TESIS DOCTORAL

VASCULAR ASPECTS OF ALZHEIMER'S DISEASE: ROLE OF OXIDATIVE STRESS ON VASCULAR MIOCYTES ß-AMYLOID PRODUCTION AND ß-AMYLOID-INDUCED TOXICITY IN ENDOTHELIAL CELLS

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Que la següent Tesis Doctoral titulada "Vascular aspects of Alzheimer's disease: Role of oxidative stress on vascular miocytes β-amyloid production and β-amyloid-induced toxicity in endothelial cells", presentada per Mireia Coma Camprodon, Llicenciada en Bioquímica per la Universitat de Barcelona, ha estat realitzada sota la seva direcció y reuneix tots els requisits necessaris per ser jutjada, autoritzant la seva presentació per optar al grau de Doctor per la Universitat Pompeu Fabra.

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Barcelona, 17 d' abril de 2007

Als meus pare,
a la Marta i
a en Pau

No puc plasmar en una, dues, tres ni quatre pàgines les experiències viscudes en aquests anys al grup de Fisiologia. Tot i així, amb aquestes quatre línies vull expressar el meu agraïment a totes els que d'una manera o altre m'han acompanyat, ajudat i estimat en aquest grata experiència de Tesis Doctoral.

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Index

I. INTRODUCTION 5

1. PATHOLOGICAL HALLMARKS OF ALZHEIMER'S DISEASE ………..............8

2. PROTEOLYTIC APP PROCESSING ……………………………………………9

2.1. Secretases ………………………………………………………….......10

2.1.1. α-secretase ………………………………………….............10

2.1.2. ß -secretase …………………………………………………12

2.1.3. γ-secretase ……………………………………….……..........14

2.1.4. Other secretases ……………………………………………..15

2.2 APP trafficking ……………………………………………………........16

3. THE VASCULAR SIDE OF ALZHEIMER'S DISEASE …………………………17

3.1. Vascular brain system …………………………………………………17

3.2. Cerebral amyloid angiopathy (CAA) ……………………..……........ 18

3.2.1. CAA Aß-type ……………………………………….........20

3.2.1.1. Sporadic CAA ……………………………........21

3.2.1.2. Hereditary or familial CAA ……………………........22

3.3. Pathomechanisms for CAA ………………………………………........23

3.3.1. Fate of Aß …………………………………………...........24

3.3.2. Flux of Aβ across the BBB ……………………………………24

3.3.3. Proteolytic degradation ……………………………………...26

3.3.4. Which is the origin of vascular amyloid deposits? ………........27

4. OXIDATIVE STRESS 29

4.1. Reactive oxygen species (ROS) ………………………….……….....29

4.2. Reactive nitrogen species (RNS) ………………………………………30

4.2.1. Peroxynitrite formation ……………………………….…31

4.2.2. Nitration, nitrosylation or oxidation of proteins ……….……..31

4.3. Oxidative stress and Alzheimer's disease …………………………….....32
4.3.1. Oxidative modifications in lipids, proteins and DNA ………...33
4.3.2. Cellular response to oxidative stress: Mitogen activated protein kinases (MAPKs) and redox sensitive transcriptors factos….33
4.3.3. Oxidative stress in AD etiology …………….……………...36
4.3.4. Oxidative stress in AD pathophysiology …….……………….36
4.3.5. Vascular oxidative stress …………………….……………….38
4.4. Antioxidants defense systems ……………………….…………….....40
4.4.1. Antioxidant enzymes …………………….………….....40
4.4.2. Non-enzymatic Antioxidant ………………………………….41
4.4.2.1. Vitamin E ..……………………………………..42
4.4.2.2. Estrogens ……………………………………..42

II. AIMS ……………………………………………………………..……47

III. RESULTS & METHODS …..……………………..……………………...51

Chapter I: "Oxidative stress triggers the amyloidogenic pathway in human vascular smooth muscle cells"……………………………..………………………53

Chapter II: "Lack of oestrogen protection in amyloid-mediated endothelial damage due to protein nitrotyrosination"…….……………..………………………...67
1. Addendum chapter II …………………………..…………………………79
1.1. Extra figure-Addendum figure 2 ….…………..…………………………79
1.2. Extra figure-Addendum figure 3 ……..…………………………………79
1.3. Extra figure-Addendum figure 4 ………………….…………………..81

Chapter III: "Oxidative stress induces the downregulation of LRP, the β-amyloid clearance receptor in brain endothelial cells"…………………..……………….………83
1. Material and Methods ……………………………………………….……….84
1.1. Cell cultures …………………………………………….………84
1.2. Treatments ……………………………………………….………..84

1.3. Western-blot assay ……………………………………………..85
1.4. Immunoprecipitation …………………………………………..85
1.5. Immunofluorescence …………………………………………..85
1.6. Statistical analysis ……………………………………………..85
2. Results …………………………………………………………………85

IV. DISCUSSION ……………………………………………………………….91

V. CONCLUSIONS …………………………………………………….……111

VI. REFERENCES ………………………………..……………………….…...115

VII. APPENDIX ………………………………..………………………….……141

Appendix I. The protective role of vitamin E in vascular amyloid beta-
mediated damage. ………………………………………………………......143

Appendix II. The physiology and pathophysiology of nitric oxide in the brain.…..165

Appendix III. The amyloid β-protein precursor and Alzheimer's disease. ………..195

Appendix IV. The role of vitamin E on intracellular signaling pathways
in brain: molecular basis for the treatment of neurodegenerative processes. …......211

I. Introduction

Alzheimer's disease (AD) is an age-dependent neurodegenerative disorder and the most common form of irreversible late life dementia. The prevalence of the disease is estimated around 1,5% at 65 years. It rises almost logarithmically with age to reach about 30% at age 85 years. Nowadays, AD is becoming tragically common due to the increase in life expectancy whereas its etiology is still unknown and there is no cure. AD causes a chronically progressive decline in cognitive functions and others symptoms such us memory loss, confusion, impaired judgment, personality changes, disorientation and loss of language skills.

In 1906, Alois Alzheimer described for the first time the disease as a heterogeneous disorder with neuronal and vascular lesions. Although the presence of cerebrovascular disease is considered an exclusion criterion for the diagnosis of AD (McKhann et al., 1984;ROTH, 1955), the emerging view is that the cerebrovascular dysregulation is a feature not only of cerebrovascular pathology, such us stroke, but also of neurodegenerative conditions, such as AD. The most of autopsy-confirmed cases of AD have ß-amyloid deposits in brain vessels known as cerebral amyloid angiopathy (CAA) which induces brain microvessels degeneration (Rensink et al., 2003). Vascular network in AD brain is characterized by reduced microvascular density, an increased number of fragmented vessels with fewer intact branches, atrophic string vessels, increased irregularity of capillary surface, marked changes in vessel diameter, capillary basement membrane thickening and collagen accumulation in the basement membrane (Zlokovic, 2005). Recently, many epidemiological, clinical, pathological and functional studies have provided new insights supporting the involvement of vascular factors in the development and progression of AD and suggesting a strong association between cognitive decline in the elderly and cerebrovascular disorder (Iadecola, 2004;Zlokovic, 2005;de la Torre, 2002). Thus, epidemiological studies have shown common risk factors for vascular diseases and AD, including old age, hypertension, diabetes, hypercholesterolaemia, hyperhomocysteinaemia and the apolipoprotein-E_4 genotype ($ApoE_4$) (Gorelick, 2004). One of the most important evidence confirming the relationship between vascular pathology and AD rises from the Nun study (Snowdon, 2003). This study shows that patients with previous strokes require considerably less AD pathology for dementia symptoms to appear. Moreover, atherosclerosis in the base of the brain is related to AD

by impairing cerebral blood flow (Roher et al., 2003). There is demonstrated the relationship between vascular amyloid deposits and vessel dysfunction such as significant cerebral microvessel pathology (Farkas and Luiten, 2001b), the cognitive impairment, previous to dementia, is associated with amyloid angiopathy (Greenberg et al., 2004) and deficient clearance of Aß across the Blood Brain Barrier (BBB) (Deane et al., 2004a). Altogether suggest that vascular factors are playing a key role in the pathogenesis of AD.

1. PATHOLOGICAL HALLMARKS OF ALZHEIMER'S DISEASE

The brains of patients suffering from AD are characterized by neuronal loss or atrophy principally in the hippocampus and in the frontal and temporoparietal cortex associated to the presence of amyloid plaques and neurofibrillary tangles (NFT). Amyloid plaques are an extracellular accumulation of local deposits of amyloid ß-peptide (Aß) in the brain parenchyma and in cerebral blood vessels. Aß is a short peptide derived from the processing of a larger protein called amyloid precursor protein (APP). One characteristic form of amyloid plaques is referred as neuritic plaque, which are composed by an amyloidogenic core surrounded by dystrophic neurites, some of which contain NFTs, as well as by activated microglia and reactive astrocytes (Selkoe, 2004). The other important feature of AD is the neuronal intracellular accumulation of the protein tau, a microtubule-associated protein, which abnormally aggregates into paired helicoidal filaments that form the NFT (Lee et al., 2001).

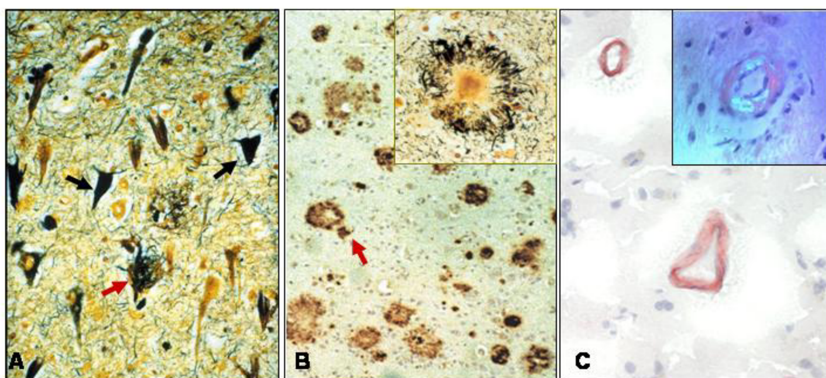


Figure 1. The pathological hallmarks of Alzheimer's disease. NFT (black arrow) **(A)**, Aß deposits in brain parenchyma (red arrow) **(A,B)**. Insert of neuritic plaque, a core of Aß surrounded by dystrophic neurites with NFT and glial cells **(B).** Aß deposits in cerebral blood vessels stained with congo red **(C).**

2. PROTEOLYTIC APP PROCESSING

APP belongs to a family of type I membrane-spanning glycoproteins constitutively expressed in many types of mammalian cells. Alternative splicing produces three major isoforms in brain, APP_{695}, APP_{751} and APP_{770}. APP_{695} is expressed in neuronal cells, whereas the APP splice forms APP_{751} and APP_{770} are widely expressed in non-neuronal cells. All the APP isoforms mature in the endoplasmic reticulum (ER) and Golgi where undergoes several posttranslational modifications such as phosphorylation, glycosylation and tyrosine-sulfation (Del Toro et al., 2005).

APP can be cleaved by two different pathways. Under normal conditions, a non-amyloidogenic pathway exists in which APP is cleaved by an enzyme with α-secretase activity belonging to the family of a disintegrin and metalloproteases (ADAMs) (Buxbaum et al., 1998;Lammich et al., 1999).α-Secretase cleaves APP within the Aß domain between Lys 16 and Leu 17 precluding Aß formation and yielding to the secreted N-terminal form, sAPPα (105-125KDa.) and the reminding C-terminal fragment of 83 aminoacids called αCTF, C83 or p3CT (10KDa) which is further cleaved by the γ-secretase identified as a heterotetrameric complex consisting of presenilin (PS)-1 or -2, nicastrin (NCT), APH-1 L (anterior pharynx-defective-1), and PEN-2 (presenilin enhancer-2) (Edbauer et al., 2003) to yield the p3 fragment (Esch et al., 1990). Alternatively, the amyloidogenic pathway produces Aß following the sequential cleavage of APP by a ß-secretase identified as ß-site APP cleaving enzyme 1 (BACE1) (Vassar et al., 1999) and a γ-secretase (Selkoe, 1998b;Wolfe et al., 1999). The initial proteolysis by ß-secretase results in the secreted form sAPPß (107-127KDa.), as well as a residual C-terminal fragment of 99 aminoacid called ßCTF or C99 (12KDa) which in turn, undergoes a second intramembrane cleavage by γ-secretase resulting in Aß release. Aß generally exists in two forms, either 40 or 42 amino acids in length that differs in their C-terminal. This difference between the two forms provides different aggregation capacity being $Aß_{1-42}$ the more fibrillogenic form (Munoz et al., 2002), being this characteristic key in the development of AD.

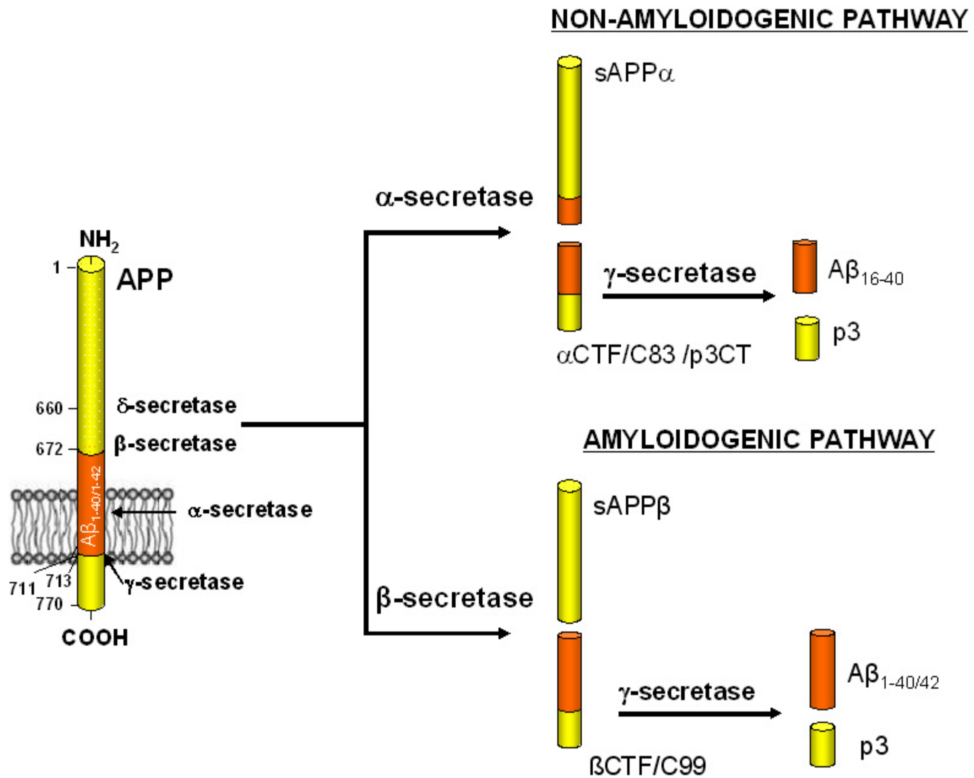


Figure 2. Proteolytic processing of amyloid precursor protein (APP). In the non-amyloidogenic pathway, APP is cleaved by the α-secretase leading to sAPPα and C83 and precluding Aß generation. C83 undergoes a second cleavage by the γ-secretase to render the p3 peptide. Alternatively, in the amyloidogenic pathway the ß-secretase carried out APP cleavage leading to sAPPß and C99. Then C99 is proteolysed by the γ-secretase to render Aß.

2.1. Secretases

2.1.1. α-secretase

Three members of the adamalysin or ADAMs family proteins, ADAM9/ meltrin γ (Koike et al., 1999), ADAM10 (Lammich et al., 1999) and ADAM17 -also called TACE (TNF-α converting enzyme) due to its first identification for the shedding of proTNF-α (Buxbaum et al., 1998;Slack et al., 2001) are the main candidates as α-secretase. Although the major APP proteolytic pathway is the non-amyloidogenic pathway, the identity of α-secretase has been controversial (Lichtenthaler and Haass, 2004). The emerging view is that there is a group of metalloproteases capable of cleaving APP at the α-secretase site. In different cell types, and possibly under

particular cellular conditions, the different members of this family contribute to a greater or lesser extent to the α-secretase cleavage of APP, in view of the fact that cell cultures from either knock out (KO) mice for ADAM9, ADAM10 and ADAM17 have still shown α-secretase activity (Hartmann et al., 2002;Weskamp et al., 2002). Nevertheless, the highest expression of ADAM10 in the central nervous system (CNS) (Karkkainen et al., 2000), the coexpression of APP, ß-secretase and ADAM10 in mouse and human brain (Marcinkiewicz and Seidah, 2000), the reduction of Aß production and its deposition in plaques in double transgenic mice overexpressing ADAM10 and APP (Postina et al., 2004) as well as, the enhancement of the number and size of senile plaques in the brains of double transgenic mice overexpressing APP and the inactive ADAM10 mutant, provide strong evidence about the key role of ADAM10 in α-secretase activity (Postina et al., 2004;Del Toro et al., 2005).

ADAMs are members of type I integral membrane proteins with common characteristic domains. It has an N-terminal signal peptide followed by a prodomain and the catalytic domain in which some ADAMs have a consensus HEXXH zinc-binding motif (X represents any amino acid) which is involved in the proteolytic activity. This prodomain contains an odd cysteine, which inhibits the catalytic site of the enzyme by its interaction with zinc. The prodomain is removed by furin-type pro-protein convertase in the Trans Golgi Network (TGN) being a prerequisite for the protease activity (Peiretti et al., 2003). Moreover, it has a cysteine-rich, disintegrin-/EGF-like domain involved in cell adhesion, a transmembrane domain and a short cytoplasmic domain. (Allinson et al., 2003). The role of the α-helical conformation and the distance (12-13 residues) of the hydrolyzed bond from the

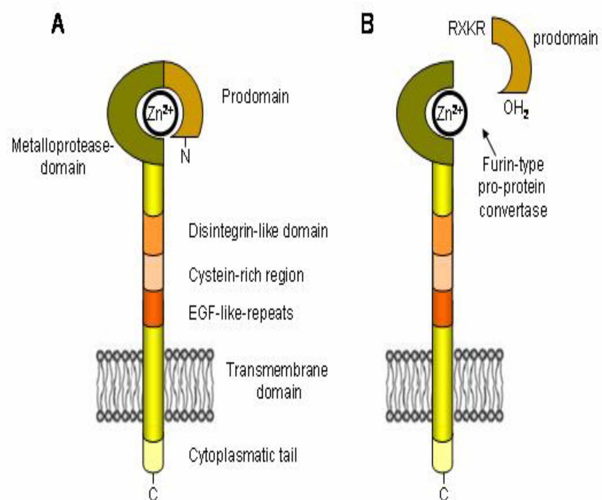


Figure 3. **ADAM multidomain structure**. Inactive (A) and active (B) form after cleavage by furin-like proprotein convertases.

membrane are the two main determinants for the non-amyloidogenic cleavage (Sisodia, 1992;Sahasrabudhe et al., 1992).

ADAMs are widely expressed and play a role in diverse biological processes such us control of growth factors, cytokines shedding, membrane fusion, cell migration as well as in fertilization, myogenesis and neurogenesis (Primakoff and Myles, 2000;Kojro and Fahrenholz, 2005). ADAM-mediated shedding of diverse membrane proteins occurs both constitutively and in response to variety of stimuli including PKC, growth factors and changes in intracellular calcium concentration. One of the main regulators for the APP shedding by ADAMs is PKC. However, since basal formation and secretion of αAPPs was unaffected in ADAM17 KO mice are believed that ADAM17 is only involved in regulated α-secretase activity (Lammich et al., 1999).

2.1.2. ß-secretase

An aspartyl protease called BACE1, ASP-2 (aspartyl protease 2) or memapsin 2 (membrane anchored aspartyl protease of the pepsin family) is the most plausible ß-secretase (Sinha et al., 1999;Vassar et al., 1999). ACE1 is a type I integral membrane protein highly expressed in brain of 501 amino acids with a N-terminal catalytic domain with two aspartyl protease active sites motifs (DTGS (residues 93-96) and DSGT (residues 289-292), a trans-membrane domain and a C-terminal cytoplasmatic domain (Vassar et al., 1999). Mutations of the catalytic active site aspartic acid residues abolish its activity, providing evidence that BACE1 is indeed an aspartyl protease. BACE1 can cleaves full-length APP at Asp1 (EVKM↓D) (or EVNL↓D, for the Swedish mutation) and at Glu11 (DSGY↓E) of the Aß sequence (Vassar et al., 1999) to release sAPPß. The Swedish APP double mutation, a substitution of a Lys^{595}-Met^{596}→Asn^{595}-Leu^{596} at the first and second positions immediately N-terminal to the ß-secretase cleavage site, allows a much more efficient ß-secretase cleavage of APP and yield to Aß which triggers to an early-onset AD (Mullan et al., 1992).

The identity of BACE1 as the ß-secretase involved in APP amyloidogenic pathway has been confirmed by several evidences; Transgenic mice overexpressing BACE1 or APP/BACE1 increases ß-secretase cleavage and Aß generation (Bodendorf et al., 2002). Conversely, BACE1 KO mice (BACE1(-/-)) (Roberds et al., 2001) and transgenic BACE1(-/-) mice coexpressing Swedish APP, has normal phenotype and

Aß production is abolished (Luo et al., 2001) in brain and fails to develop amyloid plaques with age (Luo et al., 2003). In contrast the α-secretase cleavage products (sAPPα, C83 and p3) are dramatically increased in the double transgenic mice indicating a direct competition between α- and ß-secretase for the APP (Cai et al., 2001;Luo et al., 2001). The same result has been observed in BACE1 antisense treated culture cells (Vassar et al., 1999;Yan et al., 1999). However, besides APP, BACE1 can cleaves others substrates such as the APP-like proteins 1 and 2 (Li and Sudhof, 2004;Pastorino et al., 2004), the sialyltransferase ST6Gal I (Kitazume et al., 2005;Kitazume et al., 2003), the P-selectin glycoprotein ligand-1 (PSGL-1) (Lichtenthaler et al., 2003), beta subunits of voltage gated sodium channels (Wong et al., 2005), and the LDL receptor-related protein (Von Arnim et al., 2005).

BACE1 is synthesized as a proprotein called pro-BACE1. The 24-amino-acid prodomain is required for the proper folding (Shi et al., 2001) and trafficking from the ER to the Golgi, cellular membrane and endosomal system. Pro-BACE1 is cleaved by furin and others members of the furin family of convertases to remove the 2-amino acid N-terminal region of the propeptide within the TGN. Then, BACE1 undergoes different posttranslational modifications during its maturation required for its activity. Mature BACE1 has four N-glycosilations sites at Asn153, -172, -223 and –354, and the extent of N-glycosilation determines its activity. (Charlwood et al., 2001;Huse et al., 2000). Moreover, a phosphorylation on the cytoplasmatic domain is necessary for the efficient maturation and its intracellular trafficking thought the TGN and endosomal system, where the mature BACE1 is located (Vassar et al., 1999). In addition, BACE1 is palmitoylated at three cysteine residues within its transmembrane/cytosolic tail that makes BACE1 a raft-associated protein (Benjannet et al., 2001). BACE1 has an acidic pH-optimum of 4,5 since the disruption of the intracellular pH inhibit ß-secretase activity (Haass et al., 1995;Knops et al., 1995). This finding is consistent with the idea that ß-secretase processing occurs within the Golgi, the late Golgi network and the endosomes/lysosomes (Walter et al., 2001).

BACE1 expression is strongly regulated by multiple mechanisms in a complex manner. BACE1 is regulated at transcriptional level since it has a number of putative transcription factors binding sites in the BACE1 promoter including NFκß, SP1, YY1, MZF1, HNF-3ß, PPRE, AP1 and four GATA acting as repressor or activators of

BACE1 transcription (Christensen et al., 2004;Sambamurti et al., 2004;Sastre et al., 2006). Although, alternative splicing events, muscarinic cholinergic receptor signalling, inflammatory processes and post-translational modifications can influence BACE1 concentrations and enzymatic activities in brain in a cell type-specific manner (Rossner et al., 2006).

BACE2 (also termed Asp1, memapsin 1, and DRAP), a homologue of BACE1, has an α-secretase like proteolytic cleavage within the Aß sequence at residues 19 and 20 of Aß (Farzan et al., 2000;Yan et al., 2001). This property of BACE2 provides its pathological role in Flemish mutation, in which a mutation in APP sequence (Ala to Gly at codon 692) increases the Aß production by BACE2 but not BACE1. The absence of Aß species generation in BACE1 (-/-) neurons culture indicates that BACE2 is not directly involved in APP cleavage in neurons (Cai et al., 2001).

2.1.3. γ-secretase

The first crucial clue to identify γ-secretase comes from genetic studies of human families suffering from autosomal-dominant early-onset AD. Whereas a small proportion of these cases are causes by mutations in APP itself, most are due to mutations in either of the two human PS genes, PS1 and PS2. These mutations alter the ratio of $Aß_{1-40}$/$Aß_{1-42}$, accelerating the production of the more fibrillogenic form $Aß_{1-42}$ (Selkoe, 1999).

The membrane-anchored C-terminal fragments resulting from α-secretase and ß-secretase are the direct substrates for the γ-secretase activity. This cleavage occurs within the transmembrane domain (Bergman et al., 2004) and is exerted by an aspartyl protease complex composed of the four core components PS1 or PS2, NCT, APH-1 L and PEN-2 (Edbauer et al., 2003). The functional enzyme it must assemble in the ER to produce an active complex which goes to the TGN and to the cell surface where is biologically active. PS is synthesized as single polypeptide chains with eight putative transmembrane domains. Each PS polypeptide undergoes an endoproteolysis leading to N- and C-terminal cleavage products which remain associated as stable heterodimeric integral membrane proteins (N-terminal fragment=27KDa and C-terminal fragment=17KDa) (Kopan and Ilagan, 2004). Two conserved intramembranous aspartate residues of PS led to the proposal that PS is the catalytic core of γ-secretase

complex. Mutagenesis of either of the two conserved aspartate residues (Asp257 and Asp385) of PS1 (Wolfe et al., 1999) and (Asp366) PS2 (Kimberly et al., 2000) fully inhibits γ-secretase activity. Moreover, cultured neurons from PS1 KO mice show a decreased Aß production (De Strooper et al., 1998;Naruse et al., 1998), and PS1 and PS2 KO mice completely loss Aß production (Armogida et al., 2001). NCT, APH-1 and PEN-2 are important for the maturation, trafficking and the proper enzymatic activity of the complex as well as NCT has been involved in substrate recognition (Kopan and Ilagan, 2004).

The γ-secretase renders different Aß type but mostly $Aß_{1-40}$ and $Aß_{1-42}$ triggering AD. The fact that γ-secretase can cleavage APP mostly in two separated sites (711-712 and 713-714 amino acid position referred to APP770), is associated to possible conformational changes of γ-secretase after binding APP by moving the substrate or the scissile peptide bond into the catalytic centre for hydrolysis (Fortini, 2002). Besides its implication in APP processing, γ-secretase complex is involved in the processing of a subset of type I transmembrane proteins including the ErbB4 tyrosine kinase receptor for neuregulins (Lee et al., 2002), CD44 (Murakami et al., 2003), N- and E-cadherin (Marambaud et al., 2002), low-density lipoprotein receptor –related protein (LRP) (May et al., 2002), Nectin-1R (Kim et al., 2002), Delta and Jagged (Ikeuchi and Sisodia, 2003), and Notch (De Strooper et al., 1999).

2.1.4. Other secretases

Although APP is predominantly cleaved by α-, ß- and γ-secretase, other secretase activities such as δ, ε and ζ have been identified. δ-secretase cleaves APP within the ectodomain at position -12 (referred to Aß) and has only been observed in hippocampal neurons (Simons et al., 1996). On the other hand, two PS-dependent cleavages have been described. ε-cleavage cleaves at the C-terminal end of the APP transmembrane domain between Leu49 and Val50 (Weidemann et al., 2002;Yu et al., 2001). This cleavage is a homologous cleavage to the S3 cleavage of Notch (De Strooper et al., 1999;Schroeter et al., 1998) and experiments with inactive PS1 mutants have demonstrated that ε-cleavage is dependent on PS1 activity and also on α-secretase activity (Kametani, 2004). ζ-cleavage is another PS-dependent cleavage within the transmembrane domain of APP that leads to $Aß_{1-46}$ generation (Zhao et al., 2004),

which recently has been suggested as a previous step to $Aß_{1-40}$ and $Aß_{1-42}$ generation (Zhao et al., 2007).

2.2. APP trafficking.

Regarding to the lipid composition of the plasma membrane, it is possible to define two different membrane domains, the lipid raft and the non-lipid raft domains. Lipid rafts are microdomains enriched in cholesterol, glycosphingolipids, sphingomyelin and acylated proteins. They has been implicated in a wide range of biological processes, including intracellular trafficking, transmembrane signalling, lipid and protein sorting, viral uptake and regulated proteolysis (Simons and Toomre, 2000).

The APP is localized in two cellular pools, one associated with lipid rafts, in which Aß is generated, and another outside the rafts. α-secretase is associated with non-lipid raft microdomains and cleaves APP during the trafficking and at the cell surface, whereas ß-secretase is associated with lipid raft domains where interact with APP. According to the acidic pH optimum for ß-secretase activity (Simons and Toomre, 2000;Simons and Ehehalt, 2002), ß-secretase cleavages APP after endocytosis, when internalized raft membrane can cluster and become redistributed. Supporting this theory it has been reported that a depletion of cellular cholesterol, which disrupts the structure and function of rafts, produces a decrease in Aß release and an increase in sAPPα formation (Simons and Ehehalt, 2002;Fassbender et al., 2001). Moreover, the specific targeting of BACE1 in lipid raft through replacing the transmembrane and cytosolic domain for a GPI-anchored form of BACE1, substantially up-regulated the secretion of both sAPPß and Aß, above the levels observed from cells overexpressing wild-type BACE1 (Simons et al., 2001).

Although various proteolytic functions of the γ-secretase have been assigned to the plasma membrane such as, Notch signalling and E-cadherin cleavage (Struhl and Adachi, 2000), it is still controversial if the site of ßCTF cleavage by γ-secretase overlaps with the cellular sites of Aß production. The most part of PS is retained in the ER while Aß production has been assigned within the TGN and endosomes/lysosomes. However, recently, it was shown that a fraction of PS assembles firstly with NCT and APH-1 which stabilizes PS before PEN-2 assembles and then, the complex directs to the cellular membrane where it can be biologically active (Kaether et al., 2006).

3. THE VASCULAR SIDE OF ALZHEIMER'S DISEASE

3.1. Vascular brain system

The vascular brain system includes large and small arteries, arterioles and capillaries. Large cerebral arteries branch smaller arteries and arterioles (pial arteries) running onto the surface of the brain. Penetrating intracerebral arteries are separated from the brain by the Virchow-Robin spaces and branch into smaller arteries and arterioles which gradually giving rise to intracerebral arterioles and brain capillaries (Zlokovic, 2005).

Arteries are composed by three main layers. An artery wall outer layer called tunica adventitia which consists of supportive collagen fibbers. The tunica media, the middle layer, is mostly smooth muscle cells (SMCs) with only a small number of interspersed connective tissue fibers. These SMCs regulate the local cerebral blood pressure and flow by contracting or relaxing. A layer of elastin and collagen fibres (Elastica externa) separated the tunica adventitia from the tunica media layers. The tunica intima is the luminal layer formed by endothelial cells lining, a fine connective tissue network, and a layer of elastic fibbers (Elastica interna). Arterioles branch off from the artery and give rise to capillaries.

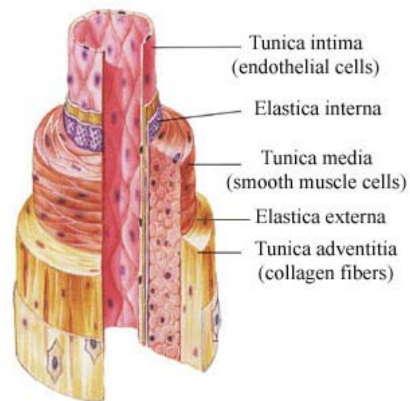
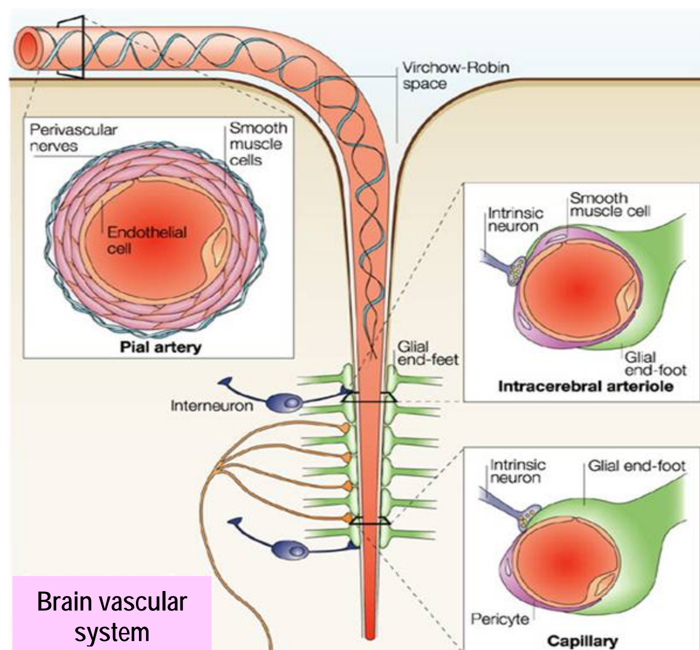


Figure 4. Artery

Capillaries form a network of tiny vessels connecting arterioles and venules and are composed of a single layer of brain endothelial cells (BECs). Capillary endothelial cells are sealed with tight junctions forming a continuous selective layer called BBB, which regulates a restrict exchange of nutrients, electrolytes and waste products between blood and brain. BECs produce powerful vasodilators and vasoconstrictors which are released by agents that activate specific receptors or by shear stress at the cell surface generated by changes in the rate of blood flow. SMCs convert this chemical signals produced mainly by BECs but also by neurons and astrocytes into changes in

vascular diameter. At the abluminal side, the basement membrane surrounds BECs and provides physical support to the microvessels, controls cellular migration, filters macromolecules, influences endothelial function, promotes cell adhesion and protects the brain against extravasated proteins (Perlmutter and Chui, 1990). Pericytes and astrocytes are found on the abluminal side of basement membrane. Pericytes maintain vascular integrity and release many biological-response modifiers that participate in vascular remodeling, angiogenic responses and regulation of blood flow in brain microcirculation and astrocytes influence angiogenesis and formation of tight junctions between BECs.

Figure 5. Brain vascular system. Pial artery**,** Intracerebral arteriole and capillary structure (From Iadecola C., 2004).



3.2. Cerebral amyloid angiopathy (CAA)

CAA is the pathological process during which amyloid progressively deposits in blood vessels walls producing the degeneration of the brain vascular cells (Vinters, 1987). CAA occurs as sporadic or familial forms with different amyloidogenic proteins being involved. Amyloid is the end product of a protein misfolding disorder, during which proteins acquire a conformation rich in ß-pleated sheet secondary structure and through protofibrillary intermediates form highly insoluble fibrils composed of protein polymers. Mutations in the precursor protein sequence, post-translational modifications,

and biochemical factors, such us protein concentration, pH and tissue factors, metal ions and amyloid associated proteins promotes the conversion of native proteins into insoluble amyloid fibrils that benefits its deposition. The amyloid is visualized by the positive staining with the Congo red dye with an apple-green colour in polarized light and with the fluorescence thioflavin S or T since both methods are dependent on the presence of ß-pleated secondary structure characteristic of Aß. CAA is classified according to the type of amyloid involved, amyloid ß-peptide(Aß), amyloid-British protein (ABri), amyloid-Danish protein (ADan), cystain C, gelsolin, prion protein (PrP) or transthyretin (TTR) (see Table I).

Amyloid peptide	**Precursor Protein**	**Disease**
ß-amyloid	Aß precursor protein (APP)	• Sporadic CAA • Hereditary or associated with chromosomal abnormalities
ABri	ABri precursor protein	FBD (Familial British Dementia)
ADan	ADan precursor protein	FDD (Familial Danish Dementia)
ACys	Cystain C	HCHWA-I (Hereditary Cerebral Hemorrahage with Amyloidosis-Icelandic type)
AGel	Gelsolin	FAF (Familial Amyloidosis, Finnish type)
PrP^{sc}	Prion protein	GSS (Gerstmann-Sträussler-Scheinker syndrome)
ATTR	Transthyretin	Meningovascular amyloidosis

Table I: Classification of CAA

3.2.1. CAA Aß-type

The most common type of CAA is caused by Aß and thus termed as CAA of the Aß type. It is associated with both sporadic and familial AD. The cortex, in particular the occipital lobe, is the brain region that is most frequently and severely affected by Aß-CAA (Attems, 2005). Hippocampus, cerebellum and basal ganglia are less affected while deep central grey matter, subcortical white matter and brain stem usually show no vascular amyloid.

CAA is characterized by the deposition of Aß mainly in the media and adventitia of small cortical and leptomeningeal arteries, arterioles, and less frequently, capillaries and venules impairing the oxygen supply to the brain and inducing the degeneration of the brain vessels (Vinters et al., 1988).

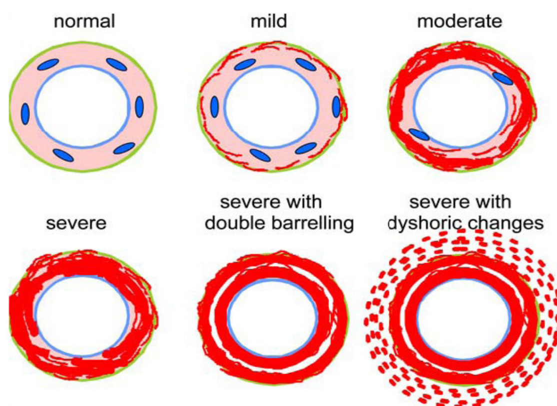


Figure 6. Progression of CAA. Aß deposits are shown in red. (From Attems J., 2005)

Pial and intracortical arteries are the most affected by CAA. According to the severity of the lesion, a neuropathological three-tiered grading system has been proposed that distinguishes between “mild”, “moderate”, and “severe” (Vinters et al., 1996;Vonsattel et al., 1991). Mild CAA is characterized by Aß deposition in the abluminal portion of the tunica media surrounding SMCs. Moderate CAA is characterized by extensive Aß deposition in the tunica media which produces loss of SMCs. In severe CAA, the vascular architecture is disrupted by a massive Aß deposition in all vessel layers with a total destruction of SMCs and degenerative changes such us double barrelling of the vessel wall, microaneurysm, fibrinoid necrosis, and perivasculat leakage of blood (Vonsattel et al., 1991;Attems, 2005). Large cerebral arteries do not present amyloid deposition but develop atherosclerosis and in consequence reduce blood supply to the brain which is a risk factor for AD developing (Zlokovic, 2005).

In affected capillaries or/and small arteries, amyloid accumulates on the outer side of basement membrane inducing the dysregulation of BBB functions and the local neuroinflammatory vascular responses including endothelium, perivascular microglia, perycites and astrocytes known as dyshoric changes (Zlokovic, 2005).

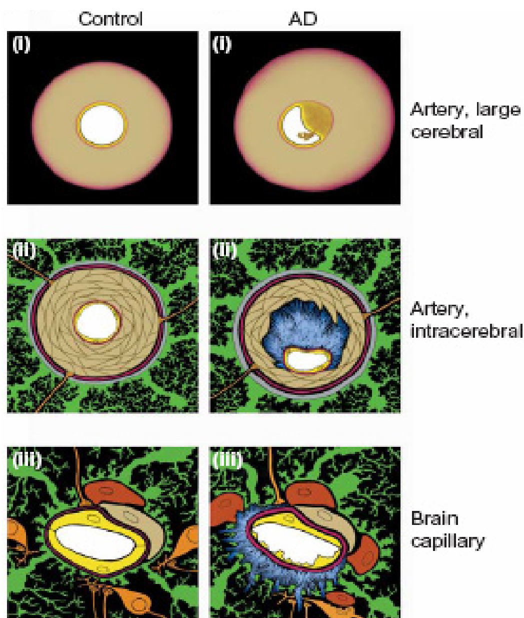


Figure 7. Vascular changes in AD. Large cerebral arteries often display atherosclerosis plaques in AD (i). Pial and intracerebral arteries (ii) develop Aß lesions (blue) and loss of SMCs (khaki), which are characteristics of CAA. At capillary level (iii) there is degeneration of endothelium (yellow), thickening of the basement membrane (dark red), Aß lesions (blue) and activation of microglia (bright red) perycites (khaki) and astrocytes (green). Local perivascular neurons and synaptic connections (orange) are severely disturbed. (Modified from Zlokovic B., 2005).

3.2.1.1. Sporadic CAA

Sporadic CAA is the most common CAA and is found in elderly individuals. Its incidence and severity are associated with aging, AD and Down syndrome (DS). Several reports have shown the association between CAA and AD demonstrating a CAA prevalence of 80 up to 100% of AD cases (Revesz et al., 2003). The most important clinical manifestation of CAA is lobar cerebral haemorrhages which represent approximately 12 to 15% of all cerebral haemorrhages, whereas other vascular pathological conditions such us inflammation/vasculitis has been observed (Attems, 2005).

The main risk factor for sporadic CAA is the presence of the Apo E_4 and the Apo E_2 allele as occurs for atherosclerotic disease. The possession of at least one ApoE_4 allele is a risk factor for both AD and CAA (Corder et al., 1993;Saunders and Roses, 1993) and it has been associated to an increased deposition of $Aß_{1-40}$ in vasculature (Chalmers et al., 2003). In addition to Apo E_4, the E_2 allele has also been demonstrated

to be associated to CAA-related cerebral haemorrhage by predisposing vessels to vasculopathic complications of CAA (Nicoll et al., 2003).

3.2.1.2. Hereditary or familial CAA

Several mutations have been identified in APP or PS genes linked to familial forms of early-onset AD and/or severe CAA. Within the Aß coding gene, five mutations localized just in the middle of Aß sequence (codon 692-694) are associated to CAA (Figure 7). They are *i)* the Flemish mutation (C to G at codon 692, A21G) (Hendriks et al., 1992), *ii)* the Dutch mutation (G to C at codon 693, E22Q) (Levy et al., 1990) *iii)* the Italian mutation (G to A at codon 693, E22K) (Tagliavini et al., 1999), *iv)* the Arctic mutation (A to G at codon 693, E22G) (Nilsberth et al., 2001) and *v)* the Iowa mutation (D to N at codon 694, D23N) (Grabowski et al., 2001).

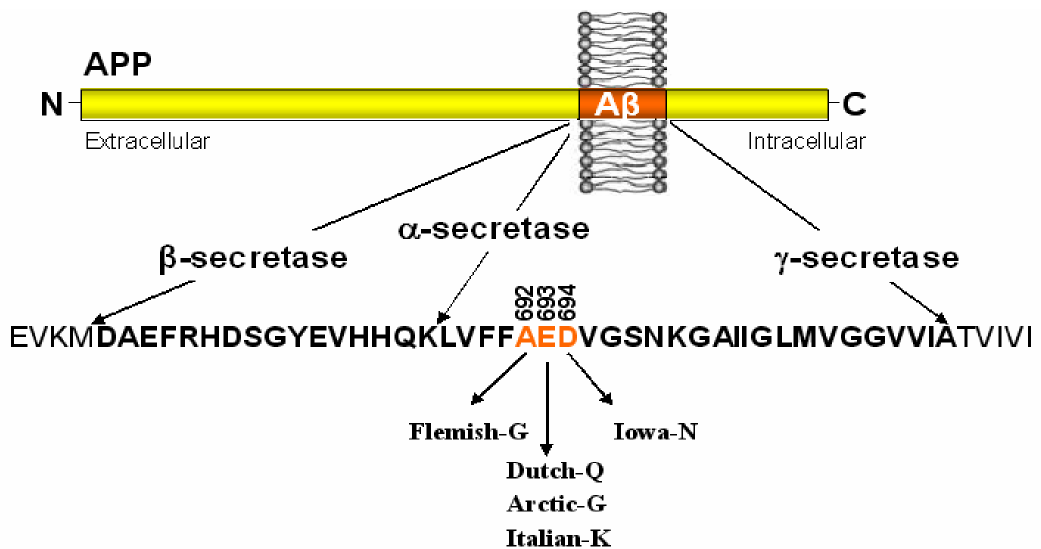


Figure 8. Schematic representation of APP mutations that induce CAA. The Aß sequence is enlarged and codon numbers and mutations are indicated.

The Flemish mutation produced by a substitution of alanine by a glycine at codon 692 is associated with early-onset AD and with severe CAA that in most cases leads to cerebral haemorrhage. The pathological phenotype shows plaques with dense amyloid core associated with or enclose vessels (Kumar-Singh et al., 2002). It seems associated to an increase of Aß production by BACE2 (Farzan et al., 2000).Hereditary cerebral haemorrhage with amyloidosis of the Dutch type (HCHWA-D) (van Duinen et al.,

1987) is an autosomal dominant disorder found in Dutch families caused by a point mutation at codon 693 in the Aß-encoding gene, resulting in the substitution of a glutamine for a glutamic (Levy et al., 1990), and rendering an Aß more fibrillogenic (Wisniewski et al., 1991). HCHWA-D patients present severe CAA and diffuse plaques in brain parenchyma but there are neither neuritic plaques nor NFT as found in AD. Clinically HCHWA-D is characterized by recurrent lobar cerebral haemorrhages and infarcts, often leading to an early death (Bornebroek et al., 1997). HCHWA-Italian type (HCHWA-I) is caused by a point mutation at codon 693, substituting a glutamic acid for a lysine. Aß deposits are found in cerebral parenchyma and in meningocortical vessels where the Aß seems to be rather amorphous than fibrillar. Clinically is associated with stroke, cognitive decline, and some of them develop seizures. At the same location, a substitution of a glutamic acid for glycine renders the Artic mutation which has been discovered in a family from northern Sweden. Patients have clinical features of early-onset AD with severe CAA that conclude in infarcts and/or ischemic lesions (Nilsberth et al., 2001). The Iowa mutation at codon 694 of APP resulted in a substitution of an asparagine for aspartic acid causes severe CAA with small cortical haemorrhages and both cortical and subcortial infarcts at mid-life. Sparse and diffuse senile plaques and either dystrophic neurites or NFT are present. Iowa mutations enhance fibrillogenic and pathogenic Aß properties (Van Nostrand et al., 2001).

Numerous mutations in PS1 and PS2 have been associated to early-onset AD. Some of them, L282V (Dermaut et al., 2001) and Q184D (Yasuda et al., 1997) PS1 mutations, PS1 deletion ΔI83/ΔM84 (Yasuda et al., 1997) and N141I PS2 mutation are also associated to severe CAA (Prior et al., 1996).

3.3. Pathomechanisms for CAA

Aß homeostasis is kept by equilibrium between Aß production and elimination. An increase of Aß production, a failure in Aß clearance, a reduction in Aß degradation or even an increased influx of Aß from blood into brain could triggers AD development.

Whereas inherited mutations in APP (e.g. Swedish mutation) or in PS genes that leads to an increase of Aß production trigger AD pathology and transgenic mice overexpressing APP develop AD, the overproduction of Aß are not the only cause of all

AD cases. Thus, defects in Aß clearance or degradation could underlie some or many cases of sporadic AD.

3.3.1. Fate of Aß

Neuronal Aß have several routes of elimination from brain. Neuronal Aß could be transported to the systemic circulation along perivascular drainage pathways via the ISF (interstitial fluid) or directly across the BBB into the bloodstream mediated mainly by the low-density-lipoprotein receptor-related protein-1 (LRP). Also, Aß can be degraded by several intracellular or extracellular proteases (Selkoe, 2001) or cleared by glia (Wyss-Coray et al., 2003) (microglia, astrocytes). When the concentration of Aß reach a critical concentration, it starts to aggregate which is favoured in vessels due to the Aß production from VSMCs and pericytes (Munoz et al., 2005b).

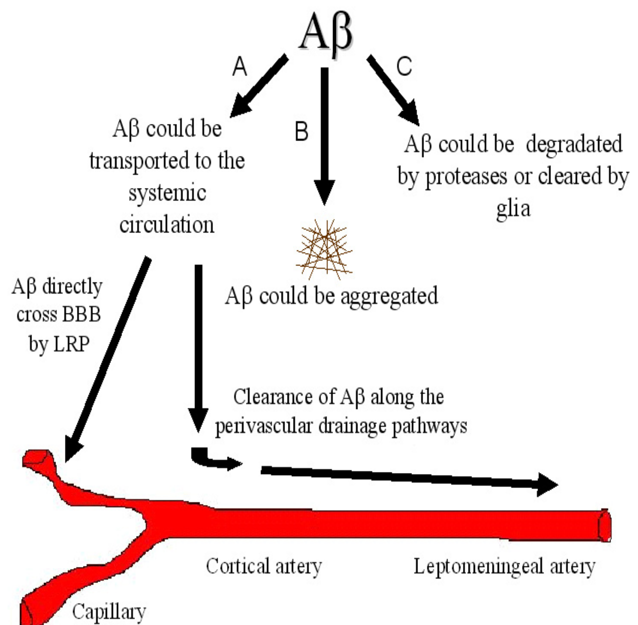


Figure 9. Fate of neuronal Aß. Aß could be transported to the systemic circulation directly by LRP receptor or along the perivascular drainage pathways **(A)**, aggregated producing Aß deposits **(B)** or degradated **(C)**.

3.3.2. Flux of Aß across the BBB

The net flux of Aß across the BBB is mainly mediated by LRP and the receptor for advanced glycation end products (RAGE). LRP controls the Aß efflux from brain to blood whereas RAGE controls the Aß influx from blood to brain (Deane et al., 2004b).

LRP, a member of the LDL receptor family, is a multiligand receptor whose physiological functions are carried out by endocytosis of ligands and activation of multiple signal transduction pathways (Herz and Strickland, 2001). LRP was first recognized as a large endocytic receptor central to transport and metabolism of

cholesterol and apoE-containing lipoproteins. LRP is synthesized as a 600 kDa transmembrane glycoprotein which is cleaved constitutively by furin proprotein convertases in the TGN to form the 515 kDa α subunit (the heavy chain of LRP) and the 85 kDa ß subunit (the light chain of LRP) that remain noncovalently associated during LRP transport to the cell membrane (Herz et al., 1990). The heavy chain of LRP contains four ligand-binding domains (clusters I–IV) that bind several ligands, some of them genetically linked to AD such us apoE, α2-macroglobulin (α2M), tissue plasminogen activator (tPA), plasminogen activator inhibitor-1 (PAI) or APP (Herz and Strickland, 2001). The light chain contains a transmembrane domain and a cytoplasmic tail that can be phosphorylated on serine and tyrosine and plays a role in intracellular signalling. A 39 kDa receptor-associated protein (RAP) is a specialized chaperone molecule that binds to LRP and regulates its proper folding (Bu, 2001).

Genetic polymorphisms in LRP gene are associated with late-onset AD (Kang et al., 1997;Hollenbach et al., 1998) and also, transgenic mice expressing low LRP-clearance mutant develops robust Aß cerebral accumulations much earlier that Tg-2576 Aß-overproducing mice (Deane et al., 2004a). However, the exact mechanism by which LRP affects the onset of the disease and Aß accumulation is still unknown. Brain efflux studies with exogenous soluble $Aß_{1-40}$, suggest that the primary clearance site for Aß is the LRP situated in brain capillary endothelium (Shibata et al., 2000). LRP interacts directly with free Aß at the abluminal side of the endothelium and through transcytosis the Aß is eliminated from the brain into the bloodstream. LRP favours the clearance of $Aß_{1-40}$ better than $Aß_{1-42}$ since the affinity of LRP for Aß is greatly reduced with high ß sheet content (Deane et al., 2004a). Aß may also interact with LRP via chaperone molecules as apoE, apo J and α2M (Herz and Marschang, 2003) since double deletion of the genes encoding apoJ and apoE accelerates Aß pathology in APP-overexpressing mice (DeMattos et al., 2004).

The ABC transporter p-glycoprotein (p-gly) known as MDR1 has been also proposed to contribute in Aß efflux at the BBB (Lam et al., 2001).

RAGE is a multiligand receptor member of the immunoglobulin superfamily first identified as a cell surface receptor for the products of nonenzymatic glycation and oxidation of proteins, the advanced glycation end products (AGEs). RAGE besides AGEs can bind a plethora of ligands such us Aß, the S100/calgranulin family of

proinflammatory cytokine-like mediators, and the high mobility group 1 DNA binding protein amphoterin (Yan et al., 2000). Whereas RAGE is present at high levels during development, especially in the CNS, its levels decline during maturity. However, RAGE expression is up-regulated by the presence of its ligands (Yan et al., 1994). Thus, RAGE levels are overexpressed in Aß-treated endothelial cells (BECs), brain vasculature in transgenic APP models and in AD (Deane et al., 2003;Deane et al., 2004a), and furthermore, RAGE was colocalized with Aß in human AD brain tissues, in neurons, microglia and vascular elements (Yan et al., 1994;Yan et al., 2000).

Another candidate that mediates the Aß flux from brain-to-blood and also from blood-to-brain is the gp330/megalin or LRP2. gp330/Megalin transport Aß complexed to apoJ (Zlokovic et al., 1996). However, the contribution of gp330/Megalin in Aß influx is minor than RAGE since the presence of high levels of apoJ in plasma saturates gp330/Megalin (Zlokovic, 2004).

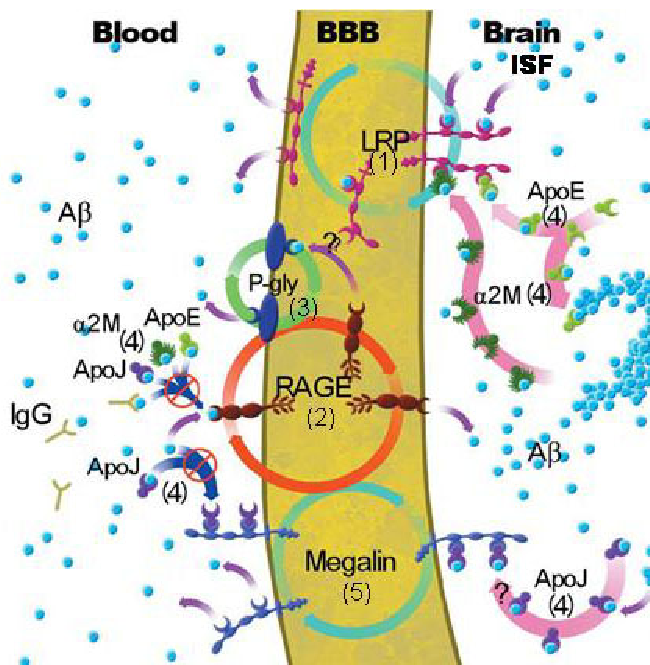


Figure 10. Transport pathways across the BBB. Aß efflux from brain to blood is mediated mainly by LRP (1) but also by p-gly and gp330/Megalin (5). Aß influx from blood to brain is mediated mainly by RAGE (2) but also gp330/Megalin (5) could contribute. Aß could bind to transport proteins such us apo E, apo J and α2M (4). This binding could influence Aß sequestration in plasma or brain. (•) Aß. (Modified from Zlokovic B., 2005).

3.3.3. Proteolytic degradation

Several enzymes have been proposed to degrade Aß peptides in brain tissue. Neprylisin (NEP), insulin degrading enzyme (IDE), insulinase, plasmin, plasminogen activator system (uPA/tPA), endothelin-converting enzyme (ECE-1) and matrix

metalloproteinases-9 (MMP9) are capable of degrading Aß (Selkoe, 2001;Dotti et al., 2004). The following table, show some properties of the proteases capable of degrading Aß.

Protease	Class	Substrates (besides Aß)	Recent Findings related to Aß Degradation
IDE	Metallo	cytosol, peroxisomes,extracellular fluid, plasma membrane, internal membranes	secreted by microglia
NEP	Metallo	plasma membrane, internal membranas	deletion causes rise in cerebral Aß levels in vivo. capable of degrading membrane-associated Aß
Plasmin	Serine	extracellular fluid	can degrade both monomeric Aß and fibrils in vitro
uPA/tPA	Serine	extracellular fluid	can be activated by Aß aggregates to generate plasmin uPA gene located near putative FAD locus
ECE-1	Metallo	plasma membrane, internal membranas	
MMP-9	Metallo	extracellular fluid	latent form of MMP-9 accumulates in AD brain

Table II. Some proteases capable to degrade Aß (Selkoe, 2001)

3.3.4. Which is the origin of vascular amyloid deposits?

The origin of Aß in blood vessel walls is poorly understood, and it is in continuous debate. Several mechanisms have been proposed grouped in three main theories termed drainage, vascular, and systemic hypothesis. However, these three theories are not necessarily thought to be exclusive and might even occur all of them.

The drainage hypothesis proposes that Aß in brain vessel walls has a neuronal origin. Neuronal Aß could be transported to the systemic circulation along perivascular drainage pathways into the cerebrospinal fluid (CSF) and from there into the bloodstream or directly across the BBB into the bloodstream mainly by LRP. This hypothesis proposes that CAA occurs due to the deposition of Aß along these drainage

pathways (Weller et al., 1998;Weller and Nicoll, 2003;Preston et al., 2003). Aß deposition could be prompt by an increase of Aß by neuronal cells and additional degenerative vascular changes, which commonly affect aged individuals (e.g., atherosclerosis, fibrohyalinosis). Neuronal production of Aß is supported by the presence of vascular amyloid deposits in transgenic mice overexpressing APP exclusively in neurons (van Dorpe et al., 2000), the capability of vascular SMCs (VSMCs) to endocytose and accumulates Aß coming from the brain parenchyma (Wisniewski et al., 2000a) and the link between reduced Aß-clearing capability and AD (Zlokovic, 2005).

The vascular hypothesis proposes that Aß is produced by VSMCs and pericytes from brain vessel. Production of Aß by VSMCs has been confirmed by cell culture studies, which showed intracellular and recently also extracellular Aß depositions (Frackowiak et al., 1994;Frackowiak et al., 2003;Frackowiak et al., 2005;Wisniewski et al., 2000a). These Aß deposits were immunoreactive for Aß sequences 1–16 and 17–24, but not 37–42, suggesting that VSMCs produce mainly $Aß_{1-40}$ (Frackowiak et al., 2005). Moreover, it was further suggested that proliferating and degenerating VSMCs (Nagy et al., 1995;Preston et al., 2003) and pericytes (Burgermeister et al., 2000) overproduce Aß. As well as, on the extracellular matrix of VSMCs has been reported a high fibrillogenic activity. (Van Nostrand et al., 2000). In fact, this hypothesis supports the idea that VSMCs secrete non-fibrillary Aß and the aggregation of monomers to fibrils is promoted by the extracellular matrix of the VSMCs.

The systemic hypothesis proposes the idea that vascular Aß deposits come from the general circulation and Aß is transported from blood to the vasculature by binding to ApoE, ApoJ and α2M by receptor mediated transport or by BBB leakage. This hypothesis is supported by the fact that APP is a ubiquitous protein and Aß could be detected in cerebral vascular wall and parenchyma after intravenous injections of Aß in rodents (DeMattos et al., 2002) and primates (Gray et al., 1987). However, there are several controversial findings against a systemic origin of vascular Aß. Firstly, the observation those arteries are more affected than veins in CAA, and also, small arteries have more severity than larger ones. Secondly, vascular amyloid deposition starts in the abluminal basement membrane of the vessels.

4. OXIDATIVE STRESS

Oxidative stress is a well-known hallmark of brain damage that includes both acute (cerebral stroke, head trauma) and neurodegenerative processes (AD, Parkinson's disease). It is cause by the generation and accumulation of reactive oxygen species (ROS) and reactive nitrogen species (RNS). ROS and RNS are either free radical or non-free radical molecules which can react with proteins, lipids and DNA producing their oxidation and leading to DNA and cell membrane damage, mitochondrial malfunction and eventually cell death. Oxidative stress describes a state of imbalance between the production and detoxification of ROS and RNS (Sies, 1991). Under normal conditions, cells are able to defend themselves against ROS and RNS damage by potent antioxidant defense systems that include biochemical structures (e.g. vitamin E) and antioxidant enzymes (e.g. superoxide dismutase). Moreover, cells can react to oxidative stress either by adaptable responses leading to activation of repair mechanisms or if the damage is severe by induction of cell death by apoptosis.

4.1. Reactive oxygen species (ROS)

ROS are natural byproduct of the normal metabolism of oxygen. There are many different sources by which ROS are generated. Endogenous sources as mitochondrial electron transport chain, peroxisomal ß-oxidation of fatty acids, stimulation of phagocytosis by pathogens or lipopolysaccharides and tissue specific enzymes such us NADPH oxidase, nitric oxide synthase (NOS), xanthine oxidase, lipoxygenases, myeloperoxidase. Also, there are exogenous source as neurotoxins or exposition to Ultraviolet light. Specific vascular oxidative stress source are explained in section 4.3.5.

Superoxide anion ($O_2^{\bullet -}$), hydrogen peroxide (H_2O_2) and hydroxyl radical ($OH^{\bullet}$) are formed by the partial reduction of oxygen (O_2). $O_2^{\bullet -}$ is highly reactive, but its damaging effects in cells are limited since diffusion across biological membranes is minimal due to the negative charge. However, others ROS are derived from $O_2^{\bullet -}$ reactions, among these are H_2O_2 which has limited reactivity but can diffuse across membranes producing damaging effects, $OH^{\bullet}$ which is an extremely reactive anion formed by the reaction between H_2O_2 and transition metals like iron and copper, as well as peroxynitrite ($ONOO^-$) a highly reactive free radical formed in a rapid reaction between $O_2^{\bullet -}$ and NO.

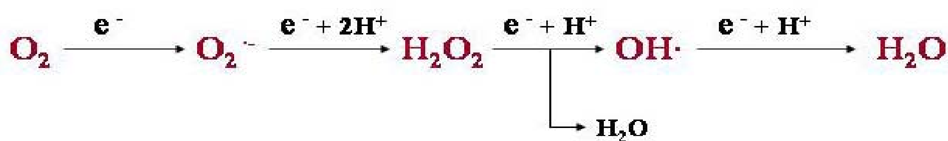


Figure 12. Reactive Oxygen Species (ROS), superoxide anion ($O_2^{\bullet -}$), hydrogen peroxide (H_2O_2), hydroxyl radical ($OH^{\bullet}$) are formed by the partial reduction of oxygen (O_2).

4.2. Reactive nitrogen species (RNS)

RNS are reactive species derived from nitric oxide (NO). NO is a molecule with 11 valence electrons, 6 from oxygen and 5 from nitrogen, with an unpaired electron in the last orbital, making NO a free radical ($^{\bullet}$NO). NO can also exist as the nitrosonium ion (NO+) depending on the cellular redox status (Stamler et al., 1992). For this reason it is termodynamically unstable and tends to react with other molecules.

NO is produced by a group of enzymes denominated NOS upon the conversion of L-Arginine to citrulline in the presence of NADPH and O_2. There are four members of the NOS family: neuronal NOS (nNOS), endothelial NOS (eNOS), inducible NOS (iNOS) and mitochondrial NOS (mtNOS). The last one is an isoform of nNOS present in the inner mitochondrial membrane (Elfering et al., 2002). They can be found in almost all the tissues and they can even co-exist in the same tissue. nNOS and eNOS are Ca^{2+}-calmodulin-dependent enzymes constitutively expressed in mammalian cells (Mungrue et al., 2003) that generate increments of NO lasting a few minutes. In contrast, iNOS is Ca^{2+}-calmodulin independent and its regulation depends on "*de novo*" synthesis (Ebadi and Sharma, 2003). iNOS is expressed following immunological or inflammatory stimulation in macrophages, astrocytes, microglia and other cells producing high amounts of NO lasting hours or days (Iadecola et al., 1995).

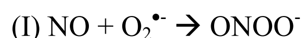
NOS isoforms have four prosthetic groups. Flavin adenine dinucleotide (FAD), flavin adenine mononucleotide (FMN) and iron protoporphyrin IX (heme) involved in the redox reactions leading to the synthesis of NO and tetrahydrobiopterin (BH4) which is absolutely necessary for NOS activity since constitute the scaffold that maintains the substrate channel. NOS structure shows two biodomains working independently. The first one consists of a C-terminal reductase domain containing sites to bind NADPH, FAD, FMN and Ca^{2+}-calmodulin. The binding of Ca^{2+}-calmodulin triggers the

activation of the enzyme opening a gate for the electron flux into the active center of the NOS. The N-terminal domain has oxygenase activity containing sites to bind BH4, heme and L-arginine (L-Arg) (Mayer et al., 1991;Guix et al., 2005).

4.2.1. Peroxynitrite formation

NO has been implicated in the physiology and pathophysiology of several systems where it exerts dual roles. Among the physiologic functions described is the relaxation of SMCs, the cytoxicity mediated by immune and glia cells, as well as, it can works as a neurotransmitter when produced by neurons (Guix et al., 2005). However NO is thermodynamically unstable and tends to react with other molecules, resulting in the oxidation, nitrosylation or nitration of proteins, with the concomitant effects on many cellular mechanisms. Much of NO mediated pathogenicity depends on the formation of secondary intermediated such us peroxynitrite anion ($ONOO^-$) and nitrogen dioxide ($^\bullet NO_2$).

NO undergoes various reactions in biological fluids resulting in the formation of nitrites (NO_2^-), and nitrates (NO_3^-). During the formation of nitrates, there are intermediate products such as $^\bullet NO_2$ and $OH^\bullet$ that are highly reactive (Beckman and Koppenol, 1996). However, many of the pathophysiologic effects of NO are mainly mediated by the highly reactive peroxynitrite anion ($ONOO^-$). $O_2^{\bullet -}$ react with NO at a higher affinity than for SOD (Huie and Padmaja, 1993) so that, when both $O_2^{\bullet -}$ and NO levels are in the nanomolar range, the former reaction will generate $ONOO^-$, while at the same time inactivates NO (Beckman and Koppenol, 1996).



$$(I)\ NO + O_2^{\bullet -} \rightarrow ONOO^-$$

4.2.2. Nitration, nitrosylation or oxidation of proteins

Under physiological conditions $ONOO^-$ has a half-life of 1–2 s and an action radius of 100 µm, being degraded into multiple toxic products (Beckman et al., 1990) or scavenged by the reaction with bicarbonate to produce nitrosoperoxycarbonate ($ONOOCO_2^-$) (Whiteman et al., 2002). Indeed low levels of $ONOO^-$ could be detoxified, under pathological conditions in which $O_2^{\bullet -}$ levels are high, exist an increase of $ONOO^-$ formation which reacts with proteins residues leading to protein nitrotyrosination. Protein nitration by $ONOO^-$ depends on its secondary products

($^{\bullet}NO_2$) formed when is protonated to the acidic form peroxynitrous acid (ONOOH) (II), (III). Nitration consists of the addition of a nitro group (NO_2) to proteins, mainly to tyrosine residues (Tyr) to give 3-nitrotyrosine. The local environment of the Tyr is important in order to be nitrated, since the proximity of negatively charged residues increases the susceptibility to nitration (Souza et al., 1999), but it is not a massive process since the nitration under inflammatory conditions affects 1–5 of every 10,000 Tyr (Radi, 2004). Protein nitration can alter protein function and conformation, impose steric restrictions and also inhibit tyrosine phosphorylation depending signaling (Radi, 2004). Moreover, some authors have proposed nitrotyrosination as a labeling for protein degradation (Grune et al., 1998).

$$\text{(II) } ONOO^- + H^+ \rightarrow ONOOH \rightarrow [^{\bullet}NO_2/^{\bullet}OH] \rightarrow NO_3^- + H^+$$

$$\text{(III) } ONOO^- + H^+ \rightarrow ONOOH + NO \rightarrow {}^{\bullet}NO_2 + NO_2$$

ONOOH and its intermediate reaction products $^{\bullet}NO_2$ and $^{\bullet}OH$ (II,III), act as oxidant agents. $^{\bullet}OH$ triggers a non-specific reaction with any cell molecule, whilst certain amino acids, such as lysines, histidines, cysteines and methionines, are more susceptible oxidation by $^{\bullet}NO_2$. On the other hand, nitrosylation is the addition of an NO group to organic molecules without producing any change in the substrate charge, resulting in C-nitroso, N-nitroso, O-nitroso, or S-nitroso derivatives.

4.3. Oxidative stress and Alzheimer's disease

Brain is particularly vulnerable to oxidative damage because it has high energy requirements and a high physiological oxygen consumption rate. Moreover, it contains high levels of transition metals which can act as catalysis for the formation of ROS and it has a relative low level of antioxidants such as glutathione, compared to other tissue in the body (Behl, 2005). In normal aging exists an imbalance between cellular antioxidants and pro-oxidants yielding to an age-related accumulation of ROS (Ames et al., 1993). Since AD is an age-associated disorder, the involvement of oxidative stress in the pathogenesis of AD disorder is further supported by the free radical hypothesis of aging. There is an increased amount of experimental and histopathological evidence that support the role of oxidative stress in the etiology and also in the pathophysiology of the disease (Miranda et al., 2000;Behl, 2005).

4.3.1. Oxidative modifications in lipids, proteins and DNA

Post mortem studies showed oxidative markers in lipids, proteins and nucleic acids from AD patients (Miranda et al., 2000). Aß induces lipoperoxidation of membranes (Sayre et al., 1997;Mark et al., 1997) which in addition to the membrane damage, this process produces 4-hydroxy-2-transnonenal (4-HNE) and malondialdehyde (MDA). This lipid peroxidation products exhibit cytotoxic properties and leads to the disruption of physiological signaling pathways. 4-HNE are capable of modifying membrane proteins (e.g. the glucose transporter, glutamate transporter) changing their functions and in turn, promoting deleterious effects for the cell (Kelly et al., 1996). As well, 4-HNE contributes to the cytoskeleton derangement characteristic of AD (Sayre et al., 1997) being involved in tau hyperfosforilation (Mattson et al., 1997). Moreover, 4-HNE levels were significantly increased in the ventricular fluid of AD patients compared with control subjects (Lovell et al., 1997). Oxidative modifications to proteins yield to the loss of their function due to oxidation of sensitive amino acids such as histidine, proline, arginine and lysine and also to the release of the toxic carbonyl radical (Stadtman, 1990). Carbonyls are increased in AD tissue, indicating an enhancement of oxidative stress in AD brain (Smith et al., 1991). Furthermore, proteins and lipids can react with monosaccarides through a non-enzymatic reaction leading to the formation of advanced glycation end products (AGEs) (Munch et al., 1997). Cytoplasmatic RNA and nuclear and mitochondrial DNA are also susceptible of oxidative modifications in AD, rendering hydroxylated products of their bases (Lovell et al., 1999). As a compensatory mechanism, the DNA repairing enzyme poly-ADP-ribose polymerase is found overexpressed in AD brains. Though, this effect depletes ATP and other crucial substrates (Soriano et al., 2001).

4.3.2. Cellular response to oxidative stress: Mitogen activated protein kinases (MAPKs) and redox sensitive transcription factors

Mitogen activate protein kinases (MAPK) pathways are the central mediators that propagate extracellular signals from the membrane to the nucleus. The three best-known MAPK pathways are extracellular signal regulated kinase (ERK) and stress activated protein kinases (SAPK), c-Jun N-terminal kinase (JNK) and p38 MAPK. ERK pathway is primarily activated by mitogen stimuli whereas JNK and p38 MAPK are

generally activated by cellular stressors such as oxidative stress. SAPK and their downstream effectors are implicated in neuroprotection or neurodegeneration depending on the cellular and environmental conditions as well as cooperation with other signalling pathways (Mielke and Herdegen, 2000). Thus in neuronal cells, potentially deleterious stimuli such as deprivation of trophic factors, UV irradiation, free radicals, hypoxia, ischemia, heat shock and cytokines provoke an intracellular stress response that either leads to apoptosis or defensive-protective adaptations. Low levels of ROS play an important role in normal cell proliferation (Stadtman, 1990) and regulate cellular signalling by the activation of MAPKs leading to induction of gene expression to protect cells. At high concentrations, these agents activate apoptosis (Kong et al., 1998). Some authors propose that low concentrations of H_2O_2 activates phosphatidylinositol-3-kinase (PI-3K) giving an increase in the cell survival by the activation of c-AMP response element binding protein (CREB) throughout the action of ERK1/2 and Akt/PKB pathways, while high concentrations of H_2O_2 are proapoptotic throughout the activation of JNK in cortical neurons (Crossthwaite et al., 2002).

In AD, all three signalling pathways, ERK, JNK and p38 MAPK are activated in accordance with the findings that propose a role for both, oxidative stress and aberrant mitotic signalling in the pathogenesis of AD (Perry et al., 1998). However, MAPKs are differentially activated during the course of the disease. Immunohistochemistry studies of brain tissue of patients with different stages of the disease according the Braak scale shown that all three kinases are activated in mild and severe cases (Braak stages III-IV) whereas in non-demented cases with limited pathology (Braak stage I and II), both ERK and JNK are activated but not p38 MAPK suggesting that both oxidative stress and abnormalities in mitotic signalling can independently serve to initiate, but both are necessary to propagate the disease pathogenesis (Zhu et al., 2001).

Concerning to SAPK, all JNK isoforms, JNK1, JNK2 and JNK3 has been related to the initial and last steps of AD pathology. JNK1 has been related to Hirano bodies (intracellular aggregates present in AD neurons and composed of many different proteins) while JNK2 and JNK3 were related to NFTs (Zhu et al., 2003). JNK is not only activated, also redistributed from nuclei to the cytoplasm in a manner that correlates with the progression of the disease. In late stage of AD, JNK is localized associated to NFT, evidencing its role in the phosphorilation of tau protein and likely in

the formation of NFTs. As well as, p38 MAPK has been also associated to neurofibrillary pathology (Zhu et al., 2004) and it has been proposed a key downstream agent in Aß-induced neuronal death (Zhu et al., 2003). AD animal models further demonstrate the involvement of JNK and p38 MAPK pathways in AD. Thus, both SAPK pathways are activated in the cerebral cortex of double transgenic mice for mutant APP (Swedish mutations) and PS1 (P264L), which produces a dramatic increase in the production and the consequent aggregation of Aß and tau phosphorilation (Savage et al., 2002).

The effect of oxidative stress on the expression and activity of transcription factors is complex and occurs at multiple levels, often in a paradoxical fashion since the same transcription factor can act as a suppressor or activator depending on the nature and duration of the stress and the cell type. A well-known redox sensitive transcription factors are the nuclear factor-κß (NF-κß) (Piette et al., 1997) and the activator protein-1 (AP-1) (Gass et al., 1992). Oxidative stress activates NF-κß and AP-1 triggering apoptosis or inducing the protection of the cells (Vollgraf et al., 1999;Bossy-Wetzel et al., 1997). AP-1 is a protein complex containing homodimers or heterodimers of c-Jun and c-Fos proteins. The three different cascades of kinases are involved in the activation of AP-1. ERK triggered by several cytokines induce c-Fos activity and JNK and p38 MAPK triggered by pro-inflammatory cytokines and cellular stress induce both, c-Fos and c-Jun activation, whereas ERK and JNK but not p38 MAPK signalling pathways have been implicated in NF-κß activation through phosphorilation of its inhibitor Iκß (Meyer et al., 1996). Its activation produces the translocation from the cytosol to the nucleus of AP-1 and NF-κß where acts as transcription factors (Behrens et al., 1999). These mechanisms have been demonstrated to occur under the effect of Aß in neurons (Kaltschmidt et al., 1997;Kaltschmidt et al., 1999;Mattson and Camandola, 2001), and in vascular cells with different pro-oxidant insults (Yin et al., 2002;Robbesyn et al., 2003).

4.3.3. Oxidative stress in AD etiology

Oxidant agents and oxidative products, such as H_2O_2 and 4-HNE, were shown to increase intracellular and secreted Aß levels in neuronal and non-neuronal cells (Paola et al., 2000;Misonou et al., 2000;Frederikse et al., 1996). H_2O_2 increase BACE1 gene expression in a dose-dependent and time-dependent manner which is traduced to an increase of secreted $Aß_{1-40}$ and $Aß_{1-42}$ in HEK293 cells overexpressing BACE1 (Tong et al., 2004). Most strikingly, expression and activity of BACE1 is increased by pro-oxidants as $H_2O_2/FeSO_4$ through HNE production in neurons (Tamagno et al., 2002). Pretreatment with antioxidants as α-tocopherol or dehydroepiandrosterone (DHEA, an adrenal steroid precursor to androgens and estrogens with antioxidant properties) is able to rescue the increase of expression, protein levels, and activity of BACE1 induced in NT2 neurons by oxidative stress (Tamagno et al., 2002;Tamagno et al., 2003b). Other studies carried out in SMCs proposed an increase on intracellular Aß accumulation in lysosomes together with an increase of the lipid peroxidation product 4-HNE after ferrous ions treatments. This effect is enhanced in apoE4 carriers (Mazur-Kolecka et al., 2004;Mazur-Kolecka et al., 2006).

Interestingly, in Down's syndrome, increased 8-OHdG and nitrotyrosine levels precede the formation of senile plaques and an increased level of isoprostane, a lipid marker for lipid peroxidation, precedes immuno-stainable amyloid plaques in transgenic mouse overexpressing APP (Pratico et al., 1998). All of this evidence suggests that oxidative stress has a causative role in AD development (Behl, 2005).

4.3.4 Oxidative stress in AD pathophysiology

Many factors have been discovered that either directly or indirectly induce disturbance of the oxidative homeostasis. However, Aß itself is one of the major sources of free radicals in AD brain. Several studies demonstrated that Aß generates H_2O_2 through metal ion reduction with concomitant TBARS (thiobarbituric acid-reactive substances) formation (Huang et al., 1999). Indeed, *in vitro* studies have demonstrated the involvement of oxidative stress in Aß-mediated cytotoxicity in neuronal (Behl, 1997) and vascular cells (Munoz et al., 2005a) since vitamin E (vit E) and other antioxidants protect against Aß-cytotoxicity. Two main theories explain how Aß fibrils induce free radicals generation. One of them proposes the oxidation of the

methionine 35 from $Aß_{1-42}$ as a mediator of free-radical-induced oxidative stress in AD brain (Butterfield and Bush, 2004;Butterfield and Boyd-Kimball, 2005). However, others propose the tyrosine 10 of Aß to play a key role in the catalytic production of H_2O_2 through copper reduction (Barnham et al., 2004). Aß aggregation induces metal-catalyzed free radical generation which contributes to crosslinking of Aß, increasing the production of Aß oligomers and large fibrils. These oligomers have themselves cytotoxic activity by interacting with RAGE, inducing an increase of intracellular ROS generation and in consequence NF-kß activation (Yan et al., 2000), or by other mechanisms which involve MAPKs activation. Free radicals cause damage to nucleic acids, membrane proteins producing carbonyl residues and protein nitration and induce membrane lipoperoxidation, (Mark et al., 1997). This damaging effect acquires much more importance in endothelial cells due to the formation of the highly reactive $ONOO^-$ by the reaction of $O_2^{\bullet -}$ with NO produced by eNOS (Guix et al., 2005). Moreover, disturbed energy metabolism is an early, predominant feature of AD. Damaged mitochondria are less efficient producers of ATP but more efficient producers of ROS (Harman, 1996;Miranda et al., 2000). Aß also disrupts cell homeostasis by impairing the function of membrane-regulatory proteins, including cation transport by ATPases and promoting the activation of N-methyl-D-aspartate (NMDA) receptors. The impairment in ATPase function, the activation of NMDA receptor plus the oxidative damage on mitochondria produces an increase of intracellular calcium levels which in addition to the oxidative damage to lipids, proteins and DNA trigger cell apoptosis (Miranda et al., 2000). As well, it has been demonstrated that $Aß_{1-40}$ and $Aß_{1-42}$ downregulate “bcl-2”, a key antiapoptotic protein, while only $Aß_{1-42}$ upregulated “bax”, a protein known to promote apoptotic cell death (Paradis et al., 1996).

Furthermore, extracellular Aß activates microglia by interacting with RAGE or the class A scavenger-receptor. Activated microglia induces an increase of free radicals mainly $O_2^{\bullet -}$ by the activation of NADPH-oxidase, NO, as well as the cytokine IL-1, as a consequence of the inflammatory process (Cras et al., 1990).

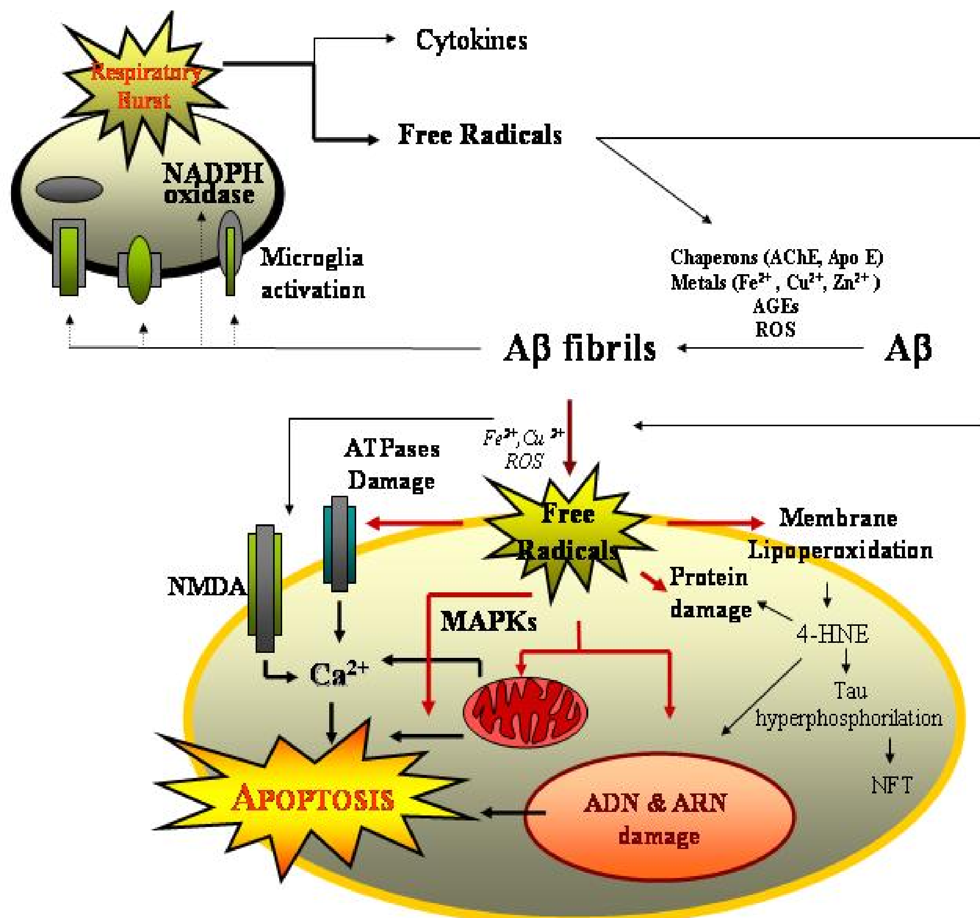


Figure 13. Aß-mediated cytotoxicity. Aß produces oxidative damage leading to the cell death. Free radicals induce membrane peroxidation with damage to both membrane lipid and protein, altering cell homeostasis by impairing ATPase and receptor function and MAPK signaling pathways activation. Although free radicals produce RNA and DNA damage too. All together produce the alteration of calcium homeostasis, which triggers cell apoptosis. Also, Aß fibrils activate microglia which in turn increases free radical formation.

4.3.5. Vascular oxidative stress

The vascular endothelium, which regulates the passage of macromolecules and circulating cells from blood to tissue, is a major target of oxidant stress, playing a critical role in the pathophysiology of several vascular diseases. In vessels the main ROS sources are xanthine oxidase (XO), mithocondria, myeloperoxidase (MPO), NADPH oxidase, uncoupled eNOS and CAA. Also, there are other sources of ROS production in vascular cells, including the mitochondrial electron transport chain, cytochrome P450 isoenzymes, lipoxygenase, cyclooxygenase, heme oxygenase, and

glucose oxidase, which contribute to a variable extent to the oxygen radical load of the vasculature.

NADPH oxidases are implicated in vascular oxidative stress associated with various vascular conditions such as hypertension and hyperhomocysteinemia. NADPH oxidase is important in vascular function for their responsiveness to a variety of agonists, such as angiotensin (Ang) II (Griendling et al., 1994).

Xantine oxidase (XO) catalyzes the oxidation of xanthine and hypoxanthine during purine metabolism using molecular oxygen and NADH to form $O_2^{\bullet-}$ and H_2O_2. XO is capable of producing large amounts of ROS under pathological conditions as ischemia/reperfusion injury, hypercholesterolemia and endothelial dysfunction in chronic heart failure. (Landmesser et al., 2002). XO is not only expressed in vascular cells but also circulates in the plasma and binds to endothelial cell extracellular matrix.

Myeloperoxidase (MPO) is a hemoprotein expressed in neutrophils and monocytes, which is secreted during activation of these cells and localizes in and around endothelial cells after leukocyte degranulation. MPO uses H_2O_2 to produce hypochlorous acid and other oxidizing species including RNS (Eiserich et al., 2002).

Uncoupled eNOS is another source of oxidative stress in endothelium when the concentration of L-arginine or BH4 is low, or if BH4 is oxidized, NOS become uncoupled and generate significant amounts of $O_2^{\bullet-}$ (Katusic, 2001). It occurs in hypertension, where activation of NADPH oxidase leads to oxidation of BH4 and production of large amounts of $O_2^{\bullet-}$ by eNOS (Eiserich et al., 2002).

As explained above in 4.3.2. Also, ROS can react with NO producing the highly reactive $ONOO^-$, which have dramatic damaging effects by the oxidation/nitration of proteins and triggering to vascular dysfunction.

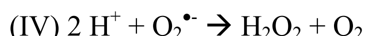
Specifically, accumulated oxidative stress in the vasculature lead to many cellular events: Interferes with NO function and impairs endothelium-dependent vasorelaxation, produce oxidative modifications to protein, lipids and nucleic acids, increases vascular endothelial permeability and promotes leukocyte adhesions and leads to alterations in vascular signal transduction, increased expression and activation of redox-sensitive genes, alters angiogenesis and reduced Cerebral Blood Flow (CBF) (Zhu et al., 2007;Taniyama and Griendling, 2003;Wassmann et al., 2004).

4.4. Antioxidant defense systems

The relevance of oxidative stress not only in the etiology but in the progression of AD has opened the possibility of antioxidant use in the treatment of this disease. Antioxidants are molecules or compounds that act as free radical scavengers. The cellular mechanisms of protection against oxidative stress damage is constituted by enzymatic and non-enzymatic antioxidants as superoxide dismutase (SOD), catalase, glutathione system, thioredoxin/thioredoxin reductase system, peroxiredoxins (Prx), glutathione- S-transferase, vitamins and estrogens.

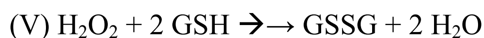
4.4.1. Antioxidant enzymes

SOD are a major cellular defense system against $O_2^{\bullet -}$ in all vascular cells, in fact, the relevance of the protective role of antioxidants in the vasculature was first evidenced by the observation that Aß-induced endothelial damage is prevented by the enzyme SOD (Thomas et al., 1996;Thomas et al., 1997;Crawford et al., 1997). SOD catalyse the breakdown of the highly reactive $O_2^{\bullet -}$ into O_2 and H_2O_2 reaction (IV) H_2O_2 has to be decomposed further by others antioxidants enzymes.



$$\text{(IV) } 2\,H^+ + O_2^{\bullet -} \rightarrow H_2O_2 + O_2$$

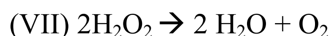
The glutathione system includes glutathione, glutathione reductase and glutathione peroxidase. This system is thought to be a major defense in low-level oxidative stress and it is considered one of the most important enzymes involved in the hydrolysis of peroxides in the brain. Glutathione peroxidase catalyzes the breakdown of H_2O_2 and lipid peroxides to water and lipid alcohols, respectively, using glutathione (GSH) as cosubstrate (V). After being oxidized, the active GSH is regenerated by the action of glutathione reductase (VI).



$$\text{(V) } H_2O_2 + 2\,GSH \rightarrow\rightarrow GSSG + 2\,H_2O$$

$$\text{(VI) } GSSG + NADPH{+}H^+ \rightarrow 2GSH + NADP^+$$

Catalase catalyses the conversion of H_2O_2 to water and molecular oxygen (VII). The enzyme catalase also plays a secondary role in the detoxification of phenols and alcohols (VIII). It is very effective in high-level oxidative stress and protects cells from H_2O_2 produced within the cell. It has been demonstrated that catalase blocks Aß toxicity (Behl et al., 1994).



$$\text{(VII) } 2H_2O_2 \rightarrow 2\,H_2O + O_2$$

(VIII) $H_2O_2 + R'H_2 \rightarrow R' + 2\ H_2O$

Further important enzymatic systems contributing to antioxidant defense include the thioredoxin/thioredoxin reductase system as well as glutathione S-transferases. Thioredoxin (Trx) plays an important role in the regeneration of proteins by the reduction of disulfide bonds formed under oxidative stress conditions. Reduced Trx is regenerated by thioredoxin reductases (Chae et al., 1999). Prx play important roles in eliminating H_2O_2 generated during cellular mechanisms using electrons from Trx. Protein levels of Prx-I and Prx-II were significantly increased in AD which induce protection against neuronal cell death, however, Prx-III, a mitochondrial protein were significantly decreased according to the mitochondrial damage described in AD (Kim et al., 2001). Glutathione S-transferases react with organic peroxides to form GSSG and the respective alcohols. Glutathione S-transferases is important in AD as is involved in detoxification of 4-HNE (Goon et al., 1993).

4.4.2. Non-enzymatic Antioxidants

Antioxidants molecules such as vitamin E, 17ß-estradiol (E_2) or melatonin contributes to antioxidant defense. The antioxidant properties of vitamin E, E_2 and melatonin are due to the OH bound to the mesomeric ring capable to react with free radicals to form innocuous end products.

The antioxidant molecules, gluthatione and Trx are explained as a part of an enzymatic system in 4.4.2.

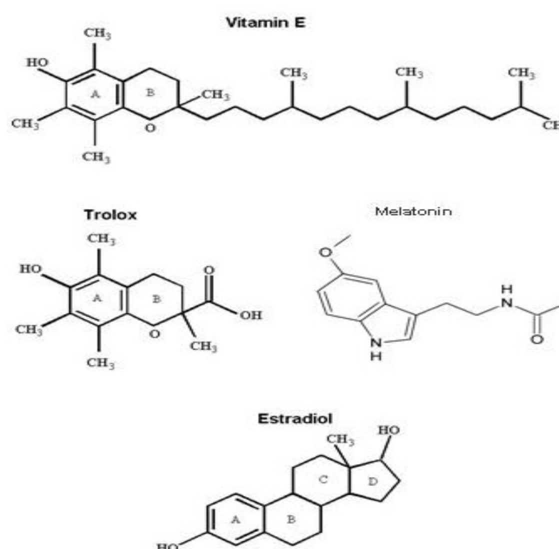


Figure 14. Molecular structure of vitamin E, trolox (a water analeg of vitamin E), 17ß-estradiol (E_2) and melatonin. The mesomeric ring bearing the reactive OH is labelled as A.

4.4.2.1. Vitamin E

Vitamin E exists in eight different forms or isomers, four tocopherols and four tocotrienols. All isomers have a chromanol ring, with a hydroxil group which can donate a hydrogen atom to reduce free radicals and a hydrophobic side chain which allows for penetration into biological membranes. These antioxidant and hydrophobic characteristics makes vitamin E the main antioxidant present in biological membranes (Perly et al., 1985) preventing lipid peroxidation by trapping peroxyl radicals (Halliwell and Gutteridge, 1984). However in vivo data has suggested that treatment with antioxidants such as vitamin E prevents learning and memory deficits caused by Aß, even though no changes in lipid peroxide in hippocampus of cerebral cortex was observed between control and treated rats (Yamada et al., 1999).Vitamin E has protective properties on neuronal cells (Behl et al., 1992) and vascular cells (Munoz et al., 2005b) against Aß- and H_2O_2-mediated cytotoxicity. The first demonstration of the protective role of vitamin E in AD patients was obtained in a clinical trial carried out with moderate to severe AD patients. This study confirmed the positive role of vitamin E in preventing AD progression showing a decline of neurological markers after 2 years of vitamin E treatment, (Sano et al., 1997). Other clinical trial suggests that use of the higher-dose vitamin E and vitamin C supplements may lower the risk of AD.

Besides the antioxidant properties of vitamin E, vitamin E has non-antioxidant actions mainly due to the modulation of several signalling pathways which contribute to its protective properties. Vitamin E has been reported to protect against oxidative stress by decreasing JNK activity and increasing the ERK activity in cardiac miocytes (Qin et al., 2003), and inhibiting caspase-3 activation in vascular endothelial cells (Uemura et al., 2002). Furthermore, the protective roles of vitamin E could also be related to other intracellular effects such as the activation of PP2A and the inhibition of alpha-PKC in VSMCs (Ricciarelli et al., 1998).

4.4.2.2. Estrogens

17ß-Estradiol (E_2) is a hormone with a wide range of cell activities and it has been proposed as a neuro- and cardiovascular protective element. The mechanisms of action of E_2 are classified as “nuclear effects” when the action of E_2 occurs at the nucleus, involving the direct participation of the estrogen receptors (ERs) as

transcription factors without other previous signalling steps required for E_2 action, or as “alternative pathways” which involve all other mechanisms of action of E_2. These alternative pathways might be initiated at either membrane or cytosolic locations and result in either direct local effects (e.g., modulation of ion channel activity and cell excitability) or effects such as the regulation of gene transcription secondary to the activation of signalling cascades (e.g., cAMP or MAPKs) (Nadal et al., 2001). The chemical phenolic structure of E_2 confers to E_2 antioxidant properties, fully independent of the activation of ERs or of any other signalling pathways. E_2 can react with peroxyl radicals (Braughler and Pregenzer, 1989) and inhibit phospholipid oxidation in cell membranes (Sugioka et al., 1987).

Several biological actions of E_2 produce a beneficiary effect in AD linked to its interaction with protective intracellular signaling pathways and its antioxidant effects. E_2 induces the decrease on Aß release (Xu et al., 1998), the enhancement of amyloid aggregates uptake by microglial cells (Li et al., 2000), the increase in the synthesis of choline-acetyltransferase (Luine, 1985) and the promotion of neuronal growth (Honjo et al., 1992). E_2 is a protective agent against Aß-mediated-toxicity in neurons (Behl, 2005).

Vascular cells express functional ERα and ERß. The binding of E_2 to ERs in vascular cells alter the expression of genes for important vasodilatory functions, such as prostacyclin synthase and eNOS (Haynes et al., 2000). E_2 accelerates endothelial cell growth and replacement in response to vascular injury, which may be partially attributed to increased local expression of vascular endothelial growth factor. However, one of the more important effects of E_2 in the vasculature is the modulation of nitric oxide (NO) bioavailability. E_2 activates eNOS via ERα-dependent manner in caveolae microdomains (Chambliss et al., 2000). Under basal conditions, eNOS is attached to caveolin-1, a scaffolding transmembrane protein in caveolae. Its activation depends on phosphorylation by phosphatidylinositol 3- kinase (PI3K)/Akt (Datta et al., 1999) and the binding of Ca^{2+}-calmodulin, which induces allosteric changes (Garcia-Cardena et al., 1997).

Binding of E_2 to the ERα induces rapid eNOS regulation. E_2 binds to ER and increases the formation of inositol 1,4,5- trisphosphate (IP3) which in turn stimulates Ca^{2+} release from the ER. Ca^{2+} forms a complex with calmodulin, which in turn binds to

and causes initial activation of eNOS, its dissociation from caveolin-1, and its translocation to intracellular sites. Also, there is a physical interaction between ER and the regulatory p85 subunit of PI3K. E_2 binding to ER directs the membrane recruitment of the catalytic subunit p110 of PI3K, that in turn phosphorylates the IP3 producing phosphatidylinositol 3,4-biphosphate (PIP2) into phosphatidylinositol 3,4,5-triphosphate (PIP3). Upon PI3K activation, a cytosolic serine/threonine protein kinase known as Akt/protein kinase B is recruited to the plasma membrane by interacting with PIP2 and PIP3 via its pleckstrin homology domain. It is then phosphorylated on threonine 308 by membrane-bound PIP3-dependent protein kinase-1 (PDK-1) and on serine 473 by either autophosphorilation or by an incomplete characterized PDK2. These Akt phosphorilation liberates the catalytic activity of Akt, which activates eNOS by a direct phosphorilation on serine-1177 (Dimmeler et al., 1999;Fulton et al., 1999) and its second translocation back to the cell membrane where it undergoes myristolation and palmytoylation, a process required for its full activation. This activation enhances calmodulin binding and produces an increase of three-fold of enzyme activity. MAPK activation could also favour eNOS phosphorilation, and hence activation. Activates eNOS promotes the transformation of L-arginine to L-citrulline (Chen et al., 1999;Simoncini et al., 2004;Orshal and Khalil, 2004).

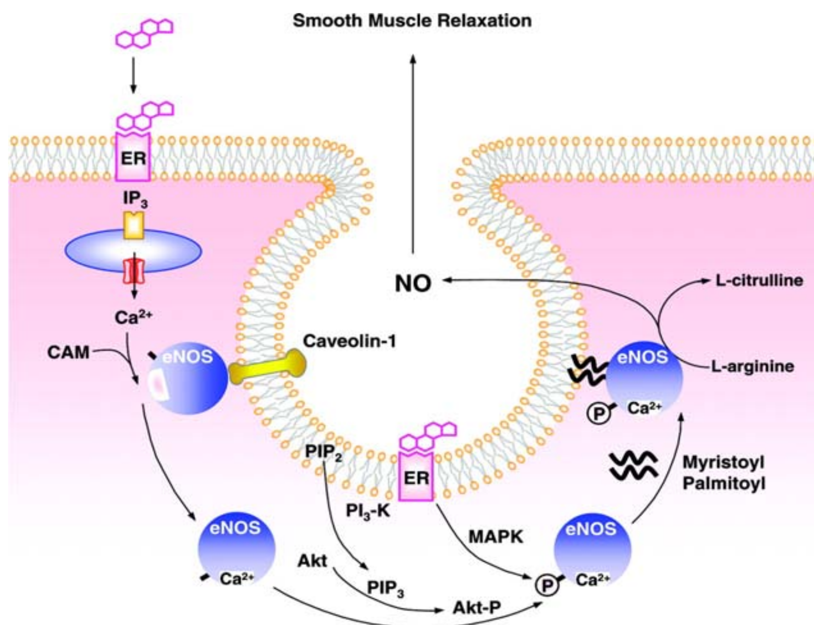


Figure 15. E_2-stimulates NO production in caveolae microdomains. (From Orshal and Khalil., 2004)**.**

Neuroprotective effects of estrogens via its antioxidant property and via its interaction in survival pathways (Behl, 2002) opens the possibility of estrogen use in the treatment of AD. Clinical trials demonstrated that women in the postmenopausal period who were treated with E_2, have a lower risk of developing AD (Kawas et al., 1997;Nadal et al., 2001) which suggest that estrogen replacement therapy (ERT) may help to prevent the occurrence of AD. On the other hand, other studies offer a less optimistic scenario. It is important to point that there is still some controversy about the adverse effects of ERT treatment linked to the increase of the risk of stroke (Gilgun-Sherki et al., 2002;Rossouw et al., 2002). Estrogen effects are complex, with plenty of bench studies praising its neuro- and cardiovascular protective effects, but with disappointing results in clinical trials.

II. Aims

General Aim

The aim of this thesis is to contribute to the knowledge of the vascular aspects of Alzheimer's disease mainly focused on the role of the oxidative stress in both the etiology and the pathophysiology of the disease. Thus, we have addressed the contribution of vascular smooth muscle cells in vascular amyloid deposits formation and also the cytotoxicity of the vascular amyloid deposits on endothelial cells. Moreover, the protective effect of antioxidants in the neurovascular dysfunction induced by vascular amyloid deposits is evaluated.

Specific aims

I- The study of the expression and activity of the secretases involved in both the non-amyloidogenic and the amyloidogenic APP processing in primary cultures of human cerebral vascular smooth muscle cells (HC-VSMCs).

II- The study of the capacity of HC-VSMCs to produce $Aß_{1-40}$ and $Aß_{1-42}$.

III- The study of the role of oxidative stress in modulating expression or/and activity of the secretases involved in APP proteolytic pathway in HC-VSMCs.

IV- The study of the role of stress activated protein kinases (SAPK) signalling pathways, JNK and p38 MAPK on oxidative stress-modulating expression or/and activity of the secretases involved in APP proteolytic pathway in HC-VSMCs.

V- The study of the protective effect of E_2 in Aß-mediated toxicity on the different brain cell types that are the pathophysiological targets in AD: neuronal, glial and vascular cells (endothelial and smooth muscle cells).

VI- The study of the role of nitric oxide (NO) in the Aß-mediated toxicity on the different brain cell types involved in AD pathology.

VII- The study of the role of the estrogen receptor in the effect of E_2 on vascular cells challenged with Aß fibrils, searching for the relationship between E_2, acting in an alternative pathway different to that described in neurons, and NO on the cytotoxicity induced by oxidative stress.

VIII- The study of the presence of nitrotyrosination in protein tyrosine residues induced by peroxynitrites and the identification of the main nitrotyrosinated proteins.

IX- Attending to the fact that reduced levels of LRP, the main Aß clearance receptor, is observed in AD patients and patients with cerebrovascular ß-amyloidosis, we study the involvement of oxidative stress induced by Aß or copper treatment in LRP downregulation in primary cultures of human brain vascular endothelial cells (HBECs).

X- The study of the role of NO in the Aß- and copper-mediated toxicity on HBECs also considering the putative action of peroxynitrite.

III. Results & Methods

Chapter I

"Oxidative stress triggers the amyloidogenic pathway in human brain vascular smooth muscle cells"

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Overview

As the origin of the vascular amyloid deposits is still controversial: neuronal versus vascular, in the first part of this Thesis we study the contribution of vascular smooth muscle cells in the formation of vascular amyloid deposits and consequently, in neurovascular dysfunction. In the present work, we demonstrate that primary cultures of human cerebral vascular smooth muscle cells (HC-VSMCs) have all the secretases involved in APP cleavage and produce $Aß_{1–40}$ and $Aß_{1–42}$. Since vascular aging is associated to an increase of ROS, we evaluated the role of oxidative stress in modulating expression or/and activity of all the secretases involved in $Aß_{1–40}$ and $Aß_{1–42}$ release. We have observed that APP, ADAM10, ADAM17, PS1 and PS2 are not modified by oxidative stress in HC-VSMCs, while BACE1 transcription, expression and activity were significantly augmented in VSMCs. This process is mediated by c-Jun N-terminal Kinase and p38 MAPK signalling and appears restricted to BACE1 regulation as no changes in the other secretases were observed. In conclusion, oxidative stress-mediated up-regulation of the amyloidogenic pathway in HCVSMCs may contribute to the overall cerebrovascular amyloid angiopathy observed in AD patients.

Coma M, Guix FX, Ill-Raga G, Uribesalgo I, Alameda F, Valverde MA, Munoz FJ.

Oxidative stress triggers the amyloidogenic pathway in human vascular smooth muscle cells

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Chapter II

"Lack of oestrogen protection in amyloid-mediated endothelial damage due to protein nitrotyrosination

M. Coma, F. X. Guix, I. Uribesalgo, G. Espuña, M. Solé, D. Andreu and F. J. Muñoz

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Overview

In the second part of this thesis, we have studied the cytotoxicity effects of vascular amyloid deposits through free radical generation. Antioxidants have shown neuroprotective activities against Aß-induced cytotoxicity. Among the different antioxidants used both in "*in vitro*" and "*in vivo*" studies, estrogen (E_2) has garnered the most attention. Consequently, in the present work we evaluated the protective effect of E_2 front Aß-mediated oxidative stress in neurons and vascular cells. Since E_2 induced the production of nitric oxide (NO), and NO can react with superoxide yielding to the formation of peroxynitrite, a special interest of this work was addressed to the study of NO and the consequent nitrotyrosination of proteins. In the present work, we demonstrate that E_2 attenuated $Aß_{E22Q}$-induced toxicity in neurons and vascular smooth muscle cells but failed to protect endothelial cells through a mechanism which involves peroxynitrite formation and protein nitration. Our data highlight the potential damaging consequences of E_2 in vascular disorders dealing with oxidative stress conditions, such as cerebral amyloid angiopathy, stroke and ischemia-reperfusion conditions.

Coma M, Guix FX, Uribesalgo I, Espuna G, Sole M, Andreu D, Munoz FJ.
Lack of oestrogen protection in amyloid-mediated endothelial damage due to protein nitrotyrosination
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Chapter III

"Oxidative stress induces the downregulation of LRP, the main Aß clearance receptor in brain endothelial cells"

Overview

The third part of this thesis was performed in collaboration with Dr. Berislav Zlokovic in the Frank P. Smith laboratories for Neuroscience and Neurosurgical Research, University of Rochester Medical Center in Rochester, New York. Since an increased production of Aß and/or a decreased clearance from the brain could be the scenario that triggers AD, the aim of this collaboration was the study of the role of oxidative stress on the low-density lipoprotein receptor related protein-1 (LRP) in primary cultures of human brain vascular endothelial cells (HBECs). This work is base in the previous findings from Dr. Zlokovic laboratory demonstrated that Aß (soluble $Aß_{1-40}$, $Aß_{1-42}$ or DI-$Aß_{1-40}$ (Dutch/Iowa-$Aß_{1-40}$) produces a dose-dependent reduction of LRP in brain endothelium HBECs. The mechanism underlying this effect seems to be the acceleration of the proteasome-dependent LRP degradation since Aß treatment did not reduce the expression of other cell surface receptors in HBECs such as transferrin receptor, and the levels of the receptor for advance glycation end products (RAGE) were increased (Deane et al., 2004a). These findings are consistent with reduced brain capillary LRP levels in Aß-accumulating transgenic mice, AD patients and patients with cerebrovascular ß-amyloidosis. It is well-known that Aß mediates cytotoxicity through oxidative stress and the fact that Aß induces a proteosome-dependent downregulation of LRP in HBECs suggests that Aß may reduce brain Aß clearance across the BBB through a mechanism which could involve oxidative stress. To explore this hypothesis we evaluated the role of oxidative stress in HBECs under different oxidative stress insults, $Aß_{1-42}$ and Copper (Cu^{2+}). In the present work, we have demonstrated that under conditions of oxidative stress, LRP levels were decreased in HBECs through a mechanism that involves nitric oxide (NO). Peroxynitrite ($ONOO^-$) induces the downregulation of LRP, as well as Cu^{2+} and Aß, and it could be prevented using an

inhibitor of eNOS. Our data highlight the potentially adverse consequences of oxidative stress in vascular disorders that cause LRP downregulation and reduce Aß clearance from brain, which contributes to AD.

1. Material and Methods

1.1 Cell cultures

Primary human brain endothelial cells (Kindly provided by Socratech, Rochester, NY) were grown in RPMI 1640 media supplemented with 20% fetal bovine serum (FBS), 30µg/ml endothelial cell growth supplement (ECGS), 5 U/ml heparin, 1% sodium pyruvate, 1% Minimum essential media Non-essential Amino Acid Solution (MEM-NEAA), 1% MEM vitamin and 1% antibiotics (penicillin/streptomycin).

1.2. Treatments

Cells were seeded in 6-well plates at density of $3x10^5$ cells/well and treated in 0,1% FBS media with 10 µM soluble $Aß_{1-42}$, 200 nM Cu^{2+} for 48h or 10^{-5} M 3-morpholinosydnonimine (SIN-1; donor of peroxynitrite) for 12h. Pre-treatment of 10^{-4} M N^G-nitro-L-Arginine (L-NNA; inhibitor of eNOS) was added to culture media 1h before $Aß_{1-42}$ or Cu^{2+} exposure. Determination of $Aß_{1-42}$, Cu^{2+}, and SIN-1 sublethal concentration was obtained running dose-response cell viability assays using the Cell Counting Kit- 8 (CCK-8) (Dojindo, Gaithersburg, MD).

1.3. Western-blot assay

Protein lysates were obtained from treated cell cultures. Samples were analyzed by SDS/PAGE using 10% tris-glycine gels. The primary antibodies (Ab) used: 4 µg/ml anti-mouse LRP ß chain antibody (Ab) (5A6) (Calbiochem), 4 µg/ml anti-mouse LRP α-chain Ab (8G1) (Calbiochem) and 0,4µg/ml anti-goat ß-actin Ab (I-19) (Santa Cruz Biotechnology). Secondary antibodies used: 0,55 µg/ml anti-mouse IgG/HRP Ab (DakoCytomation), 0,55 µg/ml anti-rabbit IgG/HRP Ab (Dakocytomation) and 0,2µg/ml anti-goat IgG-HRP Ab (Biotechnology).

1.4. Immunoprecipitation:

5 µg of anti-rabbit nitrotyrosine Ab (Molecular proves) for 50 µg of protein lysate was used for the immunoprecipitation of nitrated proteins with an immunoprecipitation kit from Roche. LRP ß subunit (LRP-85) was detected as described above in part 2.3.

1.5. Immunofluorescence:

Mice were treated with 0.12 mg/l of $CuSO_4$ in double distilled water. Control mice were treated with double distilled water. Brain sections were stained with 2µg/ml anti-rabbit-nitrotyrosine Ab for nitrotyrosine detection and 7,8 µg/ml anti-mouse CD31 (PECAM1) Ab (BD Pharmigen) for microvessels detection. Secondary antibodies used were anti-rabbit Alexa 488 Ab and anti-mouse Rhodamine Ab from Molecular Proves.

1.6. Statistical analysis

Data are expressed as the mean ± SEM of the values from the number of experiments as indicated in the corresponding figures. Data were evaluated statistically by using the Student´s *t*-test or the one way ANOVA test followed by Bonferroni's post-hoc analysis. The level of significance was $p < 0.05$.

2. Results

Oxidative stress, a harmful condition that increases with advanced age, has been implicated in the etiology and pathophysiology of AD. Many factors have been discovered that either directly or indirectly induce disturbance of the oxidative homeostasis in AD. With age, transition metals, such us copper (Cu^{2+}), iron and zinc, accumulate in brain endothelial cells and can act as catalysts for the formation of ROS which promotes vascular dysfunction. Aß *per se* has been reported to induce oxidative damage in both neurons and vascular cells, particularly in endothelial cells. In the present work, we have studied the role of oxidative stress induced by the transition metal Cu^{2+} or $Aß_{1-42}$ on LRP expression in brain endothelial cells. HBECs were treated with 200 nM Cu^{2+} or 10 µM $Aß_{1-42}$. These sublethal concentrations were previously adjusted carrying out dose-response cell viability assay (data not shown). Both chains

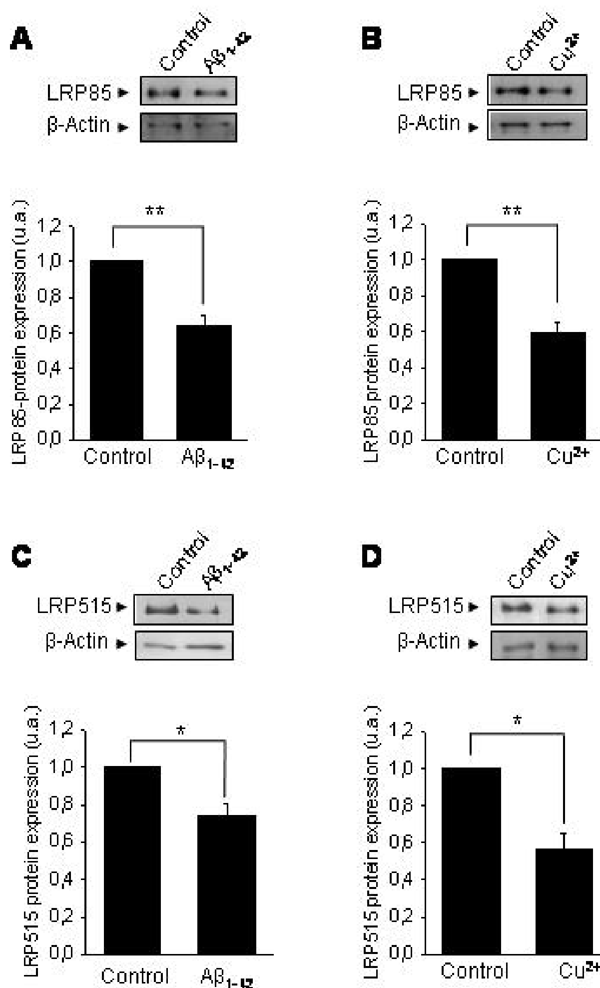

Figure 1. Downregulation of LRP1 by oxidative stress. Immunoblots of LRP-85 **(A,B)** and LRP-515 **(C,D)** after 48hr of incubation with 10 µM sAß$_{1-42}$ **(A,C)** or 200 nM Cu^{2+} **(B,D)** in HBECs. p<0.01/p<0.05 by student's t-test.

of LRP, LRP ß subunit (LRP-85) (Fig.1, upper panels) and the α subunit (LRP-515) (Fig.1, lower panels) were clearly reduced in HBECs exposed to Aß$_{1-42}$ (Fig.1 A,C) or Cu^{2+} (Fig.1 B, D).

These findings could be related to the production of peroxynitrites in a pro-oxidant environment. In endothelial cells, NO is produced by endothelial NO synthase (eNOS). Under conditions of oxidative stress, NO rapidly reacts with superoxide ($O_2^{\cdot-}$) to form the strongly reactive peroxynitrite anion ($ONOO^-$), which in turn increases oxidative/nitrative modifications in proteins. In chapter II, we demonstrated that vascular amyloid deposits correlate with nitrotyrosination in brain vessels from AD patients (Coma. et al. Brain, 2005. Figure 3E). Therefore, we also evaluated the role of Cu^{2+} as inducer of protein nitrotyrosination (Fig. 2. right panels) in microvessels (Fig. 2. middle panel) of copper-treated mice by confocal immunofluorescence. An increase of nitrotyrosine proteins has been observed in microvessels of copper-treated mice.

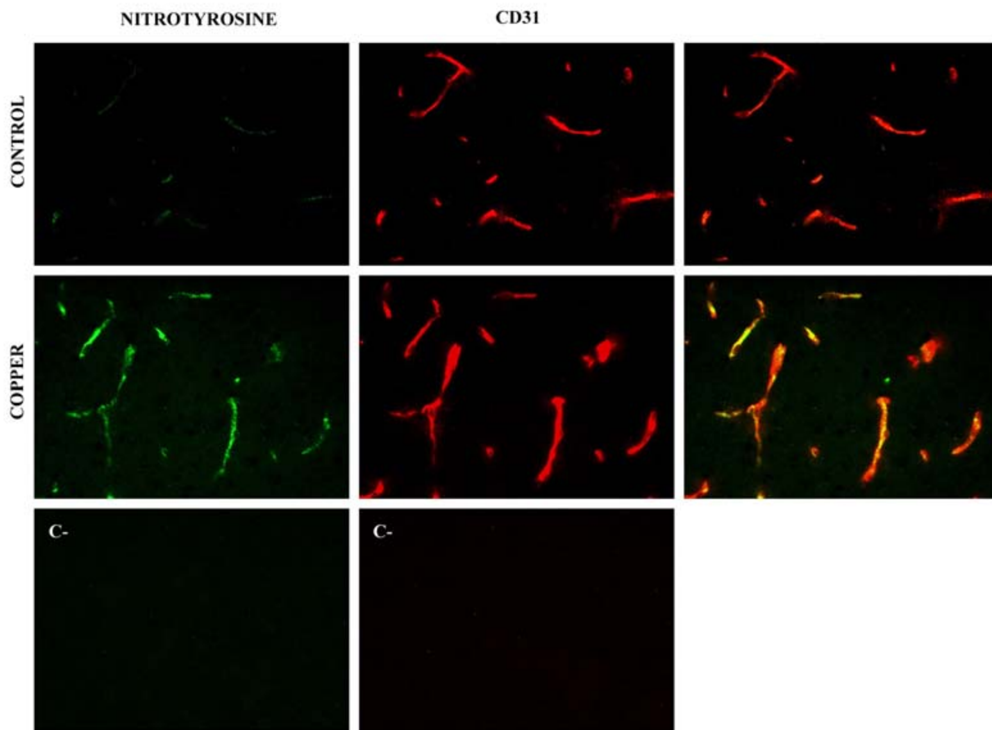

Figure 2. Increased nitrotyrosine protein staining in microvessels of copper-treated mice. Nitrotyrosine-specific fluorescence is shown as green staining using anti-rabbit nitrotyrosine Ab. Microvessels are stained in red with anti-mouse CD31 (PECAM1) Ab. Negative control for nitrotyrosine staining (right panels) and CD31 staining (middle panels). Merge (left panels).

In order to investigate the effect of peroxynitrite in LRP downregulation, we treated HBECs with the peroxynitrite donor SIN-1. HBECs were treated with the sub-lethal concentrations of 10^{-5} M SIN-1 for 12h previously adjusted carrying out dose-response cell viability assay (data not shown). SIN-1 treatment produces a downregulation of LRP-85 (Fig. 3A) and LRP-515 (Fig. 3B) in HBECs.

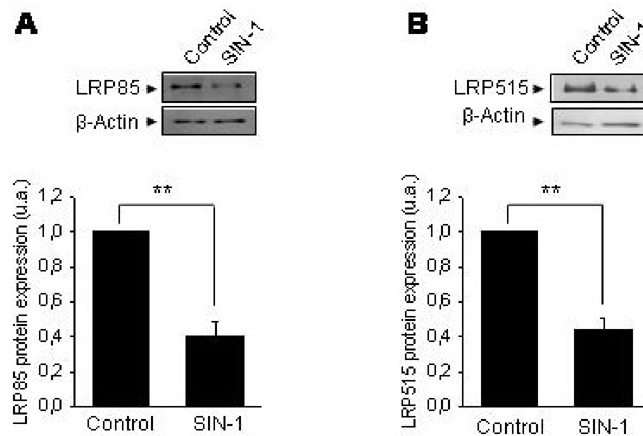

Figure 3. Downregulation of LRP by peroxynitrite. Immunoblots of LRP-85 **(A)** and LRP-515 **(B)** after 12h incubation with the peroxynitrite donor SIN-1 in HBECs. $p<0.05$ by Student's t-test

To further demonstrate the role of NO in LRP expression under oxidative stress conditions, L-NNA an inhibitor of eNOS, were challenged with $Aß_{1-42}$ and Cu^{2+}. 1h of 10^{-4} M L-NNA pre-treatment is able to prevent $Aß_{1-42}$ (Fig. 4A) and Cu^{2+} (Fig 4B)-dependent LRP-85 downregulation.

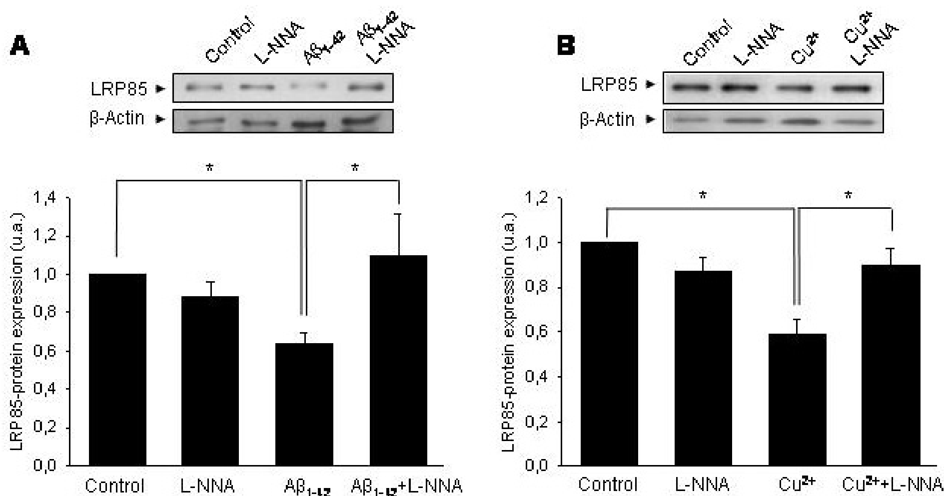

Figure 4. eNOS inhibitor is able to prevent oxidative stress-mediated LRP downregulation. Effect of NOS inhibitor 10^{-4} M L-NNA on 10 µM $sAß_{1-42}$ **(A)** or 200 nM Cu^{2+} **(B)** -mediated-LRP-85 downregulation in HBEC. $p<0.05$ by one-way ANOVA test.

Nitrotyrosination of proteins has been reported as a marker for proteosome-dependent degradation (Grune et al., 1998). Accordingly to previous studies of Dr. Zlokovic laboratory which demonstrated that Aß promotes proteosome-dependent LRP degradation without affecting LRP internalization or synthesis, we evaluated whether LRP is nitrotyrosinated under a peroxynitrite treatment as a mimic of the reaction of $O_2^{\cdot -}$ produces by Aß or Cu^{2+} with NO produced by endothelial cells. HBECs were treated with the peroxynitrite donor, 10^{-5} M SIN-1 for 12h and nitrotyrosinated proteins were immunopreciptated using an antibody anti-nitrotyrosination. Nitrated LRP was blotted using anti-LRP ß chain Ab (LRP-85) (Fig. 5B). While no nitrated LRP was detected at control conditions, nitrated LRP was detected under peroxynitrite treatment in HBECs. The significance of nitrated LRP increases, when we compare with the low levels of LRP observed under the peroxynitrite treatment by western-blot analysis of the same protein lysate (Fig. 5A).

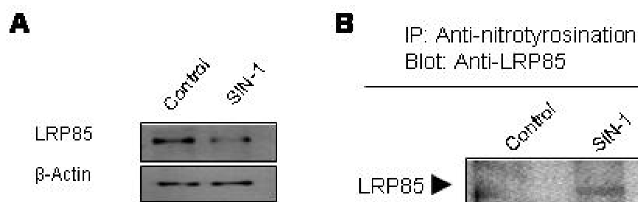

Figure 5. Identification of nitrated LRP1. Immunoblots of LRP-85 **(A)** and immunoprecipitation of nitrated proteins and blotted with anti-LRP ß chain antibody (LRP-85) Ab after 12h incubation with the peroxynitrite donor SIN-1 in HBECs **(B)**.

IV. Discussion

Life expectancy in industrial countries has increased, nonetheless over quality of life and healthcare expenditures associated with late life, prompts a dramatically increase in the number of individuals suffering aging related disorders such AD, which is the most prevalent form of senile dementia. The dementia is considered a direct effect of the neuronal damage but growing evidence suggests that CAA could play a crucial role in AD pathology (Haglund et al., 2004;Zlokovic, 2005;Iadecola, 2004). In fact, 80–90% of brains from AD patients show at least a mild degree of CAA, and about 25% shows moderate to severe CAA, involving cerebral vessels in one or more cortical regions (Rensink et al., 2003). Unfortunately, the contribution of CAA to the neurodegenerative progression of AD is still now poorly understood. This thesis is contributing to increase the knowledge of the relationship between amyloid and vessels, not only focused in the pathophysiology but also in the etiology of the disease. In normal aging, there is an imbalance between cellular antioxidants and pro-oxidants resulting in age-related accumulation of ROS that will increase the damage on the macromolecules (Ames et al., 1993). Local imbalances of ROS production can trigger or contribute to diseases. In fact, when we talk about the etiology and pathophysiology of AD, necessarily we have to talk about oxidative stress: Aß and oxidative stress are unavoidably linked since oxidants increase Aß production in neurons (Misonou et al., 2000;Paola et al., 2000;Tamagno et al., 2005), oxidative stress promotes Aß aggregation *in vitro* (Siegel et al., 2007), and fibrillar Aß induce oxidative stress *in vitro* and *in vivo* (Harkany et al., 2000).

Role of oxidative stress on Aß production by vascular smooth muscle cells

CAA appears in more than 90% of AD cases, however the source of vascular Aß remains unclear being the center of a lively debate with two possible origins: the accumulation of neuronal Aß in the course of perivascular drainage and the production of Aß within the vessel walls. The most widely accepted view proposes a neuronal origin for the vascular Aß deposits. Nevertheless, some evidences support the possibility of a relevant contribution of brain vessels in the formation of vascular Aß deposits (Frackowiak et al., 2005;Wisniewski et al., 1995). Thus cell culture studies have confirmed an intracellular pool of Aß in brain VSMCs from dogs, human and Swedish APP transgenic mice (Wisniewski et al., 1995;Frackowiak et al., 1995;Mazur-

Kolecka et al., 2003;Frackowiak et al., 2004). Other study carried out in human brain VSMCs from autopsies of AD patients demonstrated that VSMCs secreted $Aß_{1–40}$ but not $Aß_{1–42}$ (Frackowiak et al., 2005). Noteworthy, several reports demonstrated that both $Aß_{1–40}$ and $Aß_{1–42}$ are present in CAA vessel walls (Shinkai et al., 1995) being $Aß_{1–40}$ the most abundant one (Frackowiak et al., 2005). Regarding to the origin of amyloid aggregation, $Aß_{1-42}$ has been proposed to be deposited before any appreciable amount of $Aß_{1-40}$ is present in brain parenchyma acting as a seed for $Aß_{1–40}$ aggregation (Hardy and Selkoe, 2002;Harper and Lansbury, Jr., 1997). Interestingly, secretases involved in APP processing have not been previously described in VSMCs and regarding to the origin of $Aß_{1-42}$ in brain vessels, the arising question is: How does the low soluble $Aß_{1–42}$ enter into the perivascular drainage? If vascular $Aß_{1-42}$ has a neuronal origin, it should aggregate close to its production source due to its highly fibrillogenic nature. Thus, it is possible that the $Aß_{1–42}$ present in the vascular deposits is derived from a local Aß production.

In order to answer the former questions, in this thesis we have characterized the contribution of VSMCs to Aß production. Firstly we identify in VSMCs all the secretases involved in the neuronal APP processing. Since ADAM10 and ADAM17 are the most likely candidates for the α-secretase activity involved in the non-amyloidogenic processing of APP (Buxbaum et al., 1998;Lammich et al., 1999), we have demonstrated that both enzymes are constitutively present in VSMCs. Additionally, α-secretase activity was addressed throughout the identification of the p3CT, the C-terminal APP fragment resulting from its activity also called C83 or αCTF. Alternatively, the expression and activity of both enzymes implicates on the APP amyloidogenic pathway, ß-secretase (BACE1) and a γ-secretase (PS1 and PS2) (Selkoe, 1998a;Vassar et al., 1999) are also demonstrated in VSMCs. We obtain that under physiological conditions in culture, VSMCs produce $Aß_{1-40}$ that can be secreted or accumulated intracellularly, although the presence of $Aß_{1-42}$ is practically negligible.

Besides aging affects all the tissue in the body, the vasculature network is one of the most exposed to incessant external stresses such as oxidized lipids and proteins in the plasma as well as the usual increase on endogenous free radicals generation observed in advance ages. Oxidative stress has been implicated in the etiology of AD as it induces an increase of expression and activity of BACE1 in neurons (Tong et al.,

2005;Tamagno et al., 2002). In this thesis we evaluated the role of oxidative stress on APP and secretase expression and also its activity on HC-VSMC. Interestingly APP, ADAM10, ADAM17, PS1 and PS2 are not modified by oxidative stress in HC-VSMCs, while BACE1 transcription, expression and activity were significantly increased in VSMCs. This finding is crucial since BACE1 is a key enzyme in the production of Aß. Consistent with the role of oxidative stress in the APP amyloidogenic pathway, a pre-treatment with the antioxidant trolox, a water-soluble analogue of vitamin E that shares its antioxidant properties but not its non-antioxidant actions (McClain et al., 1995), abolishes the oxidative stress-dependent BACE1 up-regulation. The pathophysiological relevance of these findings is demonstrated by studies showing that both BACE1 protein concentrations and enzymatic activities are increased in AD brain (Fukumoto et al., 2002;Holsinger et al., 2002;Yang et al., 2003).

To further evaluate the mechanistic sequence of events that links vascular oxidative stress and BACE1 up-regulation in VSMCs, we have studied the role of stress-activated protein kinases, JNKs and p38 MAPK which are markedly up-regulated in AD (Zhu et al., 2004) and are activated by a variety of stress signals, including oxidative stress (Selkoe, 1998a). In accordance with previous studies carried out in neuronal cell lines demonstrating that hydrogen peroxide induces BACE1 promoter activity (Tong et al., 2004), Aß production (Tamagno et al., 2003a) and the activation of JNK and p38 MAPK signalling kinases (McDonald et al., 1998;Tamagno et al., 2003b), we have found that JNK and p38 MAPK are directly involved in the increased expression of BACE1 in VSMCs using specific pharmacological inhibitors of these two pathways. This process is independent of the proapoptotic signaling described for both JNK and p38 MAPK (Mielke and Herdegen, 2000) since the viability of VSMCs was not affected by the sub-lethal oxidative stress concentration used in this study. Accordingly, trolox pre-treatment prevents the activation of JNK and p38 MAPK, and in consequence BACE1 up-regulation. Despite the fact ERK signaling pathway is markedly increased in early stages of AD (Zhu et al., 2001;Perry et al., 1999), we have no found ERK involvement in the oxidative stress-dependent BACE1 up-regulation in VSMCs using specific pharmacological inhibitor of ERK pathway. These data do not avoid a putative role of ERK in amyloid pathology (Medina et al., 2005).

BACE1 expression is strongly regulated by multiple mechanisms in a complex control pattern. In addition to a number of factors acting as activators or repressors of BACE1 transcription, alternative splicing events, muscarinic cholinergic receptor signalling, inflammatory processes and post-translational modifications can influence BACE1 concentrations and enzymatic activity in brain (Rossner et al., 2006). Likewise it has previously seen in HEK transfected cells (Tong et al., 2004) or NT2 neuronal cell line (Tamagno et al., 2003a), in HC-VSMCs the oxidative stress induces an increase in BACE1 mRNA expression that correlates with an increase in BACE1 protein expression. Accordingly, our findings suggest a transcriptional regulation of BACE1 under oxidative stress in VSMCs. It is likely that some transcription factors, downstream of JNK and p38 MAPK pathways, are promoting the increase in BACE1 expression. Among all the putative transcription factors binding sites in BACE1 promoter some of them such as SP1, AP1, NF-κß and PPARγ are linked to oxidative stress. Mutations on the NF-κß binding sites have shown a bifunctional response of NF-κß in BACE1 expression. While NF-κß is stimulatory in activated astrocytes and soluble Aß–exposed neuronal cells, under basal conditions has a suppressor role (Bourne et al., 2007). This scenario becomes even more complex when taking into consideration the transcription factor Sp1, which is an important activator of the transcriptional regulation of BACE1 expression (Christensen et al., 2004). An interesting finding comes from the co-expression of APP and SP1 in HEK293 which produces an increase of BACE1 protein and in turn, the accumulation of the ß-secretase processed APP fragment, C99 and Aß (Christensen et al., 2004). In addition, Sp1 also is known to interact with NF-κß which could interfere in its transcriptional activity. Mutations on the PPARγ binding site of BACE1 promoter suggest a repressor function of PPARγ (Sastre et al., 2006;Sastre et al., 2003). However, beside acting as a transcriptor factor regulating gene expression, PPARγ can repress gene expression by antagonizing the activities of other transcription factors, such as STAT-1, NFκß and AP1 (Rossner et al., 2006). Furthermore the expression of the PPAR-γ protein is significantly decreased in primary culture of cortical neurons exposed to H_2O_2 for 24 h (Wang et al., 2007). Interestingly, PPARγ expression was found 40% reduced in the frontal cortex of AD brain and in transgenic mice (Sastre et al., 2006;Sastre et al., 2003), as well as, its binding in the BACE1 gene promoter. It has been demonstrated

that JNK but not p38 MAPK phosphorylates PPARγ and negatively regulates its transcriptional activity in 293T cell (Camp et al., 1999). AP1 could also play an important role in oxidative stress-dependent BACE1 transcriptional activity while both JNK and p38 MAPK are involved in AP1 activation. Nevertheless, further experiments are necessary to understand the specific mechanisms by which H_2O_2 through JNK and p38 MAPK participates in the transcriptional regulation of BACE1; what part of the BACE1 promoter contains the cis-acting element responsible for its oxidative stress mediated transcriptional regulation and/or other factors involved in this signalling pathway in the AD pathogenesis.

To further evaluate the functional significance of increased BACE1 after an oxidative insult, we quantitatively analyzed the intra- and extra- cellular levels of $Aß_{1-40}$ and $Aß_{1-42}$. Oxidative stress increased markedly the secretion of $Aß_{1–40}$ and $Aß_{1–42}$ but no changes have been observed in the intracellular levels of $Aß_{1–40}$ and $Aß_{1–42}$ in HC-VSMCs. Secreted $Aß_{1–40}$ reached a threefold increase ($p < 0.05$) and $Aß_{1–42}$ a five-fold increase ($p < 0.05$) compared to control conditions which could be reverted by an antioxidant pre-treatment. These findings strengthen the implication of oxidative stress in the induction of APP amyloidogenic pathway and highlight the crucial role of VSMCs in the origin and development of CAA. $Aß_{1–42}$ usually represents less then 20% of the total Aß secreted, however is both the earliest form and the predominant species deposited in the brain parenchyma (Golde et al., 2000). The key role of $Aß_{1-42}$ in AD has come from the study of mutations in the APP and PS genes that cause early-onset familial forms of AD due to a selectively increase of $Aß_{1–42}$ (Selkoe, 1998b). Although $Aß_{1–40}$, the most secreted Aß peptide from cells, is the predominant species aggregated in the amyloid deposits in the cerebral vasculature (Gravina et al., 1995;Iwatsubo et al., 1994), $Aß_{1-42}$ has been suggested to be deposited before any appreciable amount of $Aß_{1-40}$ is present (Hardy and Selkoe, 2002;Harper and Lansbury, Jr., 1997). Transgenic mouse studies using mutant APP and PS transgenes have provided some insights into the damaging effects on the ratio of $Aß_{1–40}$ and $Aß_{1–42}$ and the consequences on the onset of the deposition, type of deposit (e.g., diffuse versus compact), and extent of CAA (Borchelt et al., 1997;Herzig et al., 2006;Holcomb et al., 1998). Noteworthy, murine models expressing either $Aß_{1–42}$ or $Aß_{1–40}$ clearly show that both CAA and amyloid plaques require the presence of $Aß_{1–42}$ and that $Aß_{1–40}$ alone is not sufficient to

generate these lesions (McGowan et al., 2005). These data contribute to the seeding theory of Aß based on the fact that depositions of the less soluble $Aß_{1–42}$ precede and serve as a base for the subsequent deposition of the soluble $Aß_{1–40}$.

We believe that the overall results presented here, highlight the pathophysiological relevance of oxidative stress within brain vessels and strengthen the hypothesis of the contribution of VSMCs in the origin of the CAA. Those evidences strongly indicate that the drainage plus vascular hypothesis greatly influence brain Aß deposition. A possible scenario based in existing evidence (Attems, 2005;McGowan et al., 2005;van Dorpe et al., 2000;Rensink et al., 2003) might be as follows: With the onset of AD, $Aß_{1-40}$ and $Aß_{1-42}$ production by neurons increases and $Aß_{1-40}$ but rarely $Aß_{1-42}$ because of its highly fibrillogenic nature, enters to the perivascular drainage. In the course of ISF drainage, neuronal $Aß_{1-40}$ accumulates in blood vessel walls. This accumulation is probably facilitated by both pre-existing Aß derived from VSMCs and degenerative vascular changes (e.g., atherosclerosis). Taking into consideration that the highly fibrillogenic $Aß_{1-42}$ should aggregate in the proximity of the cells that produce it, we propose that the production of $Aß_{1-42}$ by VSMCs might act as a local seed (McGowan et al., 2005;Harper and Lansbury, Jr., 1997) to aggregate the less fibrillogenic $Aß_{1-40}$ produced by HC-VSMCs, as well as the $Aß_{1-40}$ produced in the brain parenchyma that arrives to the vasculature due to its perivascular drainage. In agreement with this hypothesis, a high fibrillogenic activity has been reported to occur in the extracellular matrix of VSMCs (Van Nostrand et al., 2000), which could contribute to Aß accumulation in the vessel wall and to CAA development. Also, the internalization of Aß produced by neurons plus the initial Aß deposition will cause more damage in VSMCs which together with degenerative changes in pericytes and endothelium produced by Aß deposition induces an impaired of BBB. In turn an impairment of BBB can lead to incorporation of peripheral Aß, causing further deposition and vascular degeneration (Attems, 2005;Rensink et al., 2003).

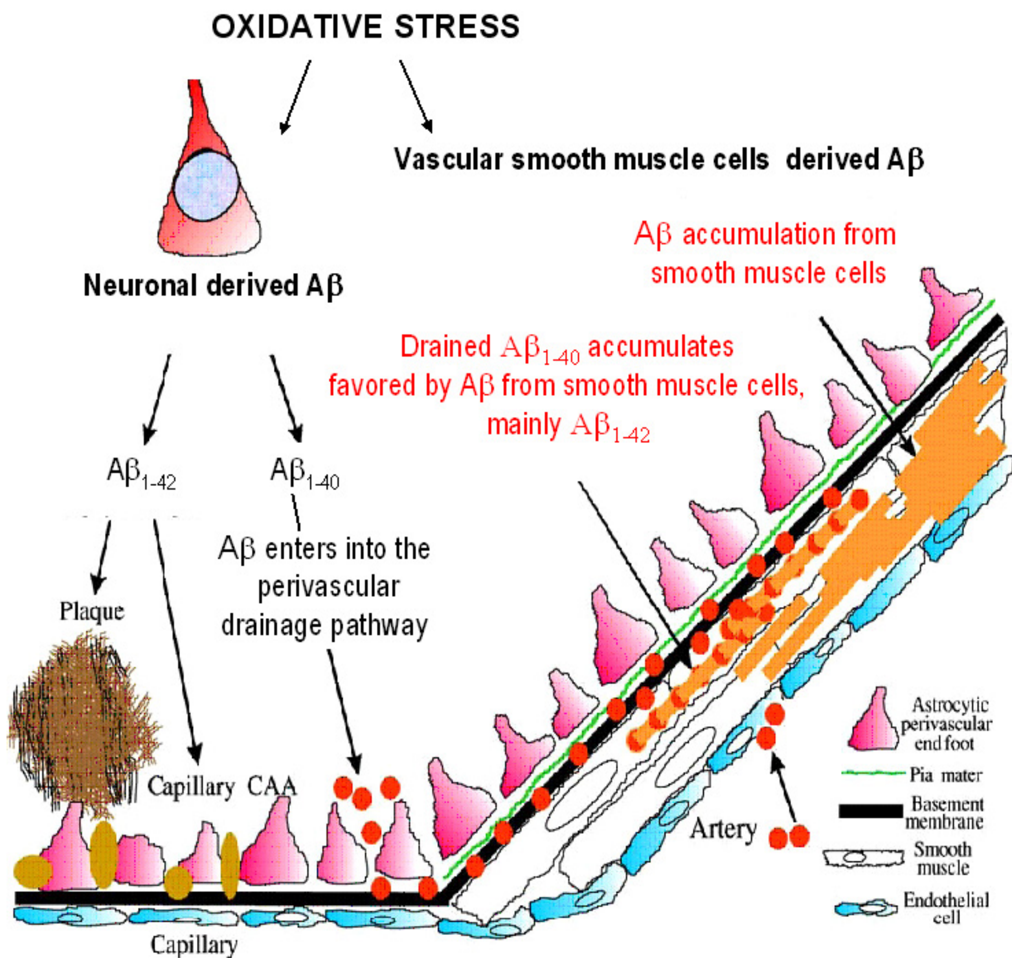

Figure 16. CAA is the product of the initial production of Aß by VSMCs induced by an increased vascular oxidative stress and the posterior aggregation of the neuronal Aß from the perivascular drainage (Modified from Attems J., 2005)

In summary, we propose that brain vascular smooth muscle cells directly contribute to the formation of vascular amyloid deposits and consequently to the neurovascular dysfunction. Increased oxidative stress in vessels of the aging brain or from those suffering hypertension, atherosclerosis, and/or ischemic processes could enhance the onset of CAA. Moreover, since Aß is producing oxidative stress on cells, the accumulation of parenchymal $Aß_{1-40}$ due to its poor drainage may increase BACE1 activity and also the release of Aß from smooth muscle cells derived, and hence, activates the etiopathogenic loop generated by Aß and oxidative stress at the vascular level.

Role of oxidative stress in Aß-induced toxicity in endothelial cells

Aß accumulation in the brain is considered the key pathogenic event that triggers brain dysfunction underlying the AD dementia (Hardy and Selkoe, 2002). The pathological mechanisms of Aß include oxidative stress, ionic homeostasis alteration, inflammation and apoptosis. Among them, oxidative stress plays an important role in AD dementia and mostly in brain vessel damage. Accordingly, APP transgenic mice exhibit vascular oxidative stress at 3-6 months age, when there is no evidence of ROS production in other brain cells (Park et al., 2004). Interestingly, ultrastructural features of vascular lesions and mitochondrial alterations indicative of oxidative stress damage have been also demonstrated in brain vascular cells from brains of AD patients and in APP transgenic mice (Aliev et al., 2002). The relevance of oxidative stress in the progression of AD (Behl et al., 1994) opens the possibility for the use of antioxidants in the treatment of this disease. Estrogens (E_2) due to its antioxidants and neurotrophic non-antioxidants properties have been proposed as neuroprotective agents in a wide number of *in vivo* and *in vitro* studies (Behl et al., 1992;Mendelsohn, 2002a). However, clinical trials have raised some controversy about the risks and benefits of the hormone replacement therapy (HRT). While several clinical trials have associated estrogens with the retardation of the onset and progression of AD (Kawas et al., 1997;Tang et al., 1996), the most rigorous Women's Health Initiative's Study (WHI) highlighted the risk of stroke associated to long-term HRT (Grodstein et al., 2000;Rapp et al., 2003).

In this thesis we have studied the protective role of E_2 against Aß-mediated cytotoxicity in different brain cell types targeted by Aß in AD. Our studies have shown a cell type-dependent protective effect of E_2 against Aß-mediated cytotoxicity. E_2 is able to protect cortical neurons, glial cells and smooth muscle cells against Aß, but fails to protect endothelial cells. Regarding to the cytotoxic effects of Aß in endothelial cells, the question that arises is: Is Aß induced cytoxicity mediated by oxidative stress in endothelial cells? Likewise in Aß treatments, E_2 is not able to protect endothelial cells from H_2O_2-mediated toxicity while the antioxidant trolox is able to protect endothelial cells against Aß-mediated cytotoxicity. Altogether these findings give enough evidence for searching other factors in the lack of E_2 protection in endothelial cells. One possible explanation is related to the presence of nitric oxide (NO).

Under physiological conditions NO is a powerful vasodilator whereas increased productions of NO coupled with elevated reactive oxygen species (ROS) scavenging NO, can lead to reduced bioavailavility of NO simultaneously with an increased oxidative stress damage through peroxynitrite formation. While E_2 is a well-known activator of endothelial nitric oxide synthase (eNOS) (Chen et al., 1999), the endothelial lack of protection by E_2 could be related to the E_2-dependent activation of eNOS and the production of NO. The use of antagonists of ER, an inhibitor of eNOS or a NO scavenger enables the protective effect of E_2 on endothelial cells challenged by Aß or H_2O_2. Moreover, confirming this hypothesis, the stereoisomer 17α-estradiol which shares with 17ß-estradiol its antioxidant properties but not its capacity to binds to ER is able to protect endothelial cells of Aß-induced cytotoxicity. Thus these results further confirm that the protective role of E_2 in a pro-oxidant environment is fully independent of the activation of ERs while is directly related to its antioxidant properties. *In vitro* the effective concentration of E_2 acting as an antioxidant is in the micromolar range (Behl, 2002), while the treatment with nanomolar concentrations of E_2 did not conferred protection against $Aß_{E22Q}$. As the effective concentration for the antioxidant properties of E_2 is much higher than the physiological levels of E_2, it seems unlikely that its antioxidant activity could be physiologically relevant, nevertheless estrogen levels *in vivo* are highly variable depending on the menstrual cycle and can reach nanomolar concentrations. However due to its hydrophobic nature it accumulates in cellular membranes in microenvironment where E_2 concentration could reach the micromolar range (Behl, 2002). In addition, unpublished data of our laboratory shows that the effective concentration of E_2 could be reduced within the physiological range in the presence of supporting antioxidants such as glutathione.

A key step by which Aß induces vascular dysfunction is through the reaction between superoxide anion with NO to form the highly reactive peroxynitrite (Radi, 2004). This reaction impairs vascular function by: i) reducing the amount of NO available for vasodilatation, ii) since NO is able to outcompete superoxide dismutase (SOD), it reduces SOD bioavailability for ROS scavenging iii) it produces oxidative/nitrosative damage to crucial enzymes for vascular function. Nitrotyrosination of protein residues is a well known damaging effect of peroxynitrite. Interestingly, an increase of nitrotyrosination of protein residues in neurons and glial cells of AD brains

has been described as a marker of cell damage (Castegna et al., 2003). Our results had shown a correlation between vascular amyloid deposits with nitrotyrosination in brain vessels from AD patients. These findings are in agreement with previous studies describing a significant increase in morphological alterations in cerebral capillaries (Farkas and Luiten, 2001a) and dysfunction of the blood-brain barrier in AD (Wisniewski et al., 2000b). Since Aß fibrils act as a source of superoxide anion (Butterfield and Bush, 2004;Butterfield and Bush, 2004), which can react with the basal levels of NO, due to the high affinity of NO for the superoxide anion (Huie and Padmaja, 1993), triggering nitrotyrosination, we have observed protein nitrotyrosination in endothelial cells exposed to Aß fibrils. However, higher levels of nitrotyrosination were observed in endothelial cells exposed to Aß plus E_2, an effect reverted by PTIO and L-NNA. Interestingly, no increase in cell viability was seen in the presence of Aß and PTIO or L-NNA suggesting that the main source of cell damage is provided by the Aß-induced oxidative stress, rather than nitrotyrosination. We think that it might be necessary to reach a nitrotyrosination threshold in order to produce cell death, as previously suggested (Paris et al., 1998).

We have identified the main proteins nitrotyrosinated by Aß plus E_2 in endothelial cells. Interestingly, proteins involved in the regulation of energy metabolism, cytoskeleton integrity, protein turnover and protection against oxidative stress are nitrotyrosinated. The functions of these proteins should be inhibited since nitrotyrosination has been mainly associated with the loss of function and subsequent labelling for degradation via the proteasome (Grune et al., 1998). One of the most striking proteins to be nitrotyrosinated is triose phosphate isomerase (TPI) associated to energy metabolism. TPI is involved in the interconversion of dihydroxyacetone phosphate (DHAP) and glyceraldehyde-3-phosphate (GA3P) in the glycolytic pathway. Interestingly, if TPI function is altered, DHAP will be accumulated in the cell and through its subsequently hydrolytic breakdown is produced the toxic methylglyoxal, which have been related to cell damage in AD by the action of advance glycation end products (Munch et al., 2003). Additionally, inherited TPI deficiency leads to abnormal accumulation of DHAP and chronic neurodegeneration and has been also associated to degeneration of vascular endothelial cells (Ahmed et al., 2003). It has also been

proposed that a defective TPI could form pathological aggregates with microtubules (Orosz et al., 2000).

Other protein nitrotyrosinated is the mitochondrial heat-shock protein 75 (mtHsp75) which is involved in energy metabolism and also in defense against oxidative stress. MtHSP75 is a member of the Hsp 70 chaperone family with ATPase activity, implicated in the import and folding of proteins into the mitochondria as well as in the folding of proteins altered by oxidative injury (Matouschek et al., 2000;Voos and Rottgers, 2002). The inactivation of this enzyme plays a key step in mitochondrial impairment, failure of energetic metabolism and cerebral hypoperfusion (Aliev et al., 2003).

Linked to defensive effect against oxidative stress is the non-selenium GluPx (or 1-cys peroxiredoxin). Its nitrotyrosination could produce an increase in the oxidative injury since peroxiredoxin could promote restoration of membrane function and integrity by cleavage and elimination of peroxidized phospholipids by combining its peroxidase and PLA_2 enzymatic activities (Fisher et al., 1999). Interestingly, peroxiredoxin knock-out mice display significantly lower survival rates, severe tissue damage and higher protein oxidation levels (Wang et al., 2003).

Other proteins undergoing nitrotyrosination are involved in cytoskeleton integrity as metavinculin or the T-complex protein one I (TCPß). Metavinculin is noteworthy for its presence in cell adhesion plaques that may attach to actin microfilaments, as well as for having been found in the Hirano bodies of AD patient brains. Also relevant is the ß subunit of the T complex protein, for its essential role in keeping the native folding of actin, tubulin and vinculin and thus contributing to the preservation of cytoskeletal integrity. In endothelial cells the abnormal folding of cytoskeletal proteins acquire more significance since alterations in cell adhesion triggers to loss of the blood-brain barrier selectivity and endothelial apoptosis (Li et al., 1999). Moreover protein turnover could be also altered in endothelial cells since proteins related to protein synthesis or degradation are nitrotyrosinated. One of them are the eukariotic translation elongation factor (EF-2) involved in the elongation phase of protein synthesis. It has been reported that oxidative stress reduces protein synthesis (Patel et al., 2002), thereby nitrotyrosination of EF-2 might result in reduced protein synthesis. Finally, 26S proteasome is one of the main degradation systems inside the

cell (Goldberg, 2003). The nitrotyrosination of 26S proteasome could alter the degradation of proteins in a critical situation as oxidative stress.

Unfortunately, limitations of the method used do not allow the identification of nitrative modifications in proteins that have high molecular weight or membrane proteins essentials for the maintaining of vascular integrity. Additionally, apart from protein nitration, substantial amounts of peroxynitrite can be protonated at physiological pH to form peroxynitrous acid, a strong oxidant itself, which in turn can yield the highly reactive $OH^{\bullet}$ producing proteins oxidations which simultaneously contributes to vascular dysfunction observed in this pro-oxidant environment (Goldberg, 2003). However, further studies are necessary to identified peroxynitrite dependent-oxidated proteins in endothelial cells under Aß plus E_2 treatment. It is also interesting to note that peroxynitrite contributes to vessels dysfunction by the inhibition of prostacyclin synthase, which synthesizes the vasodilator prostacyclin, thereby reducing the ability of vessels to dilate (Zou et al., 1999a;Zou et al., 1999b) and also, either peroxynitrite and ROS produce DNA strand breaks and activate the DNA repair enzyme poly-ADP-ribose polymerase, which mediates endothelial dysfunction by depleting ATP and other crucial substrates (Soriano et al., 2001). In accordance with the vascular oxidative stress associated to AD, both ROS and peroxynitrite oxidize the cofactor of the NOS enzymes tetrahydrobiopterin (BH4) which in turn leads to the uncoupling of NOS favouring the superoxide and hydrogen peroxide production and in consequence, contributing to the oxidative stress environment (Aziz et al., 1983;Barford et al., 1984). As well as, recent findings suggest that accelerated catabolism of tetrahydrobiopterin in arteries exposed to oxidative stress may contribute to pathogenesis of endothelial dysfunction present in arteries exposed to hypertension, hypercholesterolemia, diabetes, smoking and ischemia-reperfusion. (Katusic, 2001). In fact, the metabolism of BH4 is also disturbed in AD patients (Aziz et al., 1983;Barford et al., 1984).

In conclusion, our study shows that the beneficial effect of E_2 against Aß-mediated cell damage in endothelial cells is ER-independent, while its endothelial harmful effect is through its interaction with ER, via NO production and protein nitrotyrosination. Although there is not increased cell death in the presence of Aß and E_2 compared to Aß alone, there is a significant increase of nitrotyrosination in enzymes

involved in glucose metabolism, energetic balance, repairing systems, protein degradation and cytoskeleton, which most likely compromise cell functions.

E_2 effects are complex, with plenty of preliminary studies praising its neuro- and vascular-protective effects (Behl, 2002;Mendelsohn, 2002b) whereas clinical trial have yielded disappointing results (Grodstein et al., 2000;Rapp et al., 2003;Hippisley-Cox et al., 2003;Viscoli et al., 2001). Our data suggest possible damaging effects of E_2 in vascular disorders dealing with oxidative stress conditions, such as cerebral amyloid angiopathy (Munoz et al., 2002), stroke and ischemia-reperfusion conditions (Gilgun-Sherki et al., 2002), where an overproduction of NO can be harmful (Hobbs et al., 1999). In situations with an increased vascular production of ROS as hypertension, hyperhomocysteinaemia and hypercholesterolemia, ROS could impair endothelium-dependent relaxation in a similar way to Aß. We believe that the overall results presented might also cast light on the mechanisms that will explain the reported worsening of the injury caused by recurrent cerebral ischemia in women undergoing hormone replacement therapy (Viscoli et al., 2001;Rossouw et al., 2002).

Role of oxidative stress in Aß brain efflux impairment

An increased production of Aß and/or a decreased clearance from the brain could be the scenario that triggers AD. Thus our interest was finally focused in the study of the effect of oxidative stress on the main clearance receptor of the BBB, the low-density lipoprotein receptor 1 (LRP) in human brain vascular endothelial cells (HBECs). Previous works have reported that reduced brain capillary LRP levels have been observed in Aß-accumulating transgenic mice, AD brains and brains from patients with cerebrovascular amyloidosis. The origin of this decrease in LRP could be directly induced by Aß since soluble $Aß_{1-40}$, $Aß_{1-42}$ or DI-$Aß_{1-40}$ (Dutch/Iowa-$Aß_{1-40}$) produces the downregulation of LRP in HBECs neither effecting LRP internalization nor the synthesis while it promotes proteosome-dependent LRP degradation in HBECs (Deane et al., 2004a). Although the mechanism involved in this downregulation is not clear, it could be mediated by oxidative stress since oxidative stress plays a key role on the cerebrovascular dysfunction produced by Aß. In this sense, in this part of the thesis we have studied the role of oxidative stress in LRP-downregulation in HBECs. Since soluble Aß produces LRP downregulation and soluble Aß is not inducing oxidative

stress, it would be expected that the source of oxidative stress comes from the internalization of Aß into endothelial cells where it could be aggregated. As aging is related to oxidative stress and also, with age, transition metals, such us copper (Cu^{2+}), iron and zinc, accumulate in brain endothelial cells acting as catalysts for the formation of ROS (Miranda et al., 2000), we evaluated the role not only of Aß but also of Cu^{2+} as a source of oxidative stress.

As commented before, a key mechanism utilized by ROS to damage endothelium is through the reaction between superoxide anion with NO producing peroxynitrite, and in consequence increasing oxidative/nitrosative modification to proteins. Accordingly to our aim, both $Aß_{1-42}$ and Cu^{2+} induce LRP downregulation through a mechanism which involves oxidative stress while cells treated with the peroxynitrite donor SIN-1, clearly also shows LRP downregulation as demonstrated for LRP-85 ß subunit and LRP-515 α subunit. Consistent with this finding, cells treated with an inhibitor of eNOS and challenged with $Aß_{1-42}$ or Cu^{2+} did not have LRP downregulation. It strengthens the hypothesis of the pathological role of Aß, and hence pro-oxidant environments in endothelial cells. Interestingly, the present data suggest that the common accumulation of transitional metals in endothelial cells observed in advances ages may produces an impaired Aß flux caused by decreased LRP levels. In agreement with the importance of LRP in Aß clearance, transgenic mice expressing low LRP-clearance mutant develops robust Aß cerebral accumulations much earlier that Tg-2576 Aß-overproducing mice (Deane et al., 2004a). Thus, it would be expected that with the onset of AD, Aß loads increases which will in turn strengthens the impairment of Aß clearance by increasing the reduction of LRP levels. It is known that RAGE which mediates a continuous influx of circulating Aß into the brain is overexpressed in Aß-treated HBECs, brain vasculature in transgenic APP models and in AD (Deane et al., 2004a). Accordingly, with the course of the disease, a further increasing of the damage would occur by the internalization of circulating Aß by RAGE.

As already commented in the introduction section, nitrotyrosination of proteins residues has been reported as a marker for proteosome-dependent degradation (Grune et al., 1998). Accordingly to the fact that Aß promotes proteosome-dependent LRP degradation without affecting LRP internalization or synthesis (Deane et al., 2004a), we evaluated whether LRP is nitrotyrosinated under a peroxynitrite treatment representing

the reaction of superoxide from Aß or Cu^{2+} with NO produced by endothelial cells. As cited before, we have found that vascular amyloid deposits correlate with nitrotyrosination in brain vessels from AD patients (article II) and also an increase of nitrotyrosine proteins has been observed in microvessels of copper-treated mice. Since LRP is a membrane protein with a high molecular weight, limitations of the method used to detect nitrotyrosinated proteins in HUVECs do not allow the identification of nitrated LRP. Therefore, we carry out the immunoprecipitation of all nitrated proteins in HBECs under the peroxynitrite treatment. Among them the light chain of the LRP was found. This finding may explain the reduced LRP levels observed in endothelial cells treated with Aß or Cu^{2+}, as well as, the reduced brain capillary LRP levels in Aß-accumulating transgenic mice, AD, and patients with cerebrovascular amyloidosis (Deane et al., 2004a). However further experiments are necessary to confirm this hypothesis using the pro-oxidant insults, Aß or Cu^{2+}. As well as, since peroxynitrite produces protein nitrations and oxidations, the carbonyl production have to be studied to further confirm the mechanism by which peroxynitrite produces the down-regulation of LRP1.

Among the harmful effects of oxidative stress in the vasculature, an impaired Aß efflux caused by reduced LRP levels would create a positive feedback amplification mechanism for Aß vascular amyloid deposition in AD and in cerebrovascular amyloidosis triggering vascular dysfunction and contributing to AD development.

General discussion

In view of the items discussed above, we propose a pathological oxidative stress-dependent loop which likely plays a crucial role in the origin and development of vascular Aß deposits which in turn leads to cerebrovascular dysfunction as well as to the neurodegenerative process. Aging and AD are unavoidably linked to oxidative stress. In advances ages, degenerative vascular changes (e.g. atherosclerosis), the accumulation of transition metals in endothelial cells, as well as increased production of neuronal Aß that enters to the perivascular drainage pathway produces an increase of vascular oxidative stress unable to be compensated with the physiological antioxidant mechanisms. This increase in vascular oxidative stress induces an increase of BACE1

activity and the release of secreted $Aß_{1-40}$ and $Aß_{1-42}$ by VSMCs. With the onset of AD development, cortical Aß load increases and $Aß_{1-40}$, but rarely $Aß_{1-42}$, because of its highly fibrillogenic nature, enters to the perivascular drainage pathway while neuronally derived $Aß_{1-42}$ fibrillizes into plaques and deposits on capillary walls/precapillary spaces (Attems, 2005). Therefore, according to the seeding amyloid hypothesis, we propose that the increase of $Aß_{1-42}$ produced by VSMCs acts as a seed which trap the more soluble form $Aß_{1-40}$ producing the accumulation of $Aß_{1-40}$, neuronal and non-neuronal derived, in the course of the perivascular drainage pathway. In view of the fact, that oxidative stress impairs Aß efflux by reducing LRP levels, probably simultaneously with an increased of RAGE levels as described in Aß-treated HBECs, brain vasculature in transgenic APP models and in AD (Deane et al., 2003;Deane et al., 2004a), an imbalance between LRP-mediated and RAGE-mediated Aß transport at the BBB could enhance vascular Aß accumulation. Thus it would be expected that vascular Aß deposition would create a pathological oxidative stress positive feedback which in turn, produces much more release of $Aß_{1-40}$ and $Aß_{1-42}$ by VSMCs, together with nitrative/oxidative modifications on key proteins for cell integrity, neurovascular repair, cellular protection and Aß elimination.

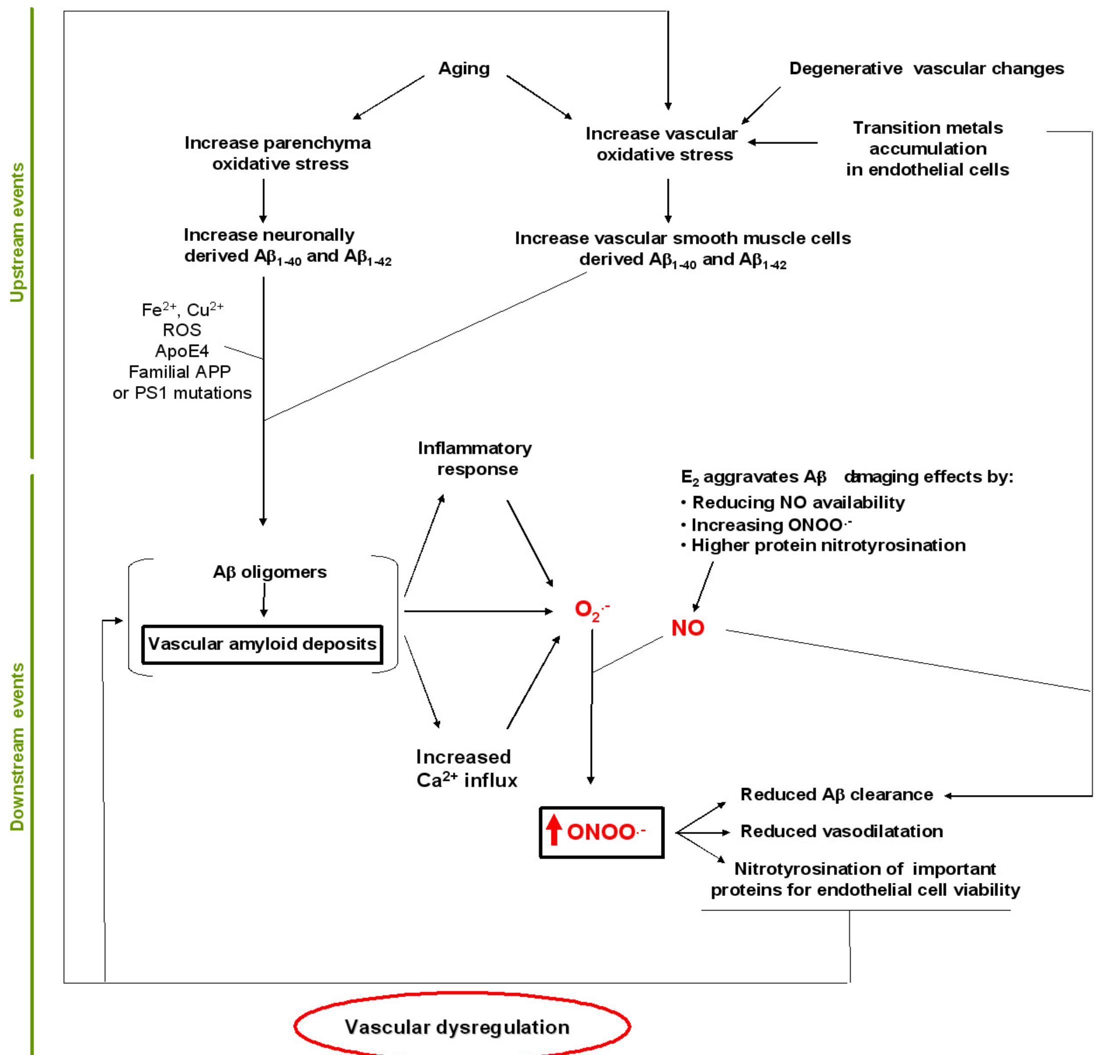

Figure 17. Schematic representation of the main findings of this thesis. Effect of oxidative stress in the etiology and pathophysiology of vascular amyloid deposits.

The relationship between CAA and AD is yet to be resolved; however this thesis gain new insights about the key role of oxidative stress in the etiology and pathophysiology of vascular amyloid deposits which could have direct effects in the cognitive dysfunction associated to AD.

V. Conclusions

1) Vascular smooth muscle cells contributes to vascular amyloid deposits formation and consequently, to neurovascular dysfunction associated to AD.

1.1. Primary cultures of human cerebral vascular smooth muscle cells (HC-VSMCs) have all the secretases involved in APP cleavage and produce $Aß_{1\text{–}40}$ and $Aß_{1\text{–}42}$.

1.2. Oxidative stress up-regulates the APP amyloidogenic pathway since up-regulates BACE1 transcription, expression and activity while it has no effect on the modulation of APP, ADAM10, ADAM17, PS1 and PS2 in VSMCs.

1.3. Oxidative stress increases secreted $Aß_{1\text{–}40}$ and $Aß_{1\text{–}42}$ but not intracellular $Aß_{1\text{–}40}$ and $Aß_{1\text{–}42}$ in HC-VSMCs.

1.4. Oxidative stress dependent BACE1 up-regulation is mediated by JNK and p38 MAPK signalling pathways.

1.5. Oxidative stress-mediated up-regulation of the amyloidogenic pathway in HC-VSMCs may contribute to the overall cerebrovascular amyloid angiopathy observed in AD patients.

2) Lack of estrogen protection in Aß-mediated endothelial damage due to protein nitrotyrosination.

2.1. Although estrogen has plenty of beneficiary effects in neuronal and smooth muscle cells, our results shows that E_2 is not able to protect endothelial cells from Aß-mediated-oxidative stress.

2.2. The absence of protection is due to an enhancement of peroxynitrite formation linked to an increase of protein nitrotyrosination that concludes in cell damage. We have identified several modified proteins which play a key role in energy

metabolism, cytoskeleton, protection against oxidative stress and protein turnover which could explain the harmful effect of HRP in post-menopausic women attending to the increase in stroke.

3) Oxidative stress induces the downregulation of LRP, the main ß-amyloid clearance receptor in brain endothelial cells through a mechanism that involves peroxynitrite formation.

3.1. Aß induces a decrease in the LRP levels on human brain endothelial cells (HBECs), which should contribute to increase the Aß load inside the brain.

3.2. Copper and peroxynitrite induce the same effect on the LRP as Aß.

VI. References

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VII. Appendix

Appendix I

"The protective role of vitamin E in vascular amyloid beta-mediated damage."

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Chapter 7

The Protective Role of Vitamin E in Vascular Amyloid β-Mediated Damage

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Abstract: Amyloid β peptide (Aβ) accumulation produces the senile plaques in the brain parenchyma characteristic of Alzheimer's Disease (AD) and the vascular deposits of Cerebral Amyloid Angiopathy (CAA). Oxidative stress is directly involved in Aβ-mediated cytotoxicity and antioxidants have been reported as cytoprotective in AD and CAA. Vitamin E has antioxidant and hydrophobic properties that render this molecule as the main antioxidant present in biological membranes, preventing lipid peroxidation, carbonyl formation and inducing intracellular modulation of cell signalling pathways. Accordingly, vascular damage produced by Aβ and prooxidant agents can be decreased or prevented by vitamin E. The protective effect of vitamin E against Aβ cytotoxicity in vascular cells in comparison to the neuronal system is reviewed in this chapter.

Key words: Amyloid β-peptide, Cerebral Amyloid Angiopathy, vitamin E, vascular cells, antioxidants, oxidative stress.

1. VASCULAR AMYLOIDOSIS

The different systemic diseases generally termed *amyloidosis* are all characterized by the misfolding of proteins into β-pleated sheet-rich structures that in turn leads to the aggregation and fibrillogenesis of the proteins, triggering pathological processes in the tissues. The most representative pathologies affecting the brain vessels are those produced by the aggregation of cystatin C (hereditary cerebral hemorraghe with

amyloidosis of the Icelandic type; HCHWA-I), transthyretin (familial transthyretin amyloidosis; TTR), gelsolin (familial amyloidosis of the Finnish type; FAF), prion protein (Gerstmann-Sträussler-Scheinker syndrome; GSS), ABri (familial British dementia; FBD), ADan (familial Dannish dementia) and the amyloid β-peptide (Alzheimer disease and cerebral amyloid angiopathy; AD and CAA).

2. CEREBRAL AMYLOID ANGIOPATHY

CAA is present in most cases of AD and it is characterized by the deposition of amyloid β-peptide (Aβ) in the media and adventitia of both leptomeningeal arteries and intracortical arterioles and capillaries (Figure 1), and less frequently in veins (Vinters *et al.*, 1988; Calhoun *et al.*, 1999). These vascular deposits are mostly composed by $A\beta_{1\text{-}40}$ wild type (Castaño *et al.*, 1996) and produce degeneration of vascular smooth muscle cells (VSMCs) from the media (Wisniewski and Wegiel, 1994; Zhang *et al.*, 1998) and endothelial cells from the intima (Wisniewski *et al.*, 1992; Kalaria, 1997). A variant of CAA with an early onset of the disease is hereditary cerebral haemorrhage with amyloidosis of the Dutch type (HCHWA-D). HCHWA-D is caused by Aβ-encoding gene point mutation which produces substitution of Glu→Gln at the position 22 (Levy *et al.*, 1990) resulting in a peptide with increased ability to form amyloid fibrils (Wisniewski *et al.*, 1991). Although HCHWA-D patients show diffuse amyloid deposition in the brain parenchyma, the main hallmarks of AD (mature senile plaques and neurofibrillary tangles) are not observed (Timmers *et al.*, 1990; Maat-Schieman *et al.*, 1994). In both CAA and HCHWA-D, the vascular amyloid deposits contain extracellular matrix molecules and other common components of senile plaques from the neuropil of AD patients (Snow *et al.*, 1988; Verbeek *et al.*, 1998; Mesulam *et al.*, 1992; Van Duinen *et al.*, 1995). Significantly, as in senile plaques (Vehmas et al., 2003), the vascular deposits show the presence of reactive glia (Uchihara et al., 1997).

3. ORIGIN OF VASCULAR Aβ

The amyloid precursor protein (APP) is present in VSMCs, pericytes and endothelial cells (Schmechel *et al.*, 1988; Shoji *et al.*, 1990; Tagliavini et al., 1990; Wisniewski and Wegiel, 1994), but the origin of vascular Aβ deposits

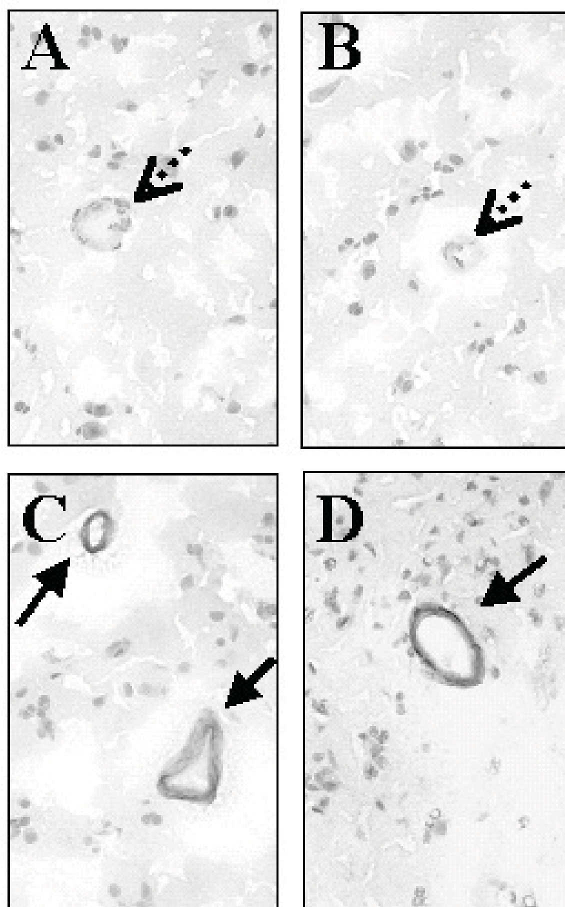

Figure 1. Amyloid deposits identified by Congo red staining in brain samples from the frontal cortex of control (A,B) and AD patients (C,D) with AD in the VI stage. Arrows show the blood vessels. The samples from AD patients show that most of the blood vessels are Congo red-positive.

is controversial (Weller *et al*., 1998). VSMCs are able to produce Aβ (Frackowiak *et al*., 1995), which has been identified even in intracellular compartments of VSMCs (Mazur-Kolecka *et al*., 1995; Wisniewski *et al*., 2000). Nevertheless, transgenic mice overexpressing neuronal mutated APP (Dutch-Iowa-Swedish mutations) also develop CAA (Davis *et al*., 2004), indicating that neuronal Aβ is deposited in the vessels. It could be due to the flux from the neuronal Aβ drainage, but the contribution of VSMCs to the vascular Aβ deposits could be also relevant by producing seeds for the fibrillation of the Aβ coming from the neurons. It could be due to the flux from the neuronal Aβ drainage, but the contribution of VSMCs to the vascular Aβ deposits has been demonstrated in cell cultures (Frakcowiak *et al*., 2004) and human brain vessel cultures (Mazur-Kolecka *et al*., 2004). Moreover, if neurons were the producers of all the vascular Aβ, a gradient of

immature Aβ deposits should be shown from the parenchyma to the vessels and it does not occur in arteries or small arterioles, where Aβ deposits have been found. Nevertheless, such a gradient is shown in the proximity of veins, probably corresponding to the clearance of neuronal Aβ. These findings suggest that the vascular Aβ is produced by both type of cells, neurons and VSMCs, and that VSMCs play a key role in the Aβ secretion.

4. Aβ EFFECTS ON THE VESSELS

CAA is characterized by the degeneration of VSMCs and endothelial cells (Miyakawa *et al*, 1997; Kalaria, 1997). Aβ deposits are present in the tunica media of large vessels at early stages of AD. VSMCs close to Aβ deposits have swollen nuclei and express the proliferating cell nuclear antigen. When the amyloidosis is at advanced stages, the tunica media is replaced by amyloid deposits and VSMCs degenerate becoming scarce (Wisniewscki *et al*., 2000). Thus, it has been reported that there is an increase in the number of apoptotic VSMCs and endothelial cells in AD (De la Monte *et al*., 2000). A direct toxic effect of Aβ on VSMCs *in vitro* has been also demonstrated (Davis and Van Nostrand, 1996; Muñoz *et al*., 2002).

At the functional level, Aβ enhances the vessel contraction (Crawford *et al*., 1998; Suo *et al*., 2000) and decreases the endothelium-dependent vasodilatation (Thomas *et al*., 1997) despite the increased nitric oxide (NO) production by AD endothelial cells (Grammas *et al*., 2000). The lack of vasodilatatory properties of NO may be due to the sequestration of NO in a pro-oxidant environment to produce peroxynitrite, a powerful oxidant produced from the reaction of superoxide anion ($O_2^{\cdot -}$) and NO. There is also evidence of endothelial cell degeneration in CAA (Miyakawa *et al*., 1997), which produces blood vessel damage and increased permeability of the blood-brain barrier (Wisniewski *et al*., 2000).

5. OXIDATIVE STRESS IN THE ETIOLOGY OF AD

Oxidative stress could be involved in the development of AD since it has been demonstrated that the expression and activity of BACE, the proposed β-secretase for APP, is increased by oxidative stress (Tamagno *et al*., 2002), and that oxidative stress enhances the production of Aβ (Frederikse *et al*., 1996). Moreover, homocysteine, a well-known risk factor for atherogenic damage and vascular disease, has been also proposed as a risk factor for AD (Seshadri *et al*., 2002), and the deleterious effect of homocysteine on blood

vessels may be mediated by oxidative stress (Perna *et al*., 2003). Thus the putative role of homocysteine in the development of AD could be related to a pro-oxidant activity yielding to an increase in the production of Aβ, and/or because homocysteine increases Aβ-induced cytotoxicity (Mok *et al*., 2002).

6. OXIDATIVE STRESS IN Aβ-MEDIATED CYTOTOXICITY

The existence of a specific receptor mediating the cytotoxicity induced by Aβ has been proposed, but none of the putative candidates can explain all the cytotoxic effects observed. There is an increased amount of experimental and histopathological evidence suggesting that oxidative stress plays a key role in Aβ-mediated cytotoxicity (Behl *et al*. 1994; Miranda *et al*., 2000; Muñoz *et al*., 2002).

Aβ generates hydrogen peroxide (H_2O_2) through metal ion reduction (Huang *et al*., 1999) and is able to increase the free radical generation by metals such as iron, copper and zinc (Bondy *et al*., 1998), which are highly concentrated within the core and periphery of Aβ deposits (Lovell *et al*., 1998). The oxidative damage of proteins generates an increase in carbonyl groups (Stadtman, 1990). Carbonyl residues and protein nitration in AD brain may come from the action of peroxynitrites (Smith *et al*., 1997). Proteins can also be modified by non-enzymatic reaction with monosaccarides, such as the Maillard reaction, when irreversible advanced glycation end products (AGEs) are formed, concomitant with hydroxyl radical ($HO^{\cdot}$) generation (Münch *et al*., 1997a). It has been demonstrated *in vitro* that Aβ induces lipoperoxidation of membranes (Koppal *et al*., 1998, Mark *et al*., 1997) leading to the disruption of the physiological signalling pathways (Kelly *et al*., 1996). Moreover, it impairs the function of membrane-regulatory proteins, including cation transport ATPases (Mark *et al*., 1995). The impairment of ATPase function means that the intracellular calcium can not be pumped out. The progressive cytoplasmic accumulation of calcium and the oxidative damage on mitochondria and nucleic acid (Gabbita *et al*., 1998; Nunomura *et al*., 1999) trigger cellular apoptotic mechanisms. Therefore, extracellular Aβ produces a cascade of ROS which induces intracellular damage (Figure 2)

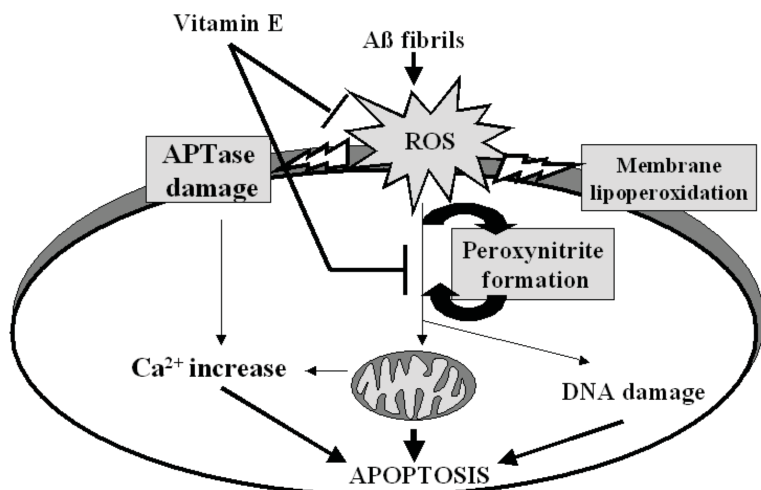

Figure 2. Aβ produces oxidative damage leading to the cell death. Reactive oxygen species (ROS) induce peroxidation with damage to both membrane lipid and protein. This event disrupts cell homeostasis. The intracellular ROS cascade is increased by the formation of peroxynitrites. Mitochondrial oxidative damage is triggering apoptotic pathways by the release of cytochrome C and the enhancement of intracellular calcium, which even activates endonucleases. Vitamin E can prevent the cell death by inhibiting the damage from extracellular ROS in the membrane and in the intracellular ROS cascade.

7. INTRACELLULAR SIGNALLING PATHWAYS INVOLVED IN THE OXIDATIVE DAMAGE

The mitogen-activated protein kinase (MAPK) pathways are involved in the deleterious effect of Aβ, but the specific role of the extracellular signal-regulated kinase (ERK), c-Jun NH_2-terminal protein kinase (JNK) and p38/RK/MpK2/CSBP kinases in the Aβ toxicity is controversial (Zhu *et al.*, 2002).

Low levels of radical/reactive oxygen species (ROS) play an important role in normal cell proliferation (Burdon, 1995; Benhar *et al.*, 2002) and regulate cellular signalling by the activation of MAPKs leading to induction of gene expression to protect cells. But at high concentrations, these agents activate ERK2 and JNK and ICE/Ced-3 caspase pathway inducing apoptosis

(Kong et al., 1998). Other authors propose that low concentrations of H_2O_2 activates phosphatidylinositol-3-kinase (PI-3K) giving an increase in the cell survival by the activation of c-AMP response element binding protein (CREB) throughout the action of ERK1/2 and Akt/PKB pathways, while high concentrations of H_2O_2 are proapoptotic throughout the activation of JNK/c-jun cascade in cortical neurons (Crossthwaite et al., 2002). In cardiac myocytes two of the MAPK pathways, JNK and p38 are reported as proapoptotic, whereas ERK pathway is considered antiapoptotic (Aikawa et al., 1997; Turner et al., 1998).

Regarding Aβ, it triggers the JNK/c-jun cascade (Figure 3) producing cell death and increasing the expression of proapoptotic molecules in neurons (Okazawa and Estus, 2002). Therefore, the inhibition of JNK protects PC12 cells against Aβ-mediated cytotoxicity (Troy *et al*., 2001). The involvement of JNK and p38 pathways in AD has been also demonstrated in an animal model. Thus, both MAPK pathways are activated in the cerebral cortex of a double transgenic mice for mutant APP (Swedish mutations) and mutated presenilin-1 (P264L), which produces a dramatic increase in the production and the consequent aggregation of Aβ (Savage *et al*., 2002). All these results show that firstly the activation of MAPK pathways rendering apoptosis or protection depends on cell type and the concentration of the prooxidant agent. Secondly, the specific role of ERK1/2 in oxidative stress/AD/CAA is the most controversial but JNK and p38 appears to be directly involved in the cell damage.

On the other hand, oxidative stress produces the activation of redox-sensitive transcription factors, such as nuclear factor-κβ (NF-κβ) (Piette *et a*l., 1997) and activator protein-1 (AP-1) (Lo *et al*., 1996; Vollgraf *et al*., 1999) triggering apoptosis or inducing the protection of the cells (Bossy-Wetzel et al., 1997). AP-1 is a protein complex containing Jun and Fos proteins or Jun dimers (Gass and Herdegen, 1995), and the activation of the migration to the nucleus of AP-1 and NF-κβ is mainly controlled by JNK and p38 pathways (Behrens *et al*., 1999). These mechanisms have been demonstrated to occur under the effect of Aβ in neurons (Kaltschmidt *et al*., 1997; Mattson *et al*., 1997), and in vascular cells with different pro-oxidant insults (Yin *et al*., 2002; Wang *et al*., 2002; Robbesyn *et al*., 2003)

8. INTRACELLULAR ANTIOXIDANT DEFENCES

The cellular mechanism of protection against oxidative stress is constituted by different intacellular enzymes, mainly catalase, superoxide

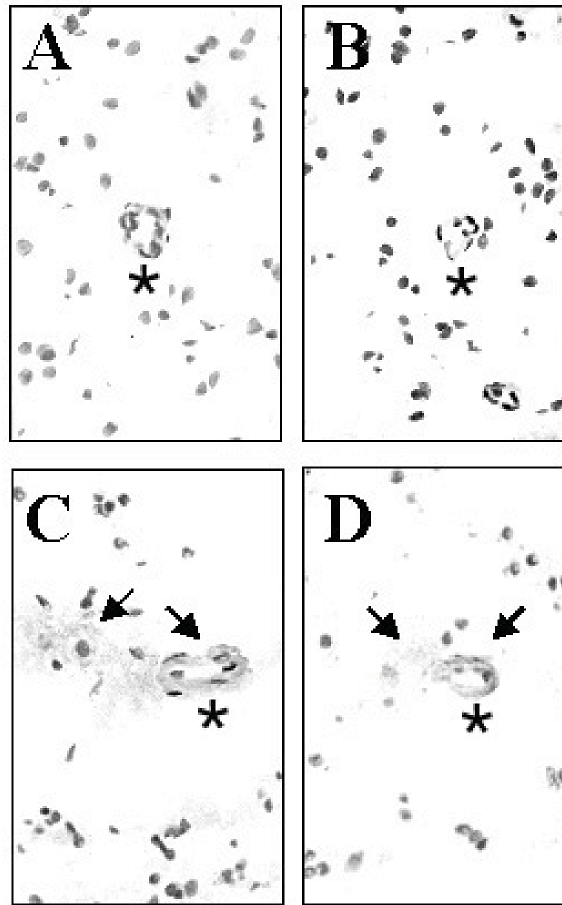

Figure 3. c-jun activation identified by immunohistochemical staining with peroxidase in brain samples from the frontal cortex of control (A, B) and AD patients (C, D) with AD in the VI stage. Asterisks indicate the presence of vessels. Arrows show the positive areas for c-jun activation into the vessels and the brain parenchyma. Positive areas for c-jun correlate with AD brain vessels and their periphery in all the samples analysed from AD patients.

dismutase (SOD), thioredoxin, the peroxiredoxins and the enzymes related to the glutathione (GSH) pathway. GSH is a tripeptide formed by glutamate, glycine and cysteine, and its antioxidant properties depends on the thiol group of the molecule. GSH-peroxidase is considered one of the most important enzymes involved in the hydrolysis of peroxides in the brain. Furthermore, different neuronal cell lines showed resistance against Aβ-mediated cytotoxicity which was directly proportional to the levels of GSH-peroxidase (Calderón *et al.*, 1999). The relevance of the protective role of antioxidants in the vasculature was first evidenced by the observation that Aß-induced endothelial damage is prevented by the enzyme SOD (Thomas *et al.*, 1996; 1997; Crawford *et al.*, 1997). These results are in agreement

with a direct effect of $O_2^{\cdot -}$ in Aβ-mediated cytotoxicity in vascular cells, as it was demonstrated by the measurement of the dihydroethidium fluorescence, an indicator of ROS, in VSMCs and endothelial cells challenged by Aβ (Muñoz *et al*., 2002).

9. PROTECTION BY ANTIOXIDANT MOLECULES

Antioxidants such as vitamin E, 17β-estradiol or melatonin have demonstrated protective properties on neuronal cells against the Aβ-mediated cytotoxicity (Behl, 2000; 2002; Behl *et al.*, 1992; 1997; Mattson and Goodman, 1995; Pappolla *et al.*, 1997; Bonnefont *et al.*, 1998), (see also Chapter 3). Vitamin E can protect VSMCs and endothelial cells against alcohol, which may induce oxidative stress (Altura and Gebrewold, 1996), and against Aβ-mediated cytotoxicity (Muñoz *et al*., 2002). Vitamin C, which shares anti-oxidant properties (Podmore *et al*., 1998), prevents β-amyloid-induced intracellular calcium increase and cell death in PC12 cells (Yallampalli *et al*., 1998). However other authors have not found any protection with antioxidants such as vitamin E, trolox (a hydrosoluble form of vitamin E), vitamin C or N-acetyl-L-cysteine (NAC) on neuronal cells (Lockhart *et al*., 1994; Pike *et al*., 1997). This lack of protection could be due to the experimental procedures. The steroidal hormone 17β-estradiol has also been proposed to play a key role in the prevention or retardation of AD related pathologies, since women treated with estrogen replacement therapy showed a lower prevalence of AD (Tang *et al.*, 1996; Kawas *et al.*, 1997) due to the pleiotropic effects of 17β-estradiol (Behl, 2002).

10. PROTECTION BY VITAMIN E

Vitamin E was discovered by Evans and Bishop in 1922. Vitamin E is a term which includes a group of tocopherols and tocotrienols, both having four isomers (alpha, beta, gamma and delta). The alpha-tocopherol is the most active in humans because of the high affinity of the tocopherol transporter protein (TTP) for this molecule (Hosomi *et al*., 1997). The relevance of this transporter is shown when there are mutations in the TTP gene. It produces a reduction of alpha-tocopherol in plasma and tissues yielding to ataxia with vitamin E deficiency (Ben Hamida *et al*., 1993). When considering the vitamin E distribution in brain, there are no specific areas of the brain or spinal cord that are richer in vitamin E than others, however, the uptake of vitamin E is considerably high in the cerebellum (Vatassery, 1992).

Due to the antioxidant and hydrophobic characteristics of vitamin E, it is the main antioxidant present in biological membranes (Perly *et al.*, 1985), preventing lipid peroxidation (Halliwell and Gutteridge, 1984) by trapping the peroxyl radicals (Naiki *et al.*, 1998). Vitamin E has protective properties on neuronal cells against the Aβ-mediated cytotoxicity (Behl *et al.*, 1992). These neuronal protective properties have been demonstrated even in synaptosomas challenged with Aβ (Koppal *et al.*, 1998). Regarding the role of vitamin E on blood vessels (Figure 4), it has been reported that alpha-tocopherol can protect VSMCs and endothelial cells against alcohol (Altura and Gebrewold, 1996) and against Aβ-mediated cytotoxicity even when both types of cells were challenged with the Dutch variant of Aβ, which is more toxic for vascular cells than the wild type Aβ (Muñoz *et al.*, 2002).

The protective effect of vitamin E against oxidative stress is not just due to the free radical scavenging activity of the molecule but also due to the modulation of signalling pathways. In fact, vitamin E has been reported to protect against oxidative stress by decreasing JNK activity and increasing the ERK activity in cardiac myocytes (Qin *et al.*, 2003), and inhibiting caspase-3 activation in vascular endothelial cells (Uemura *et al.*, 2002).

Moreover, in HeLa cells, pretreatment with free radical scavengers NAC, GSH or vitamin E, inhibited JNK pathway activation by prooxidant agents (Kong *et al.*, 1998). Furthermore, the protective roles of vitamin E could also be related to other intracellular effects such as the activation of PP2A, the inhibition of alpha-PKC in VSMCs (Ricciarelli *et al.*, 1998), the inhibition of the production of eicosanoids (Pratico *et al.*, 1998; Jialial *et al.*, 2001; Lee *et al.*, 1999) or the inhibition of inducible NO synthase (iNOS) (Badger *et al.*, 2000; Guan *et al.*, 1998) which leads to a decrease in the protein peroxynitration. On the other hand, vitamin E protects neurons and vascular cells against oxidative cell death *in vitro* by the activation of NF-κß (Behl, 2000; Li-Weber *et al.*, 2002). Alpha-tocopherol also induces the expression of connective tissue growth factor (CTGF) in VSMCs in a PKC-independent pathway (Villacorta *et al.*, 2003) and increases the synthesis of alpha-tropomyosin in VSMCs (Aratri *et al.*, 1999), suggesting an improvement in the VSMC function in the vessels. Moreover, vitamin E can be a vasodilatatory agent since thrombin-mediated PKC activation and endothelin secretion are inhibited by alpha-tocopherol in endothelial cells (Martin-Nizard *et al.*, 1998). A clinical trial confirmed the positive role of vitamin E in preventing AD in ageing people (Sano *et al.*, 1997). There are also reports suggesting that use of high vitamin E and vitamin C supplements may decrease the risk of AD (Morris *et al.*, 1998). Other studies suggest that

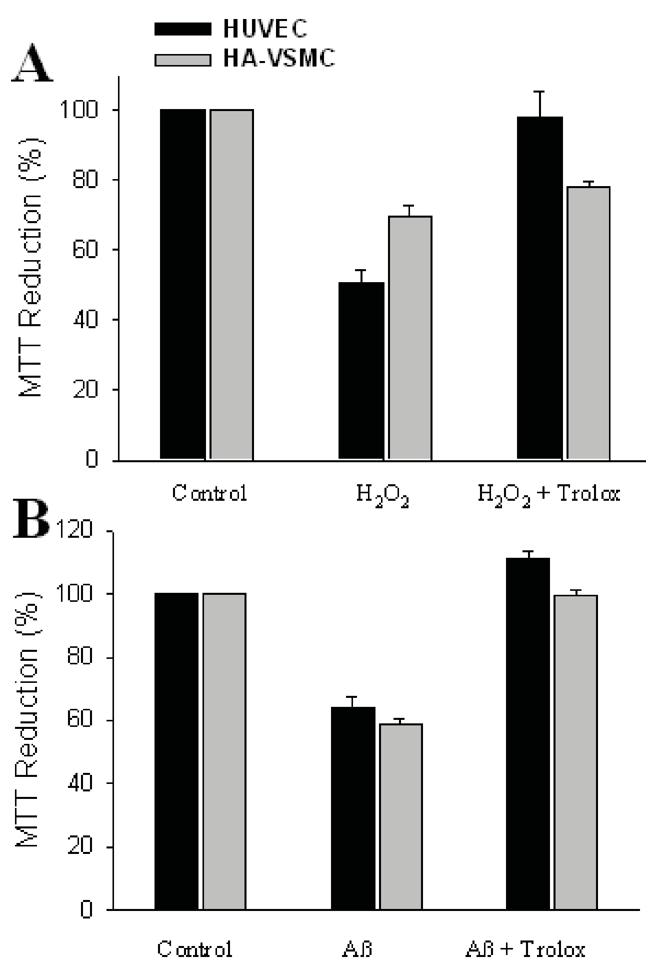

Figure 4. The water-soluble analogue of Vitamin E (Trolox) protects human umbilical vein endothelial cells (HUVEC) and human aortic vascular smooth muscle cells (HA-VSMC) from H_2O_2 and Dutch $A\beta_{1-40}$ fibrils cytotoxicity. Representative experiments were performed in quadruplicate. Cells were challenged with 4 μM H_2O_2 in HUVEC and 50 μM H_2O_2 in HA-VSMCs (A), and 0.25 μM Aβ in HUVEC and 0.125 μM Aβ in HA-VSMCs (B). For the protection studies, cells were treated with 500 μM Trolox. Cell viability was evaluated by MTT reduction after 24h of incubation. Control cells were assumed to have 100% of viability.

vitamin E could be also protective in vascular disease (*reviewed by* Steinberg, 1995; Gotto, 2003). In addition, there are experimental data demonstrating that treatment with antioxidants such as idebenone and alpha-tocopherol prevents learning and memory deficits caused by Aβ in rats (Yamada *et al*., 1999). Furthermore, decreasing serum levels of vitamin E were associated with poor memory performance in older people (Perkins *et al*., 1999), however this effect was not found in rats (Ichitani *et al*., 1992).

11. CONCLUSIONS

Oxidative stress is directly involved in Aβ-mediated cytotoxicity. Thus, free radical scavengers and antioxidants are considered key pharmacological tools against AD and CAA. Vascular damage produced by Aβ and prooxidant agents can also be decreased or avoid by vitamin E. These findings suggest that vitamin E is a good biological cytoprotective agent, but more work is needed to elucidate all the intracellular mechanism triggered by vitamin E and the changes in the expression of specific protective genes such antiapoptotic molecules. Moreover, considering that Aβ production has been related directly with oxidative stress, vitamin E could prevent the triggering of AD by decreasing the production of Aβ in vascular cells.

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***Abbreviations**:* AD, Alzheimer's disease; AGEs, advanced glycation end products; AP-1, activator protein-1; APP, amyloid precursor protein; Aß, amyloid ß-peptide; CAA, cerebral amyloid angiopathy; CREB, c-AMP response element binding protein; CTGF, connective tissue growth factor; ERK, extracellular signal-regulated kinase; GSH, glutathione; HCHWA-D, hereditary cerebral haemorrhage with amyloidosis of the Dutch type; iNOS, inducible NO synthase; JNK, c-Jun NH_2-terminal protein kinase; MPAKs, mitogen-activated protein kinases; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; NAC, N-acetyl-L-cysteine; NF-κβ, nuclear factor-κβ; NO, nitric oxide; PI-3K, phosphatidylinositol-3-kinase; ROS, radical oxygen species; SOD, superoxide dismutase; TTP, tocopherol transporter protein; VSMCs, vascular smooth muscle cells.

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Appendix II

"The physiology and pathophysiology of nitric oxide in the brain."

Guix F.X., Uribesalgo I., Coma M., Muñoz F.J.

Prog. Neurobiol., 76: 126-152 (2005)

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The physiology and pathophysiology of nitric oxide in the brain.
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Appendix III

"The amyloid β-protein precursor and Alzheimer's disease."

Del Toro D., Uribesalgo I., G., Coma M., Guix F.X., Muñoz F.J.

Therapeutic approaches. Curr. Med. Chem.-Central Nervous
System Agents,
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Appendix IV

"The role of vitamin E on intracellular signaling pathways in brain: molecular basis for the treatment of neurodegenerative processes."

Serra S., Ill-Raga G., Coma M., Guix F.X., Muñoz F.J.

Vitamin E: new research. Chapter 9. Editor: Matthew H. Braunstein, Nova Science Publishers, Inc. pags.:151-176 (2006)

Serra SA, Ill-Raga G, Coma M, Guix FX, Muñoz FJ. The role of vitamin E on intracellular signaling pathways in brain: molecular basis for the treatment of neurodegenerative processes. Dins: Braunstein MH, editor. Vitamin E: new research. New York: Nova Science Publishers; 2006. p.151-76.