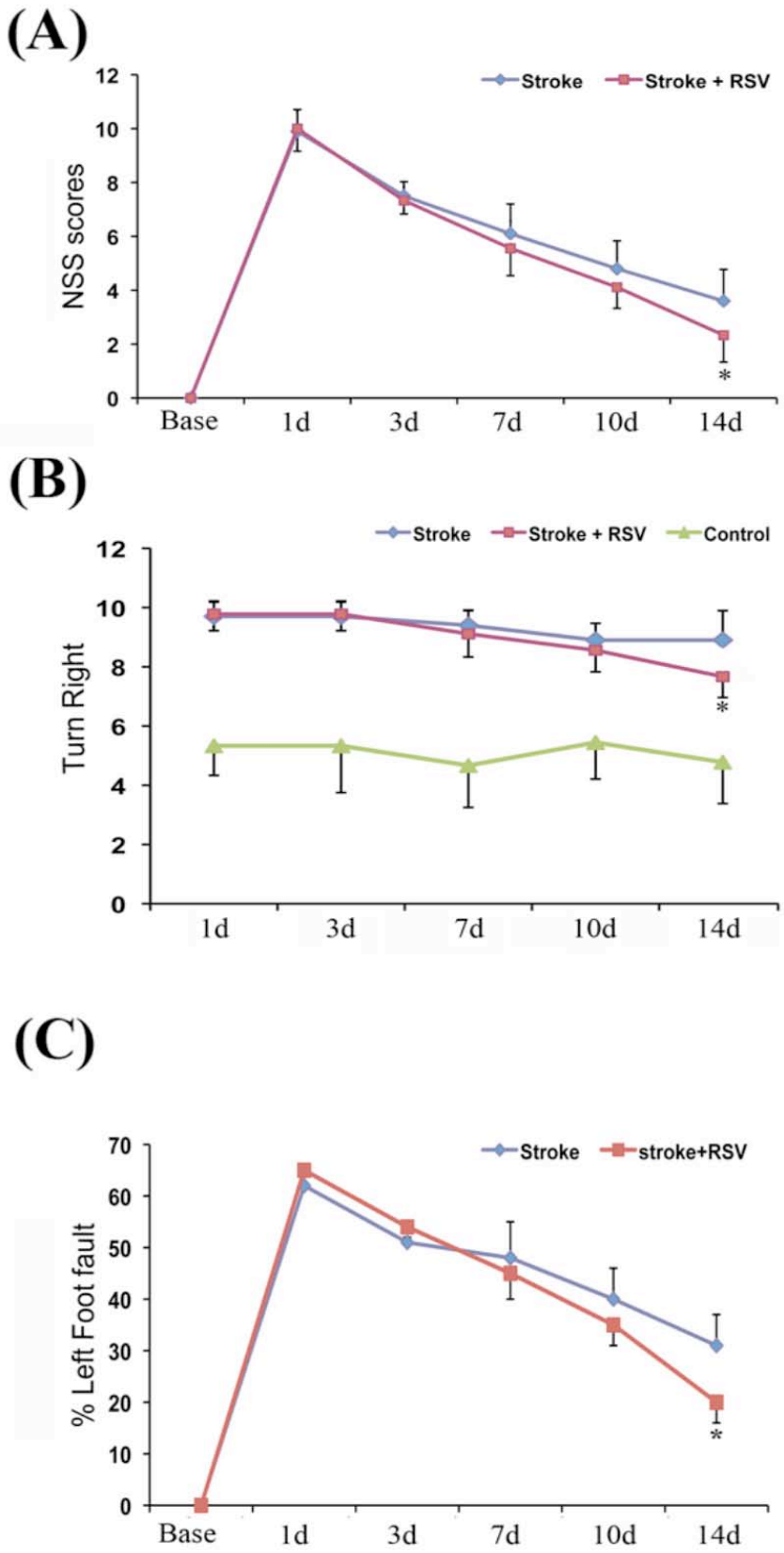
Figure 5. Resveratrol increases number of neurons *in vitro*. A through D, TuJ1 immunoreactive cells differentiated from single neural stem cell, control (A),

RSV 0.1 μM (B), RSV 0.5 μM (C) and RSV 1 μM (D). Quantitative data of TuJ1-positive cells (E), secondary neurosphere formation (F) and proliferation assay by MTT (G). *P<0.05 vs. control.

Figure 6. Effects of resveratrol on expression of neurovascular regulatory factors. The protein levels of VEGF and BDNF were detected using Western blot analysis. (A) Representative electrophoresis gels show the expression level of VEGF and BDNF in the ischemic peri-infarct region at 14 days after stroke. (B and C) Densitometry analysis for comparisons of each factor. Sample was normalized against β-actin and quantified using ImageJ software. Resveratrol enhanced the expression of BDNF (C) and VEGF (B) compared with stroke- only group. N=5 animals for each group. Data are expressed as mean ± SEM. *p< 0.05 vs. stroke group.

Figure 7. SVZ explant cell migration. C=Control; B=anti-BDNF; V=anti-VEGF; R=Resveratrol; R+B= Resveratrol + anti-BDNF; R+V= Resveratrol + anti-VEGF. (H) Quantitative data of SVZ cell migration.

Figura 1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Stroke + RSV
NSS scores
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8
6
4
2
0
Base
1d
3d
7d
10d
14d
*
(B)
Stroke
Stroke + RSV
Control
Turn Right
12
10
8
6
4
2
0
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3d
7d
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Base
1d
3d
7d
10d
14d
*

Figura 2

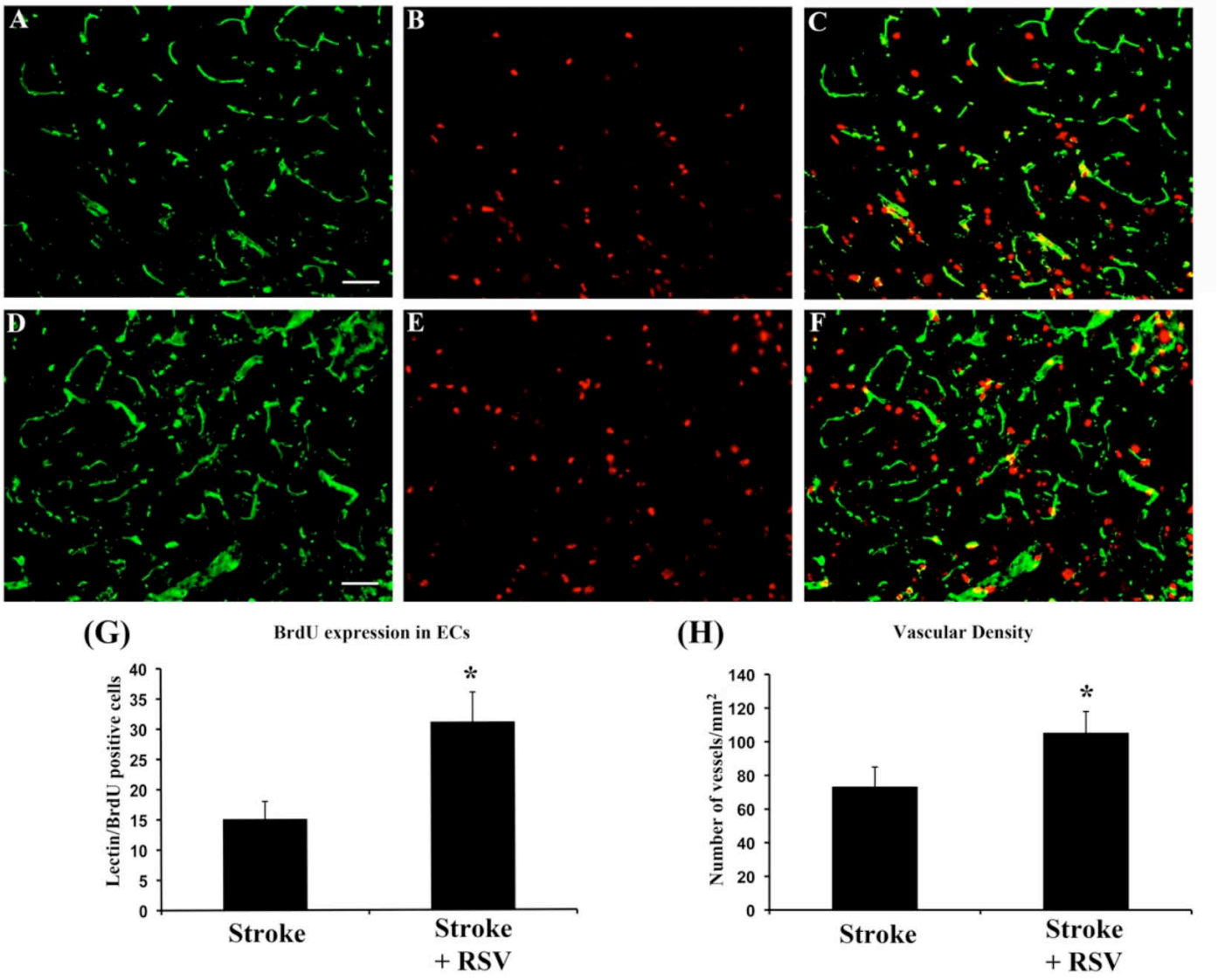

A
B
C
D
E
F
(G)
BrdU expression in ECs
Lectin/BrdU positive cells
40
35
30
25
20
15
10
5
0
*
Stroke
Stroke
+ RSV
(H)
Vascular Density
Number of vessels/mm2
140
120
100
80
60
40
20
0
*
Stroke
Stroke
+ RSV

Figura 3

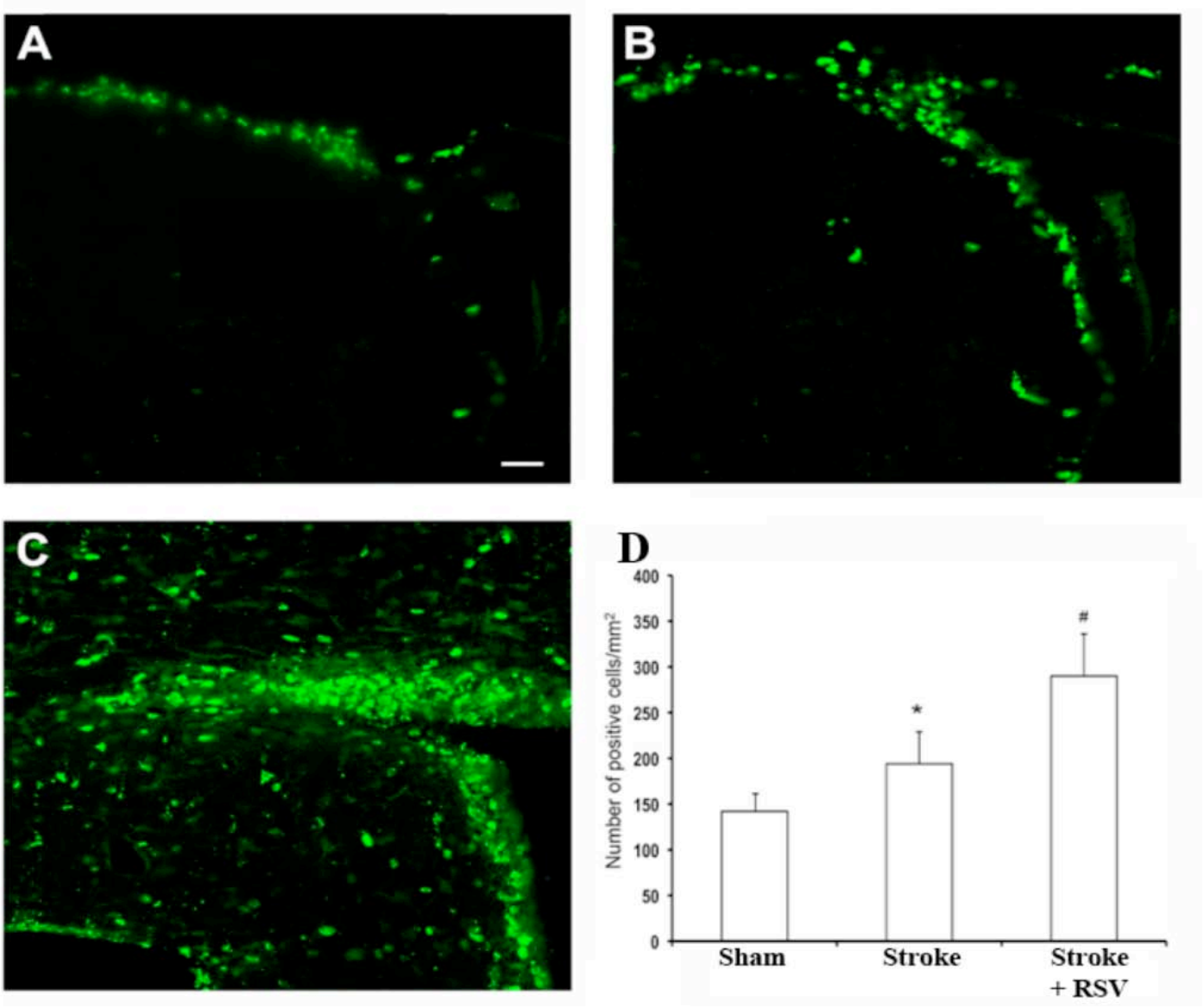

A
B
C
D
Number of positive cells/mm²
400
350
300
250
200
150
100
50
0
*
#
Sham
Stroke
Stroke
+ RSV

Figura 4

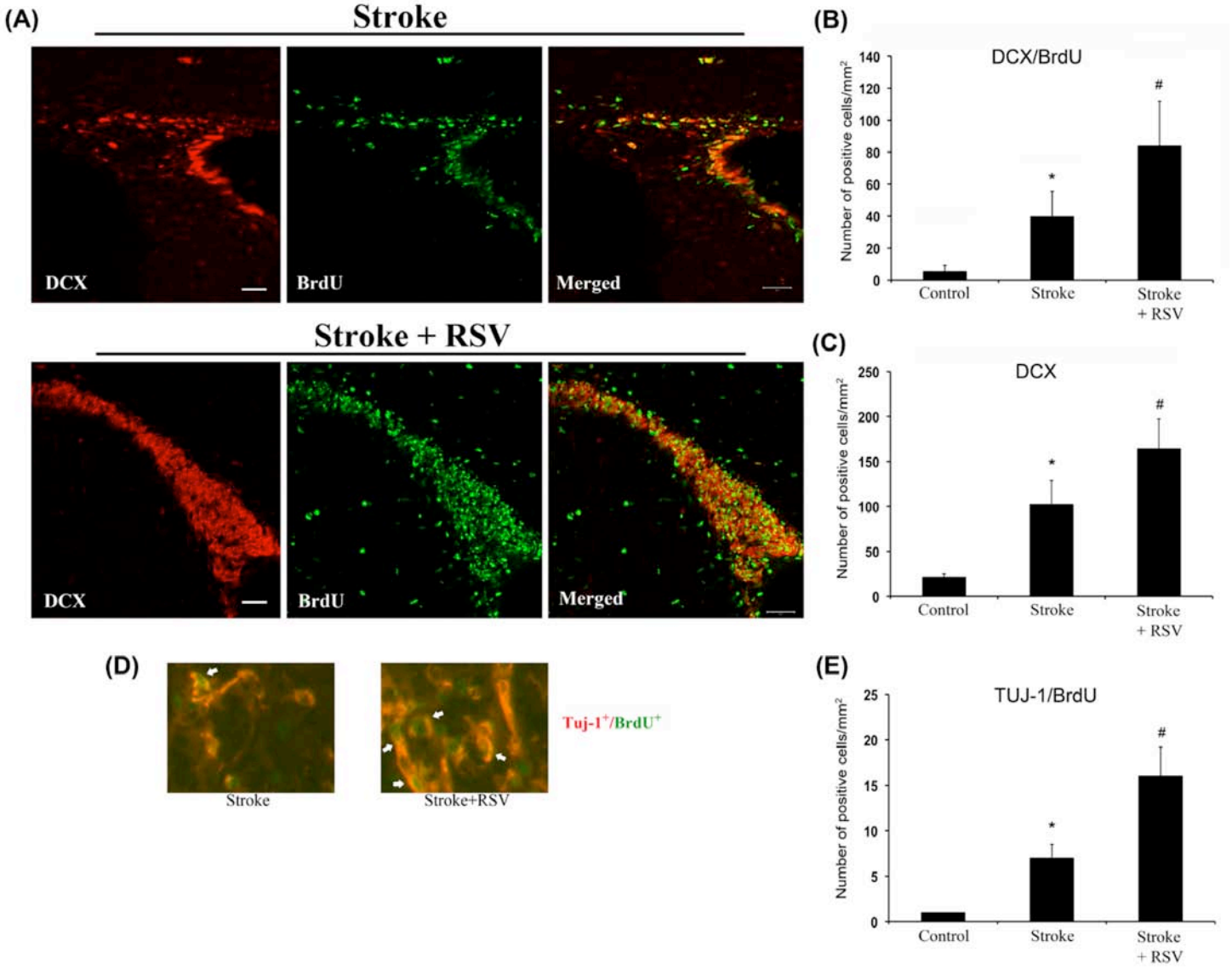

(A)
Stroke
DCX
BrdU
Merged
Stroke + RSV
DCX
BrdU
Merged
(B)
DCX/BrdU
Number of positive cells/mm²
Control
Stroke
Stroke + RSV
(C)
DCX
Number of positive cells/mm²
Control
Stroke
Stroke + RSV
(D)
Stroke
Stroke+RSV
Tuj-1⁺/BrdU⁺
(E)
TUJ-1/BrdU
Number of positive cells/mm²
Control
Stroke
Stroke + RSV

Figura 5

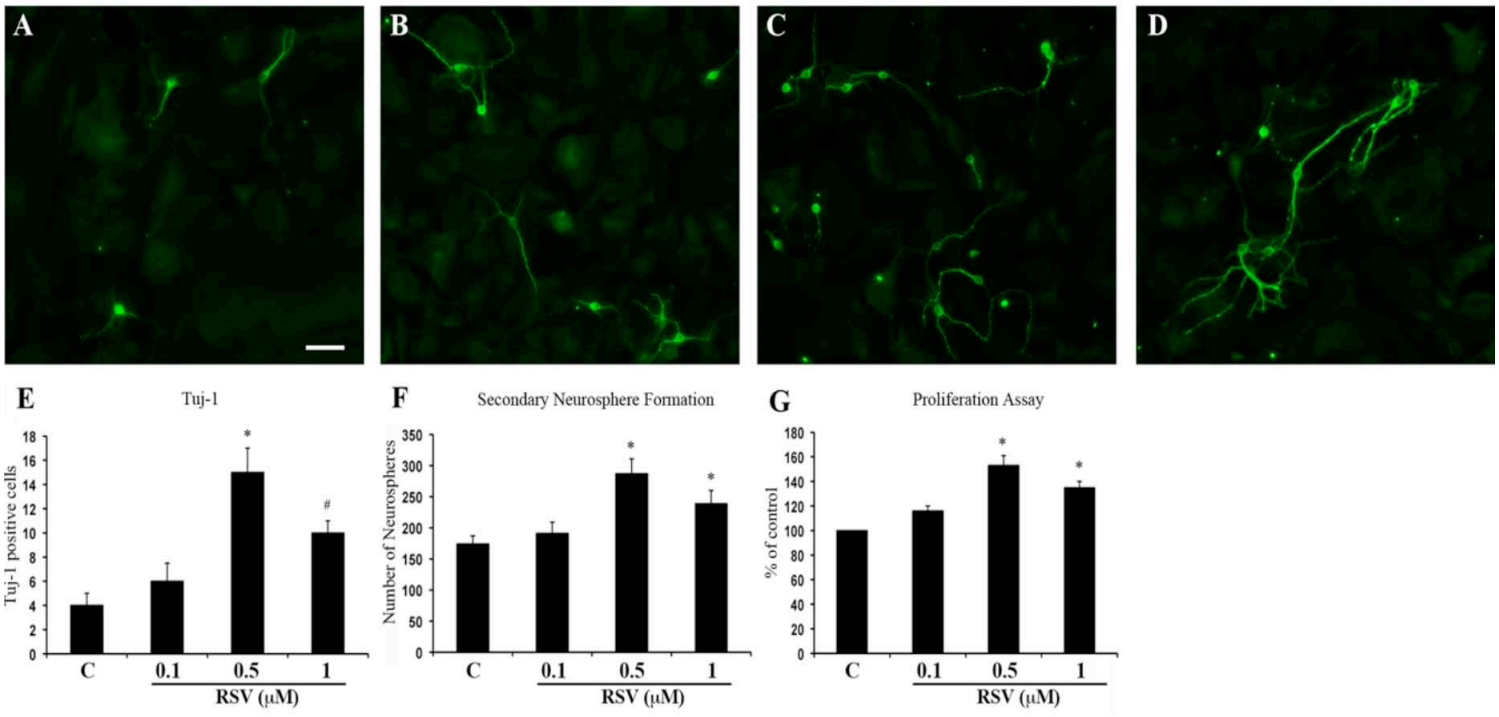

A
B
C
D
E
Tuj-1
Tuj-1 positive cells
RSV (μM)
F
Secondary Neurosphere Formation
Number of Neurospheres
RSV (μM)
G
Proliferation Assay
% of control
RSV (μM)

Figura 6

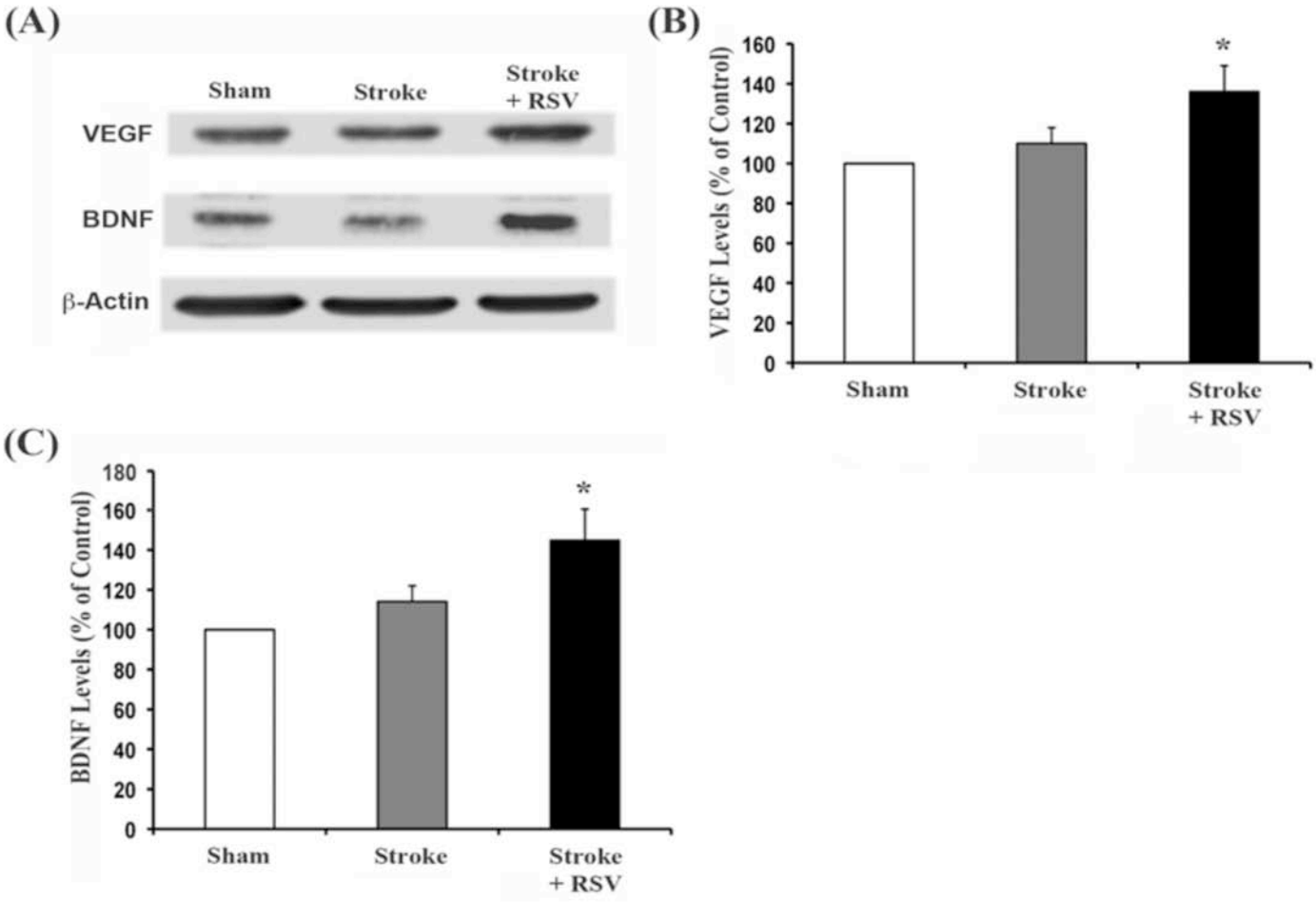
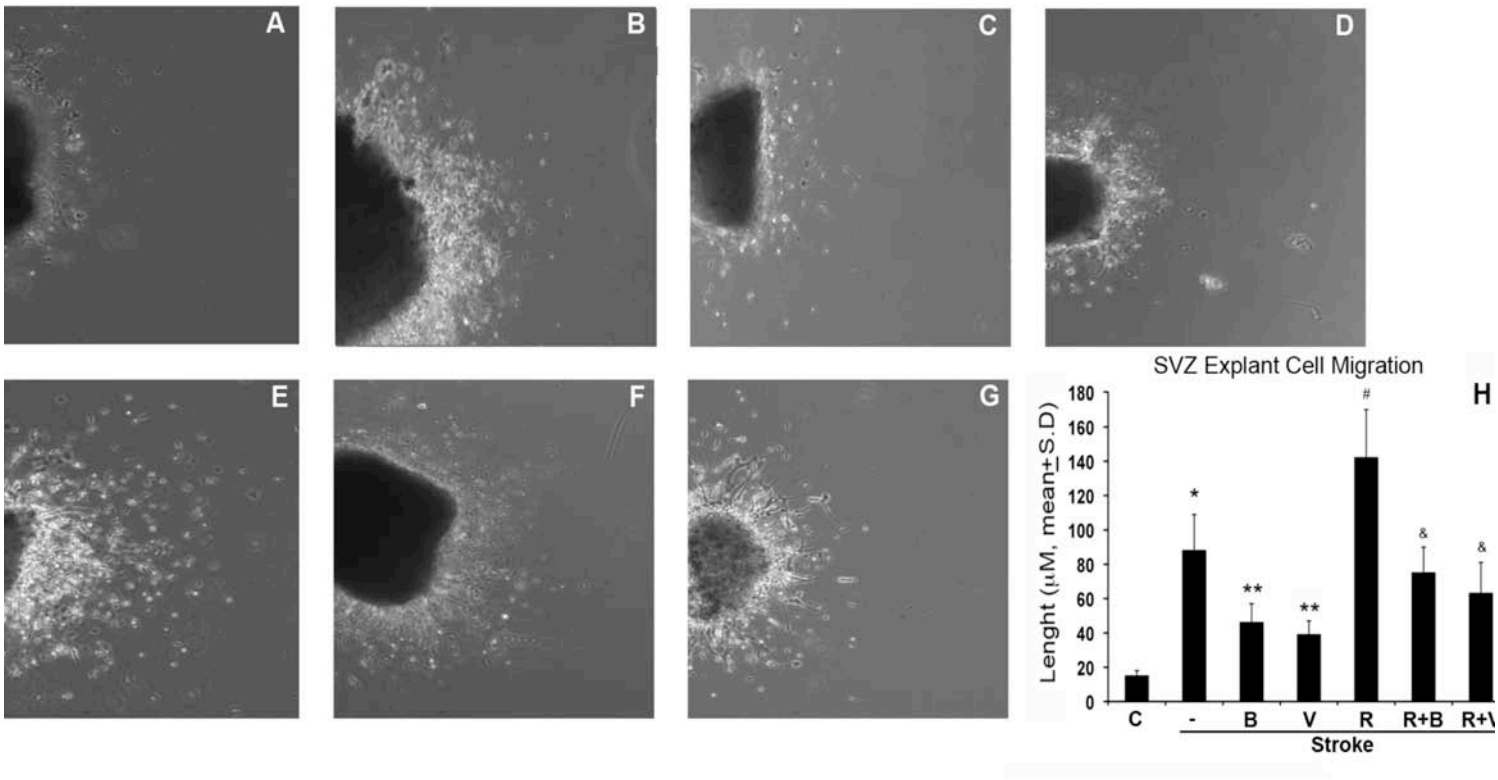

(A)
Sham
Stroke
Stroke + RSV
VEGF
BDNF
β-Actin
(B)
VEGF Levels (% of Control)
160
140
120
100
80
60
40
20
0
*
Sham
Stroke
Stroke + RSV
(C)
BDNF Levels (% of Control)
180
160
140
120
100
80
60
40
20
0
*
Sham
Stroke
Stroke + RSV

Figura 7


A
B
C
D
E
F
G
H
SVZ Explant Cell Migration
Lenght (µM, mean±S.D)
180
160
140
120
100
80
60
40
20
0
C
-
B
V
R
R+B
R+V
Stroke

9. DISCUSSÃO

Durante as últimas décadas, evidências vêm demonstrando que o resveratrol influencia o crescimento, a diferenciação, a maturação e a função de vários tecidos, além de desempenhar um potente efeito neuroprotetor. O resveratrol têm sido implicado nos efeito benéfico da ingestão prolongada de vinho tinto, na tentativa de regularizar o micro-ambiente ou de aumentar a resistência da própria célula, "proteção". Tendo em vista que a isquemia cerebral é um problema de grande relevância clínica, devido a sua alta freqüência e morbidade, bem como aos elevados custos de manutenção de pacientes com seqüelas, nossa proposta foi estudar os mecanismos envolvidos na neuroproteção e recuperação funcional promovida pelo resveratrol. Os resultados obtidos neste trabalho contribuem para avaliar os efeitos do resveratrol, em condições basais e também na isquemia cerebral experimental, visando o melhor entendimento do seu mecanismo de ação. Nesta perspectiva, nosso objetivo foi investigar os mecanismos envolvidos na neuroproteção, bem como o efeito do resveratrol na modulação da angiogênese e da neurogênese para a promoção da recuperação funcional.

Grande número de estudos animais, tanto *in vitro* como *in vivo*, têm sugerido que o resveratrol desempenha um importante papel neuroprotetor, impulsionando o interesse em determinar a sua eficácia na prevenção ou recuperação de doenças neurodegenerativas e cerebrovasculares em humanos (HUANG *et al.*, 2001a; SINHA *et al.*, 2002; WANG *et al.*, 2002; GAO *et al.*, 2006a; ZAMIN *et al.*, 2006; RAVAL *et al.*, 2008; FUKUI *et al.*, 2010). Além do efeito neuroprotetor, destacam-se sua atividade antioxidante, cardioprotetora, anti-inflamatória e antitumoral. Recentemente, têm sido sugerido que o principal mecanismo regulatório dos efeitos biológicos do resveratrol seja pela atuação na via de sinalização das sirtuínas. As sirtuínas são histonas deacetilases dependentes de NAD que participam na

fisiopatologia de uma série de modificações relacionadas à idade e que, de acordo com estudos experimentais, estariam associadas à longevidade (ANEKONDA, 2006). O resveratrol demonstrou aumentar a longevidade em diversas espécies como *Saccharomyces cerevisiae*, *Caenorhabditis elegans*, *Drosophila melanogaster* e roedores por ativar as sirtuinas (WOOD *et al.*, 2004).

Os quatro primeiros capítulos desta tese buscaram aprofundar o conhecimento das propriedades neuroprotetoras do resveratrol no modelo de isquemia cerebral global. Utilizamos o tratamento com resveratrol nos 7 dias que antecederam o evento isquêmico, como pré-tratamento, com o propósito de avaliar a neuroproteção. DAS e col. (2005c) demonstraram que um tratamento curto com resveratrol por 7 dias foi suficiente para disparar mecanismos endógenos, resultando em redução dos efeitos deletérios promovidos pela isquemia cardíaca. O pré-tratamento com resveratrol foi capaz de aumentar, de forma coordenada, a indução de iNOS-VEGF-KDR-eNOS e de promover o pré-condicionamento cardíaco, sendo que os pré-tratamentos de 3 e 5 dias não foram efetivos (DAS *et al.*, 2005c). O pré-condicionamento com resveratrol pode oferecer maior discriminação entre os mecanismos responsáveis pela neuroproteção endógena, e assim fornecer novas estratégias para tornar as células cerebrais mais resistentes à lesão isquêmica. Neste contexto, a neuroproteção vem oferecendo novas alternativas de estratégias terapêuticas que antagonizam e/ou interrompem uma seqüência de eventos bioquímicos e moleculares prejudiciais desencadeados pela isquemia. Com a proposta de neuroproteção frente a uma lesão cerebral, utilizamos o modelo de isquemia cerebral global que se caracteriza especialmente pela vulnerabilidade neuronal na região de CA1 hipocampal e no córtex cerebral. Outra característica do modelo é a morte neuronal tardia, que só acontece de 3-7 dias após o

episódio isquêmico na região de CA1 hipocampal (PULSINELLI & BRIERLEY, 1979).

Os resultados apresentados no primeiro capítulo demonstram que o pré-tratamento com resveratrol foi neuroprotetor no modelo de isquemia cerebral global em ratos, prevenindo a morte neuronal na região de CA1 e no córtex cerebral. Nosso estudo corrobora o de WANG e col. (2002), no qual o resveratrol foi neuroprotetor e utilizado em dois momentos em gerbilos: (1) durante o evento isquêmico e 24 hs mais tarde e (2) imediatamente após o evento, com uma segunda injeção também 24 hs mais tarde. Ambos os tratamentos reduziram a morte neuronal em CA1, além de diminuírem a ativação de astrócitos e de microglia. Durante o período de reperfusão pós-isquemia, aumenta o suprimento de oxigênio resultando em uma superprodução de espécies reativas de oxigênio (EROS) e nitrogênio (ERN) (CRACK & TAYLOR, 2005); tanto EROS e ERN são poderosos mediadores da lesão isquêmica e são rapidamente aumentados. Em um primeiro momento, o ambiente redox das células modulam cascatas de transdução de sinal que faz perder o balanço entre vias de sobrevivência e morte (CRACK & TAYLOR, 2005). No segundo cenário, EROS e ERN atuam diretamente como executores e operadores da morte celular (CHAN, 2001).

O cérebro é particularmente vulnerável ao ataque dos radicais devido às suas limitadas defesas antioxidantes (ADIBHATLA & HATCHER, 2010). Nossos resultados demonstraram que a isquemia cerebral global promoveu rápida geração de EROS e ERN 1 hora após a isquemia, seguida pelo aumento da peroxidação lipídica nos tempos de 1 e 24 horas em hipocampo e córtex cerebral. Uma vez que a isquemia aumentou a produção de radicais livres, espera-se que possa estar modulando as defesas antioxidantes. Neste contexto, a isquemia diminuiu as defesas antioxidantes

em ambas estruturas estudas. O pré-tratamento com resveratrol diminuiu a geração de EROS, de ERN, bem como a peroxidação lipídica nos mesmos tempos analisados para a isquemia.

A remoção de radicais livres gerados patologicamente durante o período de isquemia parece ser uma abordagem viável para neuroproteção. Com isso, o resveratrol mostrou atuar como um excelente "scavenger" de EROS e ERN, prevenindo a peroxidação lipídica, e aumentando a capacidade antioxidante. Tendo em mente que a isquemia cerebral global esta associada a um desequilíbrio da homestase iônica, avaliamos a atividade da enzima Na^+K^+-ATPase. A Na^+K^+-ATPase está localizada na membrana citoplasmática, é responsável pelo transporte ativo dos íons Na^+ e K^+ no sistema nervoso, mantendo o gradiente iônico necessário para a regulação e excitabilidade neuronal. Além disso, estudos demonstram que a enzima é sensível a ação de radicais livres. Em nosso estudo, a isquemia cerebral global diminuiu a atividade da enzima, possivelmente devido ao aumento de radicais livres e da peroxidação lipídica da membrana, componente essencial para a manutenção da enzima. O tratamento com resveratrol preveniu a inibição da atividade da Na^+K^+-ATPase no córtex cerebral e hipocampo. Tais observações sugerem que o resveratrol pode ser um agente protetor clinicamente viável para uma variedade de condições onde o dano celular é uma conseqüência do estresse oxidativo.

No segundo capítulo, propusemos investigar os efeitos do resveratrol sobre o conteúdo e a distribuição de gangliosídios, assim como as concentrações de fosfolipídios e de colesterol no córtex cerebral e no hipocampo de ratos após a isquemia cerebral global. Esta hipótese foi baseada nos resultados encontrados quanto à modulação de radicais livres e peroxidação da membrana pela isquemia, que, uma vez aumentados, podem desestabilizar componentes da membrana lipídica.

Consequentemente, pode levar a alteração da estrutura da membrana plasmática, abalando a homeostase iônica e ativando a degradação de enzimas.

Tem sido demonstrado que o cérebro contém muitos lipídeos complexos e preservam uma variedade de funções fisiológicas em neurônios. Dentre os lipídeos, os gangliosídeos estão presentes em grandes quantidades na membrana neuronal, desempenhando funções de maturação e diferenciação de sinapses, comunicação intercelular e na plasticidade neuronal (AGRANOFF & HAJRA, 1994). Além disso, os fosfolipídios realizam funções estruturais, bem como a modulação da sinalização celular. Já o colesterol controla propriedades físico-químicas e contribui para a formação do transporte (OHVO-REKILÄ *et al.*, 2002). Em nosso estudo, a isquemia cerebral global promoveu uma redução no conteúdo total de gangliosídeo, fosfolipídeos e colesterol nos tempo de 1 e 24 hs após isquemia em córtex cerebral e hipocampo. Dados da literatura descrevem que o gangliosídeo GM1 é neuroprotetor no modelo de isquemia cerebral global, além de diminuir as reações de radicais livres induzida por glutamato (MAHADIK *et al.*, 1989).

Na proposta de identificar possíveis gangliosídeos que estejam sendo modulados pela isquemia cerebral global, analisamos o perfil de diferentes espécies de gangliosídeos. A isquemia cerebral global não alterou as diferentes espécies de gangliosídeos, entretanto, a quantidade absoluta diminuiu em ambas as estruturas estudadas. O pré-tratamente com resveratrol preveniu a diminuição dos componentes de lipídios da membrana em hipocampo e córtex cerebral de ratos. Baseado em dados da literatura que descrevem que o dano celular é devido, pelo menos em parte, ao dano oxidativo em lipídios de membrana, nós propomos que o resveratrol possa estar preservando os componentes de lipídios e mantendo a estabilização da membrana através de sua função antioxidante.

Conforme mencionada na introdução, o resveratrol tem mostrado modular a função de muitas proteínas regulatórias envolvidas na transdução de sinal, no crescimento, sobrevivência, e morte celular (DAS, 2005a, b, c; ZAMIN *et al.*, 2006). Os alvos de ação compreendem, entre outros o óxido nítrico (HATTORI *et al.*, 2002; IMAMURA *et al.*, 2002; HUNG *et al.*, 2004.), receptores de adenosina (DAS *et al.*, 2005a, b), MAPK/ERK (DAS *et al.*, 2006a, b) e a via de sinalização da PI3-K/Akt (DAS *et al.*, 2006a, b).

Na tentativa de elucidar um dos possíveis mecanismos moleculares envolvidos com a via de sinalização da PI3-K, investigamos a ativação da proteína Akt. Nesta cascata de transdução de sinal, a Akt é o efetor direto, logo abaixo de PI3-K (SONGYANG *et al.*, 1997), e uma proteína cinase chave que promove a sobrevivência neuronal depois da isquemia (ZHANG *et al.*, 2004). Seguindo esta via, a GSK-3β é um substrato da Akt (CROSS *et al.*, 1995). O principal mecanismo regulatório destas enzimas é por fosforilação: a Akt é ativada enquanto que a GSK-3β é inibida por fosforilação (LI *et al.*, 2000). A ativação da GSK-3β promove a sinalização pró-apoptótica (LI *et al.*, 2000). O CREB, proteína de ligação ao elemento de resposta ao AMP cíclico, é outra molécula alvo regulada pela Akt (SHAYWITZ & GREENBERG, 1999). CREB tem sido reconhecido como um importante fator nuclear por promover a transcrição de genes envolvidos com a sobrevivência da célula, tais como o BDNF e *c-fos* (TAO *et al.*, 1998). Nossos resultados mostraram que o resveratrol induziu a fosforilação de ambas as proteínas, refletindo na ativação de Akt, inibição de GSK-3β e ativação de CREB. O aumento nos níveis de fosforilação das proteínas foi observado 1 e 4 horas após a isquemia, voltando aos níveis de base em 24 horas. O pico de fosforilação para todas as proteínas foi em 1 hora, sendo o tempo escolhido para os demais experimentos.

Com o propósito de confirmar o envolvimento da Akt na neuroproteção, foi utilizado LY294002, um inibidor direto de PI3-K e, conforme descrito no capítulo 3, este reduziu a fosforilação da proteínas Akt, GSK-3β e CREB e, paralelamente preveniu o efeito neuroprotetor do resveretrol. Este resultado descreve fortemente que as proteínas GSK-3β e CREB estejam sendo moduladas por uma via dependente da PI3-K/Akt. Portanto, esses resultados sugerem que o resveratrol possa estar ativando uma das vias de sinalização envolvidas com a sobrevivência celular através da Akt/CREB e outra, no bloqueio de uma das vias que levam a indução da apoptose pela Akt/GSK-3β.

Acredita-se que estas ações rápidas, não-transcricionais ou não-genômicas sejam de natureza modulatória, e críticas para a comunicação célula-célula que estão ocorrendo na superfície celular (GREEN *et al.*, 1996). Visto que o pré-tratamento com resveratrol inicia uma série de eventos bioquímicos que são conhecidos por aumentar a tolerância do cérebro a fatores lesivos, sugerimos que a droga pode induzir a ativação de vias de sobrevivência após o insulto isquêmico. A ativação dessas vias, que são inter-relacionadas e complexas, pode resgatar células neuronais após o insulto metabólico.

Com a finalidade de ampliar a investigação sobre os mecanismos envolvidos no efeito neuroprotetor induzido pelo resveratrol, sendo que muitos desses efeitos biológicos interagem na promoção da neuroproteção, focalizamos na ação antiinflamatória do resveratrol na isquemia cerebral global. A inflamação é uma resposta orquestrada envolvendo a regulação e ativação rápida de uma variedade de proteínas e genes. O bloqueio da inflamação tem sido descrito como atenuador do dano isquêmico cerebral (NOGAWA *et al.*, 1997; PHILLIS *et al.*, 2006). Após um insulto isquêmico, os astrócitos são ativados induzindo a um aumento na expressão da

proteína GFAP, o que caracteriza o processo denominado astrogliose reativa (PEKNY & NILSSON, 2005). Além disso, juntamente com a microglia ativada, os astrócitos secretam moléculas que são capazes de induzir excitotoxicidade, estresse oxidativo e inflamação. Dentre estas moléculas pode-se citar o glutamato, citocinas como TNF-α, IL-1β e IL-6, prostaglandinas e o radical superóxido (BLOCK *et al.*, 2007). Os resultados descritos no capítulo 4 mostraram que o tratamento com resveratrol foi capaz de reduzir a ativação glial, demonstrado pelo decréscimo no imunoconteúdo da proteína GFAP. Além disso, o tratamento com resveratrol reduziu a ativação microglial, como pode ser observado pela diminuição da reatividade da Isolectina B4. Nossos resultados nos permitem propor que o efeito neuroprotetor do resveratrol também poderia ser conseqüência da diminuição da ativação glial.

A ativação da cascata inflamatória pode desencadear danos celulares. Portanto, a lesão de qualquer constituinte da unidade neurovascular pode levar ao extravasamento vascular de células inflamatórias e proteínas que são prejudiciais para os neurônios (HUANG *et al.*, 2006). Os principais intervenientes na lesão inflamatória são citocinas, moléculas de adesão, eicosanóides e óxido nítrico, que são produzidos imediatamente após o início da isquemia e contribuem para o estabelecimento de danos irreversíveis (MEHTA *et al.*, 2007). Vários estudos sugerem que o NF-κB é o fator de transcrição mais importante na resposta inflamatória que pode ser ativado por espécies reativas de oxigênio, citocinas ou vírus.

Há um conjunto de evidências que sugerem a ativação do NF-κB após um episódio de isquemia cerebral. Muitos estudos demonstraram a ativação do NF-κB em neurônios (CLEMENS *et al.*, 1997; SCHNEIDER *et al.*, 1999; HUANG *et al.*, 2001; NURMI *et al.*, 2004). Além disso, a ativação de NF-κB também foi encontrada em

células endoteliais, astrócitos e microglia (TERAI *et al.*, 1996; HOWARD *et al.*, 1998; GABRIEL *et al.*, 1999; ZHANG *et al.*, 2007). A ativação de NF-κB envolve a translocação nuclear das subunidades p50 e p65 e aumento da ligação ao DNA de p50 e p65 (SCHNEIDER *et al.*, 1999; HUANG *et al.*, 2001). O aumento da ligação ao DNA reflete a ativação de NF-κB. A localização de p65 nuclear em nosso estudo, conforme descrito no capítulo 4, apresenta semelhança com achados anteriores no modelo de isquemia cerebral global, onde NF-κB foi localizado no núcleo das células da região de CA1 hipocampal. Nossos resultados mostraram que o tratamento com resveratrol foi capaz de prevenir significativamente a fosforilação e conseqüente ativação de NF-κB nuclear, levando os níveis de fosforilação dessa proteína a níveis comparáveis com os controles.

Vários genes-alvos de NF-κB parecem estar associados à inflamação e codificam mediadores pró-inflamatórios, tais como iNOS e COX-2 (ZHANG *et al.*, 2007a). Contribuindo para a resposta inflamatória no tecido isquêmico, a proteína iNOS é super-expressa por astrócitos e microglia em situações de estresse celular possuindo papel fundamental na neurotoxicidade. A super-expressão na iNOS contribui para a síntese de NO, que conjugado ao ânion superóxido forma peroxinitrito, composto altamente reativo também envolvido com a neurotoxicidade (XIE *et al.*, 2002). Além disso, o NO por si só é capaz de induzir a morte celular de neurônios, principalmente por sua capacidade de inibição da cadeia respiratória e pelo aumento da liberação de glutamato e conseqüente excitotoxicidade por ele desencadeada (BAL-PRICE & BROWN, 2001). Já foi demonstrado também que a iNOS pode ter a sua expressão aumentada em resposta à secreção de citocinas como TNF-α, IL-1β e IL-6 (MINGHETTI & LEVI, 1998; COMBS *et al.*, 2001). Além disso, sabe-se que o NO é capaz de aumentar a expressão de COX-2, propagando

assim a reação inflamatória (PAOLETTI *et al.*, 1998). Juntas, iNOS e COX-2, influenciam o funcionamento do cérebro através da produção de EROS e prostanóides. Alguns trabalhos têm sugerido que a indução da COX-2 contribui para o dano cerebral isquêmico (OHTSUKI *et al.*, 1996; NOGAWA *et al.*, 1997; NAKAYAMA *et al.*, 1998), enquanto que outros descrevem que a COX-2 permanece regulada em células de CA1 hipocampal mesmo em 3 dias após a isquemia cerebral global (NAKAYAMA *et al.*, 1998; KOISTINAHO *et al.*, 1999). Nossos resultados mostraram que o tratamento com resveratrol foi capaz de prevenir significativamente a expressão das proteínas pro-inflamatórias, iNOS e COX-2, levando os níveis de expressão destas a níveis basais.

A via da JNK é ativada de forma sustentada após um insulto isquêmico e exerce um papel fundamental no dano neuronal tardio (SUGINO *et al.*, 2000; NOZAKI *et al.*, 2001; FERRER *et al.*, 2003). A ativação dessa proteína leva à transcrição de fatores e citocinas pró-inflamatórias envolvidas na morte celular (XIE *et al.*, 1995). Mostramos que o tratamento com resveratrol foi capaz de prevenir significativamente a fosforilação e conseqüente ativação de JNK, levando os níveis de fosforilação dessa proteína a níveis comparáveis com os controles (capítulo 4). Considerando que citocinas pró-inflamatórias, proteínas como ERK1/2 e JNK, além da COX-2, podem ser super-expressas no período de re-oxigenação (WANG *et al.*, 2007b), acredita-se que este momento é crucial para uma intervenção farmacológica com o intuito de prevenir a morte neuronal tardia, uma característica da lesão isquêmica (MEHTA *et al.*, 2007). O conjunto de resultados apresentados no quarto capítulo permite afirmar que resveratrol foi eficaz em modular parâmetros da inflamação na isquemia cerebral global devido à ação simultânea de vários mecanismos, como: 1) a prevenção da ativação das vias NF-κB e JNK; 2) a prevenção

da ativação glial; 3) a redução nos níveis de mediadores pró-inflamatórias como iNOS e COX-2.

Embora mais estudos utilizando inibidores destas vias sejam necessários para confirmar a hipótese de trabalho, o resveratrol pode ser considerado um bom candidato para a intervenção famacológica em insultos isquêmicos por atuar bloqueando a neuroinflamação desencadeada por um evento isquêmico.

A partir dos resultados demonstrando a ação neuroprotetora do resveratrol através da modulação de vias de sinalização celular, assim como os dados da literatura mostrando a sua modulação em uma ampla variedade de fatores tróficos, passamos a investigar o efeito do resveratrol na função neurovascular. É cada vez mais aceito que as respostas neurovasculares desempenham um papel fundamental na capacidade de recuperação do cérebro após o AVE. Sinais e substratos de neurogênese e neuroplasticidade são co-regulados com a angiogênese e com o remodelamento vascular (LO, 2010). Isto pode não ser surpreendente, pois os mecanismos fundamentais da neurogênese e angiogênese são evolutivamente conservados de modo que os mediadores estão envolvidos de forma semelhante em ambos os fenômenos (CARMELIET & STORKEBAUM, 2002). Após a isquemia, neuroblastos recém-nascidos migram ao longo da rota perivascular (THORED *et al.*, 2007), e a promoção da angiogênese aumenta a neurogênese e a recuperação funcional (OHAB *et al.*, 2006). Dessa forma, terapias que promovam a remodelação neurovascular devem proporcionar novas oportunidades para reparar o tecido cerebral danificado após a isquemia (XIONG *et al.*, 2010). Dados recentes sugerem que o resveratrol, além de do efeito de neuroproteção, pode ter efeitos benéficos sobre a recuperação isquêmica (DONG *et al.*, 2008; SAKATA *et al.*, 2010; SHIN *et al.*, 2010), contudo, os mecanismos que possam mediar esses fenômenos permanecem

desconhecidos. No presente estudo, testamos a hipótese de que o resveratrol pode promover angiogênese em células endoteliais de microvasos cerebrais. Propusemos investigar as vias da PI3-K e MAPK como sinalização regulatória de óxido nítrico endotelial, e este promovendo a secreção de VEGF e ativação de MMPs na promoção da angiogênese.

A angiogênese é o processo de crescimento vascular pelo surgimento de vasos pré-existentes. Células endoteliais angiogênicas devem proliferar, produzir moléculas capazes de degradar a matriz extracelular, alterar suas propriedades adesivas, migrar e, finalmente, se diferenciam em novos tubos vasculares (MUÑOZ-CHÁPULI *et al.*, 2004). Primeiramente, nossos resultados demonstram que resveratrol promoveu proliferação, migração e induziu a formação de tubo vascular *in vitro* (Capitulo 5). Além da função da célula, o resveratrol alterou o aspecto morfológico de células endoteliais do cérebro associadas com o rearranjo do citoesqueleto e relocalização de β-catenina e VE-caderina, características de células em proliferação e migração.

A homeostase vascular endotelial é mantida em grande parte através da síntese de óxido nítrico (NO) a partir do precursor L-arginina, sob a influência da enzima óxido nítrico sintase endotelial (eNOS) (PALMER *et al.*, 1988). NO é uma molécula pleiotrópica que afeta diversos processos bioquímicos e fisiológicos, incluindo funções anti-inflamatória (LAROX *et al.*, 2001), anti-trombótico (VAN GOOR *et al.*, 2001) e anti-aterosclerose (KAWASHIMA *et al.*, 2004) dentro dos vasos sangüíneos, além da regulação do tônus e remodelamento vascular (GARTHWAITE & BOULTON *et al.*, 1995), sendo um potente alvo terapêutico para a angiogênese (ZICHE *et al.*, 2000; ZHANG *et al.*, 2003). O NO também é um mediador crítico de VEGF na angiogênese, uma vez que, camundongos knock-out para $eNOS^{-/-}$ (FUKUMURA *et al.*, 2001; MUROHARA *et al.*, 1998) perderam a habilidade de

responder ao VEGF promovendo angiogênese, além de inibidores de eNOS bloquearem a angiogênese induzida por VEGF (PAPAPETROPOULOS *et al.*, 1997; BUSSOLATI *et al.*, 2001). Na tentativa de elucidar um mecanismo para angiogênese e na regulação de eNOS, observamos que o resveratrol ativou as vias PI3-K/Akt e MAPK/ERK as quais levaram a regulação da proteína eNOS e ao aumento dos níveis de NO. O envolvimento desta via foi confirmada através do uso dos inibidores, LY294002 (PI3-K), U0126 (MAPK) e L-NAME (NO), os quais também inibiram os efeitos do resveratrol na promoção da angiogênese através da formação de tubos vasculares.

O VEGF tem a capacidade de induzir angiogênese, tanto durante o desenvolvimento normal como em condições patológicas (YANG *et al.*, 2003). Doenças cerebrovasculares como aneurismas, malformações arteriovenosas, isquemia e hemorragia levam ao aumento da expressão de VEGF (BASU *et al.*, 2001). O VEGF pode se ligar a dois diferentes receptores de tirosina cinase endotelial: VEGF-R1 (Flt-1) e VEGF-R2 (KDR). Um dos principais eixos pró-angiogênicos baseia-se na ligação de VEGF a VEGF-R2, estimulando a atividade da tirosina cinase, autofosforilação e sinalização de proteínas que regulam diversos processos incluindo a quimiotaxia, a migração, a permeabilidade vascular, sobrevivência celular e proliferação (YANG *et al.*, 2002; FERRARA *et al.*, 2005). As metaloproteinases (MMPs), um dos alvos de VEGF, são responsáveis pelo processamento proteolítico da matriz extracelular de proteínas estruturais, que regulam a migração de células endoteliais e o remodelamento da matriz durante a angiogênese (KARAGIANNIS *et al.*, 2005; SEGARRA *et al.*, 2005). Particularmente, MMP-2 e MMP-9, os principais constituintes da membrana basal, estão envolvidos na migração e invasão celular (KRÄLING *et al.*, 1999; PUYRAIMOND *et al.*, 1999). AICHER e col (2003)

relataram que camundongos knockout para eNOS tiveram a atividade basal fortemente reduzida da MMP-9. Funcionalmente, os camundongos knock-out para eNOS tiveram a mobilização de células progenitoras da medula óssea prejudicada. Um estudo realizado por WANG e col (1998) demonstrou a regulação da atividade de MMPs em células musculares lisas expostas a inibidores de VEGF e VEGF-R2. Coletivamente, estes dados demonstram de forma convincente que a ativação da MMP-9 pela liberação de NO e VEGF seja um evento crucial para a angiogênese.

A seguir, tentamos relacionar a elevação de NO induzida por resveratrol com a modulação na expressão de VEGF. O resveratrol aumentou o nível intracelular e a secreção de VEGF. Este efeito foi dependente de NO, uma vez que seu inibidor, L-NAME, reduziu os níveis de VEGF. Consistente com os níveis elevados da proteína observou-se a ativação da sinalização de VEGF em células endoteliais tratadas com resveratrol. O resveratrol também aumentou os níveis de fosforilação do receptor VEGF-R2, indicando que a sinalização de fato estava ocorrendo. Além de VEGF, outra classe importante de mediador pró-agiogênico são as MMPs. A exposição de células endoteliais cerebrais ao resveratrol aumentou a atividade de MMP-2 e MMP-9. A indução de MMPs também foi dependente de NO uma vez que o inibidor, L-NAME, preveniu os efeitos do resveratrol. Neste contexto, a capacidade do resveratrol em induzir MMPs foi prevenida pelo antagonista de VEGF (s-FLT1) e pelo bloqueador do receptor de VEGF-R2 (SU1498). Em conjunto, estes dados demonstram que o resveratrol fortemente ativa ambas as vias de VEGF e MMPs.

Em resumo, os resultados obtidos no capitulo 5 sugerem que resveratrol promove a angiogênese em células endoteliais de microvasos cerebrais através da sinalização coordenada de NO/VEGF/MMPs. Além disso, estudos *in vivo* e a exploração clínica destas vias se justifica para validar estes achados experimentais e

desenvolver o fármaco resveratrol como uma potente terapia de reparo neurovascular para o AVE e a lesão cerebral.

Com o objetivo final para testar nossa hipótese de trabalho, investigamos o efeito do resveratrol na regulação de fatores tróficos que promoveram a angiogênese, resultados apresentados no capitulo 5, agora em modelo *in vivo* de lesão isquêmica. Na última década, cresceram as evidências mostrando que o cérebro adulto de mamíferos apresenta áreas focais de neurogênese ao longo da zona subventricular e da zona subgranular. Em condições basais, esses neurônios migram para regiões olfativas e hipocampo. Após um episódio isquêmico ou hemorraágico, o nascimento de novas células parece aumentar e ocorre o redirecionado destas células para a área de tecido cerebral danificado (ARVIDSSON *et al.*, 2002; PARENT *et al.*, 2002.), talvez como parte de um mecanismo endógeno do cérebro de auto-reparo. No entanto, se esses novos neurônios contribuem significativamente para a recuperação funcional ainda não existem evidências. A resposta neurogênica pode persistir por longos períodos, mas a maioria dessas células recém-nascidas sobrevive apenas por alguns dias (ARVIDSSON *et al.*, 2002).

Em conjunto com a neurogênese e a plasticidade neural, o cérebro se recupera do acidente vascular cerebral exibindo padrões complexos de remodelamento vascular. A angiogênese e a vasculogênese em regiões peri-infarto foram observadas em modelos animais de isquemia cerebral (KRUPINSKI *et al.*, 1993), e em humanos após o AVE (KRUPINSKI et al., 1994). Na verdade, hoje já está estabelecido que as respostas angiogênicas e neurogênicas são fortemente co-reguladas após acidente vascular e/ou lesões cerebrais (ARAI *et al.*, 2009) e esta inter-relação, é mantida após AVE (THORED *et al.*, 2007). Estas evidências sugerem que terapias direcionadas para aumentar os sinais e substratos endógenos de remodelação neurovascular são

importantes, considerando a janela de intervenção terapêutica pós episódio isquêmico (ZHANG *et al.*, 2005).

No presente estudo, nos demonstramos que o resveratrol, quando administrado 72 h após o evento de oclusão da ACM, promoveu a recuperação funcional neurológica e concomitante aumento da angiogênese e da neurogênese e estes processos podem traduzir na recuperação da função neurológica. Por exemplo, camundongos com alta taxa de neurogênese no giro dentado exibiram melhor desempenho em tarefas dependentes do hipocampo, ao passo que uma diminuição da taxa de neurogênese está relacionada com menor desempenho nesta tarefa. Portanto, o aumento da neurogênese pode contribuir para a recuperação depois do tratamento com resveratrol. Demonstramos que o resveratrol foi capaz de aumentar a migração de neuroblastos na região ZSV comprovada pelo aumento de células positivas a DCX. Além disso, observamos que o resveratrol induziu o aumento de células positivas a BrdU na região ZSV e na área peri-infartada, o que sugere o aumento da proliferação de células-tronco neurais. Em paralelo, nossos resultados *in vitro* também demonstraram função na proliferação e diferenciação de cultura de neuroesferas. Resveratrol aumentou o número de neurônios tanto no modelo *in vivo* quanto no modelo *in vitro*.

Angiogênese e neurogênese, no cérebro adulto, são moduladas pelos fatores VEGF e BDNF. Após estímulo angiogênico via VEGF, células endoteliais secretam BDNF, que induz a neurogênese. Além de seu papel angiogênico, VEGF estimula a neurogênese. Nossos dados anteriores mostraram que o resveratrol, promove a angiogênese, assim como aumenta a expressão de VEGF. Nós propomos que o aumento produzido por resveratrol de VEGF pode não somente causar angiogênese, mas também proporcionar um microambiente favorável, o que pode aumentar a

plasticidade neuronal e sináptica. A neurogênese ocorre em estreita proximidade com os vasos sanguíneos, onde a expressão do VEGF é alta e está em curso na angiogênese (PALMER *et al.*, 2000). Além disso, o resveratrol aumentou a expressão do BDNF 14 dias após o AVE. A vasculatura recém-ativada e a vascularização ampliada aumentam substancialmente a produção e a liberação de BDNF, cuja indução é tanto espacialmente e temporalmente associado com o recrutamento de novos neurônios (LEVENTHAL *et al.*, 1999). Por fim, ainda no capítulo 6, observamos que o resveratrol induziu a migração neuronal no cultivo de explantes da SVZ, sendo essa migração diminuída com o uso de inibidores para BDNF e VEGF. Estes dados sugerem que a plasticidade neuronal está intimamente relacionada com a angiogênese e os fatores BDNF/VEGF podem facilitar a plasticidade neuronal induzida após AVE.

Os resultados obtidos neste trabalho descrevem que os efeitos benéficos do resveratrol podem estar associados ao aumento de fatores tróficos, tal como VEGF e BDNF, e a indução da angiogênese, neurogênese que levariam a recuperação funcional.

10. CONCLUSÕES

Os dados obtidos durante a realização deste trabalho permitem concluir que:

1- O pré-tratamento com resveratrol exerceu efeito neuroprotetor no modelo de isquemia cerebral global em ratos. Esse efeito foi mediado por um conjunto de atividades, dentre elas:

- Ação antioxidante pela prevenção do aumento do estresse oxidativo através da redução de espécies reativas de oxigênio, nitrogênio e peroxidação lipídica. Esses efeitos foram seguidos por um aumento da atividade antioxidante e preveniu a perda da atividade da $Na^{+}K^{+}$-ATPase em córtex cerebral e hipocampo. (Capítulo 1)

- Prevenção da redução dos componentes de lipídios da membrana - gangliosídeos, fosfolipídeos e colesterol - em hipocampo e córtex cerebral de ratos. (Capítulo 2)

- Envolvimento da via de sinalização da PI3-K/Akt, o qual é diretamente implicada na sobrevivência celular, que bloqueia seu substrato GSK-3β, que participa na indução da apoptose. Alem disso, PI3-K/Akt regulou o fator de transcrição CREB, que promove a transcrição de genes envolvidos com a sobrevivência celular. (Capítulo 3)

- Ação anti-inflamatória pela regulação da proteína JNK e o fator de transcrição NF-kB, responsável pela modulação de proteínas pró-inflamatórias como iNOS e COX-2 que apresentaram reduzidas. Além disso, preveniu a ativação de células gliais, astrócito e microglia, responsável pela secreção de mediadores inflamatórios. (Capítulo 4)

2- O tratamento com resveratrol em células endoteliais humanas de microvasos cerebrais promoveu a angiogênese caracterizada pelo aumento da proliferação, migração e indução da formação vascular dentro de estruturas em redes *in vitro*. Como característica da angiogênese, o aspecto morfológico foi alterado pelo resveratrol e foi associado com o rearranjo do citoesqueleto, relocalização de β-catenina e VE-caderina. A promoção da angiogênese por resveratrol ativou as vias de sinalização PI3-K/Akt e MAPK/ERK que regularam eNOS modulando os níveis de VEGF e metaloproteinases. (Capítulo 5)

3- O pós-tratamento com resveratrol promoveu a recuperação funcional, possivelmente pelo aumento da angiogênese e neurogênese em camundongos. Além disso, BDNF e VEGF podem estar modulando a plasticidade neuronal induzida por resveratrol após isquemia cerebral focal. (Capítulo 6)

11. PERSPECTIVAS

Como continuação desse trabalho, pretende-se trabalhar com os seguintes objetivos:

- Avaliar a hipótese que o tratamento com resveratrol promove plasticidade sináptica após a isquemia cerebral focal e crescimento axonal em culturas de neurônios *in vitro*.
- Testar a hipótese que resveratrol modula a via de sinalização Notch para promover a arteriogênese após isquemia cerebral focal.
- Avaliar se SDF-1a e angiopoetina-1 estao sendo modulados no dano isquêmico, assim como seus receptores CXCR4 (SDF-1a) e Tie2 (angiopoetina-1) na zona subventricular e, portanto, promover a migração do neuroblasto no modelo de isquemia focal e em cultura de neuroesferas.
- Avaliar se células-tronco neurais tratadas com resveratrol induz angiogênese *in vitro* através da produção de VEGF e seu receptor KDR.
- Avaliar se o tratamento com resveratrol após a lesão isquêmica induz neurogênese e recuperação funcional no modelo de isquemia cerebral global.

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