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Toxicidade induzida pelos peptídeos Aβ42 e Aβ25-35 em modelo de cultura organotípica de hipocampo de ratos

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Aos amores da minha vida:

À minha mãe, minha bússola, meu porto seguro, meu exemplo de pessoa.
Ao meu pai, batalhador, honesto, coração feito de pura bondade.
À minha irmã, linda, minha melhor amiga, minha querida companheira...
Ao meu irmão, querido; simplicidade e alegria estampadas na cara.
À Dinda, minha amiga, segunda mãe, companheira e fada madrinha.
Ao Pato, meu amor, por se tornar minha casa, onde quer que estejamos.

"O mistério da vida me causa a mais forte emoção. É o sentimento que suscita a beleza e a verdade, cria a arte e a ciência."

Albert Einstein

AGRADECIMENTOS

ÍNDICE

APRESENTAÇÃO

Esta dissertação está apresentada no formato de artigos científicos, organizada da seguinte forma:

PARTE I

Introdução contendo uma revisão da literatura que fundamentou nossa proposta de trabalho.

Objetivos gerais e específicos do trabalho.

PARTE II

Capítulos I e II, compreendendo dois artigos científicos, um submetido e outro a ser submetido.

PARTE III

Discussão contendo uma interpretação geral dos resultados obtidos nos diferentes trabalhos.

Conclusões.

Perspectivas.

REFERÊNCIAS BIBLIOGRÁFICAS

Referências bibliográficas citadas nas Partes I e III.

ANEXOS

ANEXO 1. Lista de figuras.

ANEXO 2. Autoria e co-autoria em artigos publicados durante o mestrado.

PARTE I

RESUMO

O crescimento da expectativa de vida média da população mundial tem sido acompanhado do aumento na prevalência de doenças neurodegenerativas, como a doença de Parkinson, isquemia cerebral e, especialmente, a doença de Alzheimer. A doença de Alzheimer (DA) caracteriza-se por um crescente declínio na função mental e memória do paciente. Estes sintomas são explicados por uma profunda perda neuronal e pela presença de alterações estruturais no tecido cerebral: as placas senis, extracelulares e os emaranhados neurofibrilares, intracelulares. O passo que desencadeia a neurodegeneração na DA é ainda objeto de estudo, mas a hipótese mais aceita atualmente é a de que a secreção anormal do peptídeo β amilóide (Aβ), principal componente das placas senis, dê início ao processo. O presente trabalho teve como objetivos investigar a toxicidade induzida pelo peptídeo Aβ1-42 e seu fragmento, o peptídeo Aβ25-35 em culturas organotípicas de hipocampo de ratos. Na primeira parte do trabalho, investigamos a toxicidade induzida pela exposição de culturas organotípicas de hipocampo de ratos a 10 ou 20 μM do peptídeo Aβ1-42, por 24 ou 72 h. Além disso, investigamos o envolvimento das proteínas iNOS e GSK-3β no mecanismo de morte celular induzida pelo peptídeo Aβ1-42. Na segunda parte do trabalho, investigamos a toxicidade induzida pelo fragmento Aβ25-35 na concentração de 25 μM, nos tempos de 1, 3, 6, 12, 24 ou 48 h de exposição às culturas organotípicas, e seu efeito sobre as proteínas Akt, GSK-3β e PTEN. A morte celular foi quantificada pela incorporação do iodeto de propídeo, corante marcador excluído de células sadias, e as proteínas foram quantificadas por imunodetecção com o uso de anticorpos específicos. Nossos resultados mostraram que o peptídeo Aβ1-42 apresentou toxicidade nas concentrações de 10 e 20 μM, induzindo a uma considerável morte celular após 72 h de exposição. A análise do imunoconteúdo da proteína iNOS mostrou um aumento significativo em relação ao controle, após 72 h de exposição ao peptídeo e na concentração de 20 μM. Os resultados obtidos na segunda parte do trabalho mostraram uma morte celular significativa apenas após 48 h de exposição ao peptídeo Aβ25-35 na concentração de 25 μM. O tratamento com peptídeo Aβ25-35 levou ao aumento no estado de fosforilação da proteína Akt após 6 h de exposição e também da proteína GSK-3β, um potencial substrato da Akt. Após 12 h de exposição ao peptídeo, observamos uma diminuição de ambas as proteínas (Akt e GSK-3β). Porém, após 24 h de exposição ao peptídeo, a proteína Akt continua menos fosforilada enquanto que a proteína GSK-3β apresenta um novo pico de fosforilação. O imunoconteúdo da proteína fosfatase PTEN apresentou um aumento significativo em 24 h e 48 h. Os resultados do presente estudo mostraram que o tratamento das culturas organotípicas de hipocampo de ratos com o peptídeo Aβ1-42 como com o fragmento Aβ25-35 apresentou toxicidade após um período de aproximadamente 48 h de tratamento, e mostrou ser um bom modelo para o estudo de sua toxicidade. Com relação ao mecanismo investigado, os dados sugerem que a proteína iNOS pode estar envolvida na toxicidade induzida pelo peptídeo Aβ1-42. Além disso, sugerem que o fragmento Aβ25-35 possa exercer sua toxicidade através da inibição da via de sobrevivência celular PI3-K, por diminuir a fosforilação/ativação da proteína Akt, parecendo envolver a proteína fosfatase PTEN, principal regulador negativo da via PI3-K/Akt.

ABSTRACT

The increase of the average life expectancy of the world-wide population has been followed by an increase in the prevalence of neurodegenerativas diseases, such as Parkinson's disease, cerebral ischemia, and especially, Alzheimer's disease. The Alzheimer's disease (AD) is characterized for an increasing decline in the mental function and memory. These symptoms are explained by a profound neuronal loss and the presence of structural alterations in the cerebral tissue: the senile plaques, extracellular, and the neurofibrilary tangles, intracellular. The step that triggers the neurodegeneration in AD is until unclear, but the hypothesis most accepted currently is that the abnormal secretion of the β amyloid peptide (Aβ), main component of the senile plaques, gives beginning to the process. The precise mechanism for which the Aβ peptide induces toxicity is still objective of study. The present work had as objective to investigate the toxicity induced by Aβ peptide Aβ1-42 and its fragment, the peptide Aβ25-35, in organotypic hippocampal cultures. In the first part of the work, we investigated the toxicity induced by the exposure of organotypic hippocampal cultures to 10 or 20 µM of the Aβ1-42 peptide, for 24 or 72 h. Moreover, we investigated the involvement of iNOS and GSK-3β proteins in the mechanism of cellular death induced by the peptide. In the second part of the work, we investigated the toxicity induced by 25 µM of the fragment Aβ25-35, at the periods of exposure 1, 3, 6, 12, 24 or 48 h to organotypic hippocampal cultures, and its effect on proteins Akt, GSK-3β e PTEN. Cellular death was quantified by the incorporation of the propidium iodide, a marker excluded from healthy cells, and the proteins had been quantified by immunodetection with the use of specific antibodies. Our results had shown that the Aβ1-42 peptide (10 and 20 µM) was toxic after a 72-h treatment. The analysis of the immunocontent of the iNOS protein showed a significant increase after a 72-h peptide treatment (20 µM). The results obtained in the second part of the work had shown a significant cellular death after a 48-h Aβ25-35 peptide treatment. The treatment with the peptide resulted to the increase in the phosphorylation state of the Akt and GSK-3β proteins after a 6-h treatment. After 12 h of treatment to the peptide Aβ25-35, we observed a reduction in the phosphorylation state of both the proteins. However, after 24 h of treatment, the phosphorylation of Akt protein continues low, while the phosphorylation of GSK-3β protein presents a new peak. The immunocontent of the PTEN protein presented a significant increase after 24 h and 48 h of treatment to Aβ25-35 peptide. The results of the present study had shown that the treatment of the organotypic hippocampal cultures with the Aβ1-42 peptide as with the fragment Aβ25-35 presented toxicity after a period approximately of 48 h of exposure, and showed to be a good model for the study of its toxicity. With relation to investigated mechanism, the data suggest that the protein iNOS could be involved in the toxicity induced by Aβ1-42. Moreover, they suggest that the fragment Aβ25-35 can exert its toxicity through the inhibition of the cellular survival PI3-K pathway, by decreasing the phosphorylation/activation of the Akt protein, seeming to involve the protein fosfatase PTEN, main negative regulator of PI3-K/Akt pathway.

LISTA DE ABREVIATURAS

Aβ1-42 – peptídeo beta amilóide contendo 42 aminoácidos

Aβ1-40 - peptídeo beta amilóide contendo 40 aminoácidos

Aβ25-35 – fragmento de 11 aminoácidos (do 25 a 35) do Aβ1-40 e Aβ1-42

Akt – proteína cinase B (homóloga celular ao oncogene viral v-Akt)

APOEε4 – apolipoproteína epsilon 4

DA – Doença de Alzheimer

BACE – β-secretase (*β-site APP-cliving enzyme*)

BAD - proteína pró-apoptótica pertencente à família das Bcl-2

Bcl-2 - oncogene da célula B

FKHR – família dos fatores de transcrição em "forquilha" (*forkhead family*)

GSK-3β – glicogênio sintase cinase-3 (*glycogen synthase kinase-3β*)

iNOS – óxido nítrico sintase induzível *(inducible nitric oxide synthase)*

NO – óxido nítrico (*nitric oxide*)

pAkt – fosfo-Akt (*phospho-Akt*)

pGSK-3β - fosfo-GSK-3β (*phospho- pGSK-3β)*

PI3-k – fofoinositol-3 cinase (*Phosphoinositide3-kinase*)

PDK – proteína cinase dependente de fosfoinositóis-3

PP2A – proteína fosfatase A (*protein phosphatase type 2A*)

PPA – proteína precursora amilóide (*amyloid precursor protein*)

PTEN – fosfatase da proteína PI3-K (*phosphatase and tensin homolog deleted on chromosome 10*)

PS – presilina

SNC – Sistema Nervoso Central

Wnt/β-catenina – via de sinalização envolvida na proliferação e diferenciação celular

INTRODUÇÃO

1. DOENÇA DE ALZHEIMER: prevalência e principais características.

O crescimento da expectativa de vida média da população mundial tem sido acompanhado do aumento na prevalência de doenças neurodegenerativas, como a doença de Parkinson, isquemia cerebral e, especialmente, a Doença de Alzheimer (DA). A DA atinge atualmente cerca de 15 milhões de pessoas acima dos 65 anos de idade em todo o mundo, podendo triplicar o número de casos até 2050 (Puglielli L. et al., 2003; Forman M.S. et al., 2004).

A DA é uma desordem neurodegenerativa progressiva associada com perda de memória e disfunção cognitiva, sendo considerada a principal causa de demência após os 60 anos de idade (Citron M., 2002; Parihar M.S. & Hemnani T., 2004). Foi primeiramente descrita pelo médico alemão Alois Alzheimer em 1907. Alzheimer estudou o cérebro *pos mortem* de uma paciente de 51 anos de idade que apresentava uma doença mental incomum, e observou uma profunda atrofia cerebral, uma morte neuronal massiva e alterações morfológicas características no tecido cerebral: as *placas senis*, que se acumulam extracelularmente, e os *emaranhados neurofibrilares*, intracelulares (Figura 1) (Sisodia S.S. & St. George-Hyslop P.H., 2002; Hamdane M. et al., 2003; Parihar M.S. & Hemnani T., 2004).

O principal componente das placas senis é o peptídeo β amilóide (Aβ), que possui de 40 a 42 aminoácidos e tem a capacidade de se autoagregar, formando filamentos cercados de neuritos deteriorados, microglia e astrócitos reativos (Selkoe D.J., 2001; Selkoe D.J. & Schenk D., 2003). Os emaranhados neurofibrilares são formados pela deposição da proteína *tau*, uma fosfoproteína constituinte da família das proteínas associadas a microtúbulos, que normalmente age como uma ponte nos microtúbulos. A proteína *tau* está altamente expressa no cérebro, essencialmente em neurônios (Parihar M.S. & Hemnani T., 2004). Na doença de

Alzheimer, a *tau* está hiperfosforilada, desprende-se dos microtúbulos, acumula-se no soma e desestrutura o citoesqueleto neuronal (Phiel C.J. et al., 2003).

Múltiplos fatores genéticos e ambientais parecem interagir no desenvolvimento da DA. Cerca de 1% a 2% dos casos deve-se a fatores genéticos, como mutações em genes específicos, representando os casos de origem familiar (Casserly I. & Topol E., 2004). A maior parte dos casos de DA (90% a 95%) é de início tardio e está relacionada a diversos fatores de risco, como o avanço da idade, o sexo feminino, o baixo nível educacional, o tabagismo, polimorfismo da apolipoproteína E (APOE) e doenças graves, como o diabete melito, hipertensão, isquemia cerebral e hipercolesterolemia (Naidu A. et al, 2002; Sinigaglia-Coimbra R. et al., 2002; Casserly I. & Topol E., 2004; Mattson M.P., 2004).
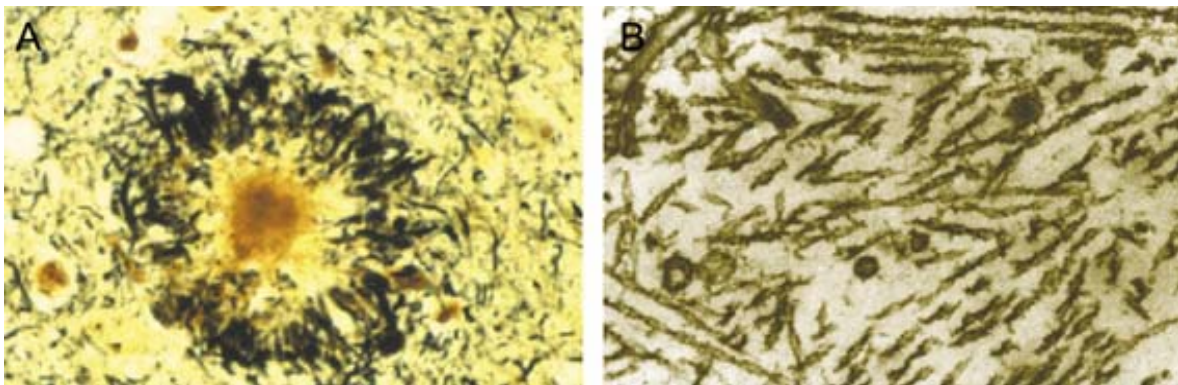
Figura 1. Alterações morfológicas características do tecido cerebral na DA. A: placas senis, extracelulares; B: emaranhados neurofibrilares, intracelulares (Adaptada de Sisodia S.S. & St. George-Hyslop P.H., 2002).

2. NEURODEGENERAÇÃO NA DOENÇA DE ALZHEIMER: principais hipóteses.

Apesar de passado quase um século desde sua primeira descrição, o passo que desencadeia a neurodegeneração na DA é ainda objeto de estudo. São três as principais hipóteses: 1) hipótese colinérgica, 2) hipótese dos emaranhados neurofibrilares, e 3) hipótese da cascata amilóide.

2.1. Hipótese colinérgica.

Os neurônios colinérgicos da região anterior cerebral, que fornece as principais fibras para o hipocampo e neocórtex, estão entre as células mais gravemente afetadas na DA (Mufson E.J. et al., 2002; Aucoin J.S. et al., 2005). Muitos estudos defendem a hipótese colinérgica (Bartus R.T., 2000; Auld D.S. et al., 2002), sugerindo que a redução de marcadores colinérgicos, como colina acetiltransferase, acetilcolinesterase e do neurotransmissor acetilcolina, é a responsável pela perda cognitiva e da memória no paciente na DA (Auld D.S. et al., 2002; Parihar M.S. & Hemnani T., 2004; Aucoin J.S. et al., 2005). Apesar do tratamento sintomático padrão atual para DA, leve à moderada, ser a administração de inibidores anticolinesterásicos, ele é limitado, e o déficit colinérgico não explica inteiramente as características neuropatológicas observadas na DA (Parihar M.S. & Hemnani T., 2004; Wu C.K. et al., 2005; Prohovnik I. et al., 2006;).

2.2. Hipótese dos emaranhados neurofibrilares.

A hipótese dos emaranhados neurofibrilares argumenta que a hiperfosforilação da proteína *tau* pode ser o "gatilho" inicial para a degeneração na DA. Sua principal função é manter os monômeros de tubulina unidos, conservando a estabilidade dos microtúbulos, que modulam a organização funcional do neurônio (Geula C. et al., 1998; Hamdane M. et al., 2003). Esta proteína, que está anormalmente fosforilada na DA, desprende-se dos microtúbulos, acumula-se no soma, forma filamentos intracelulares (emaranhados neurofibrilares), e leva à desorganização do citoesqueleto celular (Phiel C.J. et al., 2003). A hipótese dos emaranhados sugere que a neurodegeneração pode ocorrer não só pela perda de função da proteína *tau*, mas também pela toxicidade dos emaranhados formados (Trojanowski J.Q. & Lee V.M., 2005). No entanto, a disfunção da proteína *tau* e a formação dos emaranhados neurofibrilares estão presentes em outros tipos de enfermidades do sistema

nervoso central (SNC), denominadas *taupatias,* e não necessariamente aparecem em conjunto com as placas senis, características essenciais para o diagnóstico *post mortem* de DA.

2.3. Hipótese da cascata amilóide.

A hipótese mais aceita atualmente é a da cascata amilóide, que defende que o processo neurodegenerativo na DA é resultado de uma série de eventos desencadeados pelo acúmulo, agregação e toxicidade do peptídeo Aβ, iniciados com o processamento anormal da proteína precursora amilóide (PPA) (Figura 2) (St. George-Hyslop P.H. & Westaway D.A., 1999; Mattson M.P. & Chan S.L., 2003). A formação dos emaranhados neurofibrilares e o dano neuronal colinérgico também seria favorecida pelo peptídeo Aβ (Williamson R. et al., 2002; Fuentealba R.A. et al., 2004).

3. PLACAS SENIS, PEPTÍDEO β AMILÓIDE E SUA ORIGEM: hipótese da cascata amilóide.

As placas senis são alterações moleculares complexas e seu desenvolvimento temporal é parcialmente compreendido. O principal componente das placas senis é o Aβ, que possui 40 ou 42 aminoácidos. O peptídeo Aβ de 40 aminoácidos (Aβ1-40) é produzido fisiologicamente em pequenas quantidades com o envelhecimento, enquanto o Aβ de 42 aminoácidos (Aβ1-42) é produzido em excesso na DA. Ambos podem se agregar para formar placas amilóides, mas o peptídeo Aβ1-42 mostra uma maior tendência que o Aβ1-40, sendo o principal responsável pela formação das placas (Selkoe D.J. & Schenk D., 2003). O peptídeo Aβ1-42 é produzido através da clivagem anormal da PPA devido a causas genéticas e/ou ambientais. A PPA é uma proteína transmembrana, cuja função fisiológica ainda é alvo de estudo. Evidências têm sugerido um importante papel na regulação da sobrevivência

neuronal, crescimento neurítico, plasticidade sináptica e adesão celular (Mattson M.P. & Chan S.L., 2003).
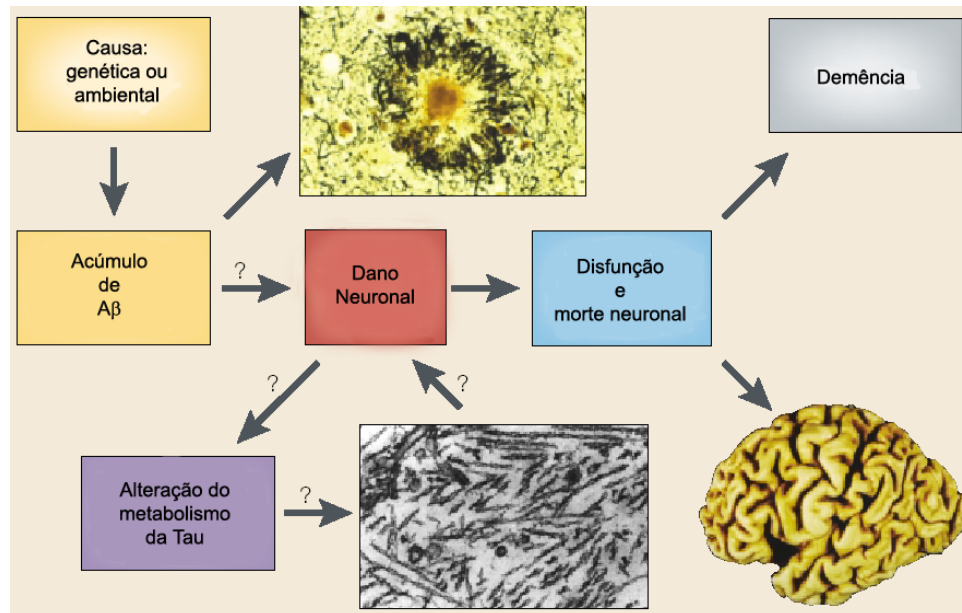
Figura 2. Esquema da hipótese da cascata amilóide. O acúmulo do peptídeo Aβ leva a uma série de eventos intracelulares que induzem ao dano neuronal, alteração do metabolismo da proteína *tau*, disfunção, morte neuronal e, por fim, à demência (Adaptada de Sisodia S.S. & St. George-Hyslop P.H., 2002).

As proteases que clivam a PPA são conhecidas como secretases. O processo normal envolve a atividade da enzima α-secretase. No processo anormal, que ocorre na DA, a atividade da enzima β-secretase ou *β-site APP-cliving enzyme* (BACE) é favorecida, seguida pela ação da γ-secretase, resultando na secreção do peptídeo Aβ1-40 ou Aβ1-42. O peptídeo formado depende da posição a PPA é clivada pela enzima γ-secretase. Se clivada no aminoácido de posição 711, é gerado o peptídeo Aβ1-40 e, se clivada no aminoácido de posição 713, é formado o peptídeo amiloidogênico e tóxico Aβ1-42 (Figura 3) (LaFerla F.M., 2002). As causas do processo proteolítico alterado da PPA e da deposição de Aβ ainda não estão completamente esclarecidas, mas podem incluir o aumento do estresse oxidativo decorrente do envelhecimento, danos no metabolismo energético, perda da homeostase iônica

celular e possíveis mutações no gene da PPA, da BACE, e das presinilinas (PSs) 1 e 2, proteínas envolvidas na atividade da enzima γ-secretase (St. George-Hyslop P.H. & Westaway D.A., 1999; Kimberly W.T. et al., 2000; Mattson M.P., 2004).
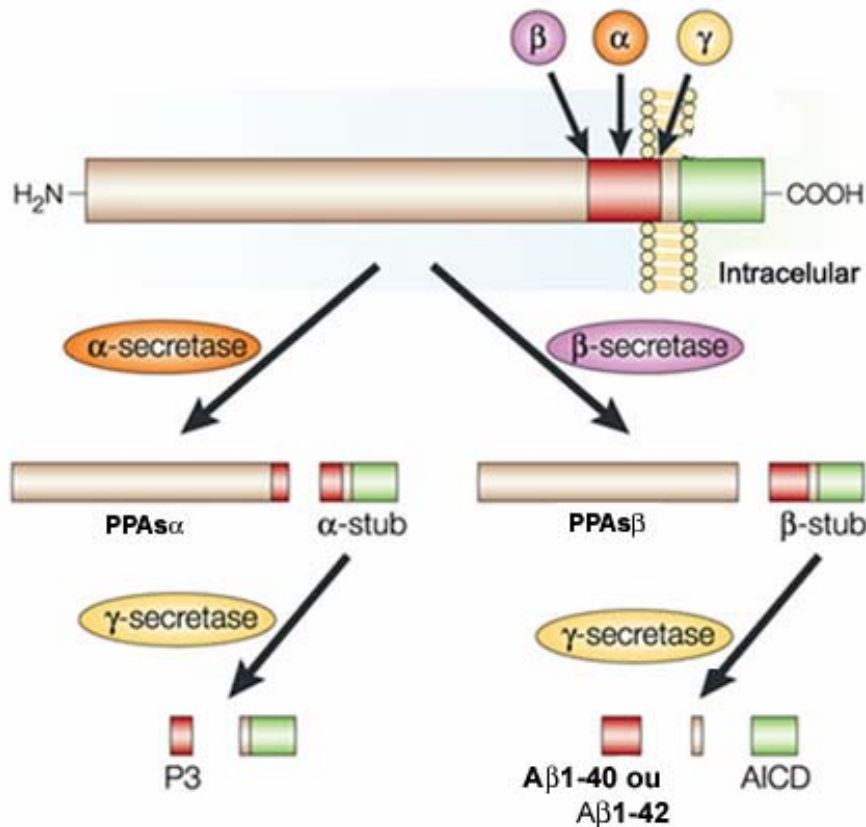
Figura 3. Esquema do processo proteolítico normal (à direita) e anormal (à esquerda) da proteína precursora amilóide (PPA) e seus principais produtos, entre eles o Aβ1-42. (Adaptada de LaFerla F.M., 2002).

Estas mutações parecem estar relacionadas especialmente com a DA de origem familiar, aumentando a proporção do peptídeo Aβ1-42 em relação ao peptídeo Aβ1-40. A primeira mutação identificada está no gene da PPA, localizado no cromossomo 21. O cromossomo 21 era visto como provável local das mutações causadoras da DA por duas razões: (1) é o local do gene da PPA; e, (2) em pessoas com síndrome de Down (trissomia do cromossomo 21), os sinais patológicos da doença se desenvolvem precocemente (Cummings J.L. et al., 1998). O fato de essas mutações aumentarem a secreção de Aβ1-42 e levarem ao

aparecimento precoce da DA fornece forte evidência à hipótese da cascata amilóide (Small D.H. et al., 2001; Hardy J. & Selkoe D.J., 2002).

A hipótese da cascata amilóide tem recebido considerável sustentação de estudos moleculares e genéticos, como o uso de animais transgênicos, com genes envolvidos na DA familiar super-expressos (Spires T.L. & Hyman B.T., 2005), além de estudos com imunização anti-Aβ, que relacionam a destruição das placas senis à diminuição do déficit da memória (Dodart J.C. et al., 2002; Schenk D., 2004; Buttini M. et al., 2005; Frenkel D. et al., 2005). Um recente estudo (Hardy J., 2006) mostra que alguns casos de DA de início precoce resultam da duplicação do lócus que codifica a PPA, fornecendo ainda mais credibilidade à hipótese da cascata amilóide.

4. MODELOS EXPERIMENTAIS

O entendimento do mecanismo neurotóxico induzido pelo peptídeo Aβ que parece iniciar a neurodegeneração na DA é essencial para o desenvolvimento de agentes terapêuticos capazes de evitar, retardar e até retroceder o processo degenerativo observado. Com o objetivo de elucidar esses efeitos, são utilizados modelos experimentais em animais *in vivo*, transgênicos ou não, e *in vitro*, com cultivo de células ou tecidos.

4.1. Animais transgênicos.

Os animais transgênicos empregados na pesquisa da DA são principalmente ratos e camundongos que superexpressam os genes envolvidos na doença de origem familiar: os genes da PPA, da BACE e das PSs. A superexpressão desses genes acelera a produção do peptídeo Aβ, induz neurodegeneração progressiva e, em alguns casos, gera fenótipos similares àqueles vistos em pacientes com DA (Gordon M.N. et al., 2002; Gotz J. et al., 2004;

Spires T.L. & Hyman B.T., 2005). Já os modelos com animais *knockout* para essas proteínas foram gerados para explorar as funções nativas destes genes (Spires T.L. & Hyman B.T., 2005). Embora nenhum dos modelos transgênicos tenha sido capaz de reproduzir exatamente a condição humana na DA, a habilidade de estudar processos patológicos similares em animais vivos forneceu respostas sobre os mecanismos da doença, especialmente referentes à hipótese da cascata amilóide (Spires T.L. & Hyman B.T., 2005).

4.2. Injeção intracerebral de peptídeos β-amilóide sintéticos.

A injeção intracerebral de peptídeos sintéticos tem sido utilizada como um modelo para observação dos aspectos comportamentais e eletrofisiológicos induzidos pela administração do peptídeo Aβ, além de um modelo para o teste de substâncias possivelmente terapêuticas. São administrados o peptídeo Aβ1-40 (Minogue A.M. et al., 2003), Aβ1-42 (Boyd-Kimball D. et al., 2005) e também fragmentos deste, como o peptídeo Aβ contendo os aminoácidos de 25 a 35 (Aβ25-35) (Freir D.B. et al., 2003; Hervas-Aguilar A. et al., 2005; Stepanichev M.Y., et al., 2006).

O fragmento Aβ25-35 corresponde a um fragmento de 11 aminoácidos dos peptídeos Aβ1-40/Aβ1-42 que tem sido amplamente utilizado na pesquisa como uma alternativa em modelos *in vitro* e *in vivo* de indução de toxicidade. O fragmento Aβ25-35 parece reter a capacidade de formar fibrilas e de induzir toxicidade como o peptídeo original, possuindo efeitos similares (Freir D.B. et al., 2003; Casal C. et al., 2004; Giunta S. et al., 2004; Hashioka S. et al., 2005; Hervas-Aguilar A. et al., 2005; Stepanichev M.Y. et al., 2006).

4.3. Cultivo primário de células.

Os modelos de cultivo primário envolvem diversos tipos celulares, como neurônios corticais e cerebelares (Dall'Igna O.P. et al., 2003; Movsesyan V.A. et al., 2004; Vaudry D. et

al., 2004; Inestrosa N.C. et al., 2005; Lee B.Y. et al., 2005; Liu M.L. & Hong S.T., 2005; Quintanilla R.A. et al., 2005), astrócitos corticais (Hu J. & Van Eldik L.J., 1999; Abe K. et al., 2003; Rodriguez-Kern A. et al., 2003; Monnerie H. et al., 2005), microglia (Nakai M. et al., 1999; Wu S.Z. et al., 2004; Hashioka S. et al., 2005), oligodendrócitos (Zeng C. et al., 2005), ou linhagens celulares, como neuroblastoma (Tamagno E. et al., 2003), e PC12 (Martín D. et al., 2001; Jang J-H & Surh Y.-J., 2005).

Apesar da facilidade em trabalhar com cultivo primário de células e linhagens celulares, os efeitos induzidos pela exposição ao peptídeo Aβ não são completos, já que demonstram a resposta em um conjunto de células isoladas, enquanto que o SNC compreende vários tipos celulares que interagem.

4.4. Cultivo de tecidos cerebrais.

Uma alternativa para o modelo *in vitro* de estudos moleculares é o cultivo de tecidos, em que a multiplicidade celular original do tecido cerebral e as conexões intraneuronais são mantidas (Holopainen I.E., 2005; Noraberg J. et al., 2005). Nestes estudos, são utilizadas principalmente as culturas organotípicas de hipocampo (Baskys A. & Adamchik Y., 2001; Dineley K.T. et al., 2001; Vincent V.A. et al., 2002; Holopainen I.E., 2005), devido à importância do hipocampo na memória e cognição, especialmente afetadas na DA.

5. MECANISMOS DE TOXICIDADE DO PEPTÍDEO β-AMILÓIDE

Com o auxílio dos modelos experimentais *in vivo* e *in vitro*, foram identificados numerosos processos bioquímicos e moleculares modificados pelo peptídeo Aβ, incluindo:
- Secreção excessiva de mediadores inflamatórios (Vincent V.A. et al., 2002; Brown G.C. & Bal-Price A., 2003; White J.A. et al., 2005);

- Produção de radicais livres de oxigênio (Tamagno E. et al., 2003; Abramov A.Y. et al., 2004; Behl, C., 2005; Moreira P.I. et al., 2005) e nitrogênio (Torreilles, F. et al., 1999; Jang J-H & Surh Y.-J., 2005; Zeng C. et al., 2005);

- Alteração na secreção e recaptação do aminoácido excitatório glutamato, favorecendo a excitotoxicidade (Harris M.E., 1996; Danysz W. & Parsons C.G., 2003; Francis P.T., 2003);

- Acúmulo de cálcio intracelular (Mattson M.P. & Chan S.L., 2003; Abramov A.Y. et al., 2004; Mattson M.P., 2004), ativando uma série de cascatas de morte celular (Orrenius S. et al., 2003);

- Favorecimento da hiperfosforilação da proteína *tau*, alterando o citoesqueleto celular (Williamson R. et al., 2002; Ávila J. et al., 2004; Fuentealba R.A. et al., 2004);

- Desequilíbrio no metabolismo energético celular, com dano mitocondrial (Byrne E., 2002; Harman D., 2002; Reddy P.H. & Beal M.F., 2005);

- Alteração na expressão e atividade de proteínas envolvidas em cascatas de morte celular apoptótica, como Akt (Martín D. et al., 2001; Ryder J. et al., 2004), GSK-3β (Ryder J. et al., 2004; Inestrosa N. et al., 2005), caspase-3 (Tamagno E. et al., 2003; Vaudry D. et al., 2004; Fifre A. et al., 2006).

Esses eventos induzidos pelo peptídeo Aβ podem aumentar diretamente a vulnerabilidade dos neurônios à morte celular, e também indiretamente através da ativação das células gliais (astrócitos e microglia, especialmente) ao estimular a secreção de substâncias tóxicas (Small D.H. et al., 2001).

6. SINALIZAÇÃO INTRACELULAR envolvida no mecanismo de toxicidade do peptídeo β amilóide.

6.1. Oxido nítrico sintase induzível (iNOS).

O óxido nítrico (NO) é um radical livre de nitrogênio que possui efeitos fisiológicos como modulador sináptico e vasodilatador (Rang H.P. et al., 2004). Ele é produzido a partir do aminoácido L-arginina pela ação de três isoformas da enzima óxido nítrico sintase (NOS): a NO sintase neuronal (nNOS), a NO sintase endotelial (eNOS) e a NO sintase induzível (iNOS), todas expressas no tecido cerebral (Parihar M.S. & Hemnani T., 2004; Jang J.H. & Surh Y.-J., 2005). As duas primeiras enzimas são constitutivas e dependentes de cálcio, enquanto a iNOS é independente de cálcio e sua síntese é induzida por diversos fatores, entre eles o peptídeo Aβ, estando super-expressa nas células gliais constituintes das placas senis (Fernandez-Vizarra P. et al., 2004; Duncan A.J. & Heales S.J., 2005; Jang J.H. & Surh Y.-J., 2005). O NO formado em excesso é considerado um potente mediador pró-inflamatório (Jang J.H. & Surh Y.-J., 2005) e modulador pró-apoptótico, através da ativação de caspases (Choi B.M. et al., 2002). O NO pode ainda combinar-se com o ânion superóxido (O_2^-), subproduto da cadeia respiratória mitocondrial, produzindo o peroxinitrito ($ONOO^-$) (Choi B.M. et al., 2002; Parihar M.S. & Hemnani T., 2004), potente agente pró-oxidante. Evidências sugerem que o $ONOO^-$ pode promover fragmentação oxidativa do DNA e peroxidação lipídica, contribuindo para o dano neuronal característico da DA (Choi B.M. et al., 2002; Fernandez-Vizarra P. et al., 2004).

6.2. Glicogênio sintase cinase-3β (GSK-3β).

A enzima glicogênio sintase cinase-3β (GSK-3β) foi originalmente identificada como um modulador do metabolismo do glicogênio (Wagman A.S. et al., 2004). Atualmente se sabe do importante papel regulatório dessa enzima em uma variedade de vias intracelulares, incluindo iniciação da síntese de proteínas, proliferação e diferenciação celular, desenvolvimento embrionário, apoptose e hiperfosforilação da proteína *tau* (Frame S. & Cohen P., et al., 2001; Wagman A.S. et al., 2004; Mulholland D.J. et al., 2006). A GSK-3β é

considerada uma enzima pró-apoptótica por inibir a ativação de uma variedade de fatores de transcrição importantes para sobrevivência celular (Li X. et al., 2002). Além de seu envolvimento na apoptose, de grande interesse na pesquisa da DA, ela é uma das principais cinases envolvidas na hiperfosforilação da proteína *tau* (Fuentealba R.A. et al., 2004; Mateo I. et al., 2006).

A GSK-3β é constitutivamente ativa, mas pode ser ativada por aumento transitório de cálcio intracelular (Hartigan J.A. & Johnson G.V. et al., 1999), e inibida pelas vias de sinalização da Wnt/β-catenina (Li X. et al., 2002; Fuentealba R.A. et al., 2004;), e através da ativação da cascata fosfatidilinositol 3-cinase/Akt (PI-3K/Akt) (Cross D.A. et al., 2001) (Figura 4).
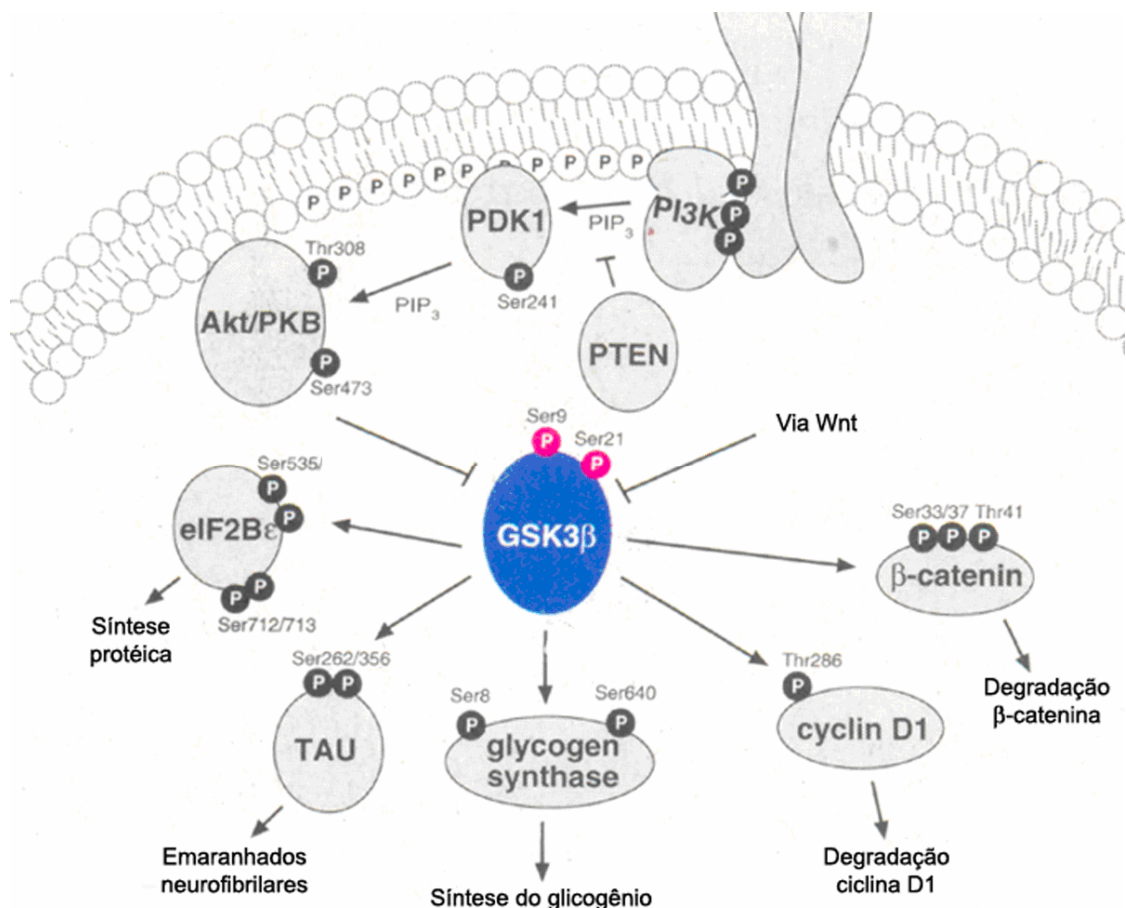
Figura 4. Esquema da via de sinalização da proteína glicogênio sintase cinase-3β (GSK-3β) (Adaptada do Catálogo da Cell Signalling Tecnology® 2005-06, página 122)**.**

6.3. Proteína cinase B (Akt/PKB).

Akt, uma cinase de serina e treonina, também denominada proteína cinase B (PKB), está altamente expressa no SNC, constituindo um promotor de sobrevivência e neuroproteção em neurônios embrionários (Brunet A. et al., 2001). A via de sinalização PI3-K/Akt possui um importante papel na sobrevivência celular em vários tipos neuronais (Hajduch E. et al., 2001; Lawlor M.A. & Alessi D.R., 2001; Kim D. & Chung J. , 2002). A Akt é ativada por insulina e vários fatores de crescimento, por intermédio da PI3-K (Brunet A. et al., 2001). Quando ativa, a Akt inibe a apoptose por fosforilar e inativar uma variedade de substratos pertencentes à maquinaria celular apoptótica, tais como a BAD - proteína pró-apoptótica pertencente à família das Bcl-2 (oncogene da célula B) (Downward J., 1999), GSK-3β (Cross D.A. et al., 2001), caspase-9, e a família de fatores da transcrição em forquilha (*Forkhead*, FKHR) (Brunet A. et al., 1999) (Figura 5). Apesar da relevância desse sistema defensivo celular, o papel da via PI3-K/Akt ainda foi pouco estudada na DA (Martín D. et al., 2001).

A Akt é ativada por fosforilação por intermédio de duas cinases dependentes de fosfoinositóis, as PDKs1 e 2 (Chan T.O. et al., 1999). Os principais reguladores negativos da Akt são duas fosfatases: a fosfatase da PI3-K (*phosphatase and tensin homolog deleted on chromosome 10*, PTEN) (Leslie N.R. & Downes C.P., 2002) e a proteína fosfatase 2A (PP2A), que diretamente a desfosforila e inativa (Millward T.A. et al., 1999; Resjo S., et al., 2002; Ugi S. et al., 2004) (Figura 5).
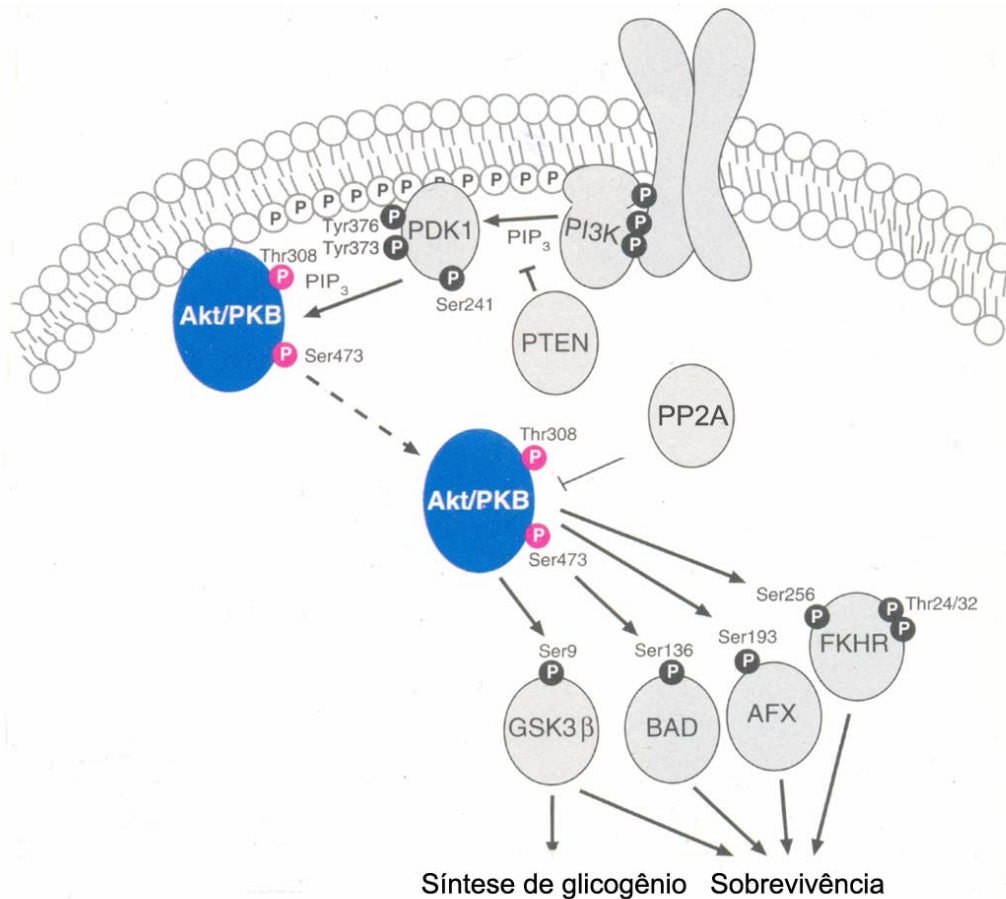
Figura 5. Esquema da via de sinalização da proteína cinase B (Akt/PKB) (Adaptada do Catálogo da Cell Signalling Tecnology® 2005-06, página 110)**.**

6.4. Fosfatase da proteína PI3-K (*phosphatase and tensin homolog deleted on chromosome 10*, PTEN).

A a fosfatase da PI3-K (*phosphatase and tensin homolog deleted on chromosome 10*, PTEN) é uma fosfatase multifuncional tendo como substratos lipídeos e proteínas (Chandrasekar B. et al., 2006). É o principal regulador negativo da via PI3k/Akt por antagonizar a atividade cinase da PI3-K (Figura 6). A PTEN age removendo diretamente fosfatos da molécula fosfatidilinositol 3, 4, 5-trifosfato (PIP3), formando PIP2 (Leslie N.R. & Downes C.P., 2002; Chow L.M.L. & Baker S.J., 2006). Originalmente foi descrita como um gene supressor de tumor, estando implicada em vários tipos de cânceres humanos

(Mulholland D.J. et al., 2006). Na DA, sua expressão e distribuição celular parecem estar alteradas, mas ainda com resultados controversos entre os estudos (Griffin R.J. et al., 2005).
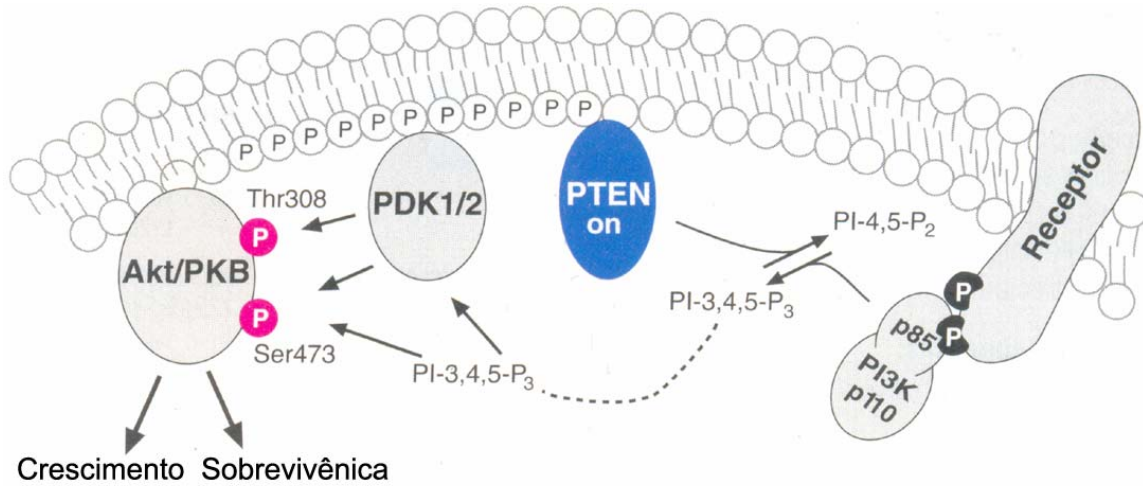
Figura 6. Esquema da alteração da fosfatase PTEN na via de sinalização da PI3-K (Adaptada do Catálogo da Cell Signalling Tecnology® 2005-06, página 127)**.**

OBJETIVOS

O objetivo geral deste trabalho foi:

- Desenvolvimento do modelo *in vitro* de toxicidade induzida pelos peptídeos Aβ1-42 e pelo fragmento de 25-35 aminoácidos (Aβ25-35) em cultura organotípica de hipocampo de ratos com a finalidade de estudar o mecanismo geral envolvido.

Objetivos específicos:

- Estabelecer uma curva de tempo de indução de morte celular pelo peptídeo Aβ1-42 e pelo fragmento Aβ25-35 em cultura organotípica de hipocampo de ratos;

- Avaliação do efeito do peptídeo Aβ1-42 sobre o imunoconteúdo da proteína iNOS e sobre o estado de fosforilação da proteína GSK-3β;

- Investigação da sinalização celular envolvida na toxicidade induzida pelo fragmento Aβ25-35, focando a via da proteína Akt, GSK-3β e PTEN.

PARTE II

CAPÍTULO I

Artigo científico submetido ao periódico

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Dear Christianne Salbego, PhD:

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Aβ$_{1-42}$ Neurotoxicity on Organotypic Hippocampal Slice Culture and Involvement of iNOS and GSK-3β proteins

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Running title: Aβ$_{1-42}$ Neurotoxicity and Involvement of iNOS and GSK-3β proteins

SUMMARY

1. Using organotypic hippocampal slice culture, we conducted the present study to investigate: (a) the neurotoxicity induced by peptide beta-amyloid ($A\beta_{1-42}$) in different concentrations (10 or 20 μM) and periods of treatment (24 or 72 h); and (b) the possible involvement of inducible nitric oxide synthase (iNOS) and glycogen synthase kinase-3β (GSK-3β) proteins.

2. In slice cultures stained with propidium iodide (PI), we could observe that the toxicity occurred only after 72 h of $A\beta_{1-42}$-exposition in both doses. Immunoblotting analysis revealed an increase of iNOS protein immunocontent only after 72 h treatment with 20 μM of $A\beta_{1-42}$. On the other hand, no significant changes in the phosphorylation or immunocontent of GSK-3β were detected.

3. These data suggest that, *in vitro*, altered iNOS expression might be involved in the $A\beta_{1-42}$ mechanism of neurotoxicity in hippocampus, but there was not found a relation with the GSK-3β phosphorylation.

Keywords: $A\beta_{1-42}$, Alzheimer's disease, organotypic culture, iNOS, GSK-3β

INTRODUCTION

Alzheimer's disease (AD) is the most prevalent of the neurodegenerative disorders correlated with advancing age that affects the cognitive function of the brain (Forman, M.S., et al., 2004). Pathological changes in AD are characterized by the formations of amyloid plaques, in which the main constituents are the aggregated 40- to 42-amino acid beta-amyloid peptides (Aβs) cleaved from the amyloid precursor protein (Selkoe, D.J., 2001). The longer peptide (Aβ$_{1-42}$) is more abundant in AD brain than in controls, and is correlated to a variety of toxic effect on cerebral cells (Dineley, K.T. et al., 2001; Molnar, Z. et al. 2004).

The precise mechanism by which Aβ induces its toxicity is still unclear, but there are a number of possible mechanisms including oxidative stress, excitotoxicity, energy depletion, inflammation and apoptosis (Parihar, M.S., and Hemnani, T., 2004). Aβ$_{1-42}$ peptide can activate a variety of cell signaling events leading to accumulation of oxygen (Behl, C., 2005; Martin, D., et al., 2001) and nitrogen free radicals (Torreilles, F. et al., 1999), rise in cytosolic calcium (Mattson, M.P., 2004) or increase in phosphorylated forms of the microtubule-associated protein tau (Ávila, J. et al., 2004, Fuentealba, R.A. et al., 2004).

In order to investigate a possible Aβ$_{1-42}$ mechanism of cellular death in organotypic slices culture we studied the relation between inducible nitric oxide synthase (iNOS) expression and glycogen synthase kinase-3β (GSK-3β) phosphorylation/inactivation. The GSK-3β protein is involved in pro-apoptotics pathways and in the tau protein hiperphosphorylation (Fuentealba, R.A. et al., 2004). Recent studies suggest an involvement between the GSK-3β, by the Wnt/β-catenina pathway, and the increased expression of iNOS (Takahashi, M. and Wakabayashi, K., 2004), enzyme associated with inflammation and nitric oxide (NO) overproduction (Torreilles, F. et al., 1999).

METHODS

Organotypic hippocampal slice culture

Organotypic hippocampal slice cultures were prepared using the method of Stoppini, L., et al. (1991). Male Wistar rats (6-8-day old) were used (according to procedures approved by the Local Committee of Animal Care). Hippocampal sections (400 μm) were prepared and transferred to ice-cold Hank's balanced salt solution (HBSS). The slices were placed on one Millicell culture insert (Millicell®-CM, 0.4 μm) and the inserts were transferred to a 6-well culture plate. Each well contained 1 mL of culture medium consisting of 50% minimum essential medium, 25% heat inactivated horse serum and 25% HBSS, supplemented with (mM, final concentration): glucose 36, HEPES 25 and $NaHCO_3$ 4; fungizone (1%) and gentamicine (36 μL/100 mL); pH 7.3. Cultures were kept at 37 ºC in an atmosphere of 5% CO_2. Culture medium was replenished every 3 days.

Aβ peptide preparation and culture treatments

Synthetic rat $Aβ_{1-42}$ was purchased from Calbiochem. $Aβ_{1-42}$ stock solutions were prepared at 100 μM in 100 mM HEPES, pH 8.5, aliquoted, and stored frozen (Dineley, K.T. et al., 2001). On the 14 day *in vitro*, the last medium change was performed and the slices were exposed to 10 or 20 μM concentrations of $Aβ_{1-42}$ for 24 or 72 hours.

Quantification of cell death

Two hours before the end of the treatments, cultures received 7.5 μM propidium iodide (PI, from Sigma) for the cellular viability evaluation by fluorescent image analysis. PI is excluded from healthy cells, but following loss of membrane integrity it enters cells, binds to DNA and becomes highly fluorescent (Noraberg, J., et al., 1999). 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).

Western blotting assay

After obtaining the fluorescent images, the 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, A. et al., 2001). Samples containing 35 μg of protein were resolved by 12% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). After electrophoresis, proteins were electrotransferred to nitrocellulose membranes using a semi-dry apparatus (Bio-Rad Trans-Blot SD). The membranes were blocked overnight with 5% powdered milk in Tris-buffered saline plus 0.1% Tween-20, followed by incubation at 4 ℃ with anti-iNOS (1:400, Santa Cruz), anti-phosphospecific GSK-3β or anti-GSK-3β antibodies, (1:1000, Cell Signaling) diluted in the same blocking solution. Subsequently, the membranes were incubated for 2 h with the horseradish peroxidase-conjugated anti-rabbit antibody (Cell Signaling) also diluted in the same blocking solution (1:1000). Immunoreactive bands were revealed by an enhanced chemioluminescence kit (ECL, Amershan Pharmacia), being detected using X-ray films. The immunoblot films were scanned and the digitized images analyzed with Optiquant software (Packard Instrument). For each experiment, the test groups were referred to control cultures not exposed to Aβ$_{1-42}$ peptide, which were considered 100%, thus assuring the same signal intensity for control and test groups.

STATISTICAL ANALYSIS

Comparisons between experimental groups were by one-way ANOVA followed by Tukey's multiple range test. All data are presented as mean±S.E.M.

RESULTS

The exposure of the cultures to 10 and 20 µM Aβ$_{1-42}$ peptide after 72 h caused a marked fluorescence in the hippocampus, indicating a high incorporation of PI, as shown in the photomicrograph in figure 1A. Quantification of PI fluorescence showed that exposition peptide 10 µM Aβ$_{1-42}$ caused about 33% of damage in hippocampus cells, and the 20 µM, about 60% a significant increase compared to control cultures (figure 1B). After 24 h of peptide exposition, there was no statistical difference, in none of the doses.

In order to investigate a possible mechanism of cellular death induced by Aβ$_{1-42}$, we examined the expression of iNOS protein. After 72 h of 20 µM Aβ$_{1-42}$ treatment, it was observed a significant increase of iNOS immunocontent, about 166% compared with the control slices (figure 2, A and B). In the 10 µM dose, there was not significant difference, as well as after 24 h of exposition. We did not observe different on the phosphorylation and immunocontent of GSK-3β protein.

DISCUSSION

AD is the most common form of dementia in the elderly people, affecting nearly 2% of the population in the industrialized countries (Mattson, M.P., 2004). Genetic, molecular, and cell biology evidence support a role for the Aβ of 40 and 42 amino acids in the pathogenesis of AD. In common with others studies (Rodriguez-Kern, A., 2004, Hirata, K et al., 2005) in this work we found that the Aβ$_{1-42}$ neurotoxic effects were observed in micromolar concentrations. The peptide post-mortem concentrations found in AD patients are generally about nanomolar (Neve, R.L. et al., 2000), and this discrepancy can be explained by the existence of other important risk factors: the apolipoprotein E polymorphism (reviewed by Herz, J., and Beffert, U., 2000), amyloid precursor protein and presenilins mutations, and chronic diseases, as hypercholesterolaemia, diabetes mellitus, hypertension (Casserly, I., and

Topol, E, 2004). We suggest due the same reasons, only after a longer period of exposition (72 h) the peptide was toxic.

Various mechanisms have been proposed to explain the pathway by which Aβ induces neuronal cell death, including intracellular calcium accumulation, reactive oxygen species and nitric oxide productions, decreased membrane fluidity, alteration of the cytoskeleton and nucleus, redox-active iron, inflammatory or autoimmune processes and increased sensitivity along an apoptosis–necrosis continuum (Mattson, M.P., 2004).

Exploring an Aβ$_{1-42}$ neurotoxic mechanism, we evaluated the iNOS immunocontent, which showed very increased after 72 h of 20 μm of Aβ$_{1-42}$. The role of iNOS on neurodegenerative diseases such as AD is suggested by the over production of NO, involved with inflammation and also can combine with superoxide anion to form peroxynitrite. NO and peroxynitrite include a variety of neurotoxic mechanism and these mechanisms are likely to be involved in the cell death and memory impairments observed in AD (reviewed by Parihar, M.S., and Hemnani, T., 2004).

We investigated a possible relation between the increased iNOS expression and GSK-3β phosphorylation. Takahashi, M. and Wakabayashi, K. (2004) showed an involvement between the GSK-3β, by the Wnt/β-catenina pathway, and the increased expression of iNOS protein in malignant cells. In our work, the rise in the iNOS immunocontent had not been followed by GSK-3β phosphorylation/inactivation. The iNOS expression increase could be stimulated by other pathways or directly by the Aβ$_{1-42}$, since its increase was observed when the peptide was exposed for a higher concentration (20 μM) and a larger period (72 h).

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Figure 1: Effect of $A\beta_{1-42}$ treatment on cellular death in organotypic hippocampal cultures. (A) Representative pictures of slices stained with PI after 24 and 72 h after peptide 10 and 20 µM exposition. (B) Quantitative analysis of hippocampus damage 24 and 72 h after exposure to 10 and 20 µM of $A\beta_{1-42}$. White columns, control slices; gray columns, slices treated with 10 µM of $A\beta_{1-42}$; and black columns, slices treated with 20 µM of $A\beta_{1-42}$. 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 hippocampus area to obtain the percentage of damage. Bars represent the mean ± S.E.M., n=6. *** Significantly different from control cultures, p<0.001. [#] Significantly different from 10 µM of $A\beta_{1-42}$, p<0.05 (One-way ANOVA followed by Tukey's, p<0.05).

Figure 2: Effect of Aβ$_{1-42}$ treatment in organotypic slices culture on the iNOS protein immunocontent. (A) Representative Western blots of the iNOS protein in rat organotypic hippocampal slice cultures treated with vehicle (control) or exposed with 10μM or 20μM of Aβ$_{1-42}$. (B) Histograms represent the quantitative Western blot analysis of iNOS immunocontent. The densitometric values obtained to iNOS from all treatments were first normalized to their respective vehicle-treated control (100%). Equal amounts of protein samples were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and immunoblotting with specific iNOS antibody. Bars represent the mean ± S.E.M., n=6. * Significantly different from control cultures, $p < 0.05$ (One-way ANOVA followed by Tukey's, $p < 0.05$).

Figure 3: Effect of Aβ$_{1-42}$ treatment in organotypic slices culture on the ratio of phosphorylated/immunocontent GSK-3β protein. (A) Representative Western blots of phospho-GSK-3β and total GSK-3β protein in different treatments. (B) 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 (100%). Equal amounts of protein samples were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and immunoblotting with specific antibody. Bars represent the mean ± S.E.M., n=6.
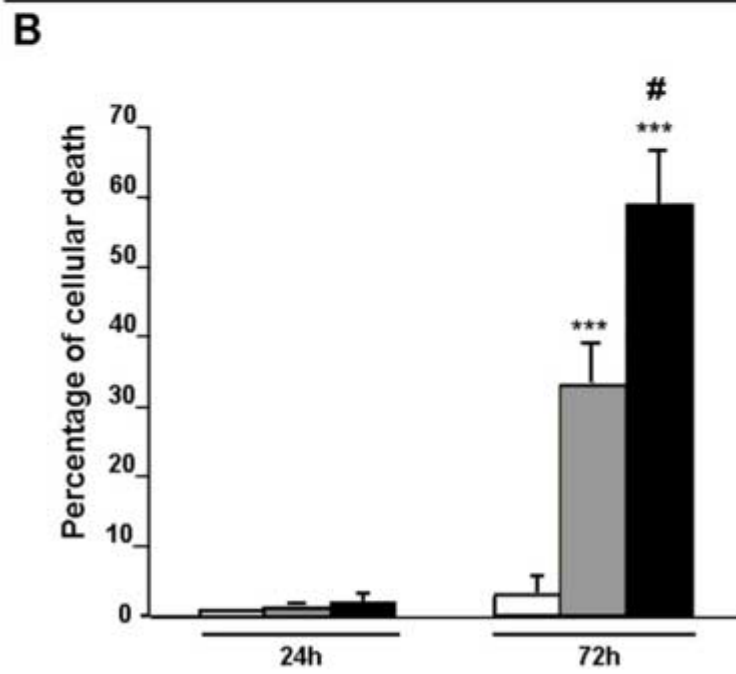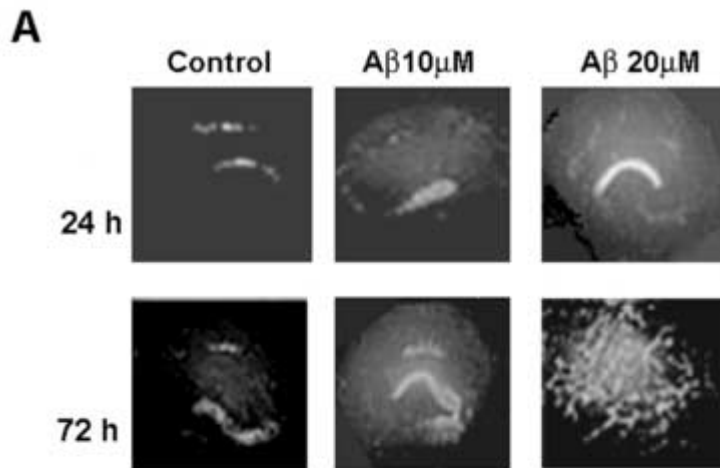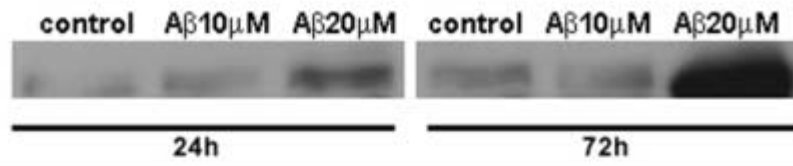
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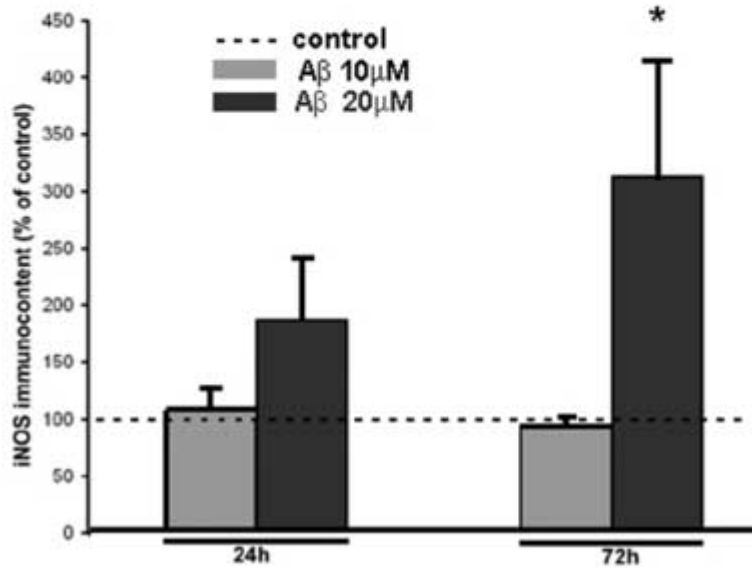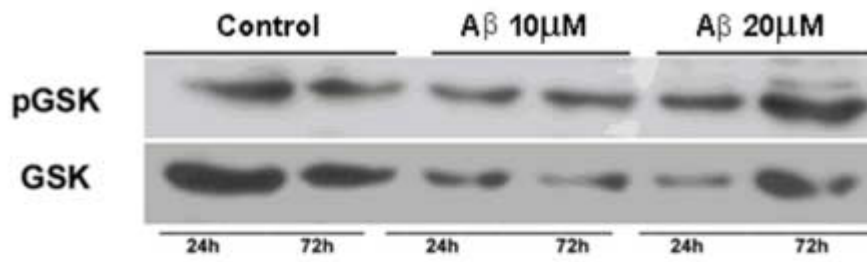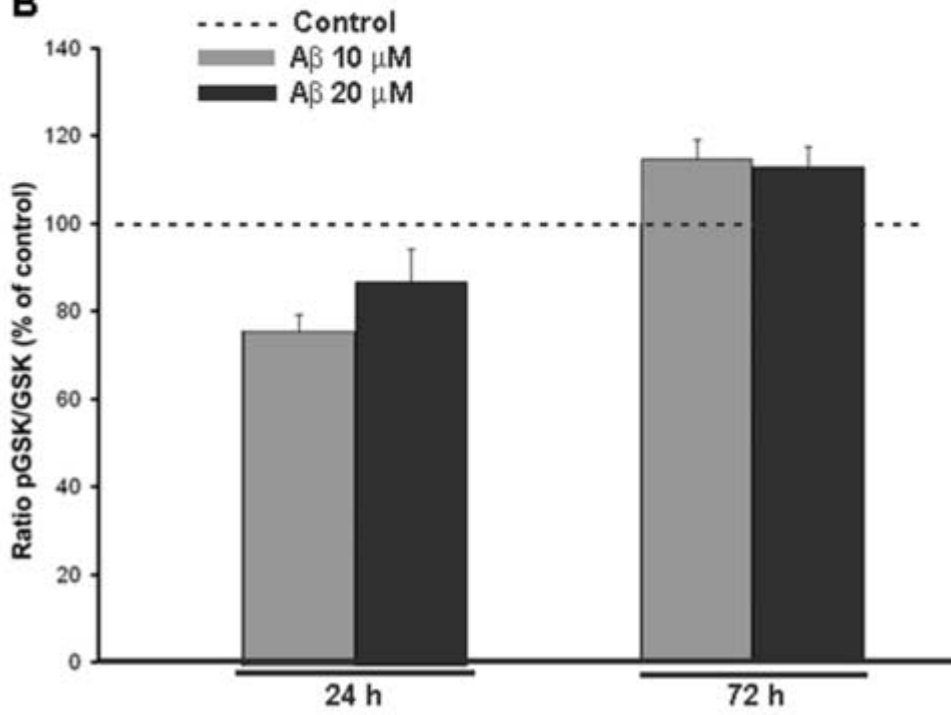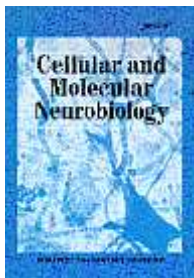
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40

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41

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Contribution to a Book
Cotman, C. W., and Hamberger, A. (1977). Glutamate as a CNS transmitter: properties of release, inactivation and biosynthesis. In Fonnum, F. (ed.), Amino Acids as Chemical Transmitters, Plenum Press, New York, pp. 379−412.

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CAPÍTULO II

Artigo científico a ser submetido ao periódico

Akt/PKB, GSK-3β, and PTEN involvement in β-amyloid peptide toxicity in organotypic hippocampal slice culture

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Running title: Aβ25-35 Toxicity: involvement of Akt and PTEN proteins.

Abstract

Alzheimer's disease is an irreversible neurodegenerative disorder associated with progressive cognitive and memory loss. Genetic and molecular evidences support a role for peptide β-amyloid (Aβ) in the pathogenesis of Alzheimer's disease. In the present study we investigated the toxicity induced by the exposition of organotypic slice culture to Aβ25-25 (25 μM) for 1, 3, 6, 12, 24 and 48 h. Also, we investigated the involvement of PI3-K pathway proteins Akt/PKB, GSK-3β, and PTEN on the toxicity induced by the peptide. Cellular death was quantified by the propidium iodide uptake and the proteins quantified by immunoblotting. Our results had shown a significant cellular death after a 48-h Aβ25-35 peptide exposition. The treatment with the peptide resulted to the increase in the phosphorylation state of the Akt and GSK-3β proteins after a 6-h treatment. After 12 h of treatment, we observed a reduction in the phosphorylation state of both the proteins. However, after 24 h of treatment, the phosphorylation of Akt protein continues low, while the phosphorylation of GSK-3β protein presents a new peak. The immunocontent of the PTEN protein presented a significant increase after 24 h and 48 h. The results of the present study suggest the involvement of Akt dephosphorylation/inactivation in the toxicity induced by the Aβ25-35 peptide in organotypic slice hippocampal culture, probably induced by an increase of PTEN immunocontent, main negative regulator of PI3-K/Akt pathway. Taken together, our results provide important leads for pursuing a more complete understanding of the molecular events of Aβ peptide.

Keywords: Alzheimer's disease, β-amyloid, organotypic culture, Akt, GSK-3β, PTEN

Alzheimer's disease (AD) is a progressive neurodegenerative disorder that currently affects nearly 2% of the population of industrialized countries (Mattson, 2004). The risk of AD dramatically increases in individuals beyond the age of 70 and it is predicted that the incidence of AD will increase three–fold within the next 50 years (Forman et al., 2004). The main pathological features of AD are massive neuronal loss, intracellular tangles, and extracellular senile plaque, which major constituent is the β-amyloid (Aβ), a peptide composed of 39-43 amino acid residues (Selkoe, 1999). These Aβ peptide depositions have been implicated in neuronal and vascular degeneration, potentially contributing to progressive dementia (Selkoe, 2000). The 11-residue amyloid β 25-35 fragment (Aβ25-35) retains activities of the full-length peptide rapidly forming fibrillar aggregates highly cytotoxic to neuronal cells (Yankner et al., 1990; D'Ursi et al., 2004; Giunta et al., 2004), being widely used in both in vitro and in vivo neuroscience researches.

The precise mechanism by which Aβ peptide induces toxicity is still unclear, but it may activate a variety of cell signaling events, including activation of mitogen-activated protein (MAP) kinase cascades including extracellular signal-regulated kinase (ERK), c-Jun N-terminal kinase (JNK/SAPK), and p38 (Dineley et al., 2001; Abe et al., 2003; Nicotra et al., 2005). However, the effect of Aβ peptide on regulation of the phosphoinositide 3-kinase (PI3-k)-Akt/PKB survival pathway, particularly on hippocampal slices cultures, has not been especially explored.

PI3-k pathway is believed to be an important anti-apoptotic signal pathway in neurons (Yuan and Yankner, 2000). Akt, also known as protein kinase B, is the main downstream kinase of PI3-k that promotes cell survival by means of its ability to phosphorylate and inactivate several pro-apoptotic targets, including the Bcl-2 family member BAD, the forkhead transcription factors, and the glycogen synthase kinase-3β (GSK-3β) (Pap and Cooper, 1998; Brunet et al., 1999; Downward, 1999; Kim and Chung, 2001). Besides the

GSK-3β pro-apoptotic role, it is the main protein kinase involved in the hiperphosphorylation of tau, promoting the tangles formation on DA brain (Inestrosa et al., 2002; Fuentealba et al., 2004; Mateo et al., 2006). The Akt kinase is activated through phosphorylation mediated by two phosphoinositide-dependet kinases, PDK 1 and 2 (Chan et al., 1999). The downregulation of this serine/threonine kinases by phosphatases that catalyze corresponding Akt dephosphorylation is still not fully understood. Recently, Akt has been shown to be dephosphorylated and inactivated in vitro by the protein phosphatase 2A (PP2A) (Ugi et al., 2004) and indirectly by the PI3-K phosphatase PTEN (Leslie and Downes, 2002).

Organotypic hippocampal slice cultures are a valuable alternative to animal experiments. It is important to mention that cultured slices maintain their cell architecture and interneuronal connections, allowing an extended survival and molecular mechanism studies (Stoppini et al., 1991; Holopainen, 2005; Noraberg et al., 2005). It has been used to investigate molecular mechanisms involved in citotoxicity, such as during oxygen and glucose deprivation (Valentin et al., 2003; Cimarosti et al., 2005; Horn et al., 2005).

The aim of this study was to investigate the involvement of Akt, GSK-3β, and PTEN proteins on the toxicity induced by the peptide Aβ25-35 in organotypic hippocampal slice culture.

Experimental procedures

1. Organotypic hippocampal slice cultures

All animal use procedures were approved by local Animal Care Committee and were in accordance with the NIH Guide for the Care and Use of Laboratory Animals. Organotypic hippocampal slice cultures were prepared according to the method of Stoppini et al. (1991), with modifications (Valentim et al., 2003; Cimarosti et al. 2005; Horn et al., 2005). Briefly,

400 μm thick hippocampal slices were prepared from 6-8-day-old male *Wistar* rats using 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% (Gibco, Grand Island, NY, USA) and gentamicine 36μL/100mL (Schering); pH 7.2. The slices were placed on Millicell culture insert (Millicell®-CM, 0.4 μm, Millipore®) and the inserts were transferred to a 6-well culture plate (Cell Culture Cluster, Costar®). Each well contained 1mL of tissue culture medium consisting of 50% minimum essential medium (MEM)(Gibco), 25% HBSS (Gibco), 25% heat inactivated horse serum (Gibco) supplemented with (mM, final concentration): glucose 36, HEPES 25 and $NaHCO_3$ 4; fungizone 1% (Gibco) and gentamicine 36μL/100mL (Schering); pH 7.3. Organotypic cultures were maintained in a humidified incubator gasified with 5% CO_2 atmosphere at 37°C for 11 days. Culture medium was changed three times/week.

2. Aβ25-35 peptide preparation and culture treatments

Synthetic rat Aβ25-35 was purchased from Sigma Chemical (St. Louis, Mo, USA). Aβ25-35 stock solutions of 1.5 mM were prepared in sterile distilled water, stored at -20°C, and, 7 days before use, incubated in 37°C for activation (Casal et al., 2004). On the 11th day in vitro, the medium was replaced by a serum free medium in which was added or not (control slices) 25 μM of Aβ25-35. The cellular death was examined by fluorescent image analysis of propidium iodide (PI) uptake after a 1-, 3-, 6-, 12-, 24-, and 48-h peptide exposition. Following the images captured after each treatment, the slices were solubilized for Western blotting assay.

3. Quantification of cellular death

Cellular damage was assessed by fluorescent image analysis of propidium iodide (PI) uptake (Noraberg et al., 1999). One hour before the end of the treatments, 7.5 μM propidium iodide (PI, Sigma Chemical) was added to the medium. PI is excluded from healthy cells, but following loss of membrane integrity it enters cells, binds to DNA and becomes highly fluorescent (Macklis and Madison, 1990). Cultures were observed with an inverted microscope (Nikon Eclipse TE 300) using a standard rhodamine filter set. Images were captured and then analyzed using Scion Image software (www.scioncorp.com). The area where PI fluorescence was detectable above background was determined using the "density slice" option of Scion Image software and compared to the total slice area to obtain the percentage of damage (Valentim et al., 2003).

4. Western blotting assay

After obtaining the fluorescent images, the slices were homogenized in lysis buffer (4 % sodium dodecylsulfate (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%. Samples containing 35μg of protein were resolved by 10% sodium dodecyl sulfatepolyacrylamide gel electrophoresis (SDS-PAGE). Then, proteins were electrotransferred to nitrocellulose membranes using a semi-dry transfer apparatus (Bio-Rad Trans-Blot SD). After 2 h incubation at 4°C in blocking solution containing 5% powdered milk 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: anti-phospho Akt (Ser473) (pAkt, 1:1000; Cell Signaling Technology), anti-Akt (1:1000; Cell Signaling Technology), anti-phospho GSK-3β (Ser9) (pGSK-3β, 1:1000; Cell Signaling Technology), anti-GSK-3β (1:1000; Cell Signaling Technology), and anti-PTEN (1:1000; Cell Signaling Technology).

The membranes were then incubated with horseradish peroxidase-conjugated anti-rabbit antibody (1:1000; Amersham Pharmacia Biotech). The chemioluminescence (ECL, Amersham Pharmacia Biotech) was detected using X-ray films (Kodak X-Omat). The films were scanned and the percentage of band intensity was analyzed using Optiquant software (Packard Instrument). For each experiment, the test groups were referred to control cultures not exposed to Aβ25-35, which were considered 100%, thus assuring the same signal intensity for control and test groups. The data are expressed as percentage of phosphorylated protein for Akt and GSK, which was obtained by the ratio of the immunocontent of phospho-protein (pAkt or pGSK-3β) with the whole amount of the protein (Akt or GSK-3β). The PTEN data are expressed by the immunodetection assay of the total antibody.

5. Statistical analysis

Data are expressed as mean ± S.E.M. One-way analysis of variance (ANOVA) was applied to the means to determine statistically significant differences between experimental groups. Post hoc comparisons were performed with Student-Newman-Keuls test for multiple comparisons. Differences between mean values were considered significant when *P* < 0.05.

Results

1. Toxicity induced by Aβ25-35 peptide in organotypic hippocampal slice cultures.

The exposition of the cultures to the Aβ25-35 peptide (25 μM) caused a marked fluorescence in the hippocampus after a 48-h treatment, indicating a high incorporation of PI, that means an increase in the cellular death, as presented in the photomicrographs in figure 1A. Quantification of PI fluorescence showed that the Aβ25-35 peptide caused about 15% of damage in hippocampus after a 48-h tratment, a significant increase compared to control

cultures (about 4% of cellular damage) (figure 1B). It was not observed any significant cell death after a 24-h exposure or less.

2. Effect of Aβ25-35 peptide on the phosphorylation/dephosphorylation of Akt protein.

The status of Akt phosphorylation was examined after a 1-, 3-, 6-, 12-, 24-, and 48-h Aβ25-35 peptide treatment by Western blotting with antibodies against the active form of Akt, phosphorylated at Ser473, as well as its total immunocontent. Aβ25-35 peptide (25μM) significantly increased the percentage of pAkt in cultures exposed for 6 h, compared to control cultures and to cultures exposed to peptide for the others periods (1, 3, 12, 24 and 48 h) (figure 2B). However, there was a significant decrease on the phosphorylated Akt after 12-, 24-, and 48-h exposure compared to respective control cultures and cultures treated at shorter intervals.

3. Effect of Aβ25-35 peptide on the phosphorylation/dephosphorylation of GSK-3β protein.

When Akt is activated (i.e. phosphorylated), it phosphorylates and inactivates GSK-3β. Therefore, GSK-3β phosphorylation was also evaluated after a 1-, 3-, 6-, 12-, 24-, and 48-h Aβ25-35 peptide treatment. We found that Aβ25-35 peptide increased the GSK-3β phosphorylation in cultures exposed for 6 h (figure 3B), according to pAkt increase for the equivalent period (figura 2B). Following, after 12 h, there was a significant decreased in the GSK-3β phosphorylation, as observed in Akt at the equivalent period. On the other hand, after 24 h, there was an increase on the pGSK/GSK ratio (figure 3B), unlike pAkt/Akt ratio at the same period (figure 2B). At 48-h exposure, the pGSK/GSK ratio returned to ordinary levels.

4. Effect of Aβ25-35 peptide on the immunocontent of PTEN protein.

Due the Akt dephosphorylation/inactivation observed after 12-, 24-, and 48-h Aβ25-35 peptide treatment, we decided investigate the effect of the Aβ25-35 peptide on the PI3-K phosphatase PTEN which indirectly downregulates Akt. As presented in figure 4B, there was a trend towards an increase time-dependent of the PTEN immunocontent. After a 24- and 48-h Aβ25-35 peptide tratment, there was a significantly increase. At these periods, there was a negative relation between the PTEN immunocontent to pAkt/Akt ratio, as shown in figure 2.

Discussion

Aβ peptide is the major component of senile plaques, which has been considered to play a causal role in the development and progress of AD. In the present study, we investigated the effect of Aβ25-35 peptide on hippocampal slices survival and on PI3-K substrates, Akt and GSK-3β proteins. Additionally, we studied the effect of this peptide on the PI3-K phosphatase PTEN, an indirect Akt downregulating agent, using organotypic hippocampal slice cultures. Organotypic cultures are a good alternative to in vivo models, since they provide a good experimental access to mimic pathophysiological pathways in living tissues, which should meet the requirements for well-preserved representation of various cell types (Stoppini et al., 1991; Tavares et al., 2001; Cimarosti et al., 2005; Holopainen et al., 2005; Horn et al. 2005).

Using this model, we have shown here that the Aβ25-35 peptide induced cell death after a 48-h peptide exposure, but not 24-h exposure or less. It can indicates that a longer period of incubation is required to Aβ25-35 to exert significant toxic effects in vitro, data in accordance with others studies (Baskys and Adamchik, 2001; Dall'Igna et al., 2003; Lu et al., 2004).

In spite of a long period of exposure to be necessary for significant toxic effect, the Aβ25-35 peptide can induce intracellular signals after shorter periods in contact with the tissue, and the toxic effect could be a result from these previous signaling events. The

phosphorylation/activation state of Akt shows to be increased after a 6-h exposure, and significantly decreased after a 12-, 24- and 48-h exposure. At 6 h, the increase of pAkt could be a cellular response to the peptide presence, as others studies also showed (Martín et al., 2001; Wei et al., 2002). The observed decrease of Akt phosphorylated state at 12, 24, and 48 h indicated an inactivation of Akt protein and should trigger the delayed cell death observed at 48 h, according to recent studies (Ryder et al., 2004), since the Akt has an essential role on neuronal survival, inhibiting several substrates from the apoptosis machinery (Brunet et al., 1999; Downward, 1999; Kim and Chung, 2001; Pap and Cooper, 1998).

One important downstream substrate from Akt is the GSK-3β. Following an increase of Akt phosphorylation at 6 h, the phosphorylated GSK-3β is increased, indicating that GSK-3β is inactivated. At 12 h of peptide exposure, the phosphorylated state of both enzymes is again interrelated, but, in this case, the phosphorylation of Akt and GSK-3β were significantly decreased. After 12 h of Aβ25-35 peptide exposure, it was observed a significant decrease in Akt phosphorylation. This effect was maintained and was observed 24- and 48-h Aβ25-35 peptide exposure. This effect could be due, at least in part, to an increase in the phosphatase activity, since we detected an increase in the immunocontent of PI3-K phosphatase PTEN a negative regulator of Akt (Leslie and Downes, 2002). There is a small amount of information relating PTEN to AD. Most studies focused PTEN role as a tumor suppressor (Chow and Baker, 2006). In this work, we showed, for the first time, that there was a trend towards an increase time-dependent of the PTEN immunocontent in organotypic slice cultures exposed to Aβ25-35 peptide, and, significantly at long intervals of exposure (24 and 48 h). The increase of PTEN immunocontent might promote an increase of the activity of PTEN and, as a consequence, the decrease of the Akt activity and the resulting cell death, which we showed in our work. The activity of phosphatase PTEN might be increased even before its immunocontent increase, at the 6- and 12-h interval, since there was a marked decrease of the

Akt phosphorylation/activity. Other important phosphatase which could be acting as a negative regular of Akt activity at this interval is the protein phosphatase 2A PP2A (Ugi et al., 2004), or PDKs downregulators, still not fully investigated.

After a 24-h Aβ25-35 peptide exposure, the GSK-3β phosphorylation was significantly increased, and, at 48-h exposure, returned to ordinary levels. Several intracellular signal transduction pathways other than that of Akt negatively regulate GSK-3β activity, such as the Wnt pathway via activation of the PKC enzyme (Cook et al., 1996; Inestrosa et al., 2002; Caricasole et al., 2003). This could explain the increase of the GSK-3β phosphorylation at 24 h, which can not be explained by the levels of Akt phosphorylation.

It is likely that Aβ peptide can modify multiple intracellular signaling pathways, which contribute to its toxicity. In our work we could show an important involvement of Akt dephosphorylation/inactivation in organotypic slice hippocampal culture exposed to Aβ25-35 peptide, probably induced by an increase of PTEN expression, which culminated in cell death. Taken together, our results provide important leads for pursuing a more complete understanding of the molecular events of Aβ peptide.

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Figure 1: Toxicity induced by the exposure of Aβ25-35 peptide (25 µM) at different times (1, 3, 6, 12, 24 and 48 h) in organotypic hippocampal cultures. A: Representative photomicrographs of slices stained with PI at the end of the each treatment. B: Quantitative analysis of hippocampus damage just after each treatment with Aβ25-35 peptide. Bars represent the mean ± S.E.M., n=12. [+] significantly different from control cultures, and from the others periods, $p<0.001$ (one-way ANOVA followed by Student-Newman-Keuls test, $P<0.05$).

Figure 2. Effect of Aβ25-35 peptide (25 µM) on the percentage of phosphorylated Akt at different times (1, 3, 6, 12, 24 and 48 h) in organotypic hippocampal cultures. A: Representative Western blottings of phospho-Akt and Akt after each treatment revealed using specifics antibodies. B: Histograms representing the quantitative Western blotting analysis of Akt phosphorylation state. The densitometric values obtained to phospho- and total-Akt were first normalized to their respective control (100%). Data are expressed as a ratio of the normalized percentages of phosphor-Akt and Akt. Bars represent the mean ± S.E.M., n=6. [x] significantly different from respective controls cultures, and from cultures 1- and 3-h Aβ25-35 peptide exposure, $p < 0.01$; [+] significantly different from cultures 6-h Aβ25-35 peptide exposure, $p < 0.001$ (one-way ANOVA followed by Student-Newman-Keuls test, $p < 0.05$).

Figure 3. Effect of Aβ25-35 peptide (25 μM) on the percentage of phosphorylated GSK-3β at different times (1, 3, 6, 12, 24 and 48 h) in organotypic hippocampal cultures. A: Representative Western blottings of phospho-GSK-3β and GSK-3β after each treatment revealed using specifics antibodies. B: Histograms representing the quantitative Western blotting analysis of GSK-3β phosphorylation state. The densitometric values obtained to phospho- and total-GSK-3β were first normalized to their respective control (100%). Data are expressed as a ratio of the normalized percentages of phospho-GSK-3β and GSK-3β. Bars represent the mean ± S.E.M., n=6. [&] significantly different from respective controls cultures, and from cultures 1-, 3-, 12-, and 48-h Aβ25-35 peptide exposure, $p<0.01$; [+] significantly different from respective controls cultures and 6-, and 24-h Aβ25-35 peptide exposure, $p<0.01$ (one-way ANOVA followed by Student-Newman-Keuls test, $p<0.05$).

Figure 4. Effect of Aβ25-35 peptide (25 µM) on the immunocontent of PTEN protein at different times (1, 3, 6, 12, 24 and 48 h) in organotypic hippocampal cultures. A: Representative Western blottings of PTEN protein after each treatment revealed using specifics antibodies. B: Histograms representing the quantitative Western blotting analysis of PTEN protein immunocontent. The densitometric values obtained to PTEN were first normalized to their respective control (100%). Bars represent the mean ± S.E.M., n=6. [&] significantly different from respective controls cultures, and from cultures 1-, 3-, 6-, and 12-h Aβ25-35 peptide exposure, $p < 0.01$; [+] significantly different from respective controls cultures and 1-, 3-, 6-, 12-, and 24-h Aβ25-35 peptide exposure, $p < 0.001$ (one-way ANOVA followed by Student-Newman-Keuls test, $p < 0.05$).
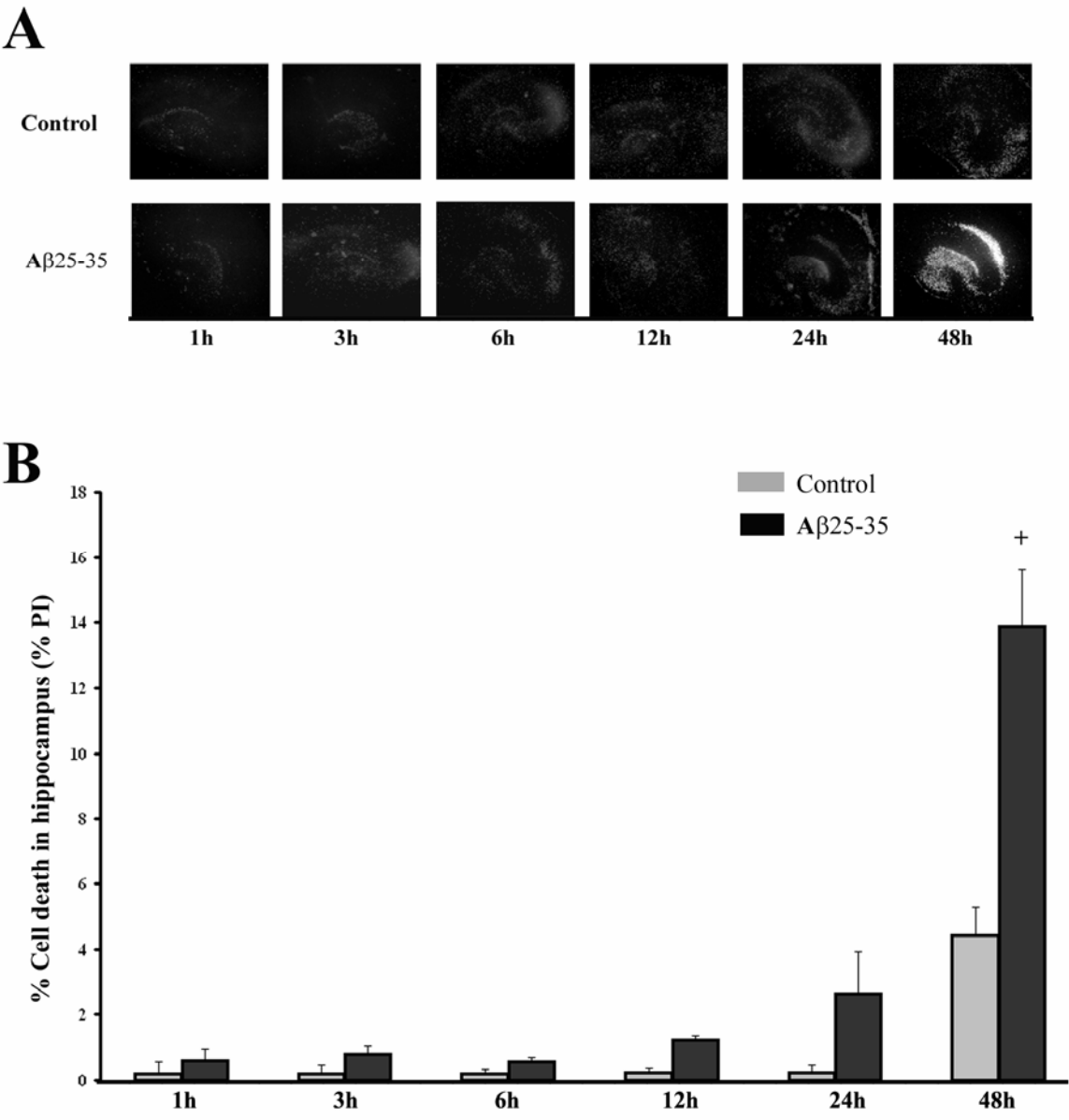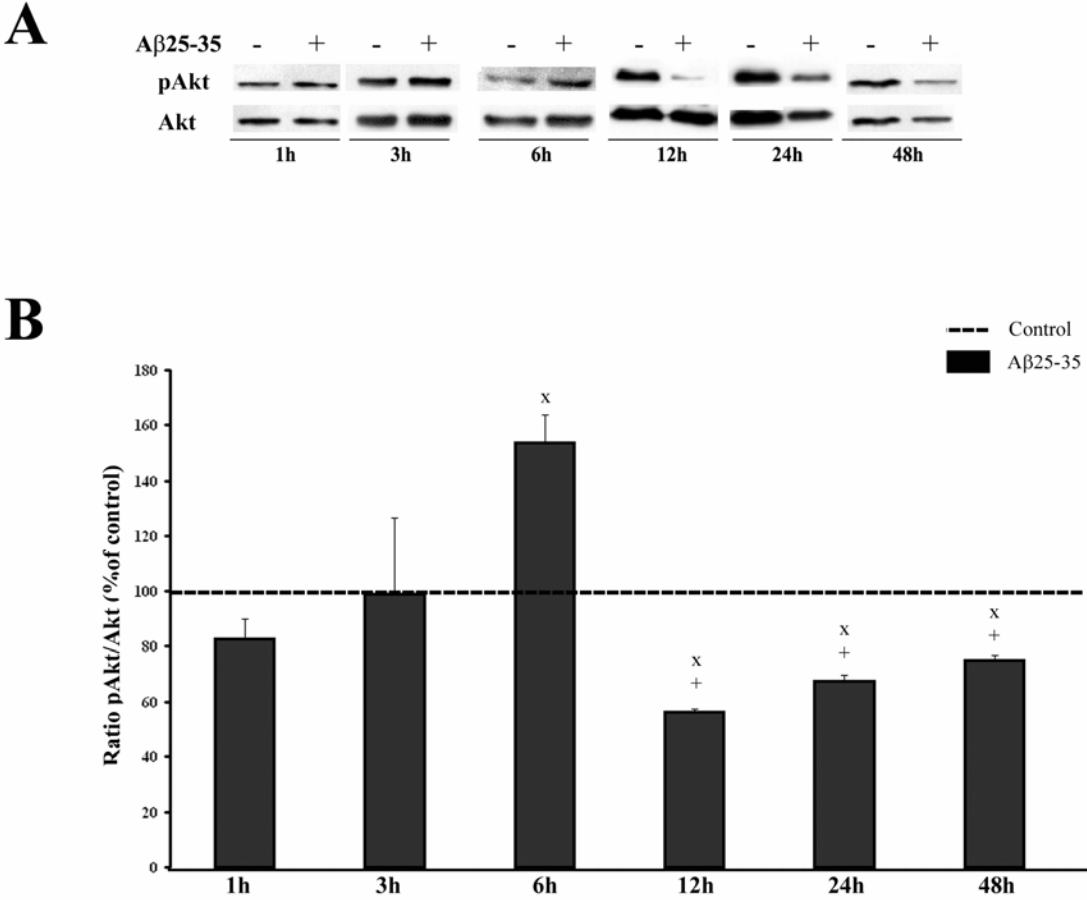
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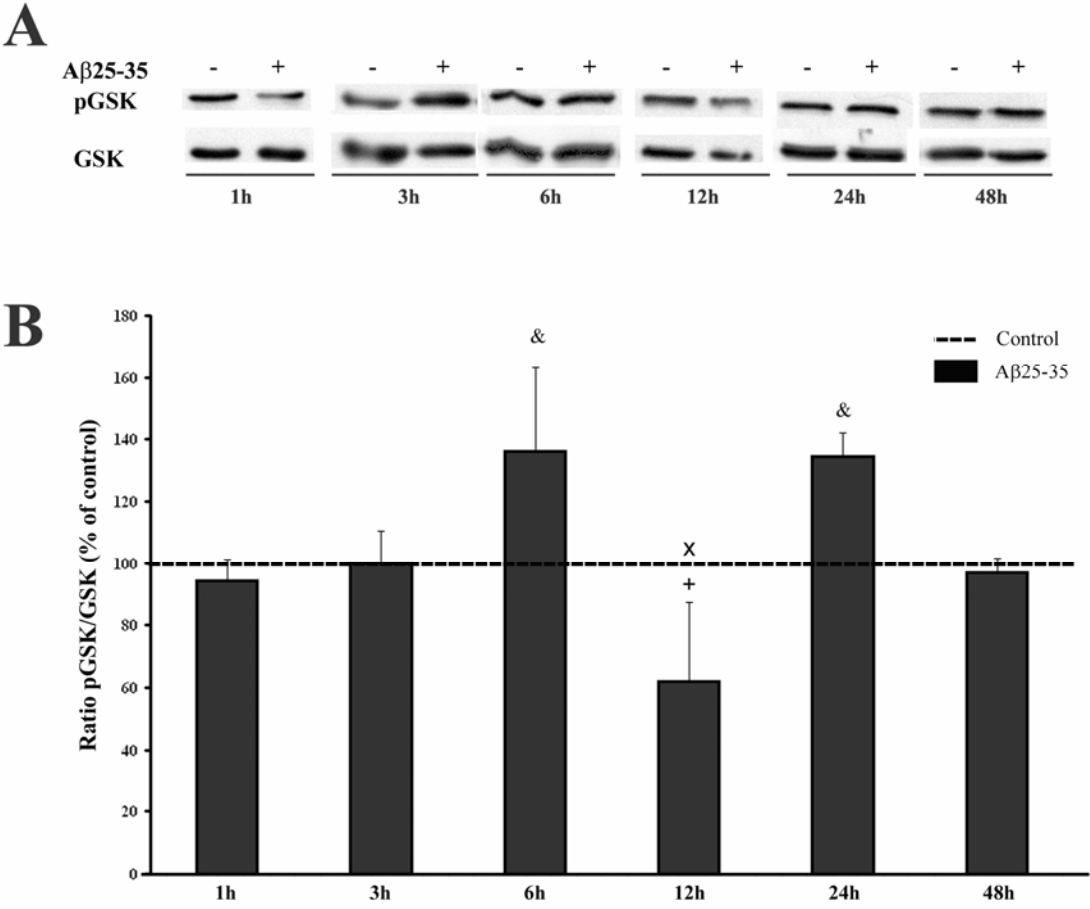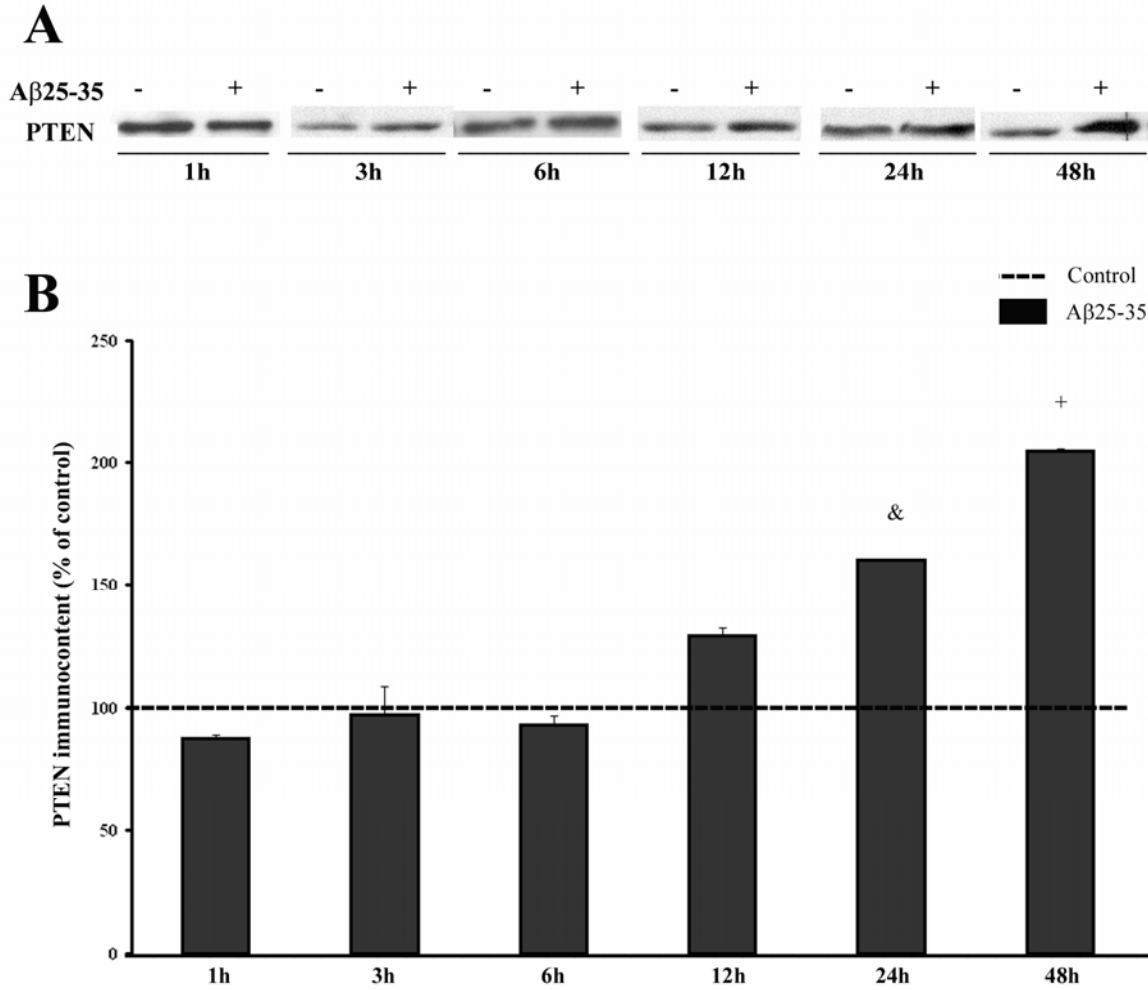
Neurochemistry International

Guide for Authors

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(a) Papers reporting original research are considered for publication provided that they describe significant, new and carefully confirmed findings and that adequate experimental details are given. All papers must be prepared according to the instructions given in Section III.
(b) Rapid Communications are those reporting original research and which are considered of such importance that **publication is necessary within the shortest possible time.** These papers should be between 1000 and 4000 words in length, and will be published within 10 weeks of *acceptance*. When submitting a manuscript for publication as a rapid communication, authors should include a brief statement justifying the reason for its inclusion in the rapid publication section of the journal.
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All manuscripts should initially be submitted to one of the Editors-in-Chief, i.e. either to Sylvester Vizi or to Roger Butterworth. To help ensure that papers are reviewed by the most suitable referees, authors can use their covering letter to provide a list of up to five candidate referees (including names, addresses, fax and e-mail) who do not have conflict of interest in the research being submitted. The Editors reserve the right to choose different referees from the ones suggested.

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Manuscripts should be accompanied by a statement that all efforts were made to minimize animal suffering, to reduce the number of animals used, and to utilize alternatives to *in vivo* techniques, if available.

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(h) Greek characters should be clearly identified.
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(a) Each paper must begin with a brief *Abstract*. It should not exceed 300 words. The first paragraph of the abstract should summarize the results obtained, the final paragraph should summarize the major conclusions in such a way that a reader not familiar with the techniques used can see any implications for his area of neuroscience. Abbreviations must not be used in the Abstract.
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(a) In general, tables and figures should be so constructed that they, together with their captions and legends, will be intelligible with minimal reference to the text.
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Betz, H., Becker, C.-M., 1988. The mammalian glycine receptor: biology and structure of a neuronal chloride channel protein. Neurochemistry International 13, 137-146.

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For example:

Baker, P. F., 1972. The sodium pump in animal tissues and its role in the control of cellular metabolism and function. In: Hopkin, L. E. (Ed.) Metabolic Pathways. Vol. 6, Academic Press, New York, pp. 243-268.

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PARTE III

DISCUSSÃO

Em um breve artigo em 1907, Alois Alzheimer descreveu a presença de placas senis e emaranhados neurofibrilares no neocórtex e hipocampo do cérebro de uma mulher de meia idade com déficit de memória e uma progressiva perda da função cognitiva. Passados quase 100 anos desde sua primeira descrição, poucos problemas neurológicos captaram tanta atenção da comunidade médica e científica como a doença de Alzheimer (DA) (Selkoe D.J. & Schenk D., 2003). A DA é a mais prevalente dentre as desordens neurológicas identificadas, atingindo atualmente cerca de 15 milhões de pessoas acima dos 65 anos de idade em todo o mundo, podendo triplicar o número de casos até 2050 (Forman M.S. et al., 2004; Puglielli L. et al., 2003).

No estudo dos mecanismos envolvidos na DA, os modelos experimentais *in vivo* e *in vitro* são ferramentas fundamentais. Dentre esses modelos, o modelo *in vitro* de fatias hipocampais, a cultura organotípica, mostra-se como uma importante alternativa ao uso de animais *in vivo* e do cultivo de células (Valentim L. et al., 2003; Cimarosti H. et al., 2005; Holopainen I.E., 2005; Horn A.P. et al., 2005; Noraberg J. et al., 2005). No presente trabalho, desenvolvemos o modelo de toxicidade do peptídeo β amilóide, Aβ1-42, e de seu fragmento Aβ25-35 em culturas organotípicas de hipocampo de ratos, uma vez que estas mantêm a multiplicidade celular original do tecido e as conexões intraneuronais (Holopainen I.E., 2005; Noraberg J. et al., 2005). Este tipo de metodologia favorece a investigação de mecanismos moleculares envolvidos com a toxicidade induzida pelo peptídeo Aβ1-42 e pelo seu derivado Aβ25-35, focando o tecido hipocampal, uma das principais regiões cerebrais afetadas na DA.

Neste trabalho, utilizamos o peptídeo Aβ1-42 (capítulo I, página 23) e seu fragmento, o peptídeo Aβ25-35 (capítulo II, página 43). Muitos trabalhos têm referido que o peptídeo Aβ1-42 tem uma grande propensão a se auto-agregar e possui grande toxicidade a neurônios, constituindo o principal componente das placas senis (Selkoe D.J. & Lansbury-Jr. P.J., 1999; Selkoe D.J. & Schenk D., 2003). Já o peptídeo Aβ25-35 é um fragmento que vem sendo

utilizado como uma alternativa ao peptídeo Aβ1-42, mostrando reter suas características de formação de fibrilas e toxicidade (Freir D.B. et al., 2003; Casal C. et al., 2004; Giunta S. et al., 2004; Hashioka S. et al., 2005; Hervas-Aguilar A. et al., 2005; Stepanichev M.Y. et al., 2006), apresentando uma vantagem financeira muito grande. Utilizamos ambos os peptídeos em concentrações micromolares, conforme descrito em vários estudos (Baskys A. and Adamchik Y., 2001; Dall'Igna O. et al., 2003; Lu X.H. et al., 2004; Rodriguez-Kern A., 2004; Hirata K. et al., 2005). Em experimentos-piloto, utilizamos concentrações na faixa de nanomolar e nenhuma toxicidade foi observada (dados não mostrados).

Na primeira parte do trabalho (Capítulo I), utilizamos o peptídeo Aβ1-42 com a finalidade de estudar sua toxicidade em culturas organotípicas de hipocampo de ratos e avaliar o envolvimento das proteínas iNOS e GSK-3β. Nossos resultados mostraram que o peptídeo apresentou toxicidade nas concentrações de 10 e 20 μM, induzindo uma considerável morte celular que chegou a 33% para a concentração de 10 μM e a 60% para a concentração de 20 μM após 72 h de exposição (capítulo I, figura 1, página 38).

A análise do imunoconteúdo da proteína iNOS mostrou um aumento significativo chegando a 200% em relação ao controle, também após 72 h de exposição ao peptídeo (capítulo I, figura 2, página 39). Estes resultados sugerem uma possível relação entre a expressão da proteína e a toxicidade induzida pelo peptídeo Aβ1-42 e estão de acordo com outros estudos *in vitro* (Akama K.T. et al., 1998; Casal C. et al., 2004; von Bernhardi R. & Eugenin J., 2004), assim como com o estudo realizado com tecido cerebral *post mortem* proveniente de pacientes com DA (Fernandez-Vizarra P. et al., 2004). O aumento da proteína iNOS levaria ao aumento de óxido nítrico, um importante mediador inflamatório (Jang J.H. & Surh Y.-J., 2005), agente neurotóxico e pró-apoptótico (Choi B.-M. et al., 2002), ou através da produção de peroxinitrito, potente agente pró-oxidante.

Ainda neste trabalho, estudamos o efeito da exposição das culturas organotípicas ao peptídeo Aβ1-42 sobre o imunoconteúdo e fosforilação da proteína GSK-3β. Em nosso modelo, não detectamos qualquer alteração nem no imunoconteúdo nem na fosforilação desta proteína (capítulo I, figura 3, página 40). Alguns autores mostraram uma possível relação entre a ativação (desfosforilação) desta proteína com a toxicidade do peptídeo Aβ (Alvarez A.R. et al., 2004; Li X. et al., 2006). Porém, estes estudos utilizaram culturas primárias de neurônios, enquanto que aqui foi utilizada cultura de tecido. Por outro lado, possíveis alterações desta proteína foram investigadas após 24 e 72 h de exposição ao peptídeo, quando o processo de morte celular já está completamente estabelecido em nosso modelo (Figura 1, página 38). O que pode ocorrer, especialmente quando há o envolvimento de vias de sinalização celular, é que as alterações em proteínas, como a fosforilação/desfosforilação da GSK-3β, podem ocorrer nos primeiros minutos ou horas após a exposição ao agente tóxico, no caso, o peptídeo Aβ1-42, e, desta forma, estar envolvida com o desencadeamento da morte tardia observada em 24 e, principalmente, em 72 h de exposição ao peptídeo.

A segunda parte de nosso estudo consistiu da investigação da toxicidade do fragmento Aβ25-35 do peptídeo Aβ1-42. Utilizamos a concentração de 25 μM, baseado em relatos da literatura (Dall'Igna O.P. et al., 2003; Malva J.O. et al., 2004), analisamos a morte celular e alterações nas vias de sinalização celular com 1, 3, 6, 12, 24 e 48 h de exposição ao peptídeo. Os resultados mostraram uma morte celular significativa apenas após 48 h de exposição ao peptídeo, não sendo observada qualquer diferença significativa entre os controles e o tratamento com o peptídeo Aβ25-35 nos tempos inferiores a este (capítulo II, figura I, página 65). Estes resultados estão de acordo com os dados do trabalho anterior com o peptídeo Aβ1-42 em que seus efeitos tóxicos aparecem a partir de uma exposição mais longa ao tecido. Estes resultados podem estar relacionados com o período crônico em que o tecido cerebral de pacientes com DA fica exposto às placas senis até o aparecimento de sintomas da demência.

No que se refere às vias de sinalização investigadas, foram detectadas alterações mais precocemente, ou seja, antes que uma morte celular fosse significativamente detectada. O conjunto de resultados que consistiu o capítulo II deste trabalho (página 43) mostraram que, após 6 h de exposição das culturas organotípicas ao peptídeo Aβ25-35, 25 μM, houve um aumento no estado de fosforilação da proteína Akt (capítulo II, figura 2, página 66) e também da proteína GSK-3β (capítulo II, figura 3, página 67), um potencial substrato da Akt. Após 12 h de exposição ao peptídeo, observamos uma diminuição de ambas as proteínas (capítulo II, figura 2, página 65 (Akt) e figura 3, páginas 66 (GSK-3β)). Estes dados sugerem uma possível relação entre a diminuição da fosforilação/ativação da proteína Akt com uma diminuição da fosforilação e conseqüente ativação proteína GSK-3β. Porém, após 24 h de exposição ao peptídeo, a proteína Akt continua menos fosforilada enquanto que a proteína GSK-3β apresenta um novo pico de fosforilação (capítulo II, figura 2, página 66 (Akt) e figura 3, páginas 67 (GSK-3β)). A GSK-3β é um importante substrato da Akt, mas pode ser negativamente regulada por outras vias, como a Wnt/β-catenina, via envolvida na proliferação e diferenciação celular, através da ativação da proteína cinase C (PKC) (Chen R.H. et al., 2000). A via da Wnt//β-catenina poderia estar envolvida neste aumento de fosforilação da GSK-3β observada após 24 h de exposição. Recentemente estudos têm buscado explorar os efeitos independentes ou comuns das vias Wnt//β-catenina e PI3-K/Akt na DA, com resultados ainda contraditórios (Chong Z.Z. & Maiese K., 2004), sendo um campo ainda em aberto para futuros estudos da DA.

A proteína Akt é uma das principais cinases celulares anti-apoptóticas, possuindo um papel central na via de sobrevivência da PI3-K (Brunet A. et al., 2001). Seu papel na DA ainda não é completamente entendido, pois alguns estudos demonstram um aumento no seu estado de fosforilação/ativação após o tratamento com peptídeos Aβ (Martín et al., 2001; Wei et al., 2002). Isso foi observado em nosso trabalho, mas apenas após 6 h de exposição ao

peptídeo. A partir do tempo de 12 h de exposição ao peptídeo Aβ25-35, o perfil de fosforilação da Akt se inverteu.

Uma vez que observamos essa diminuição no estado de fosforilação da Akt 12, 24 e 48 h pós-exposição ao peptídeo Aβ25-35 (capítulo II, figura 2, página 66), direcionamos nossa investigação sobre o que estaria levando a esta desfosforilação da Akt. Conforme referido na Introdução (página 19), uma das fosfatases envolvidas na desfosforilação e um dos principais reguladores negativos da Akt é a proteína PTEN (Leslie N.R. & Downes C.P., 2002). Nossos resultados mostraram uma tendência tempo-dependente para o aumento do imunoconteúdo desta proteína após 12 h de exposição, sendo significativamente diferente em relação ao controle as 24 e 48 h de exposição ao peptídeo, aproximadamente duas vezes em relação ao controle (capítulo II, figura 4, página 68). Nossos resultados sugerem que o peptídeo Aβ25-35 induziu o aumento do imunoconteúdo da proteína PTEN neste modelo de cultura organotípica de hipocampo de ratos, levando à desfosforilação e à conseqüente inativação da proteína de sobrevivência Akt após 12 h, especialmente após 24 e 48 h de exposição ao peptídeo. Estes efeitos provavelmente se relacionem com o mecanismo de indução de morte celular observada após 48 h de exposição ao peptídeo Aβ25-35 (capítulo II, figura I, página 65).

É provável que múltiplos fatores contribuam para a toxicidade induzida pelos peptídeos Aβ, tanto o Aβ1-42 como seu fragmento Aβ25-35 utilizados neste trabalho, o que é demonstrado pelo grande número de estudos relatando efeitos variados, tanto *in vivo* como *in vitro*. Todas as vias bioquímicas alteradas pelo peptídeo podem estar relacionadas com os sintomas observados na DA, mas os estudos acerca do mecanismo envolvido no desencadeamento da neurodegeneração na DA devem continuar para que seja possível o desenvolvimento de terapias capazes de frear e talvez retroceder o processo progressivo de degeneração observado na DA.

Os resultados obtidos neste trabalho somam-se aos de outros grupos que estudam intensamente os mecanismos envolvidos nesta doença neurodegenerativa. Nossos dados sugerem que o desequilíbrio de vias de sobrevivência intracelular, como a PI3K/Akt, pode ter papel fundamental no mecanismo de toxicidade desencadeado pelo peptídeo, e proteínas envolvidas nesta via podem ser alvos para o desenvolvimento de estratégias terapêuticas, tais como o uso de inibidores específicos de fosfatases, ou agentes que induzam a fosforilação/ativação da proteína Akt.

CONCLUSÕES

Com os dados obtidos no presente trabalho, podemos concluir:

Em relação à toxicidade induzida pelo peptídeo Aβ1-42:

1. As concentrações tóxicas observadas foram 10 μM (cerca de 33%) e 20 μM (cerca de 60%), apenas após um período de 72 h de exposição a culturas organotípicas de hipocampo de ratos;

2. O peptídeo Aβ1-42 foi capaz de aumentar o imunoconteúdo da proteína iNOS nas condições de 20 μM de concentração, após 72 h de exposição;

3. Após 24 e 72 h de exposição ao peptídeo Aβ1-42, não foi observada alteração significativa na fosforilação da proteína GSK-3β, em nenhuma das concentrações testadas (10 ou 20 μM).

Com relação à toxicidade induzida pelo fragmento Aβ25-35:

1. A concentração do peptídeo estudada foi 25 μM, que mostrou-se tóxica após 48 h de exposição às culturas organotípicas de hipocampo de ratos;

2. Após 6 h de exposição às culturas, o peptídeo aumentou o estado de fosforilação das proteínas Akt e GSK-3β;

3. Após 12 h de exposição ao peptídeo, observou-se uma diminuição no estado de fosforilação da proteína Akt, o qual foi mantido nos tempos de 24 e 48 h de exposição;

4. O peptídeo Aβ25-35 induziu um aumento no imunoconteúdo da proteína PTEN após 24 e 48 h de exposição.

Conclusão final

Os resultados do presente estudo mostraram que o tratamento das culturas organotípicas de hipocampo de ratos com o peptídeo Aβ1-42 ou com o fragmento Aβ25-35 apresentou

toxicidade após um período de aproximadamente 48 h de tratamento, e mostrou ser um bom modelo para o estudo de sua toxicidade.

Com relação ao mecanismo investigado, os dados sugerem que a proteína iNOS poderia estar envolvida na toxicidade induzida pelo peptídeo Aβ1-42. Além disso, sugerem que o fragmento Aβ25-35 possa exercer sua toxicidade através da inibição da via de sobrevivência celular PI3-K, parecendo envolver a proteína fosfatase PTEN.

PERSPECTIVAS

Os resultados obtidos neste estudo geraram muitas questões as quais abriram as seguintes perspectivas de trabalho:

– Complementar o estudo com o peptídeo Aβ1-42 em proteínas da via PI3-K em outros tempos, realizando uma curva semelhante à realizada para o peptídeo Aβ25-35;

– Comparação dos efeitos de ambos os peptídeos, Aβ1-42 e Aβ25-35, em outras proteínas envolvidas na morte apoptótica, como mTOR, caspase-3, fosfatase serina/treonina PP2A;

– Investigar o efeito do peptídeo Aβ25-35 na via de sinalização celular da MEK/ERK, via envolvida em vários processos celulares, como plasticidade neuronal;

– Investigar o efeito do peptídeo Aβ25-35 na fosforilação da proteína *tau*, com o uso de anticorpos específicos para fosfo-*tau*;

– Realizar estudos de neuroproteção contra a toxicidade induzida pelo peptídeo Aβ25-35, investigando agentes estudados em nosso laboratório, como o resveratrol, 17β-estradiol, boldina, estatinas, anticonvulsivantes.

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ANEXOS

INTRODUÇÃO

CAPÍTULO I

CAPÍTULO II

ANEXO 2. Autoria e Co-Autoria de Artigos Científicos Publicados Durante o Mestrado

1. The effects of estradiol on estrogen receptor and glutamate transporter expression in organotypic hippocampal cultures exposed to oxygen-glucose deprivation.

Helena Cimarosti, Ross D O'Shea, Nicole M Jones, Ana P. Horn, Fabrício Simão, Lauren L. Zamin, Melissa Nassif, Rudimar Frozza, Carlos A. Netto, Philip M. Beart, Christianne Salbego. Neurochem Res. 2006, *in press.*

2. Colesterol, APOEε4 e estatinas: implicações na Doença de Alzheimer.

Melissa Calegaro Nassif, Juliana Hoppe, Chistianne Gazzana Salbego. Revista Pharmacia Brasileira-Infarma, Brasília-DF, v. 17, n. 5/6 (novembro), p. 46-49, 2005.

3. Protective effect of resveratrol against oxygen-glucose deprivation in organotypic hippocampal slice cultures: involvement of PI3-K pathway

Lauren L. Zamin, Patrícia Dillenburg-Pilla, Ana Paula Horn, Fabrício Simão, Melissa Nassif, Daniéli Gerhardt, Rudimar Frozza, Christianne Salbego. Neuroscience, submetido em 2005.

4. Neuroprotection and protein damage prevention by estradiol replacement in rat hippocampal slices exposed to oxygen-glucose deprivation.

Cimarosti H., Siqueira I.R., Zamin L.L., Nassif M., Balk R., Frozza R., Dalmaz C., Netto C.A., Salbego C. Neurochem Res. 2005 Apr;30(4):583-9.

5. Estradiol protects against oxygen and glucose deprivation in rat hippocampal organotypic cultures and activates Akt and inactivates GSK-3beta.

Cimarosti H., Zamin L.L., Frozza R., Nassif M., Horn A.P., Tavares A., Netto C.A., Salbego C. Neurochem Res. 2005 Feb;30(2):191-9.

6. Estrógeno *versus* Isquemia cerebral: Hormônio Feminino como Agente Neuroprotetor.

Melissa Calegaro Nassif, Helena I. Cimarosti, Lauren L. Zamin, Christianne G. Salbego. Revista Pharmacia Brasileira-Infarma, Brasília-DF, v. 16, n. 11/12, p. 60-63, 2004.

7. Fitoestrógenos: Moléculas de Plantas Trazendo Benefícios para os Seres Humanos.

Lauren L. Zamin, Helena I. Cimarosti, Melissa Calegaro Nassif, Christianne G. Salbego. Revista Pharmacia Brasileira-Infarma, Brasília-DF, v. 16, n. 3/4, p. 75-78, 2004.