Universidade Federal do Rio Grande do Sul
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ESTRÓGENO E PRÉ-CONDICIONAMENTO:

Efeitos neuroprotetores e possíveis mecanismos de ação em modelos *in vitro* e *in vivo* de isquemia cerebral

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“Discovery consists not in seeking new landscapes but in having new eyes.”

Marcel Proust

Para o meu irmão Juliano.

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RESUMO

As bases moleculares da neuroproteção contra a isquemia mediada por estrógeno continuam obscuras, assim como os mecanismos envolvendo a tolerância ao dano isquêmico subseqüente induzida por pré-condicionamento. Neste trabalho foi estudado se as vias de sinalização celular da PI3-K (fosfatidil inositol 3-quinase) e da MEK/ERK 1/2 estariam envolvidas na neuroproteção induzida por estrógeno, bem como alguns parâmetros de estresse oxidativo, especificamente o conteúdo de radicais livres, um índice de dano oxidativo a proteínas e a capacidade antioxidante total. Também foi estudado o possível envolvimento dos transportadores de glutamato (EAAT1 e EAAT2) e dos receptores de estrógeno (ERα e ERβ) nos efeitos neuroprotetores do estrógeno e do pré-condicionamento. Para este fim, foram utilizados os modelos *in vitro* de culturas organotípicas de fatias hipocampais e fatias hipocampais preparadas a fresco expostas à privação de oxigênio e glicose (POG) e o modelo *in vivo* de hipóxia-isquemia neonatal. Em culturas tratadas tanto aguda como cronicamente com 17β-estradiol, a morte celular induzida por POG foi diminuída acentuadamente quando comparada com as culturas tratadas apenas com veículo. Este efeito neuroprotetor foi evitado por LY294002 (inibidor de PI3-K), mas não por PD98059 (inibidor de MEK/ERK 1/2). Ambos os protocolos de tratamento com estradiol induziram a fosforilação/ativação da proteína quinase B (PKB/Akt) e a fosforilação/inativação da glicogênio sintase quinase-3β (GSK-3β). Em um estudo similar, o imunoconteúdo do receptor estrogênico ERα diminuiu após POG em culturas tratadas tanto com estradiol quanto veículo, enquanto que o receptor ERβ aumentou apenas nas culturas tratadas com estradiol expostas ou não à POG. Não foram observadas alterações no imunoconteúdo dos transportadores de glutamato (EAATs) em nenhum dos tratamentos *in vitro*. Em fatias de hipocampo de cérebro de ratas ovariectomizadas que receberam reposição de estradiol, a morte celular foi reduzida em comparação ao grupo de ratas que não recebeu a reposição hormonal. Neste mesmo modelo, observou-se que a POG aumentou a produção de radicais livres nos dois grupos, porém não foram observadas diferenças na capacidade antioxidante total. Por outro lado, a reposição de estradiol evitou a redução nos conteúdos de triptofano e tirosina causada por POG. No modelo *in vivo,* o cérebro de ratos neonatos foi protegido contra a hipóxia-isquemia pelo pré-condicionamento hipóxico. Em paralelo, o pré-condicionamento aumentou o imunoconteúdo dos transportadores de glutamato EAAT2 e do receptor estrogênico ERα em córtex e diminuiu os níveis de EAAT2 em estriado, mas não afetou os níveis de EAAT1 e ERβ. Já no modelo *in vitro* de pré-condicionamento, nas culturas organotípicas de hipocampo pré-condicionadas, 15 min de POG induziu tolerância acentuada a um período subseqüente de 45 min de POG, porém não foram detectadas alterações nos transportadores de glutamato nem nos receptores estrogênicos. Juntos, os resultados sugerem que na isquemia a neuroproteção induzida por estrógeno pode envolver a via de sinalização celular da fostatidil inositol 3-quinase (PI3-K), a prevenção do dano oxidativo a proteínas e a regulação dos receptores estrogênicos ERα e ERβ, enquanto que a tolerância à isquemia cerebral induzida por pré-condicionamento pode envolver a regulação dos transportadores de glutamato EAAT2 e receptores estrogênicos ERα.

ABSTRACT

The molecular basis of estrogen-mediated neuroprotection against cerebral ischemia remains obscure, likewise the mechanisms underlying preconditioning-induced brain tolerance against subsequent ischemic insults. In this work, it was studied whether PI3-K and MEK/ERK 1/2 signaling pathways could be involved in estrogen neuroprotection, as well as some parameters of oxidative stress, specifically the free radicals content, an index of oxidative damage to proteins, and the total antioxidant capacity. It was also studied the role of glutamate transporters (EAAT1 and EAAT2) and estrogen receptors (ERα and ERβ) in the neuroprotective effects of estrogen and preconditioning. For these purposes, the *in vitro* models of organotypic hippocampal slice cultures and freshly prepared hippocampal slices exposed to oxygen and glucose deprivation (OGD) and the *in vivo* model of hypoxia-ischemia neonatal were used. In cultures treated acute or chronically with 17β-estradiol, the OGD-induced cellular death was markedly decreased, as compared to vehicle-treated cultures. This effect was prevented by LY294002 (PI3-K inhibitor), but not by PD98059 (MEK/ERK 1/2 inhibitor). Both estradiol treatment protocols induced the phosphorylation/activation of Akt and the phosphorylation/inactivation of GSK-3β. In a similar study, ERα was down-regulated after OGD in both vehicle- and estradiol-treated cultures, whereas ERβ was up-regulated in the estradiol-treated cultures exposed or not to OGD, although no significant changes in EAATs in response to estradiol and/or OGD were observed. In hippocampal slices from ovariectomized female rats receiving estradiol replacement, the cellular death was reduced, as compared to the vehicle-treated group. In both groups, OGD increased the free radical production, although no difference on total antioxidant capacity was observed. Interestingly, estradiol replacement prevented the reduction in tryptophan and tyrosine contents caused by OGD. Preconditioning with hypoxia before hypoxia-ischemia afforded brain protection in newborn rats, increasing EAAT2 and ERα levels in cortex and decreasing EAAT2 levels in striatum, although not affecting EAAT1 and ERβ levels. In preconditioned cultures, 15 min of OGD induced marked tolerance to 45 min of OGD, but no changes in EAATs or ERs were detected. Taken together, our results suggest that, regarding ischemia, estrogen neuroprotection might involve the PI3-K pathway, protein damage prevention, and regulation of ERs, whereas preconditioning-induced brain tolerance might involve regulation of EAAT2 and ERα.

APRESENTAÇÃO

Esta tese está organizada da seguinte maneira: Introdução; Objetivos; Artigos Científicos publicados e/ou submetidos, Discussão, Conclusões, Perspectivas e Referências Bibliográficas.

A **Introdução** mostra o embasamento teórico, que nos levou a formular a proposta de trabalho. Os materiais e métodos e resultados, assim como as referências bibliográficas específicas, encontram-se no corpo de cada trabalho, os quais são apresentados nos capítulos **Artigos Publicados**. O quarto trabalho foi realizado no laboratório do Prof. Philip Beart, Howard Florey Institute, University of Melbourne, Melbourne, Austrália, durante a realização do doutorado sanduíche, e submetido à revista Neuroscience Letters em fevereiro de 2005. Os demais artigos foram desenvolvidos no departamento de Bioquímica-ICBS-UFRGS.

A seção **Discussão** contém uma interpretação geral dos resultados obtidos nos diferentes trabalhos.

A seção **Conclusões** aborda as conclusões gerais da tese.

A seção **Perspectivas** discute as possibilidades de desenvolvimento de projetos a partir dos resultados obtidos durante a realização desta tese.

A seção **Referências Bibliográficas** lista as referências citadas na Introdução e Discussão da tese.

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LISTA DE ABREVIATURAS

Akt/PKB: proteína quinase B

ATP: adenosina tri-fosfato

CA1: corno de Ammon (c*ornus Ammonis*)

DG: giro denteado (*dentate gyrus*)

EAAC: carreador de aminoácido excitatório (*excitatory amino acid carrier*)

EAAT: transportador de aminoácido excitatório (*excitatory amino acid transporter*)

ER: receptor de estrogênio (*estrogen receptor*)

ERK: quinase regulada por sinais extracelulares (e*xtracellular signal-regulated kinase*)

GLAST: transportador de glutamato e aspartato (*glutamate-aspartate transporter*)

GLT-1: transportador de glutamato (*glutamate transporter*)

GSK-3β: glicogênio sintase quinase-3β (*glycogen synthase kinase-3β*)

HBSS: solução salina balanceada de Hanks

MAPK: proteína quinase ativada por mitógenos (m*itogen-activated protein kinase*)

MEM: meio essencial mínimo

NMDA: N-metil-D-aspartato

OGD: privação de oxigênio e glicose *(oxygen and glucose deprivation)*

PI3-K: fosfatidil inositol 3-quinase (p*hosphatidylinositol 3-kinase*)

PKC: proteína quinase C

POG: privação de oxigênio e glicose

SNC: sistema nervoso central

Src: co-ativador de receptor de esteróide (*steroid receptor coactivator*)

INTRODUÇÃO

1. ISQUEMIA CEREBRAL E MORTE CELULAR

As doenças neurodegenerativas afetam um amplo espectro da população e, na maioria dos casos, elas levam à incapacidade física e/ou mental, envolvendo memória, cognição, linguagem e personalidade. A neurodegeneração é um aspecto proeminente das desordens cerebrovasculares, particularmente das síndromes isquêmicas que são uma das principais causas de morbidade e mortalidade não apenas entre adultos e idosos, mas também, juntamente com a hipóxia, no período perinatal.

A isquemia é definida como uma redução severa ou um bloqueio completo do fluxo sangüíneo. A hipóxia denota a redução no conteúdo de oxigênio no sangue, que ainda circula e pode remover metabólitos (Schmidt-Kastner & Freund, 1991). Até hoje a isquemia cerebral afeta um número assustador de vítimas e o índice de mortalidade permanece em torno de 30%, sendo a terceira maior causa de disfunção de longa duração em adultos (Dirnagl *et al.*, 1999; Read *et al.*, 1999). Ambas constituem importantes condições patofisiológicas de lesão cerebral e são alvos de intensa investigação.

O cérebro 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 ou interrupção no suprimento de oxigênio e glicose que ocorre na isquemia cerebral, leva a uma intrincada cascata de eventos celulares (Figura 1), resultando em degeneração neuronal severa e, conseqüentemente, em perda das funções cerebrais (Lipton, 1999).

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, ainda não existem meios farmacológicos de prevenir nem de tratar a isquemia cerebral.

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 neuronal.

1.1. EVENTOS QUE CAUSAM A MORTE CELULAR NA ISQUEMIA

O dano cerebral isquêmico resulta de uma seqüência complexa de eventos patofisiológicos, conforme mostrado na Figura 1 (Dirnagl *et al.*, 1999).

O cérebro tem um consumo relativamente alto de oxigênio e glicose e depende quase que exclusivamente da fosforilação oxidativa para a produção de energia. Se o fluxo sangüíneo cerebral for prejudicado, haverá restrição da chegada destes substratos o que, conseqüentemente, afetará o metabolismo energético necessário para manter os gradientes iônicos (Martin *et al.*, 1994). Com a depleção dos estoques energéticos, o potencial de membrana é perdido e os neurônios e a glia despolarizam-se (Katsura *et al.*, 1994). Desta forma, os canais de Ca^{+2} dependentes de voltagem tornam-se ativados e os aminoácidos excitatórios, particularmente o glutamato, são liberados para o espaço extracelular (Choi, 1995; De Keyser *et al.*, 1999). Ao mesmo tempo, os processos dependentes de energia, tais como a captação de aminoácidos excitatórios, são impedidos, aumentando ainda mais o acúmulo de glutamato no espaço extracelular. A ativação dos receptores de glutamato ionotrópicos, particularmente do tipo NMDA, e metabotrópicos contribui para o aumento do Ca^{+2} intracelular, por abertura direta de canais de Ca^{+2} ou mediado pelas proteínas G (Nehls *et al.*, 1989). Como resultado da superativação mediada por glutamato, há aumento do influxo de Na^{+} e Cl^{-} nos neurônios através de canais iônicos, juntamente com a água, ocasionando edema celular.

O aumento nos níveis intracelulares de Ca^{2+}, um dos principais segundo-mensageiros das células, desencadeia uma série de eventos citoplasmáticos e nucleares, causando danos no tecido através da ativação de enzimas como proteases, lipases, endonucleases, entre outras, levando à morte da célula por necrose (Mitani *et al.*, 1993). A ativação da fosfolipase A_2 e da ciclooxigenase gera radicais livres acima da capacidade dos mecanismos antioxidantes endógenos, produzindo peroxidação lipídica e dano à membrana. O radical superóxido reage com o óxido nítrico gerado pela enzima óxido nítrico sintase, formando peroxinitrito e

promovendo danos ao tecido que podem levar à apoptose (Beckman & Koppenol, 1996; Iadecola, 1997).

As mitocôndrias, fontes importantes de espécies reativas do oxigênio, são afetadas pelo distúrbio da membrana mitocondrial interna mediado por radicais livres e pela oxidação das proteínas mediadoras do transporte de elétrons e da produção de ATP (Dugan & Choi, 1994). Forma-se uma espécie de poro na membrana da mitocôndria que causa um inchamento mitocondrial, a suspensão da produção de ATP e uma superprodução de radicais livres (Kristian & Siesjo, 1998). O citocromo C é liberado das mitocôndrias e esta seqüência de eventos pode desencadear a apoptose (Fujimura *et al.*, 1998).

Outro evento patofisiológico envolvido no dano cerebral isquêmico é a inflamação, que causa liberação de citocinas as quais contribuem para o aumento da morte celular (Aschner, 1998).

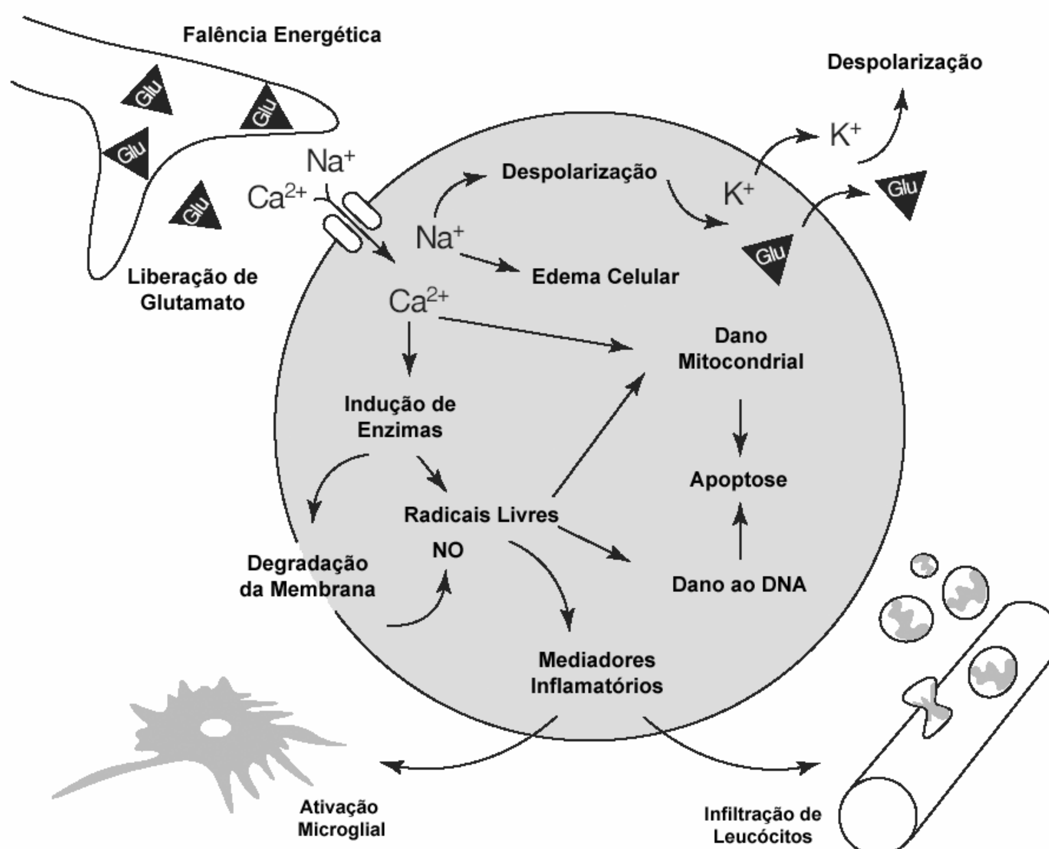

Figura 1: Visão geral simplificada dos mecanismos patofisiológicos ocorrendo no cérebro isquêmico (Modificado de Dirnagl *et al.*, 1999).

1.2. MODELOS DE LESÃO ISQUÊMICA

1.2.1. Modelos Experimentais *In Vivo*

1.2.1.1. Isquemia Global

Os insultos isquêmicos globais, que afetam todo o cérebro, são mais comumente produzidos por oclusões de vasos e menos comumente por parada circulatória cerebral completa. O modelo animal experimental mais usado nesse caso é o da oclusão dos 4 vasos (4VO), que no rato inclui a cauterização das artérias vertebrais seguida de oclusão temporária das artérias carótidas comuns (Farooqui *et al.*, 1994; Netto *et al.*, 1993; Valentim *et al.*, 1999).

1.2.1.2. Isquemia Focal

Neste modelo, artérias cerebrais específicas são interrompidas, afetando somente uma pequena parte do cérebro. Quase todos os modelos isquêmicos focais envolvem primariamente a oclusão de uma artéria cerebral média (Ginsberg & Busto, 1989), sendo que, em alguns casos, a artéria carótida também é ligada.

1.2.1.3. Hipóxia-Isquemia

A técnica, que consiste na oclusão unilateral da carótida combinada com hipóxia, tem sido adaptada com sucesso em animais neonatos, os quais podem sobreviver por dias após períodos de até 3,5 horas com oxigênio geralmente reduzido a 8% (Rice *et al.*, 1981; Roohey *et al.*, 1997; Tuor *et al.*, 1996). A hipóxia-isquemia neonatal é considerada um modelo muito bom para o estudo das principais formas de dano cerebral metabólico (Vannucci, 1990).

1.2.2. Modelos Experimentais *In Vitro*

Como modelo para o estudo da isquemia cerebral, uma alternativa para a experimentação animal *in vivo* pode ser o uso de sistemas *in vitro*, de cultivo de células ou de tecido, como as culturas organotípicas. Além disto, os modelos com o uso de fatias preparadas a fresco de regiões específicas do cérebro constituem outra alternativa. Os dois modelos consistem na exposição das culturas organotípicas ou das fatias cerebrais preparadas a fresco à privação de oxigênio e glicose (POG), por incubação das mesmas em meio livre de glicose, dentro de uma incubadora especial com atmosfera anaeróbica (Cárdenas *et al.*, 2000; Cimarosti *et al.*, 2001). Estas condições simulam *in vitro* a falta de fluxo sangüíneo da isquemia *in vivo* (Breder *et al.*, 2000; Laake *et al.*, 1999; Pringle *et al.*, 1999; Striggow *et al.*, 2000).

Os modelos *in vitro* são simples de utilizar e reproduzem vários aspectos da isquemia *in vivo*, sendo adequados para o estudo dos mecanismos envolvidos na morte celular isquêmica, bem como para a investigação de drogas com potencial neuroprotetor (Cimarosti *et al.*, 2001).

1.2.2.1. Culturas Organotípicas

As técnicas *in vitro*, por serem um modelo mais simples do que o uso do animal *in vivo*, são ferramentas importantes para os estudos moleculares de diversas doenças neurodegenerativas (Pringle *et al.*, 1997b).

As culturas organotípicas têm se tornado uma importante alternativa para estudar a morte neuronal induzida por excitotoxinas (Abdel-Hamid & Tymianski, 1997), hipóxia (Pringle *et al.*, 1997a), hipoglicemia (Tasker *et al.*, 1992) e hipóxia/hipoglicemia ou aglicemia para simular uma isquemia (Bernaudin *et al.*, 1998; Cimarosti *et al.*, 2001; Newell *et al.*, 1995).

O modelo de culturas organotípicas foi desenvolvido em 1981 por Gähwiller e modificado por Stoppini e colaboradores em 1991. Basicamente, trata-se de um método que mantém fatias de um determinado tecido em cultivo, numa interface entre o ar e o meio de cultivo, podendo permanecer por diversas semanas. Uma das principais características destas culturas é a de manter a organização do tecido tal qual ocorre *in vivo* (Buchs *et al.*, 1993; Gähwiler *et al.*, 1997; Stoppini *et al.*, 1991). A

idade das culturas é denotada em dia pós-natal equivalente (EPD – *equivalent postnatal day*), o qual corresponde à idade que o animal tinha quando as fatias foram colocadas em cultivo somado ao número de dias de permanência *in vitro* (Bruce *et al.*, 1995).

Por reproduzirem muitos aspectos da isquemia *in vivo*, tais como a morte neuronal tardia e a vulnerabilidade seletiva (Cimarosti *et al.*, 2001; Laake *et al.*, 1999; Noraberg *et al.*, 1999; Strasser & Fischer, 1995), as culturas organotípicas de fatias hipocampais são uma excelente alternativa para o estudo dos mecanismos associados à isquemia cerebral.

1.2.2.2. Fatias Cerebrais

Uma alternativa para a metodologia *in vitro* é a utilização do tecido cerebral sem cultivo prévio. Neste método utilizam-se, por exemplo, fatias de hipocampo (400 μm de espessura) que imediatamente após serem extraídas são expostas à condição de privação de oxigênio e glicose, constituindo um modelo largamente usado para o estudo do dano anóxico ou isquêmico (Kass & Lipton, 1982; Lobner & Lipton, 1993; Maarten *et al.*, 1989; Schurr *et al.*, 1988).

O uso de fatias cerebrais preparadas a fresco oferece várias das vantagens das culturas organotípicas, especialmente para experimentos agudos de inibição metabólica (isquemia *in vitro*), sendo uma técnica mais simples e economicamente vantajosa. Entretanto, por não serem mantidas em cultura, as fatias geralmente deterioram-se dentro de um período de 12-24 horas, gerando uma mistura de células danificadas e saudáveis.

Conforme descrito acima, existe uma razoável variedade de modelos experimentais disponíveis para o estudo da lesão celular induzida pela isquemia. A escolha de um modelo, ou a combinação deles, vai depender dos parâmetros a serem estudados devendo sempre ser considerada a limitação de cada método.

2. ESTRÓGENOS COMO NEUROPROTETORES

Os estrógenos são hormônios esteróides que exercem um amplo espectro de efeitos por todo o organismo, incluindo o sistema nervoso central (SNC). O potencial neuroprotetor dos estrógenos tem ganhado atenção crescente e tem sido alvo de investigação constante durante os últimos anos. Tais hormônios têm sido associados com risco diminuído, progressão retardada ou recuperação aumentada em várias doenças neurológicas, traumáticas ou crônicas, e doenças mentais em humanos, como por exemplo isquemia cerebral (Paganini-Hill, 1995), doença de Alzheimer (Costa *et al.*, 1999; Paganini-Hill & Henderson, 1996; Slooter *et al.*, 1999; Tang *et al.*,1996) e doença de Parkinson (Saunders-Pullman *et al.*, 1999; Tsang *et al.*, 2000).

Os achados sobre os efeitos neuroprotetores dos estrógenos em humanos, entretanto, permanecem controversos e o preciso mecanismo(s) de ação envolvido ainda é uma incógnita. Homens e mulheres diferem com respeito à incidência de isquemia cerebral, com as mulheres pré-menopausa exibindo uma susceptibilidade mais baixa ao dano cerebral isquêmico do que homens e mulheres pós-menopausa com idades equivalentes (Hawk *et al.*, 1998; Rusa *et al.*, 1999; Wenger *et al.*, 1993). A neuroproteção observada nas mulheres pré-menopausa pode estar relacionada a níveis mais altos de estrógenos circulantes, principalmente 17β-estradiol.

Principal estrógeno em mamíferos, o 17β-estradiol é um hormônio esteróide sintetizado nas gônadas ou convertido na periferia e no cérebro através da aromatização de andrógenos, sendo tradicionalmente associado com a reprodução feminina. Em adição ao seu papel clássico na maturação e regulação reprodutiva, vários estudos demonstram que ele desempenha importantes papéis protetores e tróficos no cérebro.

As observações a partir de estudos epidemiológicos de que a exposição moderada a estrógenos exógenos pode diminuir o risco de isquemia cerebral, junto com o achado de que o tratamento com tamoxifeno (um antagonista de receptor de estrógeno) aumenta o risco de isquemia em mulheres (Gail *et al.*, 1999), conferiram crédito à idéia de que os estrógenos desempenham um importante papel protetor na doença cerebrovascular.

Enquanto as propriedades neuroprotetoras dos estrógenos em humanos permanecem controversas, sua eficácia tem sido demonstrada em vários modelos

experimentais de neurodegeneração e dano isquêmico, tanto *in vitro* como *in vivo* (Alkayed *et al.*, 2000; Dubal *et al.*, 1998; Goodman *et al.*, 1996), sugerindo que tais hormônios possam vir a ser uma nova classe de drogas terapêuticas para a prevenção do dano neuronal associado com a isquemia cerebral. Os estrógenos têm demonstrado exercer robustos efeitos neuroprotetores contra diversos insultos tóxicos, incluindo excitotoxicidade, estresse oxidativo, toxicidade por proteína β-amilóide e isquemia (Garcia-Segura *et al.*, 2001; Goodman *et al.*, 1996; Green *et al.*, 1996; McCullough & Hurn, 2003; Singer *et al.*, 1996).

Outra questão importante a respeito dos estrógenos como neuroprotetores é a que se refere aos mecanismos pelos quais eles atuariam na isquemia. Estes efeitos neuroprotetores podem ser mediados através de diferentes mecanismos celulares e moleculares, incluindo ações dependentes e independentes de receptores estrogênicos, de forma genômica e não-genômica (Wise, 2002). Entre estes, podemos citar potencial ação antioxidante, ativação de diferentes vias de sinalização intracelular associadas à membrana e de receptores nucleares clássicos, os quais podem contribuir para a neuroproteção estrogênica (Behl & Holsboer, 1999; Gollapudi & Oblinger, 1999; Moss & Gu, 1999). Além disso, interações com neurotrofinas e outros fatores de crescimento também podem ser importantes para as propriedades neuroprotetoras destes hormônios (Cardona-Gómez *et al.*, 2001; Gibbs *et al.*, 1994). Entretanto, até o momento, os mecanismos envolvidos nos efeitos neuroprotetores dos estrógenos ainda não foram completamente esclarecidos.

2.1. MECANISMOS DE NEUROPROTEÇÃO PELOS ESTRÓGENOS

Diversos mecanismos têm sido propostos para explicar como os estrógenos podem proteger o cérebro. Estas propostas incluem um mecanismo mediado por receptor de estrógeno (ER – *estrogen receptor*), um mecanismo não-genômico envolvendo a via de sinalização da proteína quinase ativada por mitógeno (MAPK – *mitogen-activated protein kinase*) e/ou da fosfatidil inositol 3-quinase (PI3-K – *phosphatidylinositol 3-kinase*) e um mecanismo antioxidante de “varredura” de radicais livres independente de receptor. Os dois primeiros mecanismos, o genômico e o não-genômico, podem ser observados com doses fisiológicas de 17β-estradiol.

Entretanto, o terceiro mecanismo (antioxidante) é observado apenas com doses não-fisiológicas de 17β-estradiol. Assim, os dois primeiros mecanismos parecem estar envolvidos na neuroproteção fisiológica por 17β-estradiol, enquanto que o terceiro pode ser disparado com o uso de doses farmacológicas de estrogênio. Estes três mecanismos potenciais e algumas evidências suportando cada um deles são brevemente apresentados abaixo.

2.1.1. Receptores de Estrógenos

O modelo clássico de ação dos hormônios esteróides envolve ligação a proteínas receptoras intracelulares, translocação nuclear e indução de transcrição gênica (Aranda & Pascual, 2001; Hall *et al.*, 2001), sendo conhecido como via genômica ou lenta. Como outros receptores esteróides, os receptores de estrógenos (ERs – *estrogen receptors*) são fatores de transcrição nuclear.

No momento, existem dois tipos de receptores estrogênicos caracterizados: o receptor de estrógeno α (ERα) e o receptor de estrógeno β (ERβ) (Kuiper *et al.*, 1996; White *et al.*, 1987), os quais compartilham homologia em domínios de ligação ao DNA e potencialmente ativam os mesmos elementos transcricionais. Ambos ERs são expressos por todo o organismo. No cérebro a distribuição de ERα e ERβ sobrepõe-se em algumas regiões, mas é bastante distinta em outras, estando os dois presentes tanto em neurônios como em astrócitos (Garcia-Ovejero *et al.*, 2002). A densidade de ERs é mais alta no hipotálamo do que em regiões extra-hipotalâmicas, tais como o córtex cerebral e o hipocampo (Behl, 2002).

Os estrógenos, por meio dos receptores estrogênicos, podem controlar diretamente a transcrição de genes que codificam proteínas que modulam a sobrevivência neuronal. Estas proteínas podem aumentar o suporte neurotrófico, suprimir a apoptose e afetar a estrutura neuronal (Behl, 2002).

2.1.2. Sinalização Estrogênica na Neuroproteção

Além da ação genômica, cada vez mais evidências sugerem que os estrógenos podem exercer seus efeitos através de diferentes mecanismos de sinalização, os quais são conhecidos como “não-genômicos” ou “não-clássicos”.

Os efeitos estrogênicos não-clássicos são caracterizados por serem rápidos (segundos a minutos) e insensíveis a inibidores transcricionais. Estes efeitos são provavelmente mediados por receptores integrados ou associados à membrana plasmática e por ativação de distintas cascatas de sinalização intracelular (Falkenstein *et al.*, 2000; Küppers *et al.*, 2001).

Recentemente, têm sido descritos efeitos estrogênicos em uma variedade de vias de sinalização citoplásmicas, incluindo proteína quinase C (PKC – *protein kinase C*), fosfatidil inositol 3-quinase (PI3-K – *phosphatidylinositol 3-kinase*), proteína quinase ativada por mitógeno (MAPK – *mitogen-activated protein kinase*) e co-ativador de receptor de esteróide (src – *steroid receptor coactivator*) (Segars & Driggers, 2002).

Resumidamente, os estrógenos podem interagir diretamente com a via de sinalização da MAPK através da ativação das proteínas Ras, B-Raf e MEK1/2, induzindo rapidamente a fosforilação de ERK1/2. Todas estas proteínas fazem parte das cascatas de MAPK que, através de eventos de fosforilação, controlam diversos processos celulares como a proliferação, a expressão gênica, a diferenciação e a apoptose (Cano & Mahadevan, 1995; Cohen, 1997). De forma semelhante, os estrógenos interagem com a via de sinalização da PI3-K, levando a ativação do seu efetor proteína quinase B (Akt/PKB – *protein kinase B*). A PI3-K é uma enzima que fosforila fosfoinositídeos localizados na membrana e que transloca a Akt, uma serina/treonina quinase, do citoplasma para a membrana celular, o sítio de fosforilação da Akt. A Akt fosforilada (fosfo-Akt) atua de forma tanto a inibir fatores pró-apoptóticos como a estimular fatores anti-apoptóticos (Yoshimoto *et al.*, 2001). Um dos alvos centrais das vias de sinalização da MAPK e da PI3-K é a enzima glicogênio sintase quinase-3β (GSK-3β – *glycogen sinthase quinase-3β*) – um importante modulador da sobrevivência celular do SNC (Behl, 2002), que promove a apoptose quando desfosforildada mas tem seu efeito inibido por fosforilação (Pap & Cooper, 1998) (Figura 2).

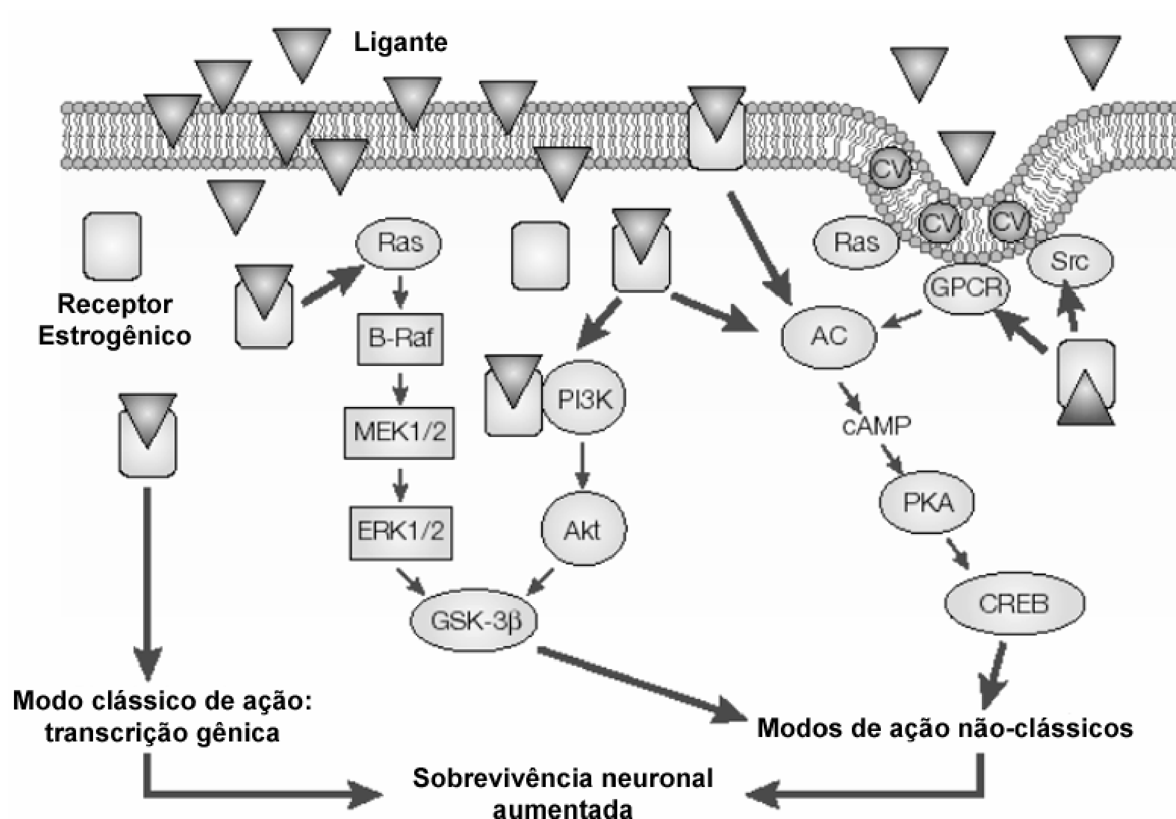

Figura 2: Mecanismos possíveis da neuroproteção mediada por estrógenos (Modificado de Behl, 2002).

2.1.3. Efeitos Antioxidantes dos Estrógenos

Os possíveis papéis dos radicais livres, do óxido nítrico e do peroxinitrito no dano celular isquêmico foram extensivamente revisados (Chan, 1996; Samdani *et al.*, 1997; Siesjo, 1992; Siesjo *et al.*, 1995). Existem várias formas pelas quais estes compostos podem ser gerados durante a isquemia e existem várias evidências obtidas a partir de diversos sistemas mostrando que estas espécies podem causar dano celular (Lipton, 1999).

Conforme já mencionado, os mecanismos envolvidos nos efeitos neuroprotetores dos estrógenos permanecem sem um esclarecimento satisfatório. Porém, uma das visões mais populares para explicar tais efeitos está relacionada à capacidade antioxidante endógena da molécula destes compostos. Diversos estudos, tanto *in vitro* como *in vivo*, demonstram que os estrógenos têm uma potente

atividade antioxidante lipídica bem estabelecida (Behl, 2002). Já foi demonstrado que o estradiol tem propriedades antioxidantes e suprime o estresse oxidativo induzido por peróxido de hidrogênio, ânions superóxido e outros pró-oxidantes em neurônios e em linhagens de células neuronais (Behl, 1999; Behl *et al.*, 1995, 1997; Bonnefont *et al.*, 1998; Mattson *et al.*, 1997; Sawada *et al.*, 1998).

Devido a estes dados, vários pesquisadores defendem a hipótese de que os estrógenos protegem as células contra o dano induzido por radicais livres, através de uma ação direta de "varredura" destes radicais livres ou da preservação de antioxidantes endógenos.

3. PRÉ-CONDICIONAMENTO HIPÓXICO-ISQUÊMICO

Curiosamente, as células neurais podem ser protegidas contra o dano cerebral quando um estresse subletal é aplicado várias horas ou dias antes de um estresse letal, um fenômeno adaptativo endógeno conhecido como tolerância. Este pré-tratamento não-letal que dispara a tolerância é denominado pré-condicionamento. O efeito neuroprotetor do pré-condicionamento contra o dano isquêmico tem sido bem estabelecido, mas os mecanismos celulares e moleculares envolvidos na indução da tolerância ainda não são completamente conhecidos.

No cérebro, a tolerância ao dano isquêmico foi demonstrada após pré-condicionamento com hipotermia subletal breve (Nishio et al., 2000), hipertermia (Ota et al., 2000), isquemia (Simon et al., 1993, Valentim et al., 2001, 2003), despolarização propagada (Kobayashi et al., 1995), inibição metabólica (Wiegand et al., 1999) e hipóxia (Jones & Bergeron, 2001, 2004).

Nos modelos *in vitro,* culturas organotípicas de fatias hipocampais de ratos, expostas previamente à privação de oxigênio e glicose por um período subletal tornam-se resistentes contra uma subseqüente privação de oxigênio e glicose letal (Valentim et al., 2003). *In vivo,* o pré-condicionamento hipóxico também reduz o dano cerebral hipóxico-isquêmico em ratos neonatos (Bergeron et al., 2000; Jones & Bergeron, 2001, 2004).

Pouco se sabe sobre os mecanismos envolvidos ou desencadeados no período de tempo entre o pré-condicionamento e o desenvolvimento de tolerância à isquemia cerebral. O entendimento das complexas respostas adaptativas a um

estresse subletal pode contribuir para o desenvolvimento de novas estratégias terapêuticas para prevenir a morte celular e promover processos de recuperação do cérebro.

4. TRANSPORTADORES DE GLUTAMATO

O L-glutamato é o principal neurotransmissor excitatório do SNC, atuando em subtipos distintos de receptores ionotrópicos, ligados a canais iônicos, e metabotrópicos, acoplados a proteína G. A ativação excessiva de receptores de glutamato ionotrópicos e metabotrópicos do grupo I, denominada excitotoxicidade, pode resultar em morte neuronal. Neste processo ocorre aumento no Ca^{+2} intracelular, ativação de uma cadeia de eventos bioquímicos potencialmente destrutivos, envolvendo quinases, lipases e proteases, geração de radicais livres tóxicos, estímulo a cascatas inflamatórias, ativação de genes e, finalmente, morte neuronal (Dunlop *et al.*, 1999). Várias condições neurodegenerativas incluindo a doença de Parkinson, a doença de Alzheimer, a isquemia cerebral, o dano cerebral traumático, a epilepsia, a doença de Huntington e a esclerose lateral amiotrófica têm sido relacionadas a distúrbios na homeostase de glutamato (Danbolt, 2001; Dunlop *et al.*, 1999; Robinson & Dowd, 1997).

As concentrações extracelulares de glutamato são mantidas dentro dos níveis fisiológicos única e exclusivamente por membros de uma família de transportadores de glutamato sódio-dependentes (também conhecidos como transportadores de aminoácidos excitatórios – EAAT – *excitatory amino acid transporters*), uma vez que não existem enzimas extracelulares capazes de metabolizar glutamato. Os EAATs, localizados tanto em neurônios como em células gliais, são responsáveis pela captação do glutamato extracelular, permitindo a transmissão excitatória normal, bem como protegendo contra a excitotoxicidade (Danbolt, 2001; Dunlop *et al.*, 1999; Robinson & Dowd, 1997) (Figura 3). Os EAATs nas células gliais também desempenham um outro papel vital, onde eles fornecem glutamato para processos metabólicos, inclusive para o ciclo glutamato-glutamina. Evidências recentes reforçam a hipótese de que alterações no metabolismo do glutamato, resultantes do seu transporte prejudicado, também podem contribuir para o dano neuronal (Rae *et al.*, 2000). Novos avanços no entendimento sobre os fatores que regulam tanto a expressão quanto a atividade dos EAATs criam a

possibilidade do uso de sua modulação como uma estratégia para o tratamento ou prevenção das patologias associadas com a excitotoxicidade (Dunlop *et al.*, 1999; Robinson, 2002).

Desde 1992, a clonagem e os estudos de biologia molecular identificaram cinco subtipos de transportadores de glutamato (EAAT1-5), possuindo farmacologia, localização celular e mecanismos modulatórios distintos. EAAT1 (GLAST) está localizado predominantemente na glia de Bergmann cerebelar, mas também está presente em células gliais por todo o SNC e, transitoriamente, em um pequeno número de neurônios (Furuta *et al.*, 1997). EAAT2 (GLT-1) é quase que exclusivamente glial e é encontrado de forma abundante por todo o cérebro frontal, cerebelo e medula espinhal (Furuta *et al.*, 1997). Os transportadores EAAT3 (EAAC1) e EAAT4 são encontrados predominantemente em neurônios, sendo EAAT3 expresso de forma abundante por todo o SNC e EAAT4 restrito a células de Purkinje do cerebelo (Furuta *et al.*, 1997). EAAT5 é expresso em células da retina (Pow & Barnett, 2000). Os significados das siglas (GLAST, *glutamate-aspartate transporter* – transportador de glutamato e aspartato; GLT-1, *glutamate transporter* – transportador de glutamato; EAAC, *excitatory amino acid carrier* – carreador de aminoácido excitatório; EAAT, *excitatory amino acid transporter* – transportador de aminoácido excitatório) não são importantes, uma vez que elas não refletem diferenças funcionais entre os transportadores. Todos os cinco transportadores catalisam o co-transporte de L-glutamato bem como L- e D-aspartato com Na^+ e K^+.

A maioria das sinapses no SNC está em justaposição com a glia e os EAATs gliais parecem ser os responsáveis pela maior parte da captação de glutamato no SNC (Rothstein *et al.*, 1996). Em condições não patológicas, o glutamato liberado durante a atividade sináptica é captado pelos astrócitos, convertido em glutamina e retornado para os neurônios a fim de restabelecer o *pool* pré-sináptico de neurotransmissor, ajudando a manter a transmissão sináptica (Sibson *et al.*, 2001).

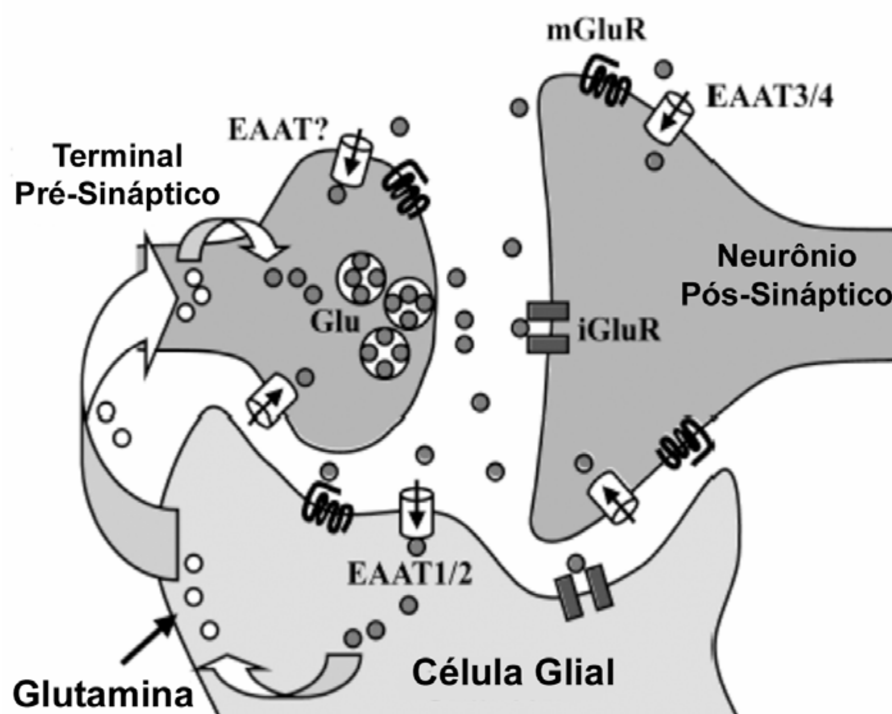

Figura 3: Diagrama de uma sinapse glutamatérgica. Glutamato (Glu – representado por círculos cinza escuro) é removido da sinapse por EAATs (representados por cilindros; as setas mostram a direção normal do transporte) localizados nos neurônios e nas células gliais, prevenindo a ativação excessiva de receptores de glutamato ionotrópicos e metabotrópicos (iGluR e mGluR, respectivamente) (Modificado de O'Shea, 2002).

Considerando que os dados na literatura referindo as propriedades neuroprotetoras do estradiol e do pré-condicionamento hipóxico-isquêmico estão bem estabelecidos em diversos modelos de isquemia cerebral, tanto *in vivo* como *in vitro*, mas que os mecanismos envolvidos ainda não são completamente entendidos, nossa hipótese de trabalho consistiu em testar o efeito neuroprotetor destas duas estratégias nos modelos de culturas organotípicas, fatias hipocampais preparadas a fresco e hipóxia-isquemia neonatal. Além disso, testar o possível envolvimento de algumas vias de sinalização, efeitos antioxidantes, receptores estrogênicos e transportadores de glutamato nos mecanismos de neuroproteção.

OBJETIVOS

OBJETIVO GERAL

Estudar os mecanismos moleculares associados à neuroproteção mediada por 17β-estradiol e por pré-condicionamento hipóxico-isquêmico contra o dano celular induzido pela isquemia cerebral.

OBJETIVOS ESPECÍFICOS

1. Investigar os efeitos neuroprotetores do 17β-estradiol em um modelo *in vitro* de lesão isquêmica, usando culturas organotípicas de fatias hipocampais de ratos expostas à privação de oxigênio e glicose (POG). Verificar se a neuroproteção mediada por estradiol estaria relacionada à via de sinalização da PI3-K e/ou da MEK/ERK 1/2, bem como a alterações no imunoconteúdo e no estado de fosforilação da Akt e da GSK-3β.

2. Investigar se um insulto isquêmico subletal poderia proteger a região CA1 do hipocampo de ratos contra um insulto isquêmico letal, usando pré-condicionamento com POG em culturas organotípicas expostas à POG subseqüente, verificando o imunoconteúdo dos transportadores de glutamato (EAAT1 e EAAT2) e dos receptores estrogênicos (ERα e ERβ). Além disso, verificar se os efeitos neuroprotetores do estradiol em culturas organotípicas expostas à POG envolvem mudanças no imunoconteúdo de EAAT1, EAAT2, ERα e/ou ERβ .

3. Investigar se a reposição de estradiol em ratas ovariectomizadas diminui a susceptibilidade do tecido cerebral a eventos isquêmicos, usando o modelo de POG em fatias hipocampais, verificando alguns parâmetros de estresse oxidativo, especificamente o conteúdo de radicais livres, um índice de dano oxidativo a proteínas e a atividade antioxidante total.

4. Investigar se o pré-condicionamento hipóxico protege efetivamente contra a morte celular em cérebro de ratos usando o modelo *in vivo* de hipóxia-isquemia neonatal, verificando o papel dos transportadores de glutamato (EAAT1 e EAAT2) e dos receptores estrogênicos (ERα e ERβ) na tolerância isquêmica induzida por hipóxia.

CAPÍTULO 1

Estradiol protects against oxygen and glucose deprivation in rat hippocampal organotypic cultures and activates Akt and inactivates Gsk-3β.

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2 # Estradiol Protects Against Oxygen and Glucose Deprivation
3 in Rat Hippocampal Organotypic Cultures and Activates Akt
4 and Inactivates GSK-3β

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8

9 *(Accepted November 18, 2004)*

10 Here we investigated the neuroprotective effect of 17β-estradiol in an *in vitro* model of
11 ischemia. We used organotypic hippocampal slice cultures, acute or chronically treated with
12 17β-estradiol (10 nM), and exposed to oxygen and glucose deprivation (OGD). Cellular death
13 was quantified by measuring uptake of propidium iodide (PI), a marker of dead cells. In OGD
14 exposed cultures, treated only with vehicle, about 70% of the CA1 area of hippocampus was
15 labeled with PI, indicating a great percentage of cellular death. When cultures were treated
16 with 17β-estradiol (acute or chronically), this cellular death was reduced to 15%. This effect
17 was prevented by LY294002 but was not by PD98059. Immunoblotting revealed that both,
18 chronic and acute, treatments with 17β-estradiol induced the phosphorylation/activation of
19 Akt and the phosphorylation/inactivation of GSK-3β. Our results show a clear neuropro-
20 tective effect of 17β-estradiol and suggest that this effect could involve PI3-K pathway.

21 **KEY WORDS:** Cerebral ischemia; 17β-estradiol; organotypic hippocampal culture; Akt; GSK-3β;
22 neuroprotection.

23

24 ## INTRODUCTION

26 Neurodegenerative diseases affect a wide spec-
27 trum of the population and, in most cases, lead to
28 physical and/or mental incapacity, involving mem-
29 ory, cognition, language and personality. Neurode-
30 generation is a prominent feature of cerebrovascular
31 disorders, particularly stroke syndromes that are a
32 major cause of morbidity and mortality in middle and
33 later life. Most strokes are caused by acute inter-
34 ruption of the brain blood supply by a thrombus,
35 leading to tissue ischemia. The brain is highly
36 dependent on continuous blood flow for the supply of
37 oxygen and glucose. The reduction in the supply of
38 these molecules to the brain that occurs in cerebral
39 ischemia leads to a complex cascade of cellular
40 events, resulting in severe neuronal degeneration and,
41 consequently, in loss of brain functions (1).

42 In the course of the investigation of cellular
43 mechanisms involved in cerebral ischemia, organo-
44 typic hippocampal slice cultures are a valuable
45 alternative to animal experiments. Therefore, orga-
46 notypic cultures have been used to study events and
47 mechanisms underlying neuronal death induced by
48 treatments such as hypoxia/aglycemia (2) and excito-
49 toxins (3). To model ischemic events organotypic cul-
50 tures are exposed to oxygen and glucose deprivation
51 (OGD) using an anaerobic chamber. We have found
52 that the response of organotypic cultured tissue to
53 injury induced by OGD is very similar to that shown

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by animals submitted to transient cerebral ischemia, suggesting the suitability of this model for the study of ischemic lesions and neuroprotective drugs (4,5).

It has been known for almost three decades that premenopausal females exhibit a lower susceptibility to stroke-related brain damage than males and postmenopausal females (6,7). The purposed neuroprotection in premenopausal females may be related to higher levels of circulating estrogens, principally 17β-estradiol. The main mammalian estrogen, 17β-estradiol, is a steroid hormone synthesized in the gonads or converted in periphery and brain through aromatization of androgens, and is traditionally associated with female reproduction. In addition to its classical role in reproductive maturation and regulation, numerous studies demonstrate that it plays an important protective and trophic roles in the brain. Recently, in a variety of models both *in vivo* and *in vitro*, steroids such as estradiol and others have been demonstrated to exert robust neuroprotective effects against diverse toxic insults, including excitotoxicity, oxidative stress, β-amyloid toxicity, metabolic inhibition and ischemia (8–14). The mechanism of neuroprotection, however, remains to be understood.

These neuroprotective effects of 17β-estradiol may be mediated through several cellular and molecular mechanisms including estrogen receptors (named ERα and ERβ) dependent and independent actions, genomic and non-genomic means (14,15). It has been shown, among many other things, that 17β-estradiol reduces the number of glutamate receptors (16), alters synthesis of proteins directly involved in the initiation of apoptotic cascade (17), upregulates protective genes in cell death pathways (18) and acts as a potent antioxidant inhibiting lipid peroxidation (19). Furthermore, estrogen has newly recognized effects on a variety of cytoplasmic signaling cascades, including protein kinase C (PKC), mitogen-activated protein kinase (MAPK), steroid receptor coactivator (src) and phosphatidylinositol 3-kinase (PI3-K) (20). In a recent study, treatment of cortical explant cultures with 17β-estradiol was also found to stimulate the phosphorylation of Akt/PKB (protein kinase B), an effector immediately downstream of PI3-K, and protect against a model of metabolic inhibition (21), suggesting that the PI3-K cascade might be involved in estrogen-induced neuroprotection. Moreover, 17β-estradiol has just been shown, both *in vivo* and *in vitro*, to decrease the activation state of glycogen synthase kinase-3β (GSK-3β), which is also a substrate of PI3-K pathway and one of the factors that triggers cell death (22).

In order to investigate the neuroprotective effects of 17β-estradiol, we used organotypic cultures of rat hippocampus exposed to an *in vitro* model of neuronal death using OGD, and we also investigated whether such neuroprotection could be related to PI3-K signaling pathway by Akt activation and GSK-3β inactivation.

EXPERIMENTAL PROCEDURE

Hippocampal Slice Cultures

Organotypic hippocampal slice cultures were prepared according to the method of Stoppini and colleagues (23). Briefly, male Wistar rat pups (postnatal day 6–8) were killed by decapitation (according to procedures approved by the Local Committee of Animal Care), the brains removed and the hippocampi dissected out. Transverse sections of 400 μm were cut with a McIlwain tissue chopper and separated in ice-cold Hank's balanced salt solution (HBSS) composed of (mM): glucose 36, $CaCl_2$ 1.26, KCl 5.36, NaCl 136.89, KH_2PO_4 0.44, Na_2HPO_4 0.34, $MgCl_2$ 0.49, $MgSO_4$ 0.44, Hepes 25; fungizone (1%) and gentamicin (36 μl/100 ml); pH 7.2. Two slices of three different animals (six in total) were placed on one Millicell culture insert (Millicell®-CM, 0.4 μm, Millipore®) and the inserts were transferred to 6-well culture plates (Cell Culture Cluster, Costar®). Each well contained one insert and 1 ml of culture medium consisting of 50% minimum essential medium (MEM), 25% heat-inactivated horse serum and 25% HBSS, supplemented with (mM, final concentration): glucose 36, HEPES 25 and $NaHCO_3$ 4; fungizone (1%) and gentamicin (36 μl/100 ml); pH 7.3. Plates were then placed in an incubator at 37°C in an atmosphere of 5% CO_2. Culture medium was changed every 3 days and slices were cultivated during 14 days *in vitro*.

Oxygen and Glucose Deprivation (OGD)

The induction of OGD to mimic ischemic injury was based on the method described by Strasser and Fisher (24), with some modifications (4). After 14 days *in vitro*, the inserts with the 6-slice cultures were transferred to a sterilized 6-well plate, rinsed twice with OGD medium consisting of HBSS lacking glucose, and then incubated with 1 ml of this medium for 15 min to deplete glucose from intracellular stores and extracellular space. After that, medium was replaced by one with the same composition but previously bubbled with N_2 for 30 min and the plate immediately transferred to an anaerobic chamber at 37°C with N_2-enriched atmosphere. Cultures were maintained in these conditions for 60 min, following which the plate was removed from the chamber, the inserts containing the slices washed twice with HBSS and fresh culture medium was added. Cultures were then placed in the incubator (37°C, 5% CO_2) for 24 h (recovery period).

The experimental treatments were: (1) Chronic estradiol group (CE) in which 17β-estradiol (10 nM) was added to the culture medium on day 7 of culture and maintained throughout the experiment; (2) acute estradiol group (AE) in which 17β-estradiol (10 nM) was added to the OGD medium and to the culture medium during the recovery period; (3) vehicle group (dimethylsulfoxide—DMSO) in which DMSO 0.01% was added to the OGD medium and to the culture medium during the recovery period; (4)

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160 LY group (LY) in which LY294002 (5 μM) was added to the OGD
161 medium and to the culture medium during the recovery period;
162 (5) AE+LY group (AE+LY) in which LY294002 (5 μM) and
163 17β-estradiol (10 nM) were added together; (6) PD group (PD) in
164 which PD98059 (20 μM) was added to the OGD medium and to
165 the culture medium during the recovery period; (7) AE+PD group
166 (AE+PD) in which PD98059 (20 μM) and 17β-estradiol (10 nM)
167 were added together.
168 Control slice cultures in 6-well plates were treated in parallel
169 to OGD slice cultures.

170 **Quantification of Cellular Death**

171 Cellular damage was assessed by fluorescent image analysis
172 of propidium iodide (PI) uptake. After a recovery period of 22 h,
173 7.5 μM PI was added to cultures and incubated for 2 h. PI is ex-
174 cluded from healthy cells, but following loss of membrane integrity
175 it enters cells, binds to DNA and becomes highly fluorescent.
176 Cultures were observed with an inverted microscope (Nikon
177 Eclipse TE 300) using a standard rhodamine filter set. Images were
178 captured and then analyzed using Scion Image software (http://
179 www.scioncorp.com). The area where PI fluorescence was detect-
180 able above background levels was determined using the 'density
181 slice' option of Scion Image software and compared to total CA1
182 area to obtain the percentage of damage (25).

183 **Western Blotting**

184 After obtaining the fluorescent images, cultured slices were
185 homogenized in lysis buffer (4% SDS, 2.1 mM EDTA, 50 mM
186 Tris). Aliquots were taken for protein determination and β-mer-
187 captoethanol was added to a final concentration of 5% (5). Samples
188 containing 35 μg of protein were resolved by 12% SDS-PAGE.
189 After electrophoresis, proteins were electrotransferred to nitrocel-
190 lulose membranes using a semi-dry apparatus (Bio-Rad Trans-Blot
191 SD). The membranes were blocked overnight with 5% powdered
192 milk in Tris-buffered saline plus 0.1% Tween-20, followed by
193 incubation at 4°C with anti-phosphospecific Akt, anti-Akt, anti-
194 phosphospecific GSK-3β or anti-GSK-3β antibodies (1:1000, Cell
195 Signaling) diluted in the same blocking solution. Subsequently, the
196 membranes were incubated for 1 h with horseradish peroxidase-
197 conjugated anti-rabbit antibody also diluted in blocking solution
198 (1:1000). Immunoreactive bands were revealed by an enhanced
199 chemiluminescence kit (ECL, Pharmacia), and detected using
200 X-ray films. The immunoblot films were scanned and the digital-
201 ized images analyzed with Optiquant software (Packard Instru-
202 ment). For each experiment, test groups were referred to vehicle
203 treated control cultures not exposed to OGD, which were consid-
204 ered 100%, thus assuring the same signal intensity for control and
205 test groups. Data are expressed as percentage of phosphorylated
206 protein, which was obtained by the ratio of the immunocontent of
207 phospho-protein (Akt or GSK-3β) with the whole amount of the
208 protein (Akt or GSK-3β) provided by the immunodetection assay
209 with total- (Akt or GSK-3β) antibody.

210 **Statistical Analysis**

211 All data are presented as mean ± S.E.M. of the indicated
212 number of experiments. One-way analysis of variance (ANOVA)
213 was applied to the means to determine statistically significant dif-
214 ferences between experimental groups; *post hoc* comparisons were
performed by Duncan's multiple range test. A level of significance 215
of $P<0.05$ was selected. 216

RESULTS 217

Exposure to OGD for 60 min caused a marked 218
fluorescence in the CA1 area, indicating a high 219
incorporation of PI, as shown in the photomicro- 220
graph in Fig. 1a (OGD DMSO group). Quantifica- 221
tion of PI fluorescence shows that OGD caused about 222
70% of damage in CA1 cells, a significant increase 223
compared to control cultures with a basal CA1 224
damage of 3% (Fig. 1b). Pretreatment with 10-nM 225
17β-estradiol for 7 days prior to OGD exposure 226
(OGD CE group) significantly reduced damage in the 227
vulnerable CA1 area from 70%, without treatment, to 228
only 15%, with treatment (Fig. 1b). When 10-nM 229
17β-estradiol was present only during the lesion 230
induction and maintained during the recovery period 231
(OGD AE group), a similar pattern of protection was 232
observed, showing a decrease in the percentage of 233
CA1 damage to only 20% (Fig. 1b). No significant 234
difference in the neuroprotective effects between 235
chronic and acute treatments with 17β-estradiol was 236
observed (Fig. 1b). Also, any difference between 237
treatments in control slices has not been detectable, 238
indicating that 17β-estradiol had no toxic effect in 239
basal conditions (Fig. 1b). 240

In order to investigate if the PI3-K signaling 241
pathway was involved in the neuroprotective effect of 242
17β-estradiol, we carried out experiments using 243
LY294002, a PI3-K inhibitor. LY294002 prevented 244
the neuroprotective effect of 17β-estradiol as shown 245
in Fig. 2b (OGD AE+LY group). As it has been 246
reported that the effects of estradiol could be medi- 247
ated through the MEK/ERK 1/2 pathway (26), we 248
further studied the effect of PD98059, an inhibitor of 249
this pathway. PD98059 did not prevent the neuro- 250
protective effect of 17β-estradiol, although PD seems 251
to slightly reduce the effect of estradiol it was not 252
statistically significant (Fig. 2b). 253

To evaluate a possible mechanism by which 254
17β-estradiol prevents cellular death in our model, we 255
analyzed the activation of Akt/PKB, a putative 256
effector of PI3-K involved in cell survival signaling. 257
This enzyme, Akt/PKB, is a serine/threonine protein 258
kinase activated by phosphorylation (27). Therefore, 259
we examined the effect of 17β-estradiol on the phos- 260
phorylation/activation of Akt using phospho- and 261
total-specific antibodies by Western blotting. In both 262
chronic and acute treatments, 10-nM 17β-estradiol 263

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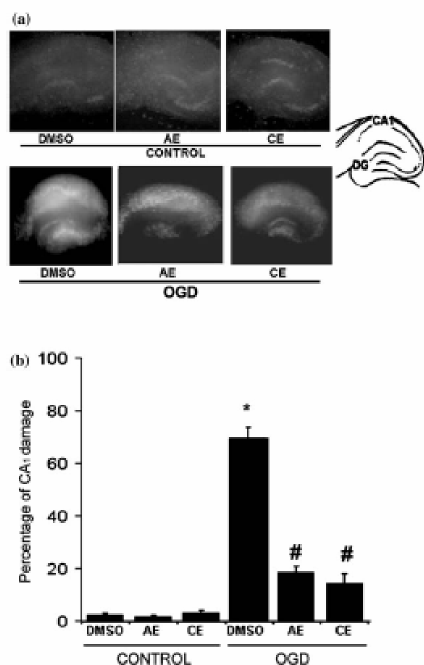

Fig. 1. Effect of 17β-estradiol on cellular damage induced by OGD for 60 min in organotypic hippocampal cultures. (a) Representative photomicrographs of cultures showing PI fluorescence 24 h after exposure to OGD. (b) Quantitative analysis of CA1 damage 24 h after exposure to OGD. Images were captured and then analyzed using Scion Image software (http://www.scioncorp.com). The area where PI fluorescence was detectable above background levels was determined using the 'density slice' option of Scion Image software and compared to the total CA1 area to obtain the percentage of damage. OGD, oxygen and glucose deprivation exposed cultures; DMSO, vehicle-treated cultures; AE, acute treatment, 17β-estradiol (10 nM) was added only at the moment of lesion induction; CE, chronic treatment, pretreatment with 17β-estradiol (10 nM) started 7 days prior to lesion induction. Bars represent the mean ± S.E.M., $n = 15$ (5 cultures from 3 different animals in duplicate). *, significantly different from control cultures; #, significantly different from control and DMSO-treated OGD cultures (one-way ANOVA followed by Duncan's test, $P < 0.05$).

264 significantly increased the percentage of phosphory-
265 lated Akt, about 31 and 29%, respectively, in both
266 control and OGD cultures. These increases were
267 already observed right after 60 min of OGD (time 0,
268 Fig. 3a) and maintained for up to 24 h (Fig. 3b). No
269 difference in the immunocontent of total Akt was
270 observed. There were no differences in the percentage
271 of phosphorylated Akt between vehicle treated con-
272 trol and OGD cultures at any time of analysis.

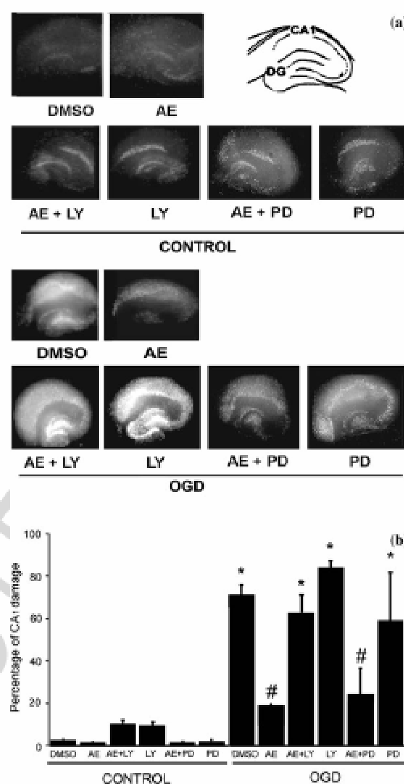

Fig. 2. Effect of LY294009 and PD98059 on neuroprotective effect of 17β-estradiol on cellular damage induced by OGD for 60 min in organotypic hippocampal cultures. (a) Representative photomicrographs of cultures showing PI fluorescence 24 h after exposure to OGD. (b) Quantitative analysis of CA1 damage 24 h after exposure to OGD. Images were captured and then analyzed using Scion Image software (http://www.scioncorp.com). The area where PI fluorescence was detectable above background levels was determined using the 'density slice' option of Scion Image software and compared to the total CA1 area to obtain the percentage of damage. OGD, oxygen and glucose deprivation exposed cultures; DMSO, vehicle-treated cultures; AE, acute treatment, 17β-estradiol (10 nM) was added only at the moment of OGD induction; LY, LY294009 (5μM) was added to OGD medium and maintained during the recovery period; AE + LY, 17β-estradiol (10 nM) and LY294009 (5 μM) were added simultaneously to OGD medium and maintained during the recovery period; PD, PD98059 (20 μM) was added to OGD medium and maintained during the recovery period; AE + PD, 17β-estradiol (10 nM) and PD98059 (20 μM) were added simultaneously to OGD medium and maintained during the recovery period. Bars represent the mean ± S.E.M., $n = 6$ (2 cultures from 3 different animals in duplicate). *, significantly different from control cultures; #, significantly different from control cultures and OGD cultures treated with DMSO, AE + LY, LY and PD (one-way ANOVA followed by Duncan's test, $P < 0.05$).

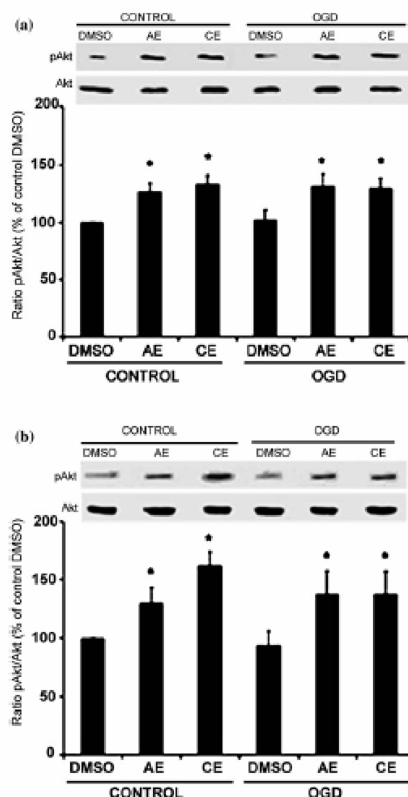

Fig. 3. Effect of acute and chronic treatments with 17β-estradiol on the percentage of phophorylated Akt in organotypic hippocampal cultures. (a) Analyzed immediately after 60 min of OGD (time 0), (b) Analyzed 24 h after the exposure to 60 min of OGD. Histograms represent the quantitative Western blot analysis of Akt phosphorylation state. The densitometric values obtained to phospho- and total-Akt from all treatments were first normalized to their respective vehicle-treated control non-exposed to OGD condition (DMSO bar) (100%). OGD, oxygen and glucose deprivation exposed cultures; DMSO, vehicle-treated cultures; AE, acute treatment, 17β-estradiol (10 nM) was added to OGD medium and maintained during the recovery period; CE, chronic treatment, pretreatment with 17β-estradiol (10 nM) started 7 days prior to lesion induction. Representative Western blots of phospho-Akt (pAkt) and Akt revealed using antibodies against phospho- and total-Akt are shown in the top of the histograms. Data are expressed as a ratio of the normalized percentages of phospho-Akt (pAkt) and Akt. Bars represent the mean ± S.E.M., $n = 15$ (5 cultures from 3 different animals in duplicate). *, significantly different from DMSO-treated control and OGD cultures (one-way ANOVA followed by Duncan's test, $P < 0.05$).

Representative Western blots are shown in the top of 273
histograms of Fig. 3a and 3b. 274
As GSK-3β is a substrate of PI3-K and MAPK 275
pathways, which are both activated by estradiol (28), 276
and since GSK-3β when phosphorylated has its pro- 277
apoptotic activity inhibited, we investigated, by the 278
same way to that performed to Akt/PKB, its phos- 279
phorylation state in our model. Compared to vehicle 280
treated control cultures (control DMSO group), chronic 281
treatment with 17β-estradiol significantly increased the 282
percentage of GSK-3β phosphorylation in both control 283
and OGD exposed cultures (50 and 33%, respectively) 284
as well as the acute treatment (43 and 30%, respectively). 285
These increases were observed immediately after the 286
OGD exposure period (time 0, Fig. 4a), being similar 287
ones observed after the 24-h recovery period (Fig. 4b). 288
No difference in the immunocontent of total GSK-3β 289
was observed. There were no differences in the per- 290
centage of phosphorylated GSK-3β between vehicle 291
treated control and OGD cultures at any time of anal- 292
ysis. Representative Western blots are shown in the 293
top of histograms on Fig. 4a and 4b. 294

DISCUSSION 295

Over the past decade, evidence has emerged 296
indicating that estrogens influence growth, differen- 297
tiation, maturation and function of many target tis- 298
sues including a neuroprotective role. The issue of 299
estrogen protection is important, as there is a dra- 300
matic age-related decline in estrogen levels in women, 301
such that postmenopausal women have estrogenic 302
levels that are approximately 1% of that observed 303
in premenopausal women. Moreover, over the past 304
century, the lifespan of women has increased to 305
more than 80 years, but the age of menopause has 306
remained fix. Consequently, women are living an 307
ever-increasing proportion of their lives in a hypoes- 308
trogenic postmenopausal state, which could contribute 309
to an increased risk of stroke, cognitive dysfunction 310
and a variety of neurodegenerative diseases (29). 311
A large number of animal studies, both *in vivo* 312
and *in vitro*, have suggested that a neuroprotective 313
role does exist for estrogen, a finding that has pro- 314
pelled interest in determining its effectiveness in the 315
prevention of neurodegenerative and cerebrovascular 316
diseases in humans (30). These studies have suggested 317
a beneficial role for estrogen in Alzheimer's disease 318
and Parkinson's disease (7) and have clearly shown 319
that estradiol exerts potent protective actions against 320
ischemic brain injury (10,14). 321

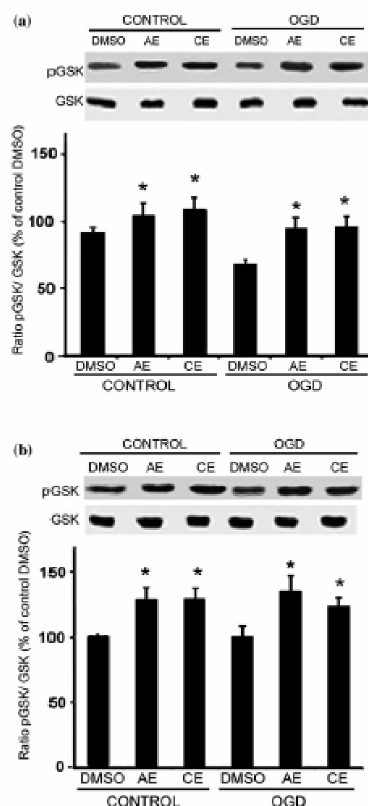

Fig. 4. Effect of acute and chronic treatments with 17β-estradiol on the percentage of phosphorylated GSK3-β in organotypic hippocampal cultures. (a) Analyzed immediately after 60 min of OGD (time 0), (b) Analyzed 24 h after the exposure to 60 min of OGD. Histograms represent the quantitative Western blot analysis of GSK-3β phosphorylation state. The densitometric values obtained to phospho- and total-GSK-3β from all treatments were first normalized to their respective vehicle-treated control non-exposed to OGD condition (DMSO bar) (100%). OGD, oxygen and glucose deprivation exposed cultures; DMSO, vehicle-treated cultures; AE, acute treatment, 17β-estradiol (10 nM) was added to OGD medium and maintained during the recovery period; CE, chronic treatment, pretreatment with 17β-estradiol started 7 days prior to lesion induction. Representative Western blots of phospho-GSK-3β (pGSK) and GSK-3β (GSK) revealed using antibodies against phospho- and total-GSK-3β are shown in the top of the histograms. Data are expressed as a ratio of the normalized percentages of phospho-GSK-3β (pGSK) and GSK-3β (GSK). Bars represent the mean ± S.E.M., $n = 9$ (3 cultures from 3 different animals in duplicate). *, significantly different from DMSO-treated control and OGD cultures (one-way ANOVA followed by Duncan's test, $P < 0.05$).

In order to further investigate the neuroprotective properties of 17β-estradiol, most potent estrogen in humans and rodents, we have used organotypic hippocampal slice cultures to examine its effects in a model of *in vitro* 'ischemic-like insult' using OGD. We have chosen this method of culturing because the maintenance of architectural integrity and communication among neurons and between neurons and glia may be important to fully understand the mechanisms of trophic and protective effects of estradiol. This is suggested by the findings that estrogen receptors exist in astrocytes (31) and some of the actions of estradiol may be very different depending on the presence or absence of these glial cells (32). Therefore, the effects of estradiol may require communication among cells of differing phenotypes. Propidium iodide uptake was used as a marker of cell death since studies have shown that, in organotypic cultures, this endpoint correlates well with the extent of cell death detected by other methods (33).

Our results have extended previous findings by demonstrating that the presence of 17β-estradiol, 7 days prior to injury or just by the time of injury, in a low dose (10 nM), exerted protective effects, preventing cell death. The present study has focused attention on the neuroprotective effects of physiological estradiol in the brain, since we believe that these levels of hormone are highly relevant to replacement therapies that women will be using. Partially in contrast to a previous work (14), in our study, pretreatment was not required for the protective effects of estradiol, since acute exposure to the same concentration of 17β-estradiol did protect. One possible explanation for this difference includes use of differing brain regions. The previous study has used cortical explant cultures, while we were using hippocampal slice cultures. Also, the injury paradigm is different; they used kainic acid or potassium cyanide/ 2-deoxyglucose exposure, while in our model we were using OGD. In this report, the authors suggest that the long-term (and the lack of short-term) protective actions of estradiol they observed involve classical nuclear estrogen receptors, possibly in a genomic fashion. In addition, Pringle and colleagues (12) suggest that conversion to 7-hydroxylated metabolites is necessary for steroid-mediated neuroprotection, what corroborates for the requirement of long incubation times and/or high steroid concentrations used in other previous studies (34,35). This would also explain why 17β-estradiol was devoid of neuroprotective efficacy when present at 100 nM pre-during and post-hypoxia

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in their model (12). On the other hand, 24-h treatment with 17β-estradiol at very low doses, such as 1 pM and 1 nM, attenuated the neuronal damage in CA1 induced by glutamate exposure (13). There have been reports of rapid, non-genomic actions of estrogens (36), which could help us to understand our result showing the short-term neuroprotective effect of low doses of estradiol. Evidence continues to accumulate supporting the existence of a membrane-bound estrogen receptor, particularly found in the brain of rats and humans (37), which has been implicated in mediating these rapid responses to estrogens.

Estrogen regulates the transcription of genes through nuclear estrogen receptors (38). In addition, increasing evidence shows that estrogen may be involved in the triggering of non-transcriptional pathways. The MAPK pathway has been suggested to be involved in the neuroprotective effect of estrogen (39). In a model of glutamate-induced neurotoxicity in cultured cortical neurons, the phophatidylinositol-3-kinase (PI3-K) pathway has also been shown to mediate the neuroprotection by estrogen (40).

To clarify the signaling pathway probably involved in the neuroprotection by estradiol observed in our model, we explored two different pathways. First, we investigated the PI3-K pathway, which mediates signaling pathways triggered by several trophic factors in a variety of cell types including neuronal cells (41). For this purpose, we used LY294002, an inhibitor of PI3-K. The results presented in Fig. 2b show that the neuroprotective effect of estradiol was almost totally prevented by this inhibitor. Second, we investigated if the MEK/ERK 1/2 pathway could be involved in the neuroprotective effect of estradiol, once it has been reported that some of its effects are mediated through this pathway (42). As shown in Fig. 2b, PD98055, an inhibitor of the MEK/ERK 1/2 pathway, did not prevent the neuroprotective effect of estradiol. These results strongly suggest that the PI3-K signaling pathway is involved in the neuroprotection by estradiol observed in our model of cellular death.

In an attempt to study the molecular mechanism that could be involved with the PI3-K signaling pathway, we investigated the activation of Akt/PKB. In this signal transduction cascade, Akt/PKB (protein kinase B), a serine/threonine kinase, is the director effector downstream of PI3-K (43), and recent studies have suggested that a signaling pathway from PI3-K to Akt/PKB mediates the cell survival signal in neuronal cells by inhibiting apoptosis (44). Following this pathway, glycogen synthase kinase 3β (GSK-3β) is a substrate of Akt/PKB (45). The main regulatory mechanism of these enzymes is by phosphorylation: Akt/PKB is activated while GSK-3β is inhibited by phosphorylation (46). Activation of GSK-3β promotes pro-apoptotic signaling (46). Recently, an inhibitor of this enzyme has been shown to reduce cell death caused by PI3-K inhibition or serum withdrawal in cortical neurons (47).

We observed that 17β-estradiol induces phosphorylation of both proteins, which is mirrored by activation of Akt and inhibition of GSK-3β, and therefore by the activation of one of the signaling pathways directly implicated in survival (Akt) and the blockade of GSK-3β, which is directly implicated in the induction of apoptosis. This increase in phosphorylated protein levels was observed either in chronic or acute treatment, suggesting that pretreatment is not necessary for the actions of estradiol on phosphorylation of these enzymes. These results seem to agree with the reported ability of estradiol to increase levels of activated Akt after 15 min (40), suggesting a non-genomic mechanism of action. These short-term, non-transcriptional or non-genomic actions are thought to be neuromodulatory in nature and critical for cell–cell communication. Such a rapid effect is not likely to be the consequence of nuclear action, but rather appears to be related to events occurring on the cell surface (28,48).

Although further work is needed to understand the precise mechanism of neuroprotection provided by 17β-estradiol, the present study adds one more evidence that the PI3-K cascade could play a pivotal role in estradiol-induced neuroprotective effect against 'ischemic-like injury'. These findings might be important for understanding the increase of the susceptibility of menopausal female to cerebral ischemia and the mechanism of neuroprotection afforded by steroid hormones.

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CAPÍTULO 2

Estradiol and preconditioning effects on glutamate transporters and estrogen receptors in organotypic cultures exposed to oxygen-glucose deprivation. Neurochemistry International, a ser submetido.

Estradiol and Preconditioning Effects on Glutamate Transporters and Estrogen Receptors in Organotypic Cultures Exposed to Oxygen-Glucose Deprivation

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Abstract

The molecular basis of estrogen-mediated neuroprotection against brain ischemia remains unclear, as well as the mechanisms by which ischemic preconditioning promotes brain tolerance against subsequent ischemic insults. In the present study, we investigated by immunoblotting the expression of estrogen receptor (ER) α and β and excitatory amino acid transporter (EAAT) 1 and 2 in organotypic hippocampal slice cultures treated with estradiol or preconditioning and exposed to oxygen-glucose deprivation (OGD). Propidium iodide (PI) staining showed that 17β-estradiol (10 nM) protected the CA1 area of hippocampus against 60 min of OGD, reducing cellular death from 69% without treatment to only 23%. In preconditioned slice cultures stained with PI, we could observe that 15 min of OGD induced marked tolerance to 45 min of OGD delivered 48 h later, affording almost 50% neuroprotection. In contrast to preconditioned cultures where the immunocontent of ERs remain unchanged, ERα was significantly down-regulated by 20% after OGD in both vehicle- and estradiol-treated cultures, whereas ERβ was significantly up-regulated by 25% in the estradiol-treated cultures exposed or not to OGD. However, no significant change in the immunocontent of EAAT1 and EAAT2 was detected in response to either estradiol treatment or ischemic preconditioning. These findings suggest that estrogen neuroprotection against ischemia might involve regulation of ERs abundance and, consequently, of the gene transcripted by these receptors.

Keywords: 17β-Estradiol; Preconditioning; Ischemia; Organotypic culture; Glutamate transporters; Estrogen receptors

1. Introduction

Epidemiological (Blanchet et al., 1999) and basic science studies (Lee and McEwen, 2001) underscore the powerful neuroprotective properties of estrogens, as well as ischemic preconditioning neuroprotection has been well established in many models of cerebral ischemia, both *in vivo* and *in vitro* (Jones and Bergeron, 2001, 2004; Kato et al., 1992; Kitagawa et al., 1990; Schurr et al., 1986; Valentim et al., 2001, 2003). In this phenomenon, brief ischemic episodes may render brain tolerant against more severe subsequent ischemic insults. The pathways in estrogen-enhancement of neuronal survival and the mechanisms underlying the increased resistance to severe ischemia by a preceding mild ischemic exposure, however, remain unclear.

Brain ischemia and several other pathological disease states involve excitotoxic cell death, which is mediated by toxic levels of extracellular glutamate that excessively activate AMPA/kainate- and/or NMDA-type glutamate receptors and initiates sodium and calcium influx (Lipton and Rosenberg, 1994). The removal of glutamate from the extracellular space, permitting normal excitatory transmission and preventing cell death due to excitotoxicity, is accomplished mainly by means of the glial glutamate transporters (also known as excitatory amino acid transporters or EAAT) subtypes: EAAT1 and EAAT2. Recent studies have shown that preconditioning can alter the transport of glutamate, either by down- or up-regulation of glutamate transporters (Douen et al., 2000; Romera et al., 2004). Estrogens may exert neuroprotective effects by directly interacting with glutamate receptors (Weaver et al., 1997; Wong and Moss, 1992), although a potential estrogen interaction with glutamate transporters has not been clarified yet.

Abundant expression of estrogen receptors (ER) is observed in the central nervous system, and it has been shown that estrogens might exert many, though not all, of their neuroprotective effects by binding to two types of these intracellular receptors: ERα and ERβ, and then inducing gene transcription (Aranda and Pascual, 2001; Hall et al., 2001). The relative contribution by either estrogen receptor subtype to estrogen-induced neuroprotection is still unresolved. Although, hypoxic tolerance by preconditioning has already been shown to be gender dependent (Kasischke et

al., 1999) and the mechanisms conferring it gender specific (von Armim et al., 2002), the effects of preconditioning on estrogen receptors have not been determined yet.

Thus, the main goal of this study was to investigate whether the neuroprotection afforded by either estrogen or ischemic preconditioning involves alteration in the abundance of glial glutamate transporters, EAAT1 and EAAT2, and estrogen receptors, ERα and ERβ.

2. Materials and methods

2.1. Materials

β-estradiol 3-benzoate, and acrylamide and bisacrylamide, used in sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE), were obtained from Sigma Chemical Co. (St. Louis, MO, USA). Polyclonal antibodies raised against rabbit EAAT1 and EAAT2 were kindly provided by Dr. D. Pow, University of Queensland; ERα was obtained from Santa Cruz Biotechnology (catalog sc-542) and ERβ was from Calbiochem (catalog PC168-50UG). A monoclonal antibody raised against mouse β-actin was obtained from Sigma (catalog A 5316). Anti-mouse IgG and anti-rabbit IgG peroxidase-conjugated and reagents to detect proteins by chemiluminescence were purchased from Amersham International. Millicell culture inserts were obtained from Millipore, culture medium and horse serum heat inactivated were obtained from Gibco. All animal use procedures were approved by local Animal Care Committee and were in accordance with the NIH Guide for Care and Use of Laboratory Animals.

2.2. Hippocampal slice cultures

Organotypic hippocampal slice cultures were prepared as described previously (Cimarosti et al., 2001; Stoppini et al., 1991). Transverse hippocampal slices (400 µm) from 6 to 8-day-old male Wistar rat pups were cut using a McIlwain tissue chopper. Slices were transferred to Millicell culture inserts (6 slices per insert) in 6-well plates, and maintained at 37ºC in an atmosphere of 5% CO_2 for 14 days prior to use. Culture medium, consisting of 50% minimum essential medium (MEM),

25% Hanks balanced salt solution (HBSS), 25% heat-inactivated horse serum, glucose 36 mM, HEPES 25 mM, $NaHCO_3$ 4mM, fungizone 1% and gentamicine 36 μL/100 mL (pH 7.3), was replaced every 3 days.

2.3. In vitro model of ischemia

Oxygen-glucose deprivation (OGD) was achieved by combining severe hypoxia with aglycemia, according to the method described by Strasser and Fisher (1995), with some modifications (Cimarosti et al., 2001). The slices were washed twice with aglycemic HBSS containing $CaCl_2$ 1.26 mM, KCl 5.36 mM, NaCl 136.89 mM, KH_2PO_4 0.44 mM, Na_2HPO_4 0.34 mM, $MgCl_2$ 0.49 mM, $MgSO_4$ 0.44 mM, Hepes 25 mM, fungizone 1% and gentamicine 36 μL/100 mL (pH 7.2), and incubated in this medium for 15 min to deplete glucose from intracellular stores and extracellular space. Then, medium was replaced by one with the same composition but previously bubbled with N_2 for 30 min and the plate immediately transferred to an anaerobic chamber at 37^oC with N_2-enriched atmosphere. Cultures were maintained in these conditions for 15 min (preconditioning) or 45 or 60 min (ischemic insult). Following OGD, the slices were washed twice with HBSS and returned to their original culture conditions (see above). Slices in the control groups, treated in parallel to slices in the OGD groups, were incubated for similar periods of time but were washed with glucose fortified medium and exposed to warmed humidified air with 5% CO_2.

2.4. Estrogen treatment

In the estradiol group (E2), 17β-estradiol (10 nM) was added to the culture medium on day 7 of culture and maintained throughout the experiment, while in the vehicle group (dimethylsulfoxide - DMSO), DMSO 0.01% was added to the OGD medium and to the culture medium during the recovery period. Cultures were exposed to 60 min of OGD followed by a 24-h recovery (Cimarosti et al., 2005).

2.5. Preconditioning protocol

In the preconditioned groups, cultures were exposed to 15 min of OGD, allowed to recover for 48 h and then exposed or not to 45 min of OGD, followed by a further 24-h recovery period (Xu et al., 2002).

2.6. Quantification of cellular death

Cellular damage was assessed by fluorescent image analysis of propidium iodide (PI) uptake. After a recovery period of 22 h, 7.5 μM PI was added to cultures and incubated for 2 h. PI is excluded from healthy cells, but following loss of membrane integrity it enters cells, binds to DNA and becomes highly fluorescent. Cultures were observed with an inverted microscope (Nikon Eclipse TE 300) using a standard rhodamine filter set. Images were captured and then analyzed using Scion Image software (www.scioncorp.com). The area where PI fluorescence was detectable above background levels was determined using the “density slice” option of the software and compared to total CA1 area to obtain the percentage of damage (Valentim et al., 2003).

2.7. Western blot analysis

After obtaining the fluorescent images, cultured slices were homogenized in lysis buffer (4% SDS, 2.1 mM EDTA, 50 mM Tris), aliquots were taken for protein determination and β-mercaptoethanol was added to a final concentration of 5% (Tavares et al., 2001). Samples containing 40 μg of protein were resolved by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Then, proteins were electrotransferred to nitrocellulose membranes using a semi-dry apparatus (Bio-Rad Trans-Blot SD). After 2 h incubation at 4°C in blocking solution containing 5% skim milk powder and 0.1% Tween-20 in Tris-buffered saline (TBS; 50 mM Tris-HCl, 1.5% NaCl, pH 7.4), membranes were incubated overnight with the appropriate primary antibody diluted in the same blocking solution. Primary antibodies against the following proteins were used: EAAT1 and EAAT2 (both

1:50000 dilution, rabbit polyclonal) were kindly provided by Dr. D. Pow, University of Queensland; ERα (1:200 dilution, rabbit polyclonal) was from Santa Cruz Biotechnology and ERβ (1:1000 dilution, rabbit polyclonal) was from Calbiochem. Subsequently, membranes were incubated for 2 h with horseradish peroxidase-conjugated secondary antibodies recognizing antigens from the same host as the corresponding primary antibody (mouse or rabbit IgG from Amersham, 1:1000 dilution in blocking solution). Immunoreactive bands were revealed by an enhanced chemioluminescence kit (ECL, Pharmacia), and detected using X-ray films. The same blots were re-probed with β-actin antibody from Sigma (1:5000 dilution, mouse monoclonal) as an internal control. As the secondary antibodies were different, rabbit for the proteins under investigation and mouse for β-actin, it was not necessary to strip the blots. The immunoblot films were scanned and the digitalized images analyzed with Optiquant software (Packard Instrument). For each experiment, test groups were referred to control groups not exposed to OGD, which were considered 100%, thus assuring the same signal intensity for all groups.

2.8. Statisical analysis

Data are presented as mean ± S.E.M. of the indicated number of experiments. One-way analysis of variance (ANOVA) was applied to the means to determine statistically significant differences between experimental groups; Duncan's multiple range test performed post hoc comparisons.

3. Results

3.1. Estradiol treatment protected against 60 min OGD

Exposure to OGD for 60 min caused a marked fluorescence in the hippocampal CA1 area, indicating a high incorporation of PI, as shown in the photomicrograph in Fig. 1A (OGD DMSO group). Quantification of PI fluorescence shows that 60 min OGD caused 69% of damage in CA1 neuronal cells (Fig. 1B), whereas in the DG area no consistent damage was evidenced (Fig. 1A).

Pretreatment with 10 nM 17β-estradiol for 7 days prior to OGD exposure (OGD E2 group) significantly reduced cellular death in the vulnerable CA1 area by 3 fold, from 69%, without treatment, to only 23%, with treatment (Fig. 1A, B).

3.2. Changes in estrogen receptors, ERα and ERβ, in response to estradiol treatment and 60 min OGD

Exposure to OGD for 60 min caused about 20% decrease in the immunocontent of ERα in cultures treated with both vehicle (81±5% control, n=10) and estradiol (79±5% control, n=12) (Fig. 2A). Pretreatment with 10 nM 17β-estradiol for 7 days prior to OGD exposure significantly increased the immunocontent of ERβ by 26% (126±10% control, n=6) in OGD non-exposed control cultures (Fig. 2B). This increase was sustained even when cultures were exposed to 60 min OGD (122±10% control, n=6). In contrast, there was no significant change in the levels of glutamate transporters, EAAT1 and EAAT2, in the cultures treated with vehicle or estradiol, exposed or not to OGD (data not shown).

3.3. Preconditioning with 15 min of OGD protected against 45 min OGD

A short period of OGD (15 min) did not cause significant neuronal death in the CA1 area of hippocampal organotypic slice cultures after 48-h recovery, as indicated by PI fluorescence (Fig. 3A). When cultures were exposed to 45 min of OGD, followed by a recovery period of 24 h, fluorescence in the CA1 area due to incorporation of PI was observed, indicating neuronal death (Fig. 3A). Quantification of PI fluorescence shows that OGD for 45 min caused about 62% of damage in the CA1 area (Fig. 3B). Similarly to 60 min OGD, no consistent damage to neuronal cells was evidenced in the DG area (Fig. 3A). Preconditioning with 15 min of OGD induced a significant tolerance to subsequent 45 min of OGD, reducing the cellular damage in the vulnerable CA1 area by almost 2 fold, from 62%, without preconditioning, to only 36%, with preconditioning (Fig. 3A, B).

3.4. Estrogen receptors, ERα and ERβ, and glutamate transporters, EAAT1 and EAAT2, remain unchanged in response to ischemic preconditioning and 45 min OGD

Despite the neuroprotection afforded by preconditioning with 15 min OGD against neuronal death caused by 45 min OGD, no significant change in the abundance of ERs and EAATs was detected (Table 1). All proteins, ERα, ERβ, EAAT1 and EAAT2, showed the same immunocontent profile in the control, only preconditioned, OGD exposed or preconditioned and OGD exposed organotypic cultures (Table 1).

4. Discussion

During ischemic preconditioning, sublethal ischemia increases the resistance of neurons to a subsequent lethal ischemia (Dirnagl et al., 2003). *In vivo* models have been shown to faithfully model ischemic preconditioning (Jones and Bergeron, 2001, 2004; Valentim et al., 2001), yet, these models are technically challenging, time-consuming and expensive. Important adjuncts to *in vivo* studies are the *in vitro* models of preconditioning that are technically easier and less expensive. Recently, organotypic slice cultures have been used to demonstrate that *in vitro* preconditioning conditions produce similar responses to the brain *in vivo* and that tolerance against neuronal death in these two models might share some common mechanistic pathways (Lange-Asschenfeldt et al., 2004; Valentim et al., 2003; Xu et al., 2002). Similarly, the neuroprotective efficacy of estrogens have been well described in *in vitro* models of cellular death (Behl, 2002; Cimarosti et al., 2005; Wilson et al., 2000).

Although neuroprotection afforded by either estradiol treatment or ischemic preconditioning have been extensively studied in a variety of *in vivo* and *in vitro* models, the specific triggering mechanisms leading to this state of protection remain unknown. We have previously shown that sublethal OGD induced tolerance to subsequent lethal OGD in organotypic cultures, which was associated with an increase in the heat shock protein 27 (HSP27) phosphorylation and immunocontent, suggesting that phosphorylated HSP27 might be involved in *in vitro* preconditioning (Valentim et al., 2003). In addition, examining the effect of 17β-estradiol in

organotypic cultures exposed to OGD, we have demonstrated that the hormone is neuroprotective and suggested that this neuroprotection could involve the recruitment of pathways involving Akt and GSK-3β that reduce apoptosis in this injury model (Cimarosti et al., 2005). In the present study, using the same experimental model, we investigated whether neuroprotection by estradiol treatment and ischemic preconditioning involve alteration in the abundance of glial glutamate transporters, EAAT1 and EAAT2, and estrogen receptors, ERα and ERβ.

Our measurements of propidium iodide (PI) uptake are consistent with others and ours previous observations of neuroprotection by estradiol treatment and ischemic preconditioning in this *in vitro* model of ischemia (Cimarosti et al., 2005; Valentim et al., 2003; Xu et al., 2002; Wilson et al., 2000). The presence of physiological levels of 17β-estradiol for 7 days prior to injury exerted protective effects against 60 min of OGD (Fig. 1A, B), as well as 15 min of OGD induced a significant tolerance to subsequent 45 min of OGD (Fig. 3A, B), preventing the cellular damage in the hippocampal CA1 region.

Western blot analysis showed that the immunocontent of ERα was significantly decreased 24 h after OGD in vehicle-treated and estradiol-treated organotypic cultures (Fig. 2A). A recent study have shown decreased nuclear ERα expression in hippocampal neurons in Alzheimer patients, suggesting that it may affect the ERα-regulated transcription (Hu et al., 2003). Our findings are in contrast with a previous report showing that ERα mRNA in the cortical region is dramatically up-regulated with *in vivo* ischemia (Dubal et al., 1999), what might be explained by experimental differences between the studies. Dubal and colleagues (1999), in the same *in vivo* study, have also shown that injury-induced down-regulation of ERβ mRNA is prevented by estradiol treatment. Although in our *in vitro* model OGD did not down-regulate ERβ, estradiol treatment induced a significant increase in the immunocontent of ERβ compared to vehicle-treated cultures, which was sustained even after the lesion induction (Fig. 2B). These data suggest that regulation of estrogen receptors abundance, particularly of ERβ, might be involved in the protective effects of estradiol observed in our *in vitro* model of cellular death.

As shown in Table 1, the immunocontent profiles of ERα and ERβ remain unchanged in response to preconditioning by a sublethal OGD followed by a lethal OGD. To our knowledge, regarding estrogen receptors and preconditioning, only a

recent study reported that the expression of ERα in hippocampus did not change in response to preconditioning with 3-nitropropionate *in vivo* (von Arnim et al., 2002).

The differential roles of estrogen receptors subtypes ERα and ERβ for neuroprotection remain unresolved. Recently, Carswell and colleagues (2004), using selective estrogen receptor agonists, showed the involvement of ERβ on neuroprotection in a mouse model of global cerebral ischemia. However, Dubal and colleagues (2001), using ERα and ERβ knockout mice, found that ERα was required for the protective effects of estradiol against brain injury. Nevertheless, other studies indicate that both estrogen receptor subtypes, ERα and ERβ, can contribute to estrogen-induced neuroprotection (Fitzpatrick et al., 2002; Zhao et al., 2004).

Dysregulation of glutamate transport may contribute to the pathogenesis of many neurodegenerative conditions and altered expression or function of EAATs has been identified in a number of these pathologies, including cerebral ischemia (O'Shea, 2002). Increasing data suggest that regulation of glutamate transport may contribute to the development of ischemic tolerance (Douen et al., 2000; Romera et al., 2004). As shown in Table 1, there were no significant change detectable in the immunocontent of EAAT1 and EAAT2 in response to preconditioning with 15 min OGD followed or not by 45 min OGD. Our results are partially confirmed by Sato and colleagues' findings (2003) that total expression of EAAT1 is not changed by estradiol.

We have recently found (unpublished data) that in response to hypoxic preconditioning in the neonatal rat brain, although the immunocontent of EAAT1 and ERβ remain unchanged, EAAT2 and ERα are up-regulated in a similar manner in cortex, while EAAT2 is down-regulated in striatum. However, the immunocontent of none of these proteins, EAAT1, EAAT2, ERα and ERβ, showed any significant changes in the hippocampus following preconditioning *in vivo*, what is in agreement with our present findings in the *in vitro* model. As organotypic cultures might respond similarly to *in vivo* ischemia, the mechanisms that result in tolerance and the response required for key proteins in determined brain regions could be also similar.

It is important to point out that although our immunoblotting study could not detect alteration in the abundance of glial glutamate transporters, either in cultures treated with estradiol or in preconditioned cultures, we cannot discard the

involvement of modulation of the activity of these transporters in response to estradiol treatment or ischemic preconditioning. The function of glutamate transporters is subject to complex modulation by a variety of endogenous factors, including glutamate itself (O'Shea, 2002). Activation of kinases appears to be a potent signal regulating the transport of glutamate: protein kinase (PK) C, PKA and phosphatidylinositol 3-kinase (PI3-K) are all implicated in increasing the activity, abundance or cell-surface expression of glutamate transporters (O'Shea, 2002). Therefore, further investigation is necessary to clarify whether neuroprotection by estrogen or preconditioning involves modulation of EAATs function.

In summary, the data reported here provide clear evidence that, in organotypic hippocampal slice cultures, ERα is down-regulated by OGD while ERβ is up-regulated by estradiol treatment, suggesting that, *in vitro*, estrogen neuroprotection against ischemia might involve regulation of ERs expression and, consequently, of the gene transcripted by these receptors. Despite the fact that significant changes in EAAT1 and EAAT2 expression were not detected, further work is needed to investigate whether the neuroprotection by estradiol or preconditioning involve modulation of their function.

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Legend to Figures

Fig. 1. Effect of 17β-estradiol on cellular damage induced by 60 min of OGD in organotypic hippocampal slice cultures. (A) Representative photomicrographs of cultures show PI fluorescence 24 h after OGD. (B) Quantitative analysis of CA1 damage in control and OGD slices treated with vehicle (DMSO) or 17β-estradiol (E2). DMSO 0.01% was added during OGD and recovery periods while 17β-estradiol (10 nM) was added 7 days prior to lesion induction and kept throughout the experiment. Images were captured and then analyzed using Scion Image software (www.scioncorp.com). The area where PI fluorescence was detectable above background levels was determined using the "density slice" option of the software and compared with the total CA1 area to obtain the percentage of damage. Data represent means ± S.E.M., n=15 slices per treatment. * Significantly different from vehicle-treated (DMSO) and estradiol-treated (E2) control cultures; # significantly different from all other groups (one-way ANOVA followed by Duncan's test, $P < 0.001$).

Fig. 2. Representative Western blots of estrogen receptors (A) ERα and (B) ERβ in rat organotypic hippocampal slice cultures treated with vehicle (DMSO) or 17β-estradiol (E2), and exposed (OGD) or not (control) to 60 min of OGD. Equal amounts of protein samples were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and immunoblotting with antibodies against ERα or ERβ and β-actin. The molecular weight of each protein (in kilodalton) is indicated on the left. Densitometric measurements were performed on individual immunoblots obtained from 6 to 12 slices per treatment for each antibody tested. The densitometric values obtained to all antibodies from all treatments were first normalized to their respective β-actin densitometric values and then expressed as percentage of their respective controls (100%). Data represent relative optical density and are expressed as the mean ± S.E.M. In (A) * Significantly different from vehicle-treated (DMSO) and estradiol-treated (E2) control cultures and in (B) * significantly different from vehicle-treated (DMSO) control and OGD cultures (one-way ANOVA followed by Duncan's test, $P < 0.05$).

Fig. 3. (A) Photomicrographs show PI fluorescence after no treatment (control), preconditioning with sublethal OGD (15' OGD), lethal OGD (45' OGD) and preconditioning with sublethal OGD 48 h before lethal OGD (15' + 45' OGD). (B) Quantification of CA1 damage following the different treatments. Images were captured and then analyzed using Scion Image software (www.scioncorp.com). The area where PI fluorescence was detectable above background levels was determined using the "density slice" option of the software and compared with the total CA1 area to obtain the percentage of damage. Data represent means ± S.E.M., n=15 slices per treatment. * Significantly different from control and 15' OGD group; # significantly different from all other groups (one-way ANOVA followed by Duncan's test, $P < 0.001$).

Table 1. Immunoblot analysis of estrogen receptors, ERα and ERβ, and glutamate transporters, EAAT1 and EAAT2, in rat organotypic hippocampal slice cultures after preconditioning with sublethal OGD (15’ OGD), lethal OGD (45’ OGD) and preconditioning with sublethal OGD 48 h before lethal OGD (15’ + 45’ OGD).

	ERα	**ERβ**	**EAAT1**	**EAAT2**
15’ OGD	107±7	104±3	94±5	109±14
45’ OGD	110±13	100±2	94±3	117±14
15’ + 45’ OGD	93±5	97±5	92±6	107±7

Data represent means ± S.E.M. of percentage of control.

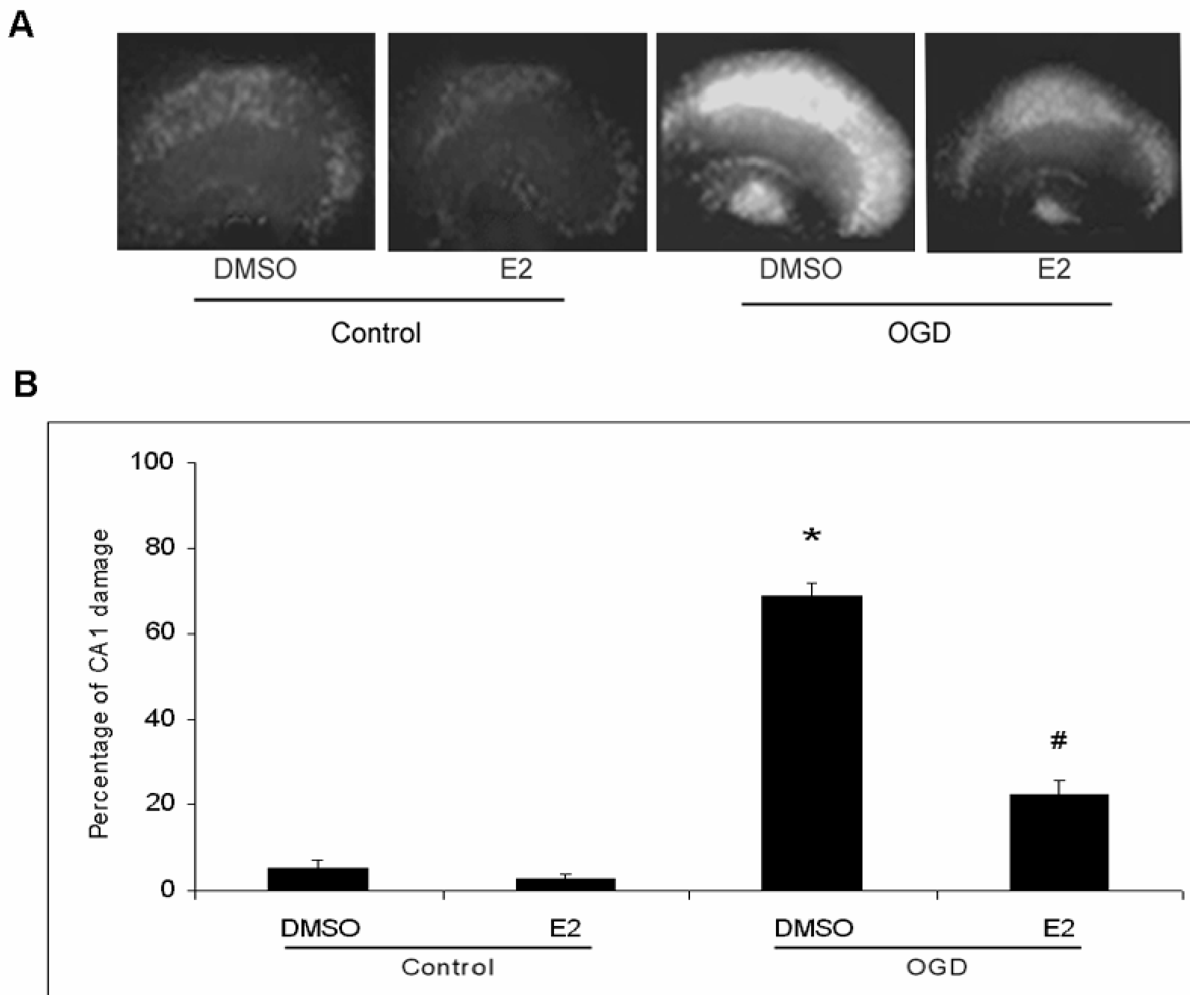

Figure 1

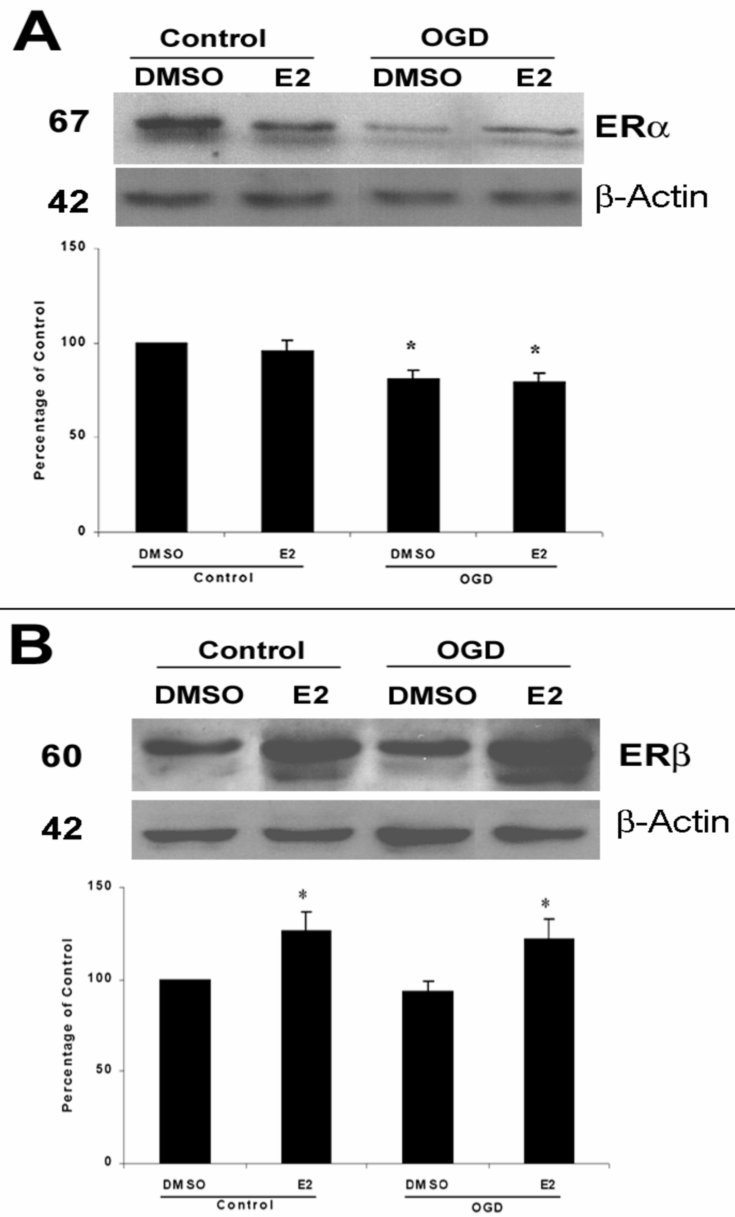

Figure 2

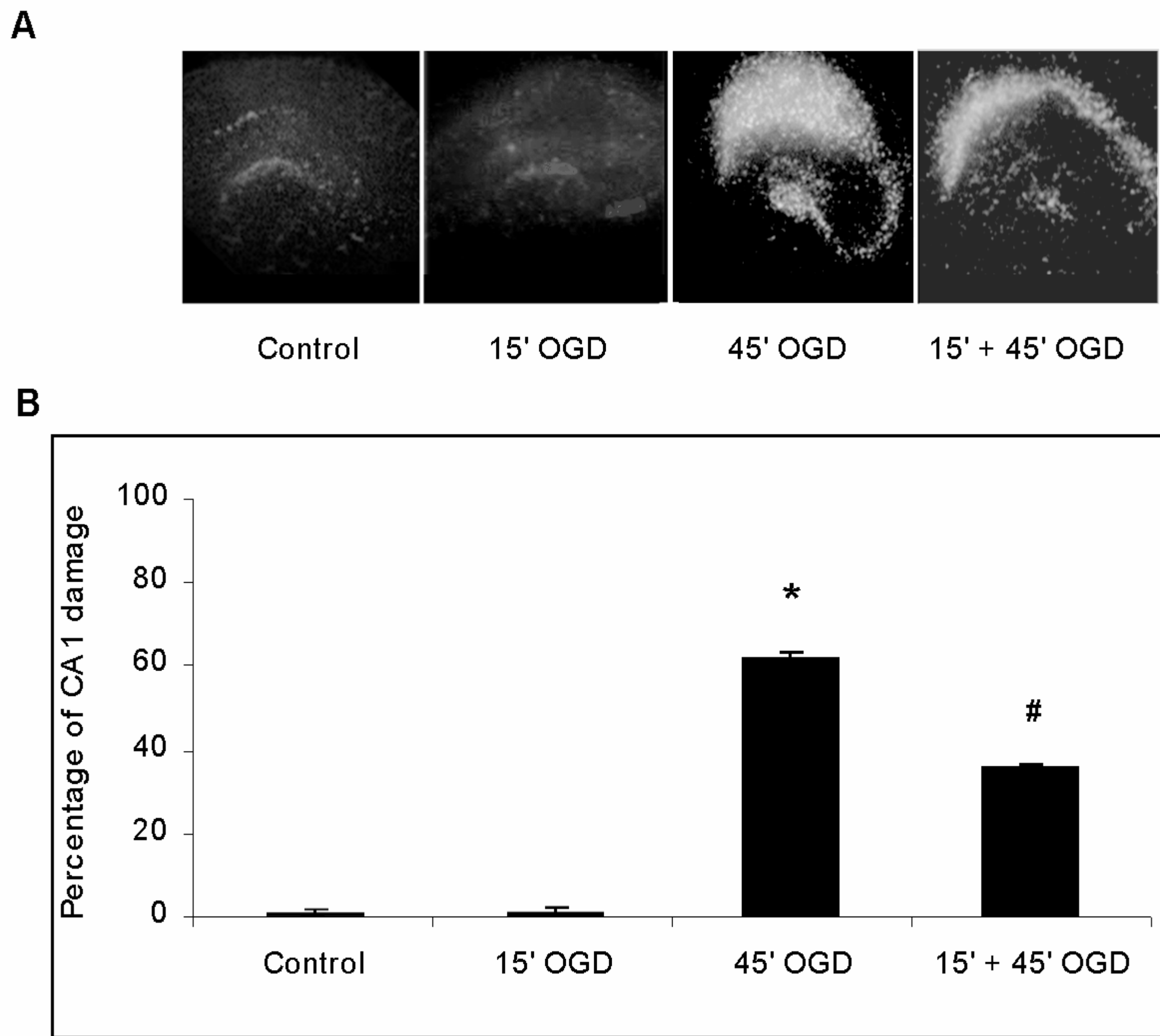

Figure 3

CAPÍTULO 3

Neuroprotection and protein damage prevention by estradiol replacement in rat hippocampal slices exposed to oxygen-glucose deprivation.
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Neuroprotection and Protein Damage Prevention by Estradiol Replacement in Rat Hippocampal Slices Exposed to Oxygen-Glucose Deprivation

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ABSTRACT

Here we investigated the effects of estradiol replacement in ovariectomized female rats using hippocampal slices exposed to oxygen-glucose deprivation (OGD). OGD induced lactate dehydrogenase (LDH) release to the incubation medium, what was assumed as a parameter of cellular death. In the estradiol-treated group the LDH release was markedly decreased by 23% as compared to the vehicle-treated group. In attempt to study a possible mechanism by which estradiol acts, we investigated some parameters of oxidative stress. In both vehicle-treated and estradiol-treated groups, OGD significantly increased the free radical production by 34% and 16%, respectively, although no significant differences on total antioxidant capacity were observed. Interestingly, estradiol replacement prevented the significant reduction in tryptophan and tyrosine contents caused by OGD observed in vehicle-treated animals. Our results show that estradiol replacement in ovariectomized female rats decreases cellular susceptibility to an ischemic-like injury and suggest a role for the hormone on protein damage prevention.

KEY WORDS: Cerebral ischemia; estradiol replacement; hippocampal slices; oxidative stress; protein damage; neuroprotection.

INTRODUCTION

Stroke ranks as the third leading cause of death and the leading cause of disability in developed countries. Needed, but currently not available, are therapies that can be administered prior to, during or after cerebral ischemia that reduce or eliminate neuronal damage from stroke.

Sex steroid hormones are believed to provide women with endogenous protection against cerebrovascular events – premenopausal women have a lower risk of stroke than men of the same age (1, 2), and the incidence of stroke in women increases rapidly after the menopause (3), coincident with diminished circulating levels of estrogen and progesterone. Several studies indicate that estrogen protects women against cerebral ischemia and others neurodegenerative diseases via various mechanisms. Recently, these have been proposed as: modification of cerebral blood flow and glucose transport; stabilization of neurons by inhibiting calcium currents; up-regulation of NGF and brain-derived neurotrophic factors (4); stimulation of endothelial and neuronal nitric oxide synthase, which produces the vasodilator molecule nitric oxide (5); regulation of levels of inflammatory mediators post-ischemia (6); anti-apoptotic properties (7, 8); protection from glutamate toxicity (9) and one of the well documented effects of estrogen is its antioxidant properties against oxidative stress (4).

The pathogenesis of cerebral ischemia/reperfusion has been associated with energy failure, release of excitatory amino acids, mitochondrial dysfunction, and excessive generation of free radicals, all of which are contributing factors to the oxidative damage (10). Oxidative stress, due to excessive formation of hydrogen peroxide and oxygen-derived free radicals, causes cell damage through chain reactions of membrane lipid peroxidation, and/or alterations in membrane fluidity.

In an attempt to contribute to experimental data of neuroprotective properties of estrogen, the aim of the present study was to investigate whether estradiol replacement in ovariectomized female rats decreases the susceptibility of brain tissue to ischemic events using the model of oxygen-glucose deprivation (OGD) in hippocampal slices. This model offers important advantages because cell composition, such as functional neurons, inflammatory competent cells, locally released effectors and intercellular connections, are preserved, allowing the

elucidation of meaningful mechanisms of cellular damage and neuroprotection (11-14).

Considering that oxidative stress has been related to brain ischemic process and evidence exists that estrogen neuroprotection is related to the antioxidant capacity of the hormone, we also investigated some parameters of oxidative stress, specifically the free radicals content, an index of oxidative damage to proteins, and the total antioxidant capacity, in the slices submitted to the same procedure.

EXPERIMENTAL PROCEDURE

Animals. Female Wistar rats (2 months) maintained under standard conditions (12-h light/dark, 22 ± 2°C) with food and water *ad libitum* were used. The Animal Care Committee approved all handling and experimental conditions. To prevent physiological hormonal oscillation, ovariectomized rats were used. For each experiment 6 ovariectomized rats treated with 17β-estradiol or just vehicle were used.

Surgery. Rats were anesthetized with 120 mg/kg ketamine HCl (Dopalen: Agribrands, Campinas, Brazil) and 16 mg/kg xylazine (Anasedan: Agribrands, Campinas, Brazil) and bilateral ovariectomy was performed through a single abdominal incision. After a recovery period of 5 to 7 days, the animals were submitted to estradiol replacement or to a sham replacement.

Estradiol Replacement. Briefly, 15 mm medical grade tubing (1.02 mm i.d. x 2.16 mm o.d.; Medicone®, Multiplast, Porto Alegre, Brazil) was filled with 10 µL of 5% (w:v) β-estradiol 3-benzoate (Sigma Chemical Co., St. Louis, MO) in sunflower oil and sealed with silicone. Capsules were soaked in sterile saline overnight and implanted subcutaneously between the scapulae under anesthesia. Sham replacement animals were implanted with capsules containing just oil.

Estradiol Levels Evaluation. The quantitative determination of serum estradiol concentration was performed with an enzyme immunoassay test kit (Genzyme Diagnostics).

Slice Preparation. After 30 days, rats were decapitated, the hippocampi were quickly dissected out and transverse sections (400 µm) were rapidly prepared using a McIlwain tissue chopper. Hippocampal slices were divided in two equal sets (control and OGD), placed into separated 24-well culture plates, and pre-incubated

for 15 minutes in a modified Krebs-Henseleit solution (pre-incubation solution, pH 7.4) containing (mM): 120 NaCl, 2 KCl, 0.5 $CaCl_2$, 26 $NaHCO_3$, 10 $MgSO_4$, 1.18 KH_2PO_4, 11 glucose, in a tissue culture incubator at 37°C with 95% air/5% CO_2 (11).

Oxygen and Glucose Deprivation (OGD) followed by Reoxygenation. After pre-incubation, the medium in the control plate was replaced with another modified Krebs-Henseleit solution (incubation solution, pH 7.4) containing (mM): 120 NaCl, 2 KCl, 2 $CaCl_2$, 26 $NaHCO_3$, 1.19 $MgSO_4$, 1.18 KH_2PO_4, 11 glucose, and incubated for 45 minutes in the tissue culture incubator. To model ischemic conditions, after pre-incubation, OGD slices were washed twice with incubation solution without glucose and incubated for 45 minutes (OGD period) at 37°C in an anaerobic chamber saturated with nitrogen, as fully detailed elsewhere (11, 13, 15).

After 45 minutes, media from both control and OGD slices were replaced by incubation solution with glucose and slices returned to the tissue culture incubator for 180 minutes (recovery period). Control and OGD experiments were run concomitantly, using six slices of the same animal in each plate.

Cellular Injury. Cellular damage was quantitatively assessed by measurement of lactate dehydrogenase (LDH) released into the medium after the recovery period (16). LDH activity was determined using a commercial kit (Doles Reagents, Goiânia, Brazil), according to the instructions of the manufacturer. Results of OGD slices from vehicle-treated rats (non-estradiol replacement) were taken as 100%.

Reactive Oxygen Species (ROS) Formation. To assess the free radical levels, 2'-7'-dichlorofluorescein diacetate (DCFH-DA, Sigma Chemical Co.) was used as probe. After the recovery period, DCFH-DA (100 μM) was added to the wells and incubated for 45 minutes at 37°C. Then, the medium was replaced by a fresh one and the plates placed on ice. The formation of the oxidized fluorescent derivative (DCF) was monitored after homogenization at excitation and emission wavelengths of 488 and 525 nm, respectively, using a fluorescence spectrophotometer Hitachi F-2000 (17). All procedures were performed in the dark and blanks containing DCFH-DA (no sample) and sample (no DCFH-DA) were processed in parallel for measurement of auto-fluorescence. The free radicals content was quantified using a DCF standard curve and results were expressed as pmol of DCF formed per milligram of protein.

Total Antioxidant Reactivity (TAR) Assay. The TAR assay, which represents the total antioxidant capacity, is based on luminol-enhanced chemiluminescence

measurement induced by an azo initiator (18, 19). The reaction mixture contained 2 mM 2,2'-azobis (2-amidinopropane) dihydrochloride (ABAP) as the source of peroxyl radicals, and 6 mM luminol in glycine buffer (0.1 M, pH 8.6). The chemiluminescence (CL) generated was measured in a scintillation counter (BECKMAN) working in the out of coincidence mode. TAR values were determined by assessing the initial decrease of luminescence, calculated as the "Io/I" ratio, where "Io" is the CL in the absence of additives, and "I" is the CL after the addition of 20 nM Trolox or samples (1 μL). TAR values were expressed as equivalents of Trolox concentration per milligram of protein.

Oxidation of Protein Tryptophan Residues. Samples were solubilized in sodium dodecyl sulfate (SDS) at a final concentration of 0.1%. The intrinsic tryptophan fluorescence was determined at excitation and emission wavelengths of 280 and 345 nm, respectively, using a fluorescence spectrophotometer Hitachi F-2000 (20).

Oxidation of Protein Tyrosine Residues. Samples were solubilized in SDS at a final concentration of 0.1%. The intrinsic tyrosine fluorescence was determined at excitation and emission wavelengths of 277 and 320 nm, respectively (21).

In these tests evaluating oxidative stress parameters, results of control slices (non-OGD) from vehicle-treated rats (non-estradiol replacement) were taken as 100%.

Protein Assay. The total protein concentrations were determined using the method described by Lowry and colleagues (22) with bovine serum albumin as the standard.

Statistical Analysis. Data were analyzed by a general factorial analysis of variance (ANOVA) followed by Duncan's multiple range test. Differences were considered significant when $P < 0.05$. Results are presented as mean ± standard error mean (S.E.M.) of 6 independent experiments.

RESULTS

Ovariectomized rats receiving estradiol replacement presented, at the end of the treatment, estradiol levels of 95±15 pg/mL, approximately the levels in the proestrus peak values (23). The estradiol serum levels were 11±3 pg/mL for ovariectomized rats receiving oil replacement.

The exposure of hippocampal slices to OGD for 45 minutes resulted in a significant increase of LDH released into the incubation medium, a marker of tissue necrosis, by 83% and 60% in vehicle-treated and estradiol-treated group, respectively, as compared to control slices. However, as can be seen in Figure 1, in the estradiol-treated group this increase was significantly lower (23% decrease) when compared to the vehicle-treated group, indicating that slices from rats that received hormone replacement were less susceptible to OGD induced damage.

In order to reinforce the importance of this neuroprotective effect induced by estradiol, we investigated its direct effect in the same model by incubating hippocampal slices from ovariectomized rats, not receiving any kind of replacement, with or without the hormone (data not shown). In these experiments concentrations of estradiol similar to those found in rats receiving hormonal replacement were added to the medium during and after the lesion induction. As we could not observe any significant neuroprotection in the slices from this “acute” estradiol treatment, we did not follow the measurement of the different parameters regarding oxidative stress.

Figures 2 and 3 illustrate the parameters of oxidative stress in slices from ovariectomized female rats receiving vehicle or estradiol replacement and exposed or not to OGD. After OGD exposure, free radical content was significantly increased by 34% and 16% in hippocampal slices from vehicle-treated and estradiol-treated rats in comparison to control slices, although DCF formation in slices from rats treated with estradiol did not significantly differ from those from vehicle-treated rats (Fig. 2A). Interestingly, no differences on total antioxidant reactivity (TAR) levels were found in any groups, non-OGD or OGD with estradiol or vehicle treatment (Fig. 2B), whereas significant changes were observed in the tryptophan and tyrosine contents (Fig. 3A, B). Both amino acids were significantly decreased by 16% in OGD slices from vehicle-treated rats, suggesting that their aromatic residues are targets of the oxidative stress induced by our model of ischemic-like injury (Fig. 3A, B). Moreover, a significant increase by 22% in the tryptophan content was seen in control slices from rats treated with estradiol (Fig. 3A). These changes suggest that the estradiol replacement increased the levels of these aromatic amino acids, and also maintained the basal levels in hippocampal slices exposed to OGD.

DISCUSSION

Recently, several studies have clearly shown that estrogen has neuroprotective effects. Our data confirm these studies: in our model of oxygen-glucose deprivation in hippocampal slices, OGD slices from rats treated with 17β-estradiol presented lower levels of LDH released into the incubation medium, a reduction of approximately 23% as compared to OGD slices from vehicle-treated rats. Considering that the amount of LDH released correlates with neuronal plasma membrane damage (24), our results shown in Figure 1 indicate that the hormone was able to protect the tissue against cellular death induced by an ischemic-like injury.

The putative mechanisms underlying the neuroprotective effects of estrogen are still obscure. Estrogen might act through estrogen receptors (ERα and ERβ) dependent and independent, genomic and non-genomic means to attenuate neural injury. Estrogen has newly recognized effects on a variety of cytoplasmic signaling cascades, including protein kinase C, phosphatidylinositol 3-kinase (PI3-K), mitogen-activated protein kinase (MAPK) and steroid receptor coactivator (src) (reviewed in 25). We have recently shown that 17β-estradiol neuroprotective effects in organotypic hippocampal slice cultures exposed to OGD might involve PI3-K pathway (8). In addition, the antioxidant activity of estrogen is one of the well-recognized estrogen receptor independent cellular mechanisms. Several studies have shown that estrogen non-specifically protects cells from oxygen radical-induced brain injury by direct radical scavenging action or through preservation of endogenous antioxidants (26-28).

The generation of free radicals is increased during cerebral ischemia followed by reperfusion. Reactive oxygen species (ROS) that are generated either early or late in the detrimental process and that escape the local defense mechanisms may lead to a multiplicity of changes in mitochondrial membranes and DNA, ultimately threatening cell viability (10). Accordingly, OGD followed by a recovery period of 180 minutes lead to increases on free radicals production, as expressed by the amount of DCF formed (Fig. 2A). Free radicals, such as hydrogen peroxide, peroxyl radical, peroxynitrite, nitric oxide, and dopamine, can oxidize DCFH, increasing DCF fluorescence and thus reflecting the overall cellular oxidative stress (29). Our results confirm previous data that exposure of hippocampal slices to an *in vitro* ischemic condition followed by reoxygenation affects cellular damage and free radical content (13, 14).

Many estrogens, including 17β-estradiol, have well established and potent lipid antioxidant activity (reviewed in 26). Fe^{2+}-induced levels of reactive oxygen species and neurotoxicity are significantly attenuated by low μM concentrations of 17β-estradiol in chick embryonic neurons, even in the presence of tamoxifen, an estrogen receptor antagonist, demonstrating that it is an effect independent of estrogen receptor (27). Murine cortical cultures are protected by estrogen, even in the presence of receptor antagonists or the protein synthesis inhibitor, cyclohexemide, suggesting a non-receptor-mediated and non-transcriptional mechanism. Estrogen also appears to spare endogenous antioxidants such as ascorbate and glutathione (28). Given these data, several investigators have hypothesized that estrogen protects cells by direct radical scavenging action. However, it is important to note that estradiol replacement did not change the free radical content (DCF formed) and the total antioxidant capacity (TAR levels) in hippocampal slices (Fig. 2A, B). These results may indicate that the effects of estradiol on antioxidant defenses might be irrelevant to the observed neuroprotective action in our model. In fact, although the evidence supporting a role for the antioxidant effects of estrogens in neuroprotection is strong, antioxidant activity of estrogens may not account for neuroprotective effects in all systems. The antioxidant effects of estrogens generally require μM concentrations, while the neuroprotective effects can often be seen with significantly lower concentrations, and this is exactly what is happening in our model: the nM concentration of estradiol assumed to be present in the slices from rats that received hormone replacement is enough to exert neuroprotective but not antioxidant actions.

Significant reductions in tryptophan and tyrosine residue contents were observed in the hippocampal slices exposed to OGD (Fig. 3A, B). These data suggest that oxidative stress, induced by oxygen-glucose deprivation, may affect aromatic residues in proteins. During the oxidation of aromatic residues, the formation of phenoxyl radicals from tyrosine, and their conversion into dityrosine and further products can occur, especially if there are no reductants to repair the tyrosyl radicals (e.g., thiols, vitamin E) and if there are vicinal tyrosyl radicals. Hydroxylation of phenylalanine, tyrosine and tryptophan is also a characteristic reaction of hydroxyl radicals (30).

It is important to point out that both long-chain acylated tyrosine and tryptophan are very rich in the transmembrane domains of all major classes of integral membrane proteins (31, 32). Tyrosine and tryptophan residues are mainly

localized in the region of the inner portion of the lipid head-groups and the beginning of the hydrocarbon tails (33). In addition, they are potent inhibitors of oxidative cell death and lipid peroxidation, probably because of their particular localization between the head and tail moieties of lipids and the ability of phenolic and indolic groups to provide hydrogen radical donors (34). The loss of these residues, as seen in vehicle-treated OGD slices (Fig. 3A, B), may alter the general dynamics of lipid bilayer membranes and indicate the loss of important antioxidants in the lipid phase of membranes. In contrast, considering the estradiol-treated group, in control slices the tryptophan content was increased, while in slices exposed to OGD the tyrosine and tryptophan contents were maintained in basal levels (Fig.3A, B). These data suggest that estradiol may protect amino group residues that when affected may contribute to the loss of enzymatic activities, as well may be involved with the lipid antioxidant action and, consequently, have neuroprotective properties.

In summary, the present study adds evidence supporting the neuroprotective properties of estradiol replacement against ischemic-like injury. However, we must mention the data now available from large, randomized, clinical trials that question the use of hormone replacement therapy for prevention of vascular disease and stroke (35, 36). Recently, increasing body of evidence has shown phytoestrogens, plant-derived compounds with partial estrogen agonist properties, as a substitute for traditional estrogen replacement therapy (37, 38). These studies suggest that phytoestrogens may be protective with regard to cardiovascular disease and breast and uterine cancer. We are currently investigating the potential neuroprotective effects of resveratrol, a phytoestrogen, in our models of brain injury.

To our knowledge, this report is the first to suggest the involvement of estradiol effects on amino acids in its neuroprotective properties. Our findings might be important for understanding the increased vulnerability of males and menopausal females to cerebral ischemia and the mechanism of neuroprotection provided by estrogen. Although, it is clear that additional work is required before a comprehensive knowledge of the potential cellular mechanisms for the neuroprotectant role of steroid hormones can be achieved.

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LEGEND TO FIGURES

Figure 1: Neuroprotective effects of estradiol on cell injury, as assessed by LDH release into the medium, of hippocampal slices from ovariectomized female rats, receiving or not hormonal replacement, exposed or not to oxygen-glucose deprivation (OGD) and reoxygenation. Data are expressed as mean ± S.E.M. of triplicates for six experiments (n=6). Values from OGD slices were considered as being 100% in each experiment for the LDH release. * Significantly different from OGD and OGD+E groups, # significantly different from all other groups, as determined by ANOVA followed by Duncan´s test ($P < 0.05$). C: control, slices from vehicle-treated rats non-exposed to OGD; C+E: control plus estradiol, slices from estradiol-treated rats non-exposed to OGD; OGD: oxygen-glucose deprivation, slices from vehicle-treated rats exposed to OGD; OGD+E: oxygen-glucose deprivation plus estradiol, slices from estradiol-treated rats exposed to OGD.

Figure 2: Effects of estradiol on **(A)** free radical content, using DCF assay, and **(B)** total antioxidant reactivity (TAR), measured by fluorimetry, were evaluated in hippocampal slices from ovariectomized female rats, receiving or not hormonal replacement, exposed or not to oxygen-glucose deprivation (OGD) and reoxygenation. Data are expressed as mean ± S.E.M. of triplicates for five experiments (n=5). Values from control slices were considered as being 100% in each experiment for the different tests. * Significantly different from C and C+E groups, for DCF test, as determined by ANOVA followed by Duncan´s test ($P < 0.05$). C: control, slices from vehicle-treated rats non-exposed to OGD; C+E: control plus estradiol, slices from estradiol-treated rats non-exposed to OGD; OGD: oxygen-glucose deprivation, slices from vehicle-treated rats exposed to OGD; OGD+E: oxygen-glucose deprivation plus estradiol, slices from estradiol-treated rats exposed to OGD.

Figure 3: Effects of estradiol on protein oxidation in **(A)** tryptophan (TRY) and **(B)** tyrosine (TYR) residues, measured by fluorimetry, were evaluated in hippocampal slices from ovariectomized female rats, receiving or not hormonal replacement, exposed or not to oxygen-glucose deprivation (OGD) and reoxygenation. Data are expressed as mean ± S.E.M. of triplicates for five experiments (n=5). Values from

control slices were considered as being 100% in each experiment for the different tests. * Significantly different from C, C+E and OGD+E groups, for TRY and TYR tests, # significantly different from all other groups, for TRY test, as determined by ANOVA followed by Duncan´s test ($P < 0.05$). C: control, slices from vehicle-treated rats non-exposed to OGD; C+E: control plus estradiol, slices from estradiol-treated rats non-exposed to OGD; OGD: oxygen-glucose deprivation, slices from vehicle-treated rats exposed to OGD; OGD+E: oxygen-glucose deprivation plus estradiol, slices from estradiol-treated rats exposed to OGD.

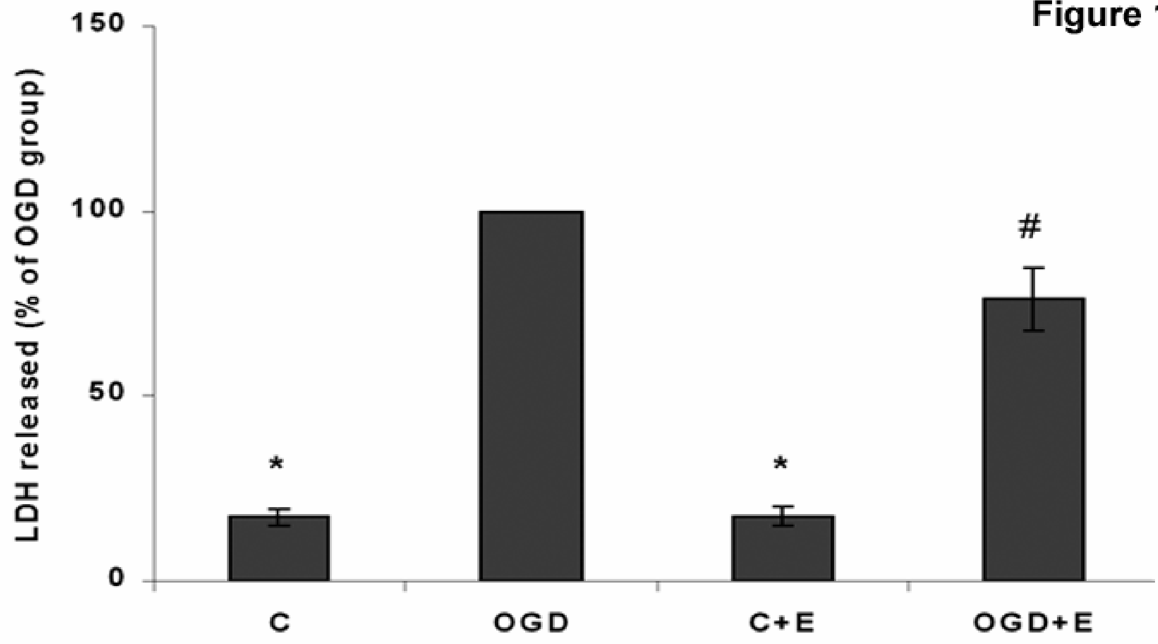
Figure 1
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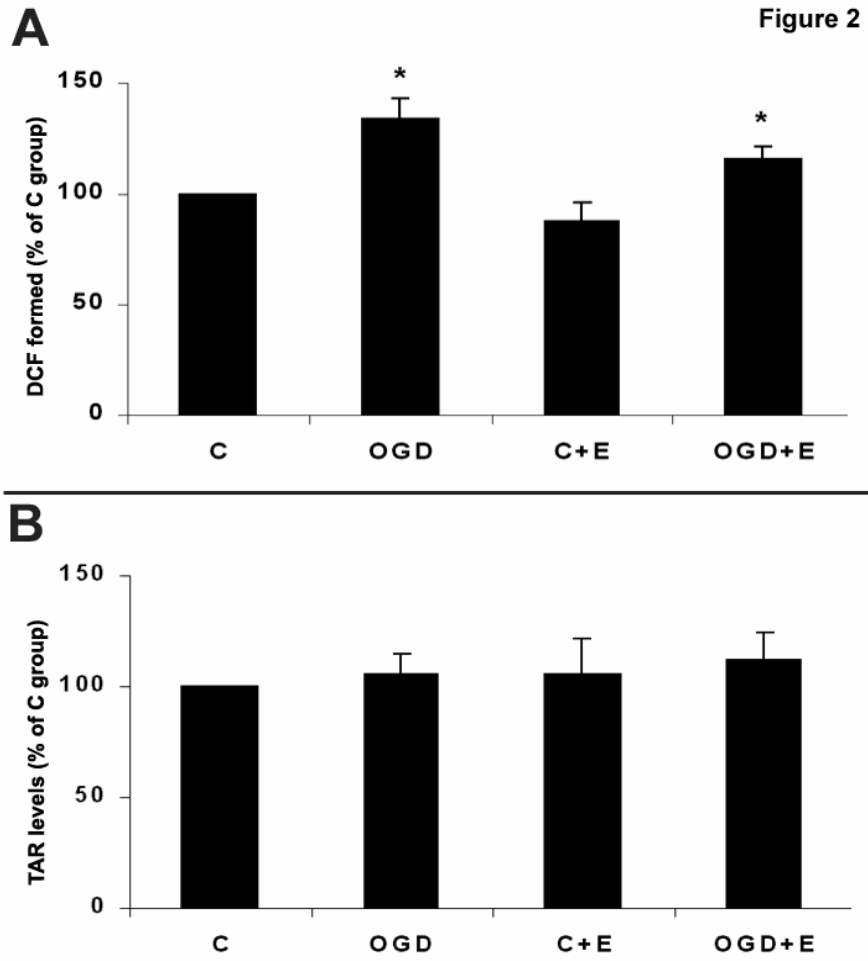
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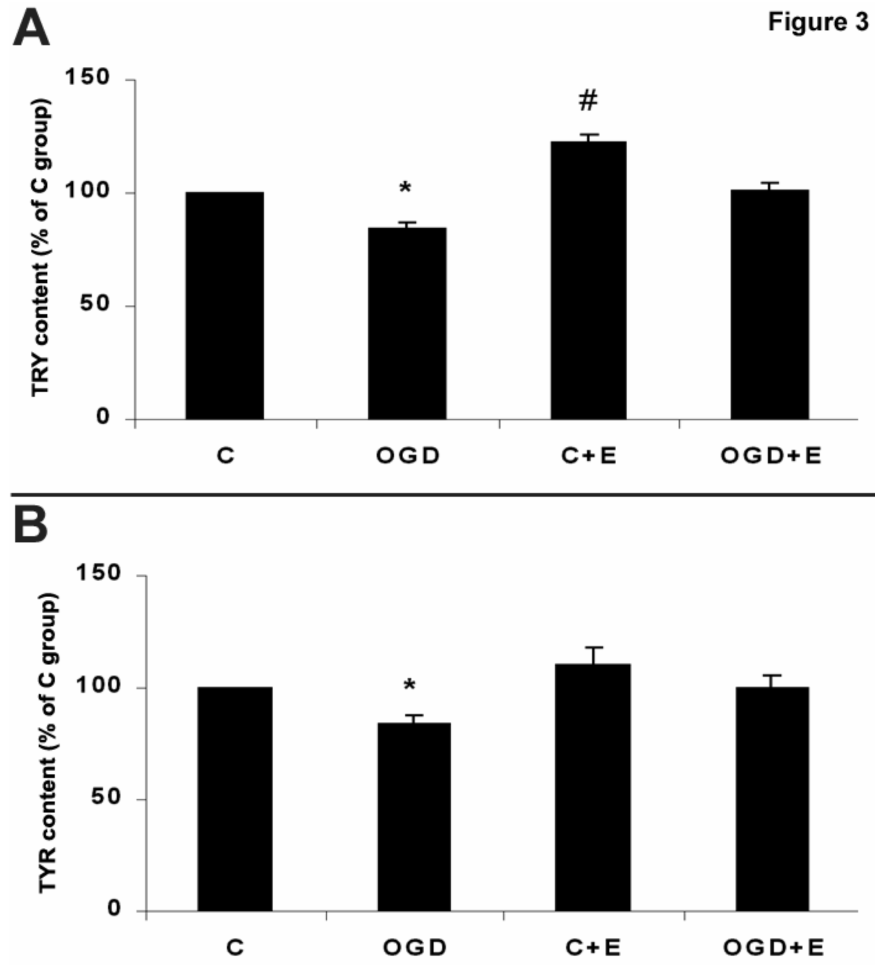
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Figure 3
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CAPÍTULO 4

Hypoxic preconditioning in neonatal rat brain involves regulation of excitatory amino acid transporter 2 and estrogen receptor alpha.

Neuroscience Letters, submetido em 16.02.2005.

Hypoxic Preconditioning in Neonatal Rat Brain Involves Regulation of Excitatory Amino Acid Transporter 2 and Estrogen Receptor Alpha

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Abstract

Exposure of the brain to a sublethal insult can protect against a subsequent brain injury. Hypoxic preconditioning induces tolerance to hypoxic-ischemic injury in neonatal rat brain and is associated with changes in gene and protein expression. To study the involvement of excitatory amino acid transporters (EAAT1 and EAAT2) and estrogen receptors (ERα and ERβ) in neonatal hypoxia-induced ischemic tolerance, we examined changes in expression of these proteins in the cortex, hippocampus and striatum of newborn rats at different time points after exposure to sublethal hypoxia (8% O_2, 3 hours). Preconditioning with hypoxia 24 hours before hypoxia-ischemia afforded marked brain protection compared with littermate control animals as determined by morphological assessment. Immunoblot analysis showed that EAAT2 and ERα were significantly increased by 55% and 49%, respectively, in cortex at 24 hours after hypoxic-preconditioning. Surprisingly, at the same time point, a significant decrease of EAAT2 by 48% in striatum was observed. In contrast, hypoxic preconditioning had no effect on the levels of EAAT1 and ERβ in any of the brain regions studied at any of the time points analyzed. The similar pattern of changes in EAAT2 and ERα levels provide the first *in vivo* indication that ERα might be a further part of some type of functional complex involving EAAT2 and Na^+/K^+ ATPase in cortex. The endogenous molecular mechanisms modulated by hypoxia preconditioning may contribute to the development of hypoxia-induced ischemic tolerance, and may provide novel therapeutic targets for the treatment of cerebral ischemia.

Theme: Disorders of the Nervous System
Topic: Ischemia

Keywords: Hypoxia; Ischemia; Preconditioning; Tolerance; Glutamate transporters; Estrogen receptors

Impaired oxygen (hypoxia) or reduced blood flow (ischemia) to the brain is a major cause of morbidity and mortality in the perinatal period, often resulting in cognitive impairment, seizures, and other neurological disabilities [8]. Although animal models of hypoxia-ischemia have increased our understanding of the processes leading to cell death, there are still no pharmacological treatments available to reduce cell death in ischemic neonatal brain.

Endogenously, the central nervous system can withstand cerebral hypoxia or ischemia for a limited amount of time, a phenomenon called primary hypoxic-ischemic tolerance. Interestingly, with an appropriate time interval and dosage, when a non-injurious hypoxic exposure (known as preconditioning) is performed before a potentially lethal hypoxic-ischemic stress, tolerance can be increased and cells protected [12, 13, 29]. Little is known about the mechanisms that occur in the time period between hypoxic preconditioning and the development of tolerance to brain ischemia, however a recent genomic study identified a number of genes which may contribute to hypoxia-induced tolerance [2].

Excitatory amino acid transporters (EAATs) in neurons and glia remove glutamate from extracellular space, thereby helping to terminate glutamatergic synaptic transmission and to prevent the extracellular glutamate concentration from rising to neurotoxic values [4, 17]. Glutamate uptake is mediated mainly by glial glutamate transporters (EAAT1 and EAAT2), and is associated with co-transport of Na^+, whose electrochemical gradient powers glutamate transport [4]. During normal conditions, the Na^+ influx accompanying glutamate uptake into astrocytes triggers activation of the Na^+/K^+-ATPase to eliminate the intracellular Na^+ load [32]. There are resultant changes in cellular energetics in both neurons and astrocytes emphasizing the key role glial EAATs play in interfacing with energy metabolism in brain. During cerebral ischemia, there is a rise in extracellular glutamate content to neurotoxic levels [4, 27], some arising from ATP-dependent presynaptic vesicular release [28], but the main source under hypoxic conditions is the reversal of EAATs induced by a rise in the level of intracellular Na^+ due to the ionic gradient disruption across the plasma membrane [23, 26, 28]. EAATs are, therefore, plausible molecular targets of preconditioning maneuvers.

Primary hypoxic tolerance is gender-dependent [1, 14] as has also been shown for the increase of hypoxic tolerance by preconditioning [14]. In recent years, several studies have been performed investigating the neuroprotective effects of

estrogen, showing that it protects against brain injury and contributes to the sex difference in ischemic vulnerability [3, 6, 10, 22]. A recent study suggested that not only is net hypoxic tolerance gender dependent, but that mechanisms conferring hypoxic tolerance are gender-specific [31].

The aim of the present study was to investigate whether glutamate transporters (EAAT1 and EAAT2) and estrogen receptors (ERα and ERβ) play a role in hypoxia-induced tolerance to ischemic injury in neonatal rat brain. Identification of key proteins involved in the complex process of induction of adaptive responses to preconditioning, and understanding changes in their expression, could reveal new molecular mechanisms of hypoxia-induced tolerance and would provide insight into cell survival and novel therapeutic approaches for stroke.

All animal work conducted in this study was approved by the Howard Florey Institute Animal Ethics and Experimentation Committee. Every measure was taken to ensure the welfare of animals at all times. Hypoxic preconditioning was performed on Sprague Dawley rat pups at post-natal day 6 (P6) as previously described [12]. Briefly, pups were placed in an 8% O_2/92% N_2 humidified atmosphere in chambers partially submerged in a water-bath maintained at the constant temperature of 37°C for 3 hours. This treatment has been shown to protect the newborn rat brain against subsequent hypoxic-ischemic brain injury [12, 13, 29]. Control animals were maintained at 37°C for 3 hours under normoxic conditions.

To determine the degree of neuroprotection afforded by hypoxic preconditioning, rats at P7 were anesthetized with 1.5% isofluorane in an O_2/N_2 mixture and underwent unilateral hypoxic-ischemic injury as described previously [12, 18]. Briefly, the left common carotid artery was exposed through a ventral midline neck incision and permanently occluded by electrocoagulation. The wound was sutured and animals returned to their mother for 1.5 hours. Pups were then placed in an 8% O_2/92% N_2 humidified chamber at a constant temperature of 37°C for 3 hours. This combined procedure is known to produce select neuronal damage or infarction in the hemisphere ipsilateral to the carotid occlusion, whereas hypoxia alone does not produce any significant brain injury [16, 18].

Three days after hypoxia-ischemia, animals were quickly decapitated' their brains removed and frozen in isopentane on dry ice. Rostro-caudal coronal sections (20 μm) were cut on a cryostat and stained with cresyl violet (Nissl) to assess the

extent of hypoxic-ischemic injury. Histopathological assessment of injury on each newborn rat brain was performed blindly as previously described [12, 29]. The following scoring system was used: 0, no histological damage; 1, minimal neuronal loss; 2, columnar cortical infarction with moderate neuronal loss; 3, severe neuronal loss and gliosis associated with extensive brain infarction.

For immunoblotting studies, a further group of animals was exposed to hypoxia preconditioning (as described above) and, at 4, 8 and 24 hours after hypoxic preconditioning, rat pups were decapitated, their brains removed and cortex, hippocampus and striatum were dissected out and frozen at -80°C until use (n= 5 to 10 animals per time point). Brains from control normoxic littermates were processed similarly. Protein samples were prepared by homogenization in ice cold lysis buffer containing 20 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1 mM EDTA, 0.5% Triton X-100, 0.1% SDS and a cocktail of protease inhibitors (Roche Molecular Biochemicals, Melbourne, Australia). Protein content was determined using a commercial assay (Biorad DC Protein Assay Kit; Bio-Rad, Sydney, Australia). Protein samples (20-80 μg) were separated by 10% sodium dodecyl sulfate – polyacrylamide gel electrophoresis following transfer to nitrocellulose membranes (0.45 μm; BioRad). After 2 hours incubation at room temperature (~ 22-25°C) in blocking solution containing 5% skim milk powder, 1% bovine serum albumin (BSA) and 0.1% Tween-20 in Tris-buffered saline (TBS; 50 mM Tris-HCl, 1.5% NaCl, pH 7.4), membranes were incubated overnight at 4°C with the appropriate primary antibody diluted in TBS containing 1% BSA and 0.1% Tween-20. Primary antibodies against the following proteins were used: EAAT1 and EAAT2 (both 1:50000 dilution, rabbit polyclonal); ERα (1:200 dilution, rabbit polyclonal; catalog sc-542) from Santa Cruz Biotechnology and ERβ (1:1000 dilution, rabbit polyclonal; catalog PC168-50UG) from Calbiochem. After washes in TBS containing 0.1% Tween-20, membranes were incubated with horseradish peroxidase-coupled secondary antibodies recognizing antigens from the same host as the corresponding primary antibody (mouse or rabbit IgG from Chemicon, Melbourne, Australia; 1:1000 dilution in TBS containing 1% BSA and 0.1% Tween-20). Bands were visualized using the Lumilight chemiluminescence detection system (Roche). The Optiquant computer-based imaging system (Packard Instrument) was used to measure the area and density of proteins bands after subtracting the background of the autoradiographic film. All blots were re-probed with

β-actin antibody from Sigma (1:10000 dilution, mouse monoclonal; catalog A 5316) as an internal control. As the secondary antibodies were different, rabbit for the proteins under investigation and mouse for β-actin, it was not necessary to strip the blots.

To determine the neuroprotective effects of the preconditioning treatment, median histopathologic score values were analyzed by unpaired two-tailed Mann-Whitney U non-parametric test. For the time-course analysis of protein immunocontent, multiple intergroup comparisons were performed by ANOVA followed by Duncan post-hoc test. Histogram data represent the mean ± S.E.M..

We first established that the preconditioning treatment used in the current study effectively protected against hypoxia-ischemia in newborn rats. The model of neonatal hypoxia-ischemia is associated with damage to the cerebral cortex, striatum, hippocampus, and thalamus in the hemisphere ipsilateral to the carotid artery occlusion [18]. Interestingly, animals subjected to hypoxic preconditioning 24 hours before hypoxia-ischemia demonstrated marked neuroprotection against hypoxia-ischemia-induced brain injury as described previously [12, 13, 29]. Brain injury in animals subjected to hypoxia preconditioning (median score=1; Fig. 1) was significantly less ($P < 0.044$) than that observed in normoxic control littermates (median score=2; Fig. 1), with protection occurring in all brain regions examined.

In addition to reducing brain damage after a hypoxic-ischemic insult, hypoxic preconditioning also reduced the mortality rate associated with the hypoxic-ischemic procedure, in agreement with previous reports [12, 13, 29]. In the present study, the mortality rate after hypoxic preconditioning was 0% compared with 33% mortality after hypoxia-ischemia alone. Mortality in the hypoxia-ischemia model has been suggested to be dependent on factors other than brain injury, which may include cardiac arrest related to increased hypotension and hypoglycemia [13, 18, 30].

Immunoblot analyses of cerebral cortex from neonatal rat pups subjected to hypoxic preconditioning showed significant increases in the levels of EAAT2, by 55% (155 ± 24% control, n=10), and ERα, by 49% (149 ± 18% control, n=10) at the 24 hour time point compared with normoxic control animals (Fig. 2A, B ; $P < 0.05$). Whereas EAAT2 and ERα were significantly altered at 24 hours after hypoxic preconditioning in cortex, both proteins remained unchanged in hippocampus (Table 1). Surprisingly, in striatum, EAAT2 was significantly decreased by 48% (52 ± 13%

control, n=8) at 24 hours after hypoxic preconditioning (Fig. 2C, $P < 0.05$), while ERα remained unchanged (Table 1). There were no significant changes compared with normoxic controls in the levels of EAAT1 and ERβ after hypoxic-preconditioning in cortex and hippocampus at any of the time points examined (Table 1).

Although other studies have reported increased tolerance to ischemia following preconditioning of the mammalian brain with exposure to moderate hypoxia, a treatment that does not produce cerebral injury, the mechanisms underlying this neuroprotection are still unclear [12, 13, 29]. The recent findings that preconditioning with cortical spreading depression and sublethal ischemia modulated intraischemic extracellular glutamate levels, coupled with the regulation of EAATs [5, 19], prompted us to further investigate the effect of hypoxic preconditioning on the abundance of EAAT1 and EAAT2 in different cerebral regions, such as cortex, hippocampus and striatum. Moreover, as we and others have reported, the neuroprotective effects of estrogen against ischemic insults [3, 15] and as primary hypoxic tolerance and preconditioning have been shown to be gender-dependent and mediated via gender-specific mechanisms [1, 14, 31], we also investigated whether estrogen receptors, ERα and ERβ, are involved in hypoxia-induced ischemic tolerance in the newborn rat brain.

Hypoxic preconditioning caused tolerance to subsequent lethal ischemia in neonatal rat brain, as previously reported [12, 13]. After hypoxic preconditioning, we did not detect changes in EAAT1 protein abundance in any of the brain regions over the time course examined, which is in agreement with the recent findings of Romera and colleagues who demonstrated that cellular expression of EAAT1 was not altered in cortical neurons exposed to ischemic preconditioning [19]. In the present study we observed changes in EAAT2 levels in brain at 24h after preconditioning with hypoxia. Specifically, EAAT2 was increased in cortical tissue, decreased in striatum and did not change in the hippocampus when compared with normoxic control animals. Similarly, EAAT2 protein levels have been shown to increase in the contralateral hemisphere of neonatal rats (exposed to hypoxia only) 24 hours after hypoxia-ischemia [9].

Previous studies have demonstrated that preconditioning with cortical spreading depression 3 days before focal ischemia down-regulated EAAT1 and EAAT2 [5]. In contrast, *in vitro* ischemic tolerance involved up-regulation of EAAT2

and EAAT3 and increased glutamate uptake in rat cortical cultures [19], suggesting that up-regulation of EAATs may mediate ischemic tolerance by decreasing excitotoxicity attributable to ischemic-induced increase in extracellular glutamate [19]. Furthermore, down-regulation of EAAT2 is thought to be associated with decreased glutamate clearance and neurotoxicity in patients with amyotrophic lateral sclerosis [21]. Although uptake through glial EAATs is the primary mechanism of cerebral glutamate clearance under normal physiological conditions, these transporters are thought to be sensitive to ionic perturbations that occur under ischemic conditions [28]. During ischemia, the sodium ionic gradient is reversed such that sodium, and concomitantly glutamate, are extruded from the cells via reversal of glutamate transporters [23, 28]. As this process exacerbates excitotoxicity during ischemia, down-regulation of EAATs following preconditioning would result in fewer EAATs available for glutamate efflux during a subsequent ischemic / excitotoxic insult, affording neuroprotection. Douen and colleagues [5] support this view, as they correlated a decrease in intraischemic glutamate content and a simultaneous down-regulation of EAAT1 and EAAT2 with the neuroprotective effect of cortical spreading depression preconditioning. A change in the abundance of glutamate transporters, therefore, does not clarify what the potential consequences since, while these transporters are fundamental to clearance of extracellular glutamate, their reversed operation accounts for most glutamate release induced by severe ischemia [11, 20]. Our data are consistent with both views: (1) the up-regulation of EAAT2 in cortex may mediate ischemic tolerance by increasing glutamate uptake and decreasing excitotoxicity, and (2) the hypoxic-mediated reduction in EAAT2 levels in striatum may reduce glutamate efflux during subsequent ischemia and contribute to the neuroprotective effect of hypoxic preconditioning.

In the context of the present studies, it is important to point out that glutamate, in addition to its receptor-mediated actions on neuronal excitability, has been reported to stimulate glycolysis – i.e., glucose utilization and lactate production – in astrocytes [32]. This metabolic action is mediated by activation of glial glutamate transporters, EAAT1 and EAAT2. The mechanism involves the Na^+/K^+-ATPase, which is activated by an increase in the intracellular concentration of Na^+ co-transported with glutamate by the electrogenic uptake system. Thus, when glutamate is released from active synapses and taken up by astrocytes, the signaling pathway described by Voutsinos-Porche and colleagues [32] provides a simple and direct

mechanism to tightly couple neuronal activity to glucose utilization. Taking into account this previous study, our findings of EAAT2 up-regulation in cortex suggest that glial glutamate transporters might be involved in some type of functional complex with Na^+/K^+-ATPase, that may be important to the development of ischemic tolerance in response to hypoxic preconditioning in the neonatal rat brain. Further studies in our laboratory are underway in an attempt to clarify this hypothesis.

With respect to estrogens receptors, the present study showed no differences in estrogen receptor β (ERβ) in any brain regions at any time points analyzed, although we did observe a significant increase in estrogen receptor α (ERα) at 24 hours after hypoxic preconditioning in cortex. This is in contrast with a recent study reporting that expression of ERα is unchanged after preconditioning with 3-nitropropionate in adult rats [31]. To our knowledge, the present study is the first investigating the effects of hypoxic preconditioning on ERβ and also the first to report increased expression of ERα after hypoxia in newborn rat brains. From experiments using knockout mice, it was demonstrated that the protective effects of estrogen could be linked to ERα, whereas ERβ was not involved in estrogen-mediated neuroprotection [7]. Data from the present study showing changes in ERα, but not ERβ, levels in association with the neuroprotective treatment of hypoxia preconditioning would support the previous findings. Indeed, pharmacologic treatment with an estrogen receptor antagonist worsened the post-ischemic outcome in the striatum of female animals via mechanisms unrelated to cerebral blood flow [25]. Direct application of estrogen has been shown to protect cortical but not striatal neurons [6]. The regulation of ERα in a similar manner to EAAT2, as shown by our results in the neonatal rat cortex, suggests that ERα might be further part of the functional complex involving EAAT2 and Na^+/K^+-ATPase, perhaps contributing to the ischemic tolerance induced after preconditioning with hypoxia. Recently, stimulation of ERα, but not ERβ, was shown to inhibit astrocytic EAAT activity and ERα was co-localized with EAAT1 in cultured astrocytes [24]. While this latter publication suggested that ERα may down-regulate EAAT1, we found no evidence for alterations to EAAT1 protein abundance in the present study.

In summary, it is likely that multiple factors contribute to neuroprotection following hypoxic preconditioning. Regulation of EAATs and estrogen receptors may be mechanisms underlying hypoxia-induced tolerance. The data from our *in vivo*

experiments provide the first evidence that EAAT2 and ERα are co-regulated in a similar manner in response to hypoxic preconditioning in the neonatal rat brain. This study suggests new mechanistic insights into preconditioning where we hypothesized that EAAT2, and ERα, might be involved in some type of functional complex with Na^{+}/K^{+}-ATPase. More studies are necessary for complete elucidation of the mechanisms regulating the acquisition of brain tolerance that could guide efforts to develop effective measures or safe pharmacological preconditioning agents to protect the brain or reduce ischemic injury.

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Legend to figures

Fig. 1. Severity of brain damage in rats subjected to hypoxia-ischemia with (white columns) or without (black columns) hypoxic preconditioning. Histopathologic scores are as follows: 0, no gross or histologic damage; 1, minimal neuronal loss; 2, columnar cortical infarction, moderate neuronal loss; 3, extensive infarction and gliosis, severe neuronal loss. Brain injury in hypoxia-treated animals (median score=1, n=19) was significantly less ($P < 0.044$, Mann-Whitney U non-parametric test) than that measured in control normoxic littermates (median score=2, n=15).

Fig. 2. Changes over time in the protein immunocontent of **(A)** excitatory amino acid transporter 2 (EAAT2) and **(B)** estrogen receptor α (ERα) in cerebral cortex, and **(C)** EAAT2 in striatum from newborn rats at 4, 8, and 24 hours after hypoxic preconditioning (8% O_2 for 3 hours). Equal amounts of protein samples were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and immunoblotting with specific antibodies. Densitometric measurements were performed on individual immunoblots obtained from 5 to 10 animals per time point for each antibody tested. The densitometric values obtained to all antibodies from all time points were first normalized to their respective β-actin densitometric values and then expressed as percentage of their respective controls (100%). The molecular weight of each protein (in kilodalton) is indicated on the right. Data represent relative optical density and are expressed as the mean $\pm$ S.E.M.. * Significantly different from all other groups, as determined by analysis of variance followed by the Duncan *post hoc* test ($P < 0.05$).

Table 1. Immunoblot analysis of estrogen receptors, ERα and ERβ, and glutamate transporters, EAAT1 and EAAT2, in the cerebral hippocampus and striatum or cortex of newborn rats at 4, 8 and 24 hours after hypoxic preconditioning (8% O_2 for 3 hours).

	ERα		**ERβ**		**EAAT1**		**EAAT2**
	hippoc.	striatum	hippoc.	cortex	hippoc.	cortex	hippoc.
4 h	123±11	122±14	85±15	106±17	77±21	105±19	81±11
8 h	112±10	143±29	109±18	122±29	79±24	81±12	102±11
24 h	125±12	128±29	101±11	138±23	76±23	88±9	80±13

Data represent means ± S.E.M. of percentage of control.

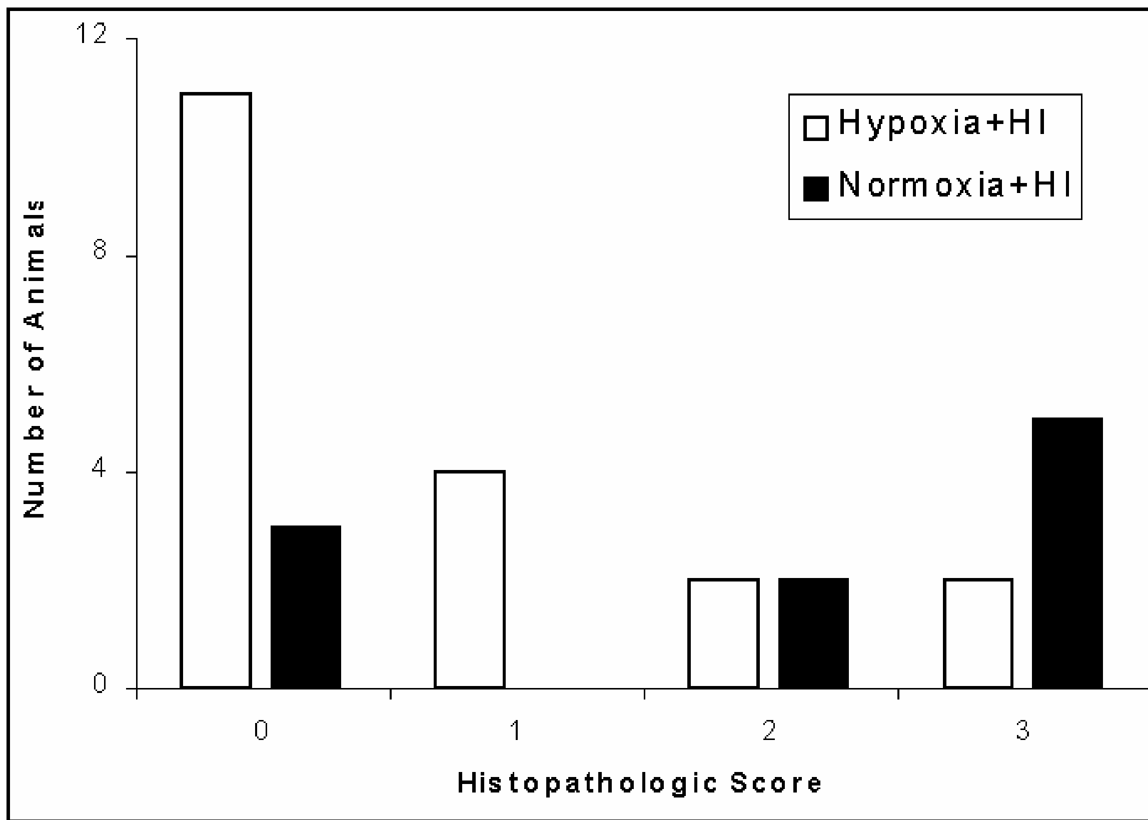

Figure 1

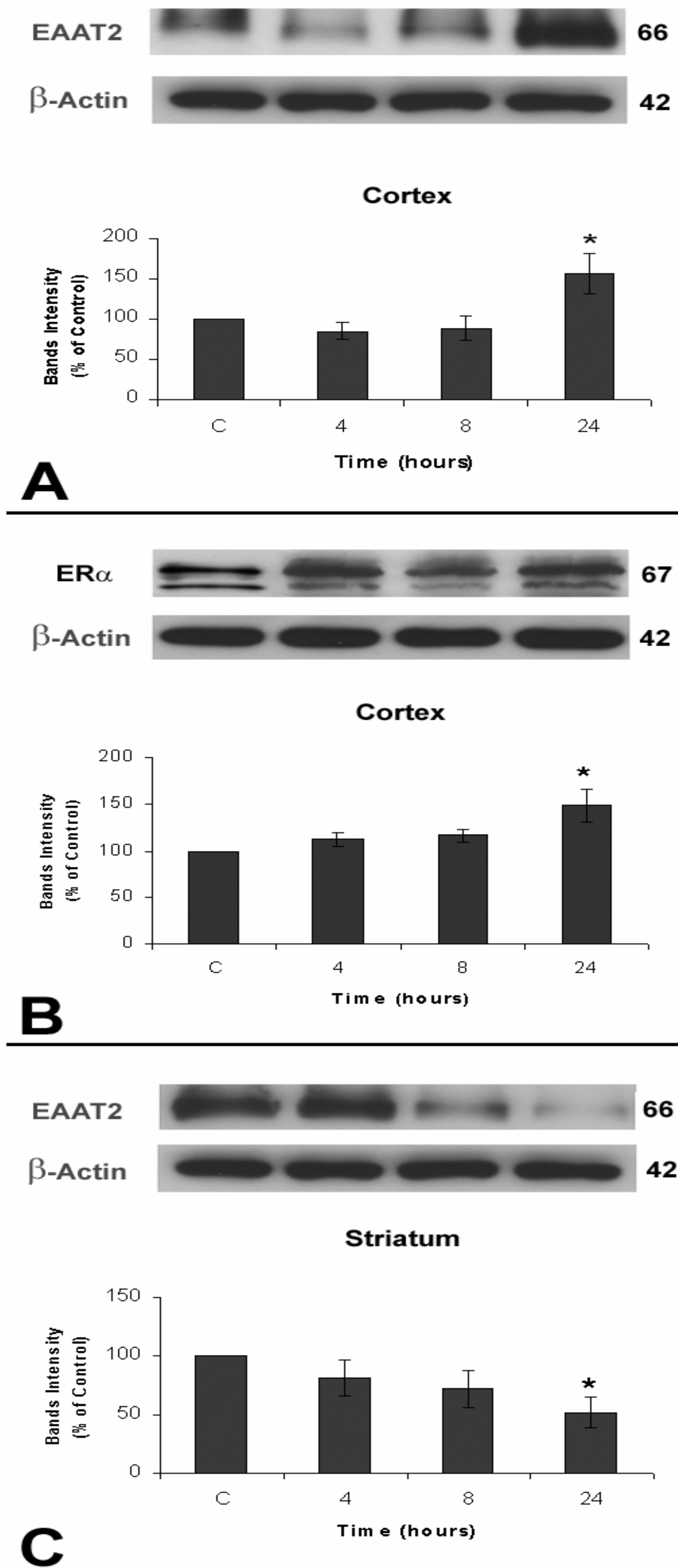

Figure 2

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DISCUSSÃO

Durante as últimas décadas, diversas evidências têm demonstrado que os estrógenos influenciam o crescimento, a diferenciação, a maturação e a função de vários tecidos-alvo, além de desempenharem um papel neuroprotetor. A questão da proteção pelos estrógenos é importante, uma vez que nas mulheres ocorre um acentuado declínio relacionado à idade nos níveis destes hormônios, de forma que na menopausa os níveis são aproximadamente apenas 1% daqueles observados na pré-menopausa. Se por um lado a expectativa de vida das mulheres vem aumentando, estando atualmente em torno de 80 anos, por outro a idade do início da menopausa manteve-se fixa. Conseqüentemente, as mulheres estão vivendo uma proporção cada vez maior de suas vidas em um estado hipo-estrogênico, o qual pode contribuir para um risco aumentado de incidência de acidente vascular cerebral, disfunção cognitiva e de uma variedade de doenças neurodegenerativas (Wise *et al.*, 2001).

Um grande número de estudos em animais, tanto *in vitro* como *in vivo*, têm sugerido que os estrógenos desempenham um papel neuroprotetor, estas evidências têm impulsionado o interesse em determinar a sua eficácia na prevenção de doenças neurodegenerativas e cerebrovasculares em humanos (Dhandapani & Brann, 2002). Estes estudos têm sugerido um papel benéfico para os estrógenos na doença de Alzheimer e na doença de Parkinson (Rusa *et al.*, 1999) e têm demonstrado claramente que o estradiol exerce ações protetoras potentes contra o dano cerebral isquêmico (McCullough & Hurn, 2003; Wilson *et al.*, 2000).

No primeiro e no segundo capítulo desta tese, a fim de investigar mais a respeito das propriedades neuroprotetoras do 17β-estradiol, o estrógeno mais abundante em humanos e roedores, foram utilizadas culturas organotípicas de fatias hipocampais expostas à privação de oxigênio e glicose (POG) como modelo *in vitro* de “dano isquêmico”. A escolha deste método de cultivo deve-se ao fato de que a manutenção da organização do tecido e da integridade das células, permitindo a comunicação entre neurônios e entre neurônios e glia, pode ser importante para melhor entender os mecanismos dos efeitos protetores do estradiol. Isto é sugerido pelos dados mostrando a existência de receptores estrogênicos em astrócitos (Azcoitia *et al.*, 1999) e pelo fato de que algumas ações do estradiol podem ser muito diferentes dependendo da presença ou ausência destas células gliais (Stone *et al.*, 1998). Neste modelo, a captação de iodeto de propídeo foi escolhida como um

marcador de células mortas uma vez que este método correlaciona-se bem com a extensão da morte celular detectada por outros métodos (Noraberg *et al.*, 1999).

Os resultados apresentados no primeiro capítulo ampliaram os achados prévios demonstrando que a presença de 17β-estradiol (10 nM), 7 dias antes (tratamento crônico) ou apenas no momento do dano (tratamento agudo), exerceu efeitos protetores, prevenindo a morte celular. Neste estudo foram utilizadas doses semelhantes à concentração fisiológica de estradiol no cérebro, por serem estas as doses de hormônio relevantes para as terapias de reposição hormonal utilizadas pelas mulheres. O pré-tratamento com estradiol não foi necessário para os efeitos protetores, uma vez que a exposição aguda à mesma concentração apresentou grau de proteção similar, o que está parcialmente em contraste com um estudo prévio mostrando que apenas o tratamento crônico foi neuroprotetor (Wilson *et al.*, 2000). Uma explicação plausível para os dados contrastantes pode ser a utilização de regiões cerebrais diferentes para o preparo das culturas, uma vez que neste estudo foram utilizadas fatias corticais enquanto nós utilizamos fatias hipocampais. Além disso, o modelo de lesão foi diferente, no estudo prévio foi utilizada a exposição do tecido ao ácido kaínico ou cianeto de potássio/2-desóxi-glicose enquanto nós utilizamos a exposição do tecido à POG. Neste estudo, Wilson e colaboradores (2000) sugerem que as ações protetoras do tratamento crônico (e a falta de ações protetoras do tratamento agudo) com estradiol envolvem receptores estrogênicos nucleares clássicos, possivelmente em um modo genômico. As ações rápidas, não-genômicas dos estrógenos (Pietras & Szego, 1980) poderiam explicar os nossos resultados mostrando o efeito neuroprotetor do tratamento agudo com doses fisiológicas de estradiol. Neste sentido, evidências sugerindo a existência de um receptor estrogênico ligado à membrana, responsável pelas respostas rápidas aos estrógenos, estão sendo descritas na literatura (Belcher & Zsarnovsky, 2001).

Além do efeito que os estrógenos apresentam de regular a transcrição de genes através de receptores estrogênicos nucleares (Smith *et al.*, 1993), cresce o número de evidências mostrando que estrógenos podem disparar vias de sinalização celular não-transcricionais. Tem sido sugerido o envolvimento tanto da via da MAPK (Singer *et al.*, 1999) como da via da PI3-K (Honda *et al.*, 2000) no efeito neuroprotetor dos estrógenos. Com o objetivo de esclarecer o mecanismo pelo qual o estradiol atuou como neuroprotetor em nosso estudo, investigamos o envolvimento destas vias de sinalização celular. Para tal, utilizando o modelo *in vitro,*

primeiramente investigamos a via da PI3-K, a qual medeia vias de sinalização disparadas por diversos fatores tróficos numa variedade de tipos celulares incluindo células neuronais (Wymann & Pirola, 1998). Utilizou-se LY294002, um inibidor direto da enzima PI3-K, e este suprimiu quase que totalmente o efeito neuroprotetor agudo do estradiol. A seguir, investigou-se o envolvimento da via da MEK/ERK 1/2, uma vez que alguns dos efeitos neuroprotetores do estradiol são mediados por esta via (Xue *et al.*, 2000). Resumidamente, os estrógenos podem interagir diretamente com a via de sinalização da MAPK através da ativação das proteínas Ras, B-Raf e MEK1/2, induzindo rapidamente a fosforilação de ERK1/2. Entretanto, PD98059, um inibidor da via da MEK/ERK 1/2, não evitou o efeito neuroprotetor do estradiol. Estes resultados sugerem fortemente que a via de sinalização da PI3-K esteja envolvida na neuroproteção por estradiol no modelo *in vitro* de morte celular.

Na tentativa de elucidar um mecanismo molecular possivelmente envolvido com a via de sinalização da PI3-K, investigou-se a ativação da enzima Akt/PKB. Nesta cascata de transdução de sinal, a Akt/PKB é o efetor direto logo abaixo de PI3-K (Songyang *et al.*, 1997) e estudos recentes sugerem que esta via de sinalização de PI3-K para Akt/PKB esteja envolvida com o sinal de sobrevivência celular em neurônios por inibição da apoptose (Crowder & Freeman, 1998). Seguindo esta via, a GSK-3β é um substrato da Akt/PKB (Cross *et al.*, 1995). O principal mecanismo regulatório destas enzimas é por fosforilação: a Akt/PKB 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). Nossos resultados mostraram que o 17β-estradiol induziu a fosforilação de ambas as proteínas, refletindo na ativação de Akt e na inibição de GSK-3β e, portanto, na ativação de uma das vias de sinalização envolvidas com a sobrevivência celular (Akt) e no conseqüente bloqueio de uma das vias que levam a indução da apoptose (GSK-3β). Estes aumentos nos níveis das proteínas fosforiladas foram observados tanto com o tratamento crônico quanto com o tratamento agudo, sugerindo que o pré-tratamento não seja necessário para as ações do estradiol sobre a fosforilação destas enzimas. Acredita-se que estas ações rápidas, não-transcricionais ou não-genômicas sejam de natureza neuromodulatória e críticas para a comunicação célula-célula. Não é provável que um efeito rápido assim seja conseqüência de ação nuclear, mas ao

invés disso parece estar relacionado a eventos que estão ocorrendo na superfície celular (Green & Simpkins, 2000; Moss *et al.*, 1997).

O conjunto de resultados apresentado no primeiro capítulo corrobora as evidências de que a via da PI3-K pode desempenhar um papel importante na neuroproteção induzida por estradiol contra o dano isquêmico. Estes achados podem ser importantes para o entendimento do aumento da susceptibilidade à isquemia cerebral observado em mulheres na menopausa e do mecanismo de neuroproteção pelos estrógenos.

No segundo capítulo, utilizando o mesmo modelo *in vitro*, foram investigados os efeitos do tratamento com estadiol sobre o imunoconteúdo dos transportadores de glutamato gliais, EAAT1 e EAAT2, e dos receptores estrogênicos clássicos, ERα e ERβ. Vários, embora não todos, efeitos neuroprotetores dos estrógenos são mediados pela ativação dos receptores estrogênicos (Kumar & Chambon, 1998; Pike, 1999; Singer *et al.*, 1999). Estes receptores ligam-se a seqüências específicas de DNA e dessa forma regulam a transcrição (Kumar & Chambon, 1998) atuando assim como fatores de transcrição ativados por ligante. O papel dos receptores estrogênicos na neuroproteção ainda não foi completamente esclarecido. Recentemente, Carswell e colaboradores (2004), utilizando agonistas seletivos de receptores estrogênicos, demonstraram o envolvimento de ERβ na neuroproteção contra a isquemia cerebral global em camundongos. Entretanto, em estudos utilizando camundongos *knockout* para ERα e ERβ, Dubal e colaboradores (2001) observaram que ERα era necessário para os efeitos neuroprotetores do estradiol contra o dano cerebral. Outros estudos sugerem que tanto ERα como ERβ podem contribuir para a neuroproteção induzida pelos estrógenos (Fitzpatrick *et al.*, 2002; Zhao *et al.*, 2004).

Neste capítulo, a análise do imunoconteúdo de ERα mostrou diminuição significativa após POG nas culturas tratadas tanto com veículo como com estradiol. Um estudo recente demonstrou que a expressão nuclear de ERα encontra-se diminuída em neurônios hipocampais de pacientes que sofrem da doença de Alzheimer, sugerindo que isto possa afetar a transcrição regulada por ERα (Hu *et al.*, 2003). Os nossos achados estão em contradição com o estudo de Dubal e colaboradores (1999), os quais demonstraram que o mRNA para ERα encontra-se aumentado com o dano isquêmico *in vivo*, o que pode ser explicado por diferenças

experimentais como o emprego do modelo *in vivo* e a análise da região cortical por estes autores. Neste mesmo estudo (Dubal *et al.*, 1999) também foi demonstrado que a diminuição do mRNA para ERβ induzida pelo dano é evitada pelo tratamento com estradiol. Se por um lado em nosso modelo *in vitro* a POG não tenha causado a diminuição de ERβ, por outro o tratamento com estradiol induziu um aumento significativo em ERβ, o qual foi mantido mesmo após a indução da lesão. Estes dados sugerem que a expressão dos receptores estrogênicos, particularmente de ERβ, pode estar envolvida nos efeitos protetores do estradiol.

Com a finalidade de ampliar a investigação sobre os mecanismos envolvidos no efeito neuroprotetor induzido pelo 17β-estradiol, focalizamos se o hormônio poderia estar atuando ao nível da liberação ou retirada do glutamato da fenda sináptica, uma vez que este tipo de lesão leva a uma descarga excessiva deste neurotransmissor. Conforme explanamos na introdução, os transportadores de glutamato gliais, EAAT1 e EAAT2, são os principais responsáveis pela remoção do glutamato do espaço extracelular, tanto em condições fisiológicas, permitindo a transmissão normal, como em condições patológicas, prevenindo a morte celular devido à ativação excessiva dos receptores de glutamato. Portanto, o transporte de glutamato desregulado pode contribuir para a patogênese de várias condições neurodegenerativas e a expressão ou a função alterada dos EAATs tem sido identificada em várias destas patologias, incluindo a isquemia cerebral (O'Shea, 2002). Apesar da importância significativa deste sistema, os efeitos dos estrógenos sobre os transportadores de glutamato ainda são pouco conhecidos.

Para determinar se o efeito neuroprotetor dos estrógenos envolve alterações na abundância de EAATs, foram analisados os imunoconteúdos de EAAT1 e EAAT2 em culturas tratadas com veículo ou estradiol e expostas ou não à POG. Não foi detectada nenhuma alteração significativa no imunoconteúdo de nenhum dos transportadores nas condições investigadas. Estes dados estão de acordo com os relatados por Sato e colaboradores (2003), mostrando que a expressão de EAAT1 não é alterada pelo estradiol. Embora não tenha sido detectada alteração significativa no imunoconteúdo de EAAT1 nem de EAAT2, não se pode excluir a possibilidade de que o estradiol esteja atuando na modulação da função destes transportadores.

Os resultados apresentados no segundo capítulo fornecem evidência clara de que a POG induz a diminuição do imunoconteúdo de ERα, enquanto o tratamento com estradiol induz o aumento do imunoconteúdo de ERβ, sugerindo que *in vitro* a neuroproteção por estradiol contra a isquemia pode envolver a regulação da abundância de ERβ e, conseqüentemente, dos genes transcritos por estes receptores, o que seria interessante de ser investigado.

Antes de utilizarmos os modelos *in vivo,* investigamos uma segunda estratégia neuroprotetora, sem o uso de droga. A indução da tolerância, observada pelo pré-condicionamento, conforme descrito na introdução, pode ser considerada como um mecanismo endógeno de resposta a um tipo de estresse (Dirnagl *et al.*, 2003). Modelos *in vivo* mimetizam fielmente o pré-condicionamento isquêmico, entretanto, estes modelos são tecnicamente desafiadores, demandam tempo e são onerosos. Adjuntos importantes para os estudos *in vivo* são os modelos *in vitro* de pré-condicionamento, os quais são tecnicamente mais fáceis e vantajosos economicamente. Recentemente, as culturas organotípicas de fatias hipocampais têm sido utilizadas para demonstrar que condições de pré-condicionamento *in vitro* produzem respostas similares ao cérebro *in vivo*, como a proteção contra a morte neuronal, e que a tolerância nestes dois modelos pode ter alguns mecanismos em comum (Jones & Bergeron, 2001; Lange-Asschenfeldt *et al.*, 2004; Valentim *et al.*, 2001, 2003; Xu *et al.*, 2002).

Ainda no segundo capítulo que compõe esta tese, foi utilizada a metodologia *in vitro* para os estudos de pré-condicionamento. Os resultados mostraram que a exposição do tecido à POG por um curto período (POG subletal) foi capaz de induzir uma tolerância significativa à POG letal subseqüente. A avaliação da morte celular, pela medida da captação do corante iodeto de propídeo, foi consistente com as observações prévias de neuroproteção já descritas neste modelo de indução de tolerância (Valentim *et al.*, 2003; Xu *et al.*, 2002).

Embora a neuroproteção por pré-condicionamento isquêmico tenha sido estudada extensivamente por mais de uma década em uma variedade de modelos *in vivo* e *in vitro* (Jones & Bergeron, 2001, 2004; Kato *et al.*, 1992; Kitagawa *et al.*, 1990; Schurr *et al.*, 1986; Valentim *et al.*, 2001, 2003), os mecanismos específicos que desencadeiam este estado de tolerância ainda não são completamente conhecidos. Dados recentes sugerem que a regulação do transporte de glutamato

pode contribuir para o desenvolvimento da tolerância isquêmica (Douen *et al.*, 2000; Romera *et al.*, 2004). Além disso, o pré-condicionamento e a tolerância hipóxica parecem ser dependentes do gênero e mediados por mecanismos gênero-específicos (Kasischke *et al.*, 1999; von Arnim *et al.*, 2002). Estas evidências nos impulsionaram na investigação de um possível envolvimento dos transportadores de glutamato gliais (EAAT1 e EAAT2) e dos receptores estrogênicos (ERα e ERβ) na tolerância isquêmica induzida por pré-condicionamento *in vitro*. Não foram detectadas alterações significativas no imunoconteúdo de EAAT1, EAAT2, ERα ou ERβ nas culturas organotípicas, o que está de acordo com os nossos achados no modelo *in vivo* (discutido abaixo). Embora a abundância dos transportadores de glutamato e dos receptores estrogênicos não tenha variado, o envolvimento da modulação da sua atividade em resposta ao pré-condicionamento isquêmico não pode ser descartado. A função dos transportadores de glutamato está sujeita a uma modulação complexa por uma variedade de fatores endógenos, incluindo o próprio glutamato (O'Shea, 2002), e os receptores estrogênicos têm a sua atividade regulada por diversas proteínas, vias e processos (McDonnell & Norris, 2002). A ativação de quinases, por exemplo, parece ser um potente sinal que leva a regulação da função dos EAATs: a PKC, a PKA e a PI3-K estão todas implicadas no aumento da atividade, da abundância ou da expressão na superfície celular destes transportadores (O'Shea, 2002).

A parte final deste estudo envolveu a utilização de metodologia *in vivo,* tanto no que se refere ao tratamento com 17β-estradiol, quanto à indução de tolerância pelo pré-condicionamento.

Os dados apresentados no terceiro capítulo foram obtidos a partir da utilização de ratas ovariectomizadas que receberam ou não reposição de 17β-estradiol. Neste conjunto de experimentos, fatias hipocampais preparadas a fresco, provenientes de animais que receberam ou não a reposição hormonal, foram expostas à POG. Para avaliação da morte celular foi utilizada a medida da atividade da enzima lactato desidrogenase (LDH) liberada para o meio de incubação do tecido (Laev *et al.*, 1993). As fatias provenientes das ratas tratadas com 17β-estradiol apresentaram níveis mais baixos de atividade de LDH no meio de incubação do que as fatias provenientes das ratas tratadas com veículo. Considerando que a quantidade de LDH liberada correlaciona-se com o dano na membrana plasmática

neuronal, estes resultados indicam que o hormônio foi capaz de deixar o tecido cerebral menos vulnerável à morte celular induzida por um insulto isquêmico. Com a finalidade de avaliar um possível mecanismo de ação para o estradiol neste modelo experimental, optamos por investigar sua ação sobre o estresse oxidativo uma vez que a geração de radicais livres aumenta durante a isquemia seguida de reperfusão. Conforme já mencionado, os estrógenos além de agirem de forma dependente e independente dos receptores estrogênicos (ERα e ERβ), de modo genômico e não-genômico, para atenuar o dano neural e de terem efeitos sobre uma variedade de cascatas de sinalização citoplasmáticas (revisado em Segars & Driggers, 2002), possuem uma atividade antioxidante bem estabelecida atuando através de mecanismos celulares independentes de receptores estrogênicos. Diversos estudos têm demonstrado que os estrógenos protegem as células do dano cerebral induzido por radicais livres através da “varredura” direta destes radicais ou da preservação de antioxidantes endógenos (Behl, 2002; Culmsee *et al.*, 1999; Kume-Kick & Rice, 1998). As espécies reativas do oxigênio geradas pela isquemia escapam dos mecanismos de defesa endógeno e podem levar a uma multiplicidade de alterações nas membranas mitocondriais e no DNA, ameaçando a viabilidade celular (White *et al.*, 2000). Nossos resultados mostraram que nas fatias hipocampais das ratas controle, a POG seguida por um período de recuperação levou ao aumento na produção de radicais livres. Nas fatias hipocampais das ratas que receberam reposição de 17β-estradiol e posteriormente foram expostas à POG, o conteúdo de radicais livres e a capacidade antioxidante total não sofreram alterações quando comparados com os controles. Estes resultados sugerem que os efeitos do estradiol sobre as defesas antioxidantes podem ser irrelevantes para a ação neuroprotetora observada neste modelo. De fato, a atividade antioxidante dos estrógenos pode não ser relevante para os seus efeitos neuroprotetores em todos os sistemas. A ação antioxidante dos estrógenos geralmente requer concentrações da ordem de μM, enquanto os efeitos neuroprotetores podem ser observados com concentrações significativamente mais baixas na ordem de nM, como as utilizadas no nosso estudo. Nas fatias hipocampais provenientes de animais tratados apenas com veículo e posteriormente expostas à POG, foram observadas reduções significativas nos conteúdos dos resíduos de triptofano e tirosina, sugerindo que o estresse oxidativo induzido por POG pode afetar os resíduos aromáticos nas proteínas. A formação de radicais livres pode levar à oxidação destes resíduos, especialmente se não

existirem redutores suficientes para repará-los. É importante destacar que tanto a tirosina como o triptofano são abundantes nos domínios transmembrana de todas as principais classes de proteínas integrais de membrana (Jones *et al.*, 1994; Von Heijne, 1994). Além disso, eles são importantes atenuadores da morte celular oxidativa e da peroxidação lipídica, provavelmente devido a sua posição particular entre os lipídeos e a habilidade dos grupos fenólicos e indólicos de doar radical hidrogênio (Moosmann & Behl, 2000). As alterações nestes resíduos podem levar a alterações na dinâmica geral das bicamadas lipídicas e indicar a perda de defesas antioxidantes importantes nas membranas. Em contraste, considerando o grupo tratado com estradiol, o conteúdo de triptofano aumentou nas fatias controle, enquanto que os conteúdos de tirosina e tiptofano mantiveram-se dentro dos níveis basais nas fatias expostas à POG. Estes dados sugerem que o estradiol pode atuar protegendo estes resíduos de aminoácidos, e desta forma pode estar envolvido com a ação antioxidante lipídica.

Os dados apresentados no terceiro capítulo reforçam os efeitos neuroprotetores da reposição estrogênica contra o dano isquêmico. Este estudo é o primeiro a sugerir o envolvimento dos efeitos do estradiol sobre aminoácidos nas suas propriedades neuroprotetoras. Estes achados podem contribuir para o entendimento da vulnerabilidade aumentada de homens e mulheres na menopausa à isquemia cerebral e o mecanismo de neuroproteção pelos estrógenos.

Embora outros estudos já tenham relatado o aumento da tolerância à isquemia seguindo o pré-condicionamento do cérebro de mamíferos com exposição à hipóxia moderada, os mecanismos envolvidos nesta neuroproteção ainda não foram completamente esclarecidos (Jones & Bergeron, 2001, 2004; Vannucci *et al.*, 1998).

Os achados recentes de que o pré-condicionamento foi capaz de modular os níveis de glutamato extracelular (Douen *et al.*, 2000; Romera *et al.*, 2004), bem como regular os transportadores de glutamato (EAATs), direcionaram a investigação do efeito do pré-condicionamento hipóxico sobre o conteúdo de EAAT1 e EAAT2 em diferentes regiões cerebrais, tais como córtex, hipocampo e estriado. Como a tolerância hipóxica primária e o pré-condicionamento podem ser dependentes de gênero e mediados via mecanismos gênero-específicos (Alkayed *et al.*, 1998; Kasischke *et al.*, 1999, von Arnim *et al.*, 2002), foi também investigado se os

receptores estrogênicos, ERα e ERβ, poderiam estar envolvidos na tolerância isquêmica induzida por hipóxia no cérebro de ratos neonatos.

Conforme relatado previamente (Jones & Bergeron, 2001, 2004), nosso estudo confirmou que o pré-condicionamento hipóxico causa tolerância à isquemia letal subseqüente (Figura 1, Anexo 1). Após o pré-condicionamento hipóxico, não foram observadas alterações em EAAT1 em nenhuma das regiões cerebrais nos tempos analisados, o que está de acordo com os achados de Romera e colaboradores (2004) de que a expressão celular de EAAT1 não estava aumentada nem diminuída em células corticais expostas ao pré-condicionamento isquêmico. Quanto ao EAAT2, foi observado um aumento do imunoconteúdo no córtex e uma diminuição no estriado, enquanto que no hipocampo não foi detectada qualquer alteração.

Dados relatados na literatura demonstraram que o pré-condicionamento *in vivo* antes da isquemia focal ocasionou diminuição de EAAT1 e EAAT2 (Douen *et al.*, 2000), enquanto que a tolerância isquêmica *in vitro* ocasionou aumento de EAAT2 e EAAT3 em culturas corticais de ratos (Romera *et al.*, 2004). Neste último estudo, a captação de glutamato também estava aumentada, levando os autores a concluírem que o aumento destes transportadores medeia a tolerância isquêmica por diminuição da excitotoxicidade atribuível ao aumento no glutamato extracelular induzido pela isquemia (Romera *et al.*, 2004). Embora a captação através dos EAATs gliais seja o mecanismo primário de *clearance* do glutamato cerebral sob condições não-patológicas, estes transportadores são sensíveis a perturbações iônicas que ocorrem sob condições isquêmicas (Szatkowski & Attwell, 1994). Durante a isquemia, o gradiente iônico de sódio é revertido, de forma que o sódio e concomitantemente o glutamato são excluídos das células via reversão dos transportadores de glutamato (Rutledge & Kimelberg, 1996; Szatkowski & Attwell, 1994). Como este processo exacerba a excitotoxicidade durante a isquemia, a diminuição dos EAATs seguindo o pré-condicionamento resultaria em menos EAATs disponíveis para o efluxo de glutamato durante um insulto subseqüente, o que garantiria a neuroproteção. Douen e colaboradores (2000) reforçam esta visão ao correlacionar um decréscimo no glutamato extracelular e uma diminuição simultânea de EAAT1 e EAAT2 com o efeito neuroprotetor do pré-condicionamento *in vivo*. Uma alteração na abundância dos transportadores de glutamato, portanto, não esclarece qual é a conseqüência porque se de um lado estes transportadores são

fundamentais para o *clearance* do glutamato extracelular, de outro a sua operação reversa é responsável pela maior parte da liberação de glutamato induzida por isquemia (Jabaudon *et al.*, 2000; Rossi *et al.*, 2000). Os dados apresentados no quarto capítulo são consistentes com as duas visões: (1) o aumento de EAAT2 em córtex pode mediar a tolerância isquêmica por aumento da captação de glutamato e diminuição da excitotoxicidade e (2) a redução mediada por hipóxia nos níveis de EAAT2 em estriado pode reduzir o efluxo de glutamato durante uma isquemia subseqüente contribuindo para o efeito neuroprotetor do pré-condicionamento hipóxico.

É importante destacar que o glutamato, além das suas ações sobre a excitabilidade neuronal mediadas por receptor, é capaz de estimular a glicólise, isto é, a utilização de glicose e a produção de lactato em astrócitos (Pellerin & Magistretti, 1994). Esta ação metabólica é mediada por ativação dos transportadores de glutamato gliais, EAAT1 e EAAT2. O mecanismo envolve a enzima Na^+/K^+-ATPase, a qual é ativada por um aumento na concentração intracelular de Na^+ co-transportado com glutamato pelo sistema de captação eletrogênico. Assim, quando o glutamato é liberado a partir das sinapses ativas e captado pelos astrócitos, a via de sinalização descrita por Pellerin e Magistretti (1994) fornece um mecanismo simples e direto para acoplar a atividade neuronal à utilização de glicose. Levando em consideração este estudo prévio, os achados de EAAT2 aumentado em córtex levaram a conjectura de que os transportadores de glutamato gliais poderiam estar envolvidos em algum tipo de complexo funcional com a Na^+/K^+-ATPase, que poderia ser importante para o desenvolvimento da tolerância isquêmica em resposta ao pré-condicionamento hipóxico em cérebro de ratos neonatos.

A respeito dos receptores estrogênicos, nosso estudo *in vivo* não demonstrou diferenças em ERβ em nenhuma das regiões do cérebro nos tempos analisados, mas ERα estava aumentado no córtex 24 horas após o pré-condicionamento hipóxico. Em contraste, um estudo recente demonstrou que a expressão de ERα permanecia inalterada no pré-condicionamento químico em ratos adultos (von Arnim *et al.*, 2002). O estudo apresentado no quarto capítulo é o primeiro investigando os efeitos do pré-condicionamento hipóxico sobre ERβ e também o primeiro a relatar uma expressão aumentada de ERα em cérebro de ratos neonatos. A regulação de ERα de uma forma similar a EAAT2, conforme

demonstrado pelos resultados na região cortical, sugere que ERα possa ser mais uma parte do complexo funcional envolvendo EAAT2 e Na^+/K^+-ATPase, talvez contribuindo para a tolerância isquêmica induzida após o pré-condicionamento com hipóxia.

É provável que fatores múltiplos contribuam para a neuroproteção seguindo o pré-condicionamento hipóxico. A regulação dos transportadores de glutamato e dos receptores estrogênicos pode ser um mecanismo envolvido na tolerância induzida por hipóxia. Os dados dos experimentos *in vivo* forneceram a primeira evidência de que EAAT2 e ERα estão co-regulados de uma maneira similar em resposta ao pré-condicionamento hipóxico em cérebro de ratos neonatos. Este estudo revelou novos conhecimentos sobre os mecanismos do pré-condicionamento, onde EAAT2 e talvez ERα possam estar envolvidos em algum tipo de complexo funcional com a Na^+/K^+-ATPase. Mais estudos são necessários para a elucidação completa dos mecanismos regulando a aquisição de tolerância cerebral que poderia guiar os esforços para desenvolver medidas efetivas ou agentes farmacológicos pré-condicionantes seguros para proteger o cérebro ou reduzir o dano isquêmico.

CONCLUSÕES

Os resultados obtidos neste trabalho permitem concluir que:

1. No modelo *in vitro* de isquemia cerebral:

1.1. No modelo de culturas organotípicas de fatias hipocampais:

- a presença de uma dose baixa de 17β-estradiol (10 nM), 7 dias antes do dano (tratamento crônico) ou apenas no momento do dano (tratamento agudo), exerceu efeitos neuroprotetores, evitando a morte celular;
- LY294002, um inibidor da via de sinalização da PI3-K, evitou quase que totalmente o efeito neuroprotetor do estradiol;
- PD98059, um inibidor da via de sinalização da MEK/ERK 1/2, não evitou o efeito neuroprotetor do estradiol;
- 17β-estradiol, crônico ou agudo, induziu a fosforilação/ativação de Akt e a fosforilação/inativação de GSK-3β.

Estes dados sugerem fortemente que a via de sinalização da PI3-K esteja envolvida na neuroproteção por estradiol, por ativação de Akt, a qual é diretamente implicada na sobrevivência celular, e bloqueio de GSK-3β, que participa na indução da apoptose.

- POG induziu uma diminuição significativa no imunoconteúdo de ERα e não alterou ERβ;
- 17β-estradiol induziu um aumento significativo no imunoconteúdo de ERβ, o qual foi mantido mesmo após a indução da lesão, e não alterou ERα;
- EAAT1 e EAAT2 não tiveram seus imunoconteúdos alterados nem por POG nem por 17β-estradiol.

Estes dados sugerem que os efeitos neuroprotetores do estradiol contra a isquemia cerebral podem envolver a regulação da abundância dos receptores

estrogênicos e, conseqüentemente, dos genes por eles transcritos, enquanto que os transportadores de glutamato gliais parecem não estar envolvidos.

- POG subletal induziu uma tolerância significativa à POG letal subseqüente;
- o pré-condicionamento isquêmico não induziu alteração significativa no imunoconteúdo dos transportadores de glutamato gliais, EAAT1 e EAAT2, nem dos receptores estrogênicos, ERα e ERβ.

Estes dados sugerem que a tolerância induzida pelo pré-condicionamento isquêmico não envolve a regulação da abundância dos transportadores de glutamato nem dos receptores estrogênicos no hipocampo.

1.2. No modelo de fatias hipocampais preparadas a fresco:

- fatias POG de ratas tratadas com 17β-estradiol liberaram menos LDH para o meio de incubação do que as fatias POG de ratas tratadas com veículo;
- a reposição de estradiol não mudou o conteúdo de radicais livres nem a capacidade antioxidante total nas fatias hipocampais;
- no grupo tratado com estradiol, o conteúdo de triptofano foi aumentado nas fatias controle, enquanto que os conteúdos de tirosina e triptofano foram mantidos dentro dos níveis basais nas fatias expostas à POG.

Estes dados sugerem que as propriedades neuroprotetoras da reposição por estradiol contra o dano isquêmico podem envolver os efeitos do estradiol sobre aminoácidos, evitando o dano em proteínas.

2. No modelo *in vivo* de isquemia cerebral:

- o pré-condicionamento hipóxico causou tolerância à hipóxia-isquemia no cérebro de ratos neonatos;

- após o pré-condicionamento hipóxico, não foram observadas alterações em EAAT1 em nenhuma das regiões cerebrais nos tempos analisados;
- um aumento de EAAT2 no córtex e uma diminuição no estriado foram observados, enquanto que no hipocampo sua abundância permaneceu inalterada;
- ERβ não demonstrou diferenças em nenhuma das regiões do cérebro nos tempos analisados;
- ERα estava significativamente aumentado no córtex 24 horas após o pré-condicionamento hipóxico;
- EAAT2 e ERα foram co-regulados de uma maneira similar em resposta ao pré-condicionamento hipóxico.

A regulação dos transportadores de glutamato, e dos receptores estrogênicos, podem ser mecanismos envolvidos na tolerância induzida por hipóxia. EAAT2, e talvez ERα, podem estar envolvidos em algum tipo de complexo funcional com a Na^+/K^+-ATPase, que pode ser importante para o desenvolvimento da tolerância isquêmica em resposta ao pré-condicionamento hipóxico no cérebro de ratos neonatos no córtex.

PERSPECTIVAS

Como continuação deste trabalho, serão realizados experimentos para:

- determinar se a neuroproteção por estradiol envolve a modulação da função dos transportadores de glutamato por exame da captação de glutamato nas culturas organotípicas após tratamento com estrógenos e exposição à POG.
- determinar se a neuroproteção por pré-condicionamento envolve a modulação da função dos transportadores de glutamato por exame da captação de glutamato nas culturas organotípicas pré-condicionadas com POG subletal e expostas à POG letal.
- investigar a hipótese de existência de algum tipo de complexo funcional entre a Na^+/K^+-ATPase e os transportadores de glutamato e os receptores estrogênicos por análise do imunoconteúdo desta enzima e também dos transportadores de glicose, GLUT1 e GLUT4, nos modelos *in vitro* e *in vivo* de pré-condicionamento.
- examinar a localização celular dos transportadores de glutamato gliais, EAAT1 e EAAT2, e dos receptores estrogênicos, $ER\alpha$ e $ER\beta$, após o pré-condicionamento hipóxico no cérebro de ratos neonatos.

ANEXOS

ANEXO 1. DADOS NÃO PUBLICADOS CITADOS EM "DISCUSSÃO"

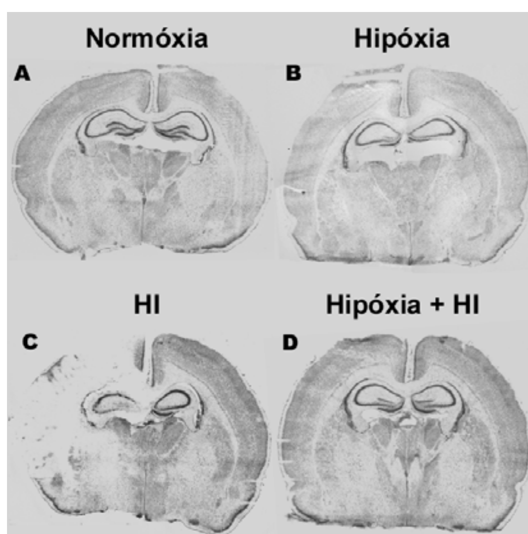

Figura 1: O pré-tratamento com hipóxia confere proteção contra o dano cerebral hipóxico-isquêmico em ratos neonatos. Os filhotes de ratos no sexto dia pós-natal (P6) foram expostos à hipóxia a 37°C (8% O_2 por 3 horas; n=19) (**B** e **D**), enquanto que os animais controles (n=15) (**A** e **C**) foram mantidos em condições de normóxia. Vinte e quatro horas após o pré-condicionamento, os filhotes foram submetidos à hipóxia-isquemia unilateral (**C** e **D**). Três dias após a hipóxia-isquemia (P10), secções coronais antero-posteriores do cérebro de cada animal foram coradas com cresil violeta e avaliadas quanto ao grau de dano.

ANEXO 2. CO-AUTORIA DE ARTIGOS PUBLICADOS DURANTE O DOUTORADO

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2. Neuroprotective effect of ebselen on rat hippocampal slices submitted to oxygen-glucose deprivation: correlation with immunocontent of inducible nitric oxide synthase, Porciúncula, L.O., Rocha, J.B., **Cimarosti, H.**, Vinadé, L., Ghisleni, G., Salbego, C., Souza, D.O., *Neuroscience Letters*, 346: 101-104, 2003.

3. Diphenyl diselenide protects rat hippocampal slices submitted to oxygen-glucose deprivation and diminishes inducible nitric oxide synthase immunocontent, Ghisleni, G., Porciúncula, L.O., **Cimarosti, H.**, Rocha, J.B., Salbego, C., Souza, D.O., *Brain Research*, 986: 196-199, 2003.

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8. Neuroprotective effect of Kava extract in hippocampal organotypic culture exposed to oxygen and glucose deprivation, Tavares, A.A., Horn, A.P., Nassif, M.C., Zamin, L.L., **Cimarosti, H.**, Salbego, C., *Toxicology In Vitro*, submetido 2005.

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