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

Mireia Coma[a], Francesc X. Guix[a], Gerard Ill-Raga[a], Iris Uribesalgo[a], Francesc Alameda[b], Miguel A. Valverde[a] and Francisco J. Muñoz[a*]

[a]Laboratory of Molecular Physiology and Channelopathies, Universitat Pompeu Fabra, Parc de Recerca Biomèdica de Barcelona; [b]Servei d'Anatomia Patològica, Hospital del Mar, Universitat Autònoma de Barcelona. Barcelona 08003, Spain.

[*] Corresponding author:

Dr. Francisco J. Muñoz

Laboratorio de Fisiologia Molecular i Canalopaties

Departament de Ciències Experimentals i de la Salut

Universitat Pompeu Fabra

C/ Dr. Aiguader, 80, Barcelona 08003, Spain,

Tel.: +34 93 542 28 84; Fax: + 34 93 542 28 02

E-mail address: paco.munoz@upf.edu

Abstract

Cerebral amyloid angiopathy, associated to most cases of Alzheimer's disease (AD), is characterized by the deposition of amyloid ß-peptide (Aß) in brain vessels, although the origin of the vascular amyloid deposits is still controversial: neuronal *vs* vascular. In the present work, we demonstrate that primary cultures of human cerebral vascular smooth muscle cells (HC-VSMCs) have all the secretases involved in amyloid ß-protein precursor (APP) cleavage and produce $Aß_{1-40}$ and $Aß_{1-42}$. Oxidative stress, a key factor in the etiology and pathophysiology of AD, up-regulates ß-site APP cleaving enzyme 1 (BACE1) expression, as well as $Aß_{1-40}$ and $Aß_{1-42}$ secretion in HC-VSMCs. This process is mediated by c-Jun N-terminal Kinase and p38 MAPK signaling 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 human cerebral vascular smooth muscle cells may contribute to the overall cerebrovascular amyloid angiopathy observed in AD patients.

Keywords: Alzheimer's disease; amyloid ß-peptide; BACE1; vascular smooth muscle cells; oxidative stress; c-JNK; p38 MAPK.

Abbreviations: Ab = antibody; Aß = amyloid ß-peptide; AD = Alzheimer's disease; ADAMs = A disintegrin and metalloproteases; APP = amyloid ß-protein precursor; BACE1 = ß-site APP cleaving enzyme type 1; CAA = cerebral amyloid angiopathy; FBS = fetal bovine serum; HA-VSMC = human aortic vascular smooth muscle cells; HC-VSMC = human cerebral vascular smooth muscle cells; hPRT = hypoxanthine phosphoribosyltransferase; JNK = c-Jun N-terminal Kinase; MTT = 3-(4,5-dimethylthiazol-

2-yl)-2,5-diphenyltetrazolium bromide; PS = presenilin; RT-PCR = reverse transcriptase-polymerase chain reaction; SAPK = stress-activated protein kinases.

1. Introduction

Patients suffering from Alzheimer's disease (AD) typically present cerebral amyloid angiopathy (CAA), characterized by amyloid ß-peptide (Aß) deposits in brain vessels [14]. The source of vascular Aß is at 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 (reviewed in [1]).

The former hypothesis states that the main source of the Aß accumulated in vessels has a neuronal origin. It is sustained by the presence of vascular amyloid deposits in transgenic mice overexpressing the amyloid ß-protein precursor (APP) exclusively in neurons [43], the capability of vascular smooth muscle cells (VSMCs) to endocytose and accumulate Aß coming from the brain parenchyma [50] and the link between reduced Aß-clearing capability and AD [56].

Despite the solid pieces of evidence supporting an important neuronal contribution to the vascular Aß, there are still opened questions related to the vascular origin of Aß and its implication in the pathophysiology of CAA. The involvement of VSMCs in the production of Aß was already hypothesized following the early observation that Aß deposits are closely associated with cerebral VSMCs [13] and the presence of APP and Aß in VSMCs [9,10,12,49]. However, several facts have rested relevance to the contribution of VSMCs to CAA: 1) most reports just mention intracellular Aß accumulation in VSMCs [49], 2) absence of $Aß_{1-42}$ production by primary cultures of VSMCs [11] and 3) the lack of a mechanism linking the production of Aß in VSMCs to the etiopathology of AD.

Neuronal APP can be cleaved by two different pathways. In the non-amyloidogenic pathway, APP is cleaved by α-secretase, belonging to the family of a disintegrin and

metalloproteases (ADAM10 and/or ADAM17) [5,19]. 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) [47] and a γ-secretase, identified as presenilin (PS) [32,53]. However, to date no reports exists on the presence and regulation of the APP-related secretases in VSMCs.

Oxidative stress, a harmful condition that increase with advancing age [21], has been implicated in the etiology of AD by increasing the expression of BACE1 in neuroblastoma cell lines [27,35]. Oxidative stress also contributes to the Aß-induced pathophysiology seen in both brain parenchyma [3] and blood vessels [8,28] of AD patients. Therefore, oxidative stress and Aß generate an etiopathogenic loop that has been well studied in neurons but not in VSMCs.

In the present work we report the production of $Aß_{1-40}$ and $Aß_{1-42}$ by human cerebral VSMCs (HC-VSMCs). Moreover, oxidative stress activates the amyloidogenic processing of APP by increasing the expression of BACE1 and, subsequently, Aß secretion, a process mediated by c-Jun N-terminal Kinase (JNK) and p38 MAPK signaling. Altogether, our data suggests that oxidative stress-dependent induction of amyloidogenic cleavage of smooth muscle APP may contribute to CAA development and vessel degeneration.

2. Materials and methods

2.1. Cell cultures

Primary cultures of HC-VSMCs were produced from cerebral arteries (basilar) obtained from autopsies of four non-demented individuals. Procedure was approved by the ethics committee of the Institut Municipal d'Investigació Mèdica and the Universitat Pompeu Fabra (IMIM-UPF). Briefly, pieces of tunica media were incubated with 0.1% collagenase type IV for 35 min at 37ºC and cultured to allow HC-VSMC migration to the flask surface. Culture media was DMEM with 4500 mg/l glucose, 25 mM HEPES, 10% fetal bovine serum (FBS), amphotericin B (2.5 µg/ml) and antibiotics (100 units/ml penicillin and 10^{-6} µg/ml streptomycin). HC-VSMCs were characterized by immunostaining with mouse anti-smooth muscle α-actin antibody (Ab) (Sigma, St Louis, MO). Cells were used up to ten passages. Human aortic VSMCs (HA-VSMCs) were kindly provided by Dr. S. Richard (Universite Montpellier, France). HA-VSMCs were grown in RPMI MCDB 131 with 5% FBS, $5x10^{-7}$ g/l EGF, $1.5x10^{-6}$ g/l b-FGF, 5 g/l insulin, 2 mM L-glutamine and antibiotics. All media, culture products and chemicals were purchased from Gibco BRL (Paisley, UK) and Sigma (St Louis, MO) unless otherwise indicated. Experiments were performed with phenol red- and serum-free media.

2.2. Brain samples

Brain tissue sections were supplied by the Banc de Teixits Neurològics (Serveis Científico-Tècnics, Hospital Clínic, Universitat de Barcelona). The procedure was approved by the ethics committee of the IMIM-UPF. Brain sections (5 µm) were obtained from the frontal cortex of control and AD patients (stage VI). Samples used in the histochemical studies were from 3 control individuals and 5 AD patients.

2.3. Treatments

Determination of the optimal H_2O_2 sub-lethal concentrations related to the higher BACE1 expression (10 µM for HC-VSMCs) were obtained running dose-response (0 to 50µM) cell viability assays using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) reduction method. The vitamin E analog, trolox (500 µM; Calbiochem, La Jolla, CA), the JNK inhibitor SP600125 (20 µM; Calbiochem) or the p38 MAPK inhibitor SB203580 (10µM; Calbiochem) were added to culture medium 1 h before H_2O_2 exposure.

2.4. RNA isolation and RT-PCR analysis

Total RNA from cell cultures was isolated using Trizol reagent (Invitrogen, Carlsbad, CA), following the manufacture's instructions. 0,5 µg of RNA were used in a reverse transcriptase-polymerase chain reaction (RT-PCR) using the OneStep RT-PCR Kit (Qiagen, Hamburg Germany). BACE1 specific primers (5'-CATTGGAGGTATCGACCACTCGCT-3' and 5'-CCACAGTCTTCCATGTCCAAGGTG-3'; Genbank accessories number: NM012104) were used for human BACE1 gene amplification and hypoxanthine phosphoribosyltransferase (hPRT) specific primers (5'-GGCCAGACTTTGTTGGATTTG-3' and 5'-TGCGCTCATCTTAGGCTTTGT-3'. Genbank accessories number: NM000194) were used as positive control. Negative control was performed in the absence of oligonucleotide primers. Results were analyzed with Image Gauge software (Fuji Photo Film Co., Tokyo).

2.5. Brain sample staining

Sections were treated with alkaline solution and stained with Congo Red. Sequential sections were treated with 4% H_2O_2 and incubated with 1:250 rabbit anti-BACE1 Ab for 2 h at room temperature or with 1:500 mouse anti-smooth muscle α-actin Ab. The secondary antibodies, rabbit biotinylated goat Ab (1:500) or mouse biotinylated goat Ab (1:250; DAKO, Glostrup, Denmark), were used for 1 h at room temperature. Slides were incubated with Streptavidin-HRP (Zymed laboratories, San Francisco, CA) and treated with Peroxidase Substrate Kit DAB (Vector, Burlingame CA). Samples were counterstained with hematoxylin, dehydrated and fixed with Eukitt (O.Kindler GmbH &CO, Fribourg, Switzerland). Representative digital images were taken with a Leica DMRB microscope and Leica DC300F digital camera. Amyloid staining is shown under polarized light with two positions of the polarized.

2.6. Identification of proteins by western-blot

Cells were lysed on ice with a solution containing 1 M Tris-HCl, 1% Nonidet P-40, 150 mM NaCl, 5 mM EDTA, 1 mM sodium orthovanadate, 1 mM dithiotreitol, pH 7.4 and a protease inhibitor cocktail (Roche, Basel, Switzerland). Protein concentration was determined by Bio-Rad protein assay (Bio-Rad, Hercules, CA). Protein samples were analyzed by using 10% Tris-glycine gels and blotted onto nitrocellulose membrane (Millipore, Bedford, MA) for secretase detection or 4-16% Tris-Tricine gels and blotted onto nylon membrane (Immobilon-P, Millipore) for C-terminal fragments detection. Membranes were incubated for 2 h at room temperature with different primary rabbit Abs: anti-BACE1 (1:500; Chemicon, Temecula, CA), anti-ADAM10 (1:1000; ProSci, Poway,

CA), anti-ADAM17 (1:500; kindly provided by Dr. J. Arribas, Hospital Vall d'Hebron, Barcelona) and anti-C-terminal of APP (1:2000; Sigma). Mouse anti-α-tubulin Ab (1:5000; Sigma) was incubated for 1h at room temperature as a loading and transfer control. The peroxidase-conjugate anti-mouse and anti-rabbit Abs were used as secondary Abs (Amersham Bioscience, Barcelona, Spain). Bands were visualized using the enhanced chemiluminescence substrate Super Signal (Pierce, Rockford, IL) or Supersignal West femto trial kit (Pierce) and Amersham Bioscience Hyperfilm ECL kit.

2.7. Immunofluorescence assays

$2x10^4$ cells were seeded onto 1% collagen I (Chemicon) (HC-VSMCs) or 1% poly-L-lysine (HA-VSMCs) coated cover slips. Treatments with H_2O_2 were carried out during 5 to 30 min for JNK and p38 MAPK detection or up to 6 h for APP and secretase detection. Cells were fixed and incubated for 1 or 2 h at room temperature with the following antibodies: rabbit anti-BACE1 Ab (1:250), rabbit anti-ADAM10 Ab (1:100), rabbit anti-ADAM17 Ab (1:100), rabbit anti-PS1 and anti-PS2 Abs (1:25; Oncogene, Boston, MA), rabbit anti-APP(CT) Ab (1:1000), rabbit anti-c-Jun N-terminal Kinase 1 (JNK1) Ab (1:50; Santa Cruz Biotechnology, Santa Cruz CA), rabbit anti-Phospho-p38 MAPK(Thr180/Tyr182)(3D7) monoclonal Ab (1:100; Cell Signaling, Beverly, MA), rabbit anti-c-jun Ab (1:20; Cell Signaling, Beverly, MA, USA), rabbit phospho-MAPKAPK-2 Ab (1:50; Cell Signaling) and mouse anti-smooth muscle α-actin Ab (1:500). The secondary Abs used was Alexa 555 goat anti-mouse and Alexa 488 goat anti-rabbit. Digital images were taken with a Leica TCS SP confocal microscope and analyzed with Leica confocal software (Heidelberg, Germany).

2.8. Aß quantification

After 24 h of treatments, media were collected and concentrated 20-folds by dialysis. For intracellular Aß detections, cells were extracted in 70% formic acid by sonication. Samples were spun at 100,000 *g* for 20 min and supernatants were removed and diluted 1:20 with 1 M Tris to neutralize the pH. Proteins were precipitated for 1 h with 24% tricloroacetic acid and centrifuged at 140,000 rpm for 15 min. Pellets were washed with ethanol:ether and diluted in assay buffer. The levels of $Aß_{1-40}$ and $Aß_{1-42}$ were measured using commercial enzyme-linked immunosorbent assay kits (IBL, Gumna, Japan). Absorbances were measured by a microplate reader Bio-Rad 680 and evaluated by microplate manager software (Bio-Rad). For intracellular Aß detection, values were normalized to total cell protein concentration by Bio-Rad DC Protein Assay (Bio-Rad).

2.9. 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 $p < 0.05$.

3. Results

3.1. Detection of APP and the secretases involved in its cleavage in HC-VSMCs

Primary cultures of HC-VSMCs were probed with antibodies against APP, BACE1, ADAM10, ADAM17, PS1 and PS2 (Figure 1A). This selection was based in the consensus that ADAM10 and ADAM17 are the most plausible candidates to be the non-amyloidogenic α-secretase. BACE1 was also studied since it has been identified as the amyloidogenic ß-secretase. PS1 and PS2 are key enzymes of the γ-secretase complex.

This is the first demonstration that all APP processing candidates are constitutively expressed in HC-VSMCs (Figure 1A, left panels). Interestingly, APP showed a particularly high level of expression, comparable to that seen in neuronal cell models (primary culture of rat embryo hippocampal neurons, the neuroblastomas N2a and SH-5YSY). Thus, HC-VSMCs present APP and all the machinery necessary to cleave APP by both the physiological non-amyloidogenic pathway through the α-secretase and the pathological amyloidogenic pathway involving the ß- and γ-secretases.

3.2. Oxidative stress increases the expression of BACE1

Aging is associated to increased oxidative stress and lower antioxidant defenses [21]. Moreover, oxidative stress has been associated to the origin and development of AD [8,32]. Particularly interesting in the context of our work are the reports claiming the modulation of APP processing by oxidative stress [36,40]. The effect of oxidative stress on the APP and secretase expression was evaluated on HC-VSMCs (Figure 1A). HC-VSMCs were exposed to sub-lethal concentrations of H_2O_2 (10 μM), previously adjusted carrying out dose-response cell viability experiments using MTT reduction assays (Figure 1E).

BACE1 expression was clearly increased in HC-VSMCs exposed to oxidative stress (Figure 1A, right panels) while the expression of APP, α-secretases (ADAM10 and ADAM17), PS1 and PS2 were not altered under the same experimental conditions.

BACE1 is the key enzyme to initiate the amyloidogenic cleavage of APP, therefore, detailed analysis of its modulation by oxidative stress was carried out in HC-VSMCs. The time course of H_2O_2 –induced BACE1 expression was examined at the transcriptional level by semiquantitative RT-PCR. Figure 1B-C demonstrates an increase of BACE1 transcript that peaks 6 h after the addition of H_2O_2 to HC-VSMCs ($p<0.05$). The increase persisted even 24 h after the initiation of the stimulus although did not reach statistical significance. Similar increase (and time course) in BACE1 mRNA expression induced by H_2O_2 was obtained using a SH-SY5Y human neuronal cell line (results not shown).

Oxidative stress conditions have been reported in AD brain blood vessels [40]. Thus, according to our results with the HC-VSMCs, BACE1 up-regulation should be detected in the brain vessels of AD patients, if it was to contribute locally to the Aß-mediated vascular pathology. To investigate further this possibility we carried out immunohistochemistry of brain sections using BACE1 and smooth muscle α-actin antibodies. This approach revealed strong BACE1 expression in brain vessels from AD patients, compared to the negligibly low staining detected in the brain sections obtained from controls (Figure 1D, upper panel). Labeling of BACE1 in blood vessels was consistent with staining of the tunica media as similar labeling was obtained with smooth muscle α-actin Ab (Fig 1D, middle panels). Analysis of consecutive sections also demonstrated the presence of mature vascular amyloid deposits (positive for Congo Red) associated to the high expression of BACE1, only in sections obtained from AD brains

(Figure 1D, lower panel).

3.3. Oxidative stress-induced production of $Aß_{1-40}$ and $Aß_{1-42}$ in HC-VSMCs

Several studies have demonstrated that both $Aß_{1-40}$ and $Aß_{1-42}$ species accumulated in CAA vessel walls [33] being $Aß_{1-40}$ the most abundant one [6]. Once we demonstrated an increase in the APP amyloidogenic pathway in HC-VSMCs exposed to oxidative stress, we set out to detect $Aß_{1-40}$ and $Aß_{1-42}$ under control conditions and following H_2O_2 treatment. We observed an increase in secreted (extracellular) $Aß_{1-40}$ and $Aß_{1-42}$ (Figure 2A) without changes in the intracellular levels (Figure 2B) detected in HC-VSMC cultures exposed to 10 µM H_2O_2. Secreted $Aß_{1-40}$ reached a threefold increase ($p<0.05$) and $Aß_{1-42}$ a fivefold increase ($p<0.05$) compared to control conditions. Increased secretion of $Aß_{1-40}$ and $Aß_{1-42}$ was reverted in HC-VSMCs pretreated with the antioxidant trolox (a vitamin E analogue) (Figure 2A). The abolition of the Aß increase by trolox can be explained by the inhibition of the oxidative-stress dependent up-regulation of BACE1 (Figure 3, left panels), an effect already reported for neuronal BACE1 [35].

3.4. Role of JNK and p38 MAPK signaling in oxidative stress-dependent up-regulation of BACE1

Oxidative stress induces the stress-activated protein kinases (SAPK) JNK and p38 MAPK [48], and both kinases are up-regulated in AD [54,55]. Accordingly, we studied whether these SAPK also mediate the up-regulation of BACE1 in HC-VSMCs exposed to oxidative stress.

Activation of JNK and p38 MAPK results in their translocation from the cytoplasm to the nucleus where they act on their targets [25]. In order to determine whether oxidative stress triggered this translocation process, we carried out immunofluorescence studies to

compare the cellular localization of JNK and p38 MAPK in HC-VSMCs exposed to 10 μM H_2O_2. Immunoflorescence images of BACE1 up-regulation were run in parallel and shown in Figure 3. JNK and p38 MAPK translocation to the nucleus occurred within 5-30 min and was abolished when HC-VSMCs were pretreated with the antioxidant Trolox. Similarly, BACE1 up-regulation was also inhibited by Trolox (Figure 3). Our results show that, similar to neuronal models [37], oxidative stress activated JNK and p38 MAPK in HC-VSMCs (and their consequent nuclear translocation), an effect upstream to the up-regulation of BACE1. Further confirmation of this mechanism was attempted using well-known inhibitors of JNK and p38 MAPK: SP600125 and SB203580, respectively [2,4]. However, unexpected high toxicity of these inhibitors prevented us to use them in the primary cultures (HC-VSMCs). In order to overcome this problem we characterized the amyloidogenic pathway in a human aortic vascular smooth muscle cell line (HA-VSMCs) amenable to the use of these inhibitors.

HA-VSMCs exposed to 25 μM H_2O_2 showed increased expression of BACE1, determined both by western blot (Figure 4A) and confocal immunofluorescence microscopy (Figure 4E). Similarly to HC-VSMCs, the increase was specific for the ß-secretase, without changes in α-secretases (ADAM10 or ADAM17) expression levels (Figure 4A), and it was prevented by trolox. Quantitative analysis of the western blot experiments are shown for BACE1 (Figure 4B), ADAM10 (Figure 4C) and ADAM17 (Figure 4D). The time course of BACE1 up-regulation in HA-VSMCs (Figure 4F) was slightly faster than in HC-VSMCs (see Figure 1). To study BACE1 activity we detected the direct product of BACE1 cleavage at the APP C-terminus (C99 fragment) and as a control, the product of α-secretase activity (p3CT fragment). Figure 4G shows a significant increase

of C99 fragment after 3 h exposure of HA-VSMCs to H_2O_2 (p<0.05), without changes in the p3CT fragment. Moreover, the increment in C99 (Figure 4G) paralleled the time course of BACE1 up-regulation (Figure 4B). Thus, HA-VSMCs resulted an appropriated model to study the oxidative stress-dependent induction of the amyloidogenic pathway in vascular smooth muscle cells.

Pharmacological inhibition of JNK (SP600125) and p38 MAPK (SB203580) and its impact upon oxidative stress-activation of BACE1 in HA-VSMCs was evaluated by immunofluorescence (Figure 5A-B) and quantified by western blot (Figure 5C-D-E). Different concentrations of the SAPK inhibitors, SP600125 (20, 10 and 5 μM) and SB203580 (10, 5 and 2.5 μM) were performed to reach the maximal inhibition without affecting cell viability (Figure 5A). After 3h of 25 μM H_2O_2 treatment, the inhibitory effects of SP600125 (20 μM) were evaluated looking at the expression of c-jun, the downstream effect of the activation of JNK signalling pathway [41] (Figure 5B, upper panel). SP600125 pre-treatment reduced c-jun protein expression induced by H_2O_2 whereas no effect has been observed with SB203580 pre-treatment. Phosphorilation of MAPKAPK-2, a stress-activated enzyme downstream of p38 MAPK, was used as a test to evaluate the inhibitory effect of SB203580 [30] following 15 min of 25 μM H_2O_2 treatment (Figure 5B, lower panel). Pre-treatment with SB203580 reduced the phosphorilation of MAPKAPK-2 induced by H_2O_2 but not the SP600125 pre-treatment. Inhibition of either JNK (Figure 5A-5C) or p38 MAPK (Figure 5A-5C) reverted the up-regulation of BACE1 in HA-VSMCs, an effect that reached statistical significance (p<0.05) (Figure 5E). The increased BACE1 protein expression with H_2O_2 and its reduction by pharmacological inhibitors might reflects changes at the transcriptional level as the same results were obtained when testing BACE1 mRNA by RT-PCR in response to H_2O_2 and in the presence or absence of SP600125 and

SB203580 inhibitors (results not shown). The participation of other putatively relevant signaling pathways in the amyloidogenic pathway of VSMCs was also evaluated. Extracellular responding kinase (ERK) is markedly increased in AD [29,54]. Using the ERK1-2 inhibitor, PD98059, we demonstrated that, similar to neuronal models [37], the ERK pathway is not involved in BACE1 up-regulation in oxidative stress conditions in VSMCs (data not show). Altogether, the experiments carried out on HC-VSMCs and HA-VSMCs revealed that activation of JNK and p38 MAPK by oxidative stress is required for the up-regulation of BACE1 and, consequently, the amyloidogenic pathway.

4. Discussion

AD is characterized by neuronal and vascular lesions produced by Aß deposition [39]. How both lesions are related to each other and how they contribute to the neurodegenerative process are aspects poorly understood that have originated a lively debate. The most widely accepted view proposes a neuronal origin for the vascular Aß deposits, although, some evidences support the possibility of a relevant contribution of brain vessels in the formation of vascular amyloid deposits [11,49].

Characterization of the vasculotropic Dutch (E22Q) and Iowa (D23N) mutations in the APP were originally viewed as models pointing to the importance of VSMCs to CAA. These mutations induce an early onset CAA with mature vascular amyloid deposit but just diffuse plaques in brain parenchyma [15,20,44], which was interpreted as the result of the more fibrillogenic nature of the mutants [42,51] and their aggregation in the proximity of the cells that produce them (i.e., vascular smooth muscle) [46]. Although more recently is being favored the hypothesis involving the poor clearance of neuronal Aß [17]. The identification of the Aß species relevant to CAA is another interesting point for discussion. The generation of a murine model expressing either $Aß_{1-42}$ or $Aß_{1-40}$ clearly shows 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 [23]. A possible scenario based in existing evidence [1,6,23,43] might be as follows: the more fibrillogenic $Aß_{1-42}$ acts as a seed facilitating the accumulation of neuronal $Aß_{1-40}$ that has entered the perivascular drainage [23]. This raises the question of how the low soluble $Aß_{1-42}$ enters the perivascular drainage.

In the present work, we have addressed the study of Aß production, secretion and its regulation by oxidative stress in human cerebral VSMCs (HC-VSMCs). We have demonstrated that HC-VSMCs produce and release $Aß_{1-40}$ and $Aß_{1-42}$. Our data also shows a

higher secretion of $Aß_{1-40}$ than $Aß_{1-42}$ by HC-VSMCs. Based in our study, the contribution of HC-VSMCs to CAA pathophysiology acquires an important role due to their ability to produce and secrete $Aß_{1-42}$. 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 [16,23] to aggregate the less fibrillogenic $Aß_{1-40}$ produced by HC-VSMCc 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 [45], which could contribute to Aß accumulation in the vessel wall and, in fact, to CAA development.

Another important aspect in the characterization of VSMC contribution to the pathophysiology of CAA is the regulation of Aß production. To address this question we have identified in HC-VSMCs the machinery responsible for APP cleavage and its regulation under conditions of increased oxidative stress. The non-amyloidogenic processing of APP is due to the action of α-secretase. As ADAM10 and ADAM17 are the most likely candidates [5,19], we have demonstrated that both enzymes are constitutively present in HC-VSMCs and also in human aortic vascular smooth muscle cells (HA-VSMCs). These enzymes, together with γ-secretase, a protein complex including presenilin (PS) [32,52], are responsible for the non-amyloidogenic APP cleavage, which results in the release of extracellular αAPP and intracellular p3CT fragments. Alternatively, the amyloidogenic pathway produces Aß following the sequential cleavage of APP by a ß-secretase (BACE1) and a γ-secretase [32,47]. We have detected in HC-VSMCs and HA-VSMCs all the enzymes involved in APP processing (BACE1, PS1, PS2, ADAM10 and

ADAM17) and their activity tested following the identification of the APP fragments produced.

One of the main factors that shifts the processing of APP towards the amyloidogenic pathway is oxidative stress [34]. Oxidative stress, a harmful condition that increases with advancing age, has been implicated in the etiology of AD as a result of the increased expression of BACE1 in neurons [35,40]. Oxidative stress, in addition to promote the expression of neuronal BACE1 may also contribute to the Aß-induced pathophysiology seen in both brain parenchyma [26] and blood vessels [8,39] of AD patients. Therefore, oxidative stress and Aß generate an etiopathogenic loop that has been well studied in neurons but still not characterized in the second most important Aß-producing cell within the brain, the vascular smooth muscle. 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 was significantly augmented in VSMCs. BACE1 is strongly regulated at the transcriptional level and post-translational level. Binding sites for several transcription factors have been identified in BACE1 promoter region, being particularly interesting those related to its tissue specific distribution, being maximal in CNS and its modulation by oxidative stress {1138,1139}. In accordance with previous studies carried out in neuronal cell lines demonstrating that hydrogen peroxide induces BACE1 promoter activity [40], Aß production [36] and the activation of JNK and p38 MAPK signaling kinases [22,38], we have found that JNK and p38 MAPK are directly involved in the increased expression of BACE1 in VSMCs. This process is independent of the proapoptotic signaling described for both JNK and p38 MAPK [25] as viability of VSMCs was not affected by the sub-lethal oxidative stress utilized in this study. These

findings suggest that some transcription factors, downstream of these pathways, are promoting the increase in BACE1 expression, and consequently the dramatic increase in Aß secretion. In fact, several transcriptions factors induced by JNK and p38MAPK can bind to the promoter of BACE1 gene as AP1, AP2, Sp1 and NFκß [7,31].

More importantly, oxidative stress increased markedly the secretion of $Aß_{1-40}$ and $Aß_{1-42}$ by HC-VSMCs. The antioxidant Trolox inhibited the oxidative stress-dependent BACE1 up-regulation and secretion of $Aß_{1-40}$ and $Aß_{1-42}$ in HC-VSMCs, confirming the implication of oxidative stress in the induction of the APP amyloidogenic cleavage pathway. In fact, our data highlights the pathophysiological relevance of oxidative stress within brain vessels and its implication in the development of brain CAA [39]. In this context, recent reports have pointed to the importance of neurovascular dysfunction in AD [18] and that vascular deposition of Aß is determinant in the neuroinflammatory process and dementia in AD [24].

In summary, we propose that human brain vessels may be contributors to the formation of vascular amyloid deposits and, consequently, neurovascular dysfunction. Moreover, increased oxidative stress in vessels of the aging brain or those suffering hypertensive, atherosclerotic and/or ischemic conditions, as well as the accumulation of parenchymal $Aß_{1-40}$ due to its poor drainage, may increase BACE1 activity and the release of Aß, and hence, activating at the vascular level the etiopathogenic loop generated by Aß and oxidative stress.

Acknowledgements

This work was supported by grants from FIS (Ministerio de Sanidad, Spain; grant nº PI041242; and Red HERACLES) and Generalitat de Catalunya (GSR2005-266).

Disclosure Statement

All the authors of the present manuscript disclose any actual or potential conflicts of interest including any financial, personal or other relationships with other people or organizations within three years of beginning the work submitted that could inappropriately influence (bias) their work.

Ethical Statement

All the procedures of the present work using human samples have been previously approved by the Ethical Commite of the Institut Municipal de Investigació Mèdica and University Pompeu Fabra (IMIM-UPF).

Figure Legends

Fig. 1 Detection of APP processing enzymes in human vascular smooth muscle. APP and secretases expression in primary cultures of human cerebral vascular smooth muscle cells (HC-VSMCs). The presence of APP, presenilins (PS1, PS2), α-secretases (ADAM10, ADAM17) and ß-secretase (BACE1) were analysed by confocal immunofluorescence microscopy in HC-VSMCs under control conditions (A; left panel) and in the presence of 10 μM H_2O_2 for 6 h (A; right panel). Time-course of BACE1 mRNA expression following H_2O_2 treatment (10μM) obtained by RT-PCR (B). Optical density values of BACE1 mRNA levels were normalized against hPRT (C). Data are the mean ± SEM values of 4 independent experiments; * $p<0.05$. Colocalization of vascular amyloid deposits (lower panel) with BACE1 (D; upper panel) and smooth muscle α-actin (D; middle panel) was performed in control and AD human brain tissues. Cell viability assay (MTT) performed in increasing H_2O_2 concentrations (0-50μM) at 6 h or 24 h (E).

Fig. 2 Secretion of $Aß_{1-40}$ and $Aß_{1-42}$ by human cerebral vascular smooth muscle cells. Oxidative stress induces $Aß_{1-40}$ and $Aß_{1-42}$ secretion in HC-VSMCs. Secreted Aß (A) and intracellular Aß (B) were measured in HC-VSMCs after treatment with 10 μM H_2O_2 for 24 h. The antioxidant Trolox (500 μM) was added to the culture media 2 h prior to the oxidative challenge. Data are mean ± SEM values of 6 independent experiments; * $p<0.05$.

Fig. 3 BACE1 up-regulation and SAPK signaling pathways in HC-VSMCs. Confocal immunofluorescence microscopy shows up-regulation of BACE1 expression by oxidative stress (10 μM H_2O_2 for 6 h) in HC-VSMCs (left panels). Nuclear translocation of JNK

(middle panels) and p38 MAPK (right panels) in response to HC-VSMCs exposure to 10 μM H_2O_2 for 15 min. Pre-incubation with Trolox (500 μM) prevented BACE1 up-regulation and nuclear translocation of JNK and p38 MAPK induced by H_2O_2. Pictures are representative of three to five independent experiments under each condition.

Fig. 4 Oxidative stress increases the amyloidogenic APP pathway in HA-VSMCs. (A) Immunoblots of BACE1, ADAM10 and ADAM17 after 3 h treatment with 25 μM H_2O_2 alone or with 500 μM Trolox pre-treatment. (C) Averaged band density for BACE1 (B), ADAM10 (C) and ADAM17 (D) under the experimental conditions shown in A. Confocal immunofluorescence analysis of BACE1 (E). BACE1 (F) and its C-terminal APP products, C99 (G), significantly increased after H_2O_2 treatment. p3CT was not increased by oxidative stress (G). Optical density values of BACE1, ADAM10, ADAM17, C99 and p3CT levels were normalized by α-tubulin. Data are mean ± SEM values of 4-7 independent experiments; * $p<0.05$.

Fig. 5 JNK and p38 MAPK signaling pathways mediate up-regulation of BACE1 expression in HA-VSMCs. BACE1 immunofluorescence images after 3 h H_2O_2 (25μM) alone or H_2O_2 (25μM) with decreasing concentrations of JNK inhibitor, SP600125 (20, 10 and 5 μM) or with p38 MAPK inhibitor, SB203580 (10, 5 and 2.5 μM) (A). Figure B shows immunofluorescence images of c-jun protein expression (after 3 h) (upper panels) and MAPKAPK-2 phosphorilation (lower panel) (after 15 min) with H_2O_2 (25μM) alone or with SP600125 (20 μM) and SB203580 (10 μM). Immunoblots show the effect of JNK inhibitor, SP600125 (20 μM) (C) or p38 MAPK inhibitor, SB203580 (D) in oxidative

stress-dependent up-regulation of BACE1 protein expression (25 μM H_2O_2 treatment for 3 h) (E) Optical density values of BACE1 levels were normalized to α-tubulin. Data are mean ± SEM values of 4-6 independent experiments; * $p<0.05$.

References

[1] Attems J. Sporadic cerebral amyloid angiopathy: pathology, clinical implications, and possible pathomechanisms. Acta Neuropathol (Berl) 2005;110:345-359.

[2] Badger AM, Bradbeer JN, Votta B, Lee JC, Adams JL, Griswold DE. Pharmacological profile of SB 203580, a selective inhibitor of cytokine suppressive binding protein/p38 kinase, in animal models of arthritis, bone resorption, endotoxin shock and immune function. J Pharmacol Exp Ther 1996;279:1453-1461.

[3] Behl C, Davis JB, Lesley R, Schubert D. Hydrogen peroxide mediates amyloid beta protein toxicity. Cell 1994;77:817-827.

[4] Bennett BL, Sasaki DT, Murray BW, O'Leary EC, Sakata ST, Xu W, Leisten JC, Motiwala A, Pierce S, Satoh Y, Bhagwat SS, Manning AM, Anderson DW. SP600125, an anthrapyrazolone inhibitor of Jun N-terminal kinase. Proc Natl Acad Sci U S A 2001;98:13681-13686.

[5] Buxbaum JD, Liu KN, Luo Y, Slack JL, Stocking KL, Peschon JJ, Johnson RS, Castner BJ, Cerretti DP, Black RA. Evidence that tumor necrosis factor alpha converting enzyme is involved in regulated alpha-secretase cleavage of the Alzheimer amyloid protein precursor. J Biol Chem 1998;273:27765-27767.

[6] Castano EM, Prelli F, Soto C, Beavis R, Matsubara E, Shoji M, Frangione B. The length of amyloid-beta in hereditary cerebral hemorrhage with amyloidosis, Dutch type. Implications for the role of amyloid-beta 1-42 in Alzheimer's disease. J Biol Chem 1996;271:32185-32191.

[7] Christensen MA, Zhou W, Qing H, Lehman A, Philipsen S, Song W. Transcriptional regulation of BACE1, the beta-amyloid precursor protein beta-secretase, by Sp1. Mol Cell Biol 2004;24:865-874.

[8] 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. Brain 2005;128:1613-1621.

[9] Coria F, Moreno A, Torres A, Ahmad I, Ghiso J. Distribution of Alzheimer's disease amyloid protein precursor in normal human and rat nervous system. Neuropathol Appl Neurobiol 1992;18:27-35.

[10] Davis-Salinas J, Van Nostrand WE. Amyloid beta-protein aggregation nullifies its pathologic properties in cultured cerebrovascular smooth muscle cells. J Biol Chem 1995;270:20887-20890.

[11] Frackowiak J, Potempska A, LeVine H, Haske T, Dickson D, Mazur-Kolecka B. Extracellular deposits of A beta produced in cultures of Alzheimer disease brain vascular smooth muscle cells. J Neuropathol Exp Neurol 2005;64:82-90.

[12] Frackowiak J, Sukontasup T, Potempska A, Mazur-Kolecka B. Lysosomal deposition of Abeta in cultures of brain vascular smooth muscle cells is enhanced by iron. Brain

Res 2004;1002:67-75.

[13] Frackowiak J, Zoltowska A, Wisniewski HM. Non-fibrillar beta-amyloid protein is associated with smooth muscle cells of vessel walls in Alzheimer disease. J Neuropathol Exp Neurol 1994;53:637-645.

[14] Ghiso J, Frangione B. Cerebral amyloidosis, amyloid angiopathy, and their relationship to stroke and dementia. J Alzheimers Dis 2001;3:65-73.

[15] Grabowski TJ, Cho HS, Vonsattel JP, Rebeck GW, Greenberg SM. Novel amyloid precursor protein mutation in an Iowa family with dementia and severe cerebral amyloid angiopathy. Ann Neurol 2001;49:697-705.

[16] Harper JD, Lansbury PT, Jr. Models of amyloid seeding in Alzheimer's disease and scrapie: mechanistic truths and physiological consequences of the time-dependent solubility of amyloid proteins. Annu Rev Biochem 1997;66:385-407.

[17] Herzig MC, Van Nostrand WE, Jucker M. Mechanism of cerebral beta-amyloid angiopathy: murine and cellular models. Brain Pathol 2006;16:40-54.

[18] Iadecola C. Neurovascular regulation in the normal brain and in Alzheimer's disease. Nat Rev Neurosci 2004;5:347-360.

[19] Lammich S, Kojro E, Postina R, Gilbert S, Pfeiffer R, Jasionowski M, Haass C, Fahrenholz F. Constitutive and regulated alpha-secretase cleavage of Alzheimer's amyloid precursor protein by a disintegrin metalloprotease. Proc Natl Acad Sci U S A 1999;96:3922-3927.

[20] Levy E, Carman MD, Fernandez-Madrid IJ, Power MD, Lieberburg I, van Duinen SG, Bots GT, Luyendijk W, Frangione B. Mutation of the Alzheimer's disease amyloid gene in hereditary cerebral hemorrhage, Dutch type. Science 1990;248:1124-1126.

[21] Mattson MP, Magnus T. Ageing and neuronal vulnerability. Nat Rev Neurosci 2006;7:278-294.

[22] McDonald DR, Bamberger ME, Combs CK, Landreth GE. beta-Amyloid fibrils activate parallel mitogen-activated protein kinase pathways in microglia and THP1 monocytes. J Neurosci 1998;18:4451-4460.

[23] McGowan E, Pickford F, Kim J, Onstead L, Eriksen J, Yu C, Skipper L, Murphy MP, Beard J, Das P, Jansen K, Delucia M, Lin WL, Dolios G, Wang R, Eckman CB, Dickson DW, Hutton M, Hardy J, Golde T. Abeta42 is essential for parenchymal and vascular amyloid deposition in mice. Neuron 2005;47:191-199.

[24] Miao J, Vitek MP, Xu F, Previti ML, Davis J, Van Nostrand WE. Reducing cerebral microvascular amyloid-beta protein deposition diminishes regional neuroinflammation in vasculotropic mutant amyloid precursor protein transgenic mice. J Neurosci 2005;25:6271-6277.

[25] Mielke K, Herdegen T. JNK and p38 stresskinases--degenerative effectors of signal-transduction-cascades in the nervous system. Prog Neurobiol 2000;61:45-60.

[26] Miranda S, Opazo C, Larrondo LF, Munoz FJ, Ruiz F, Leighton F, Inestrosa NC. The role of oxidative stress in the toxicity induced by amyloid beta-peptide in Alzheimer's

disease. Prog Neurobiol 2000;62:633-648.

[27] Misonou H, Morishima-Kawashima M, Ihara Y. Oxidative stress induces intracellular accumulation of amyloid beta-protein (Abeta) in human neuroblastoma cells. Biochemistry 2000;39:6951-6959.

[28] Munoz FJ, Opazo C, Gil-Gomez G, Tapia G, Fernandez V, Valverde MA, Inestrosa NC. Vitamin E but not 17beta-estradiol protects against vascular toxicity induced by beta-amyloid wild type and the Dutch amyloid variant. J Neurosci 2002;22:3081-3089.

[29] Perry G, Roder H, Nunomura A, Takeda A, Friedlich AL, Zhu X, Raina AK, Holbrook N, Siedlak SL, Harris PL, Smith MA. Activation of neuronal extracellular receptor kinase (ERK) in Alzheimer disease links oxidative stress to abnormal phosphorylation. Neuroreport 1999;10:2411-2415.

[30] Rouse J, Cohen P, Trigon S, Morange M, Alonso-Llamazares A, Zamanillo D, Hunt T, Nebreda AR. A novel kinase cascade triggered by stress and heat shock that stimulates MAPKAP kinase-2 and phosphorylation of the small heat shock proteins. Cell 1994;78:1027-1037.

[31] Sambamurti K, Kinsey R, Maloney B, Ge YW, Lahiri DK. Gene structure and organization of the human beta-secretase (BACE) promoter. FASEB J 2004;18:1034-1036.

[32] Selkoe DJ. The cell biology of beta-amyloid precursor protein and presenilin in Alzheimer's disease. Trends Cell Biol 1998;8:447-453.

[33] Shinkai Y, Yoshimura M, Ito Y, Odaka A, Suzuki N, Yanagisawa K, Ihara Y. Amyloid beta-proteins 1-40 and 1-42(43) in the soluble fraction of extra- and intracranial blood vessels. Ann Neurol 1995;38:421-428.

[34] Smith MA, Nunomura A, Zhu X, Takeda A, Perry G. Metabolic, metallic, and mitotic sources of oxidative stress in Alzheimer disease. Antioxid Redox Signal 2000;2:413-420.

[35] Tamagno E, Bardini P, Obbili A, Vitali A, Borghi R, Zaccheo D, Pronzato MA, Danni O, Smith MA, Perry G, Tabaton M. Oxidative stress increases expression and activity of BACE in NT2 neurons. Neurobiol Dis 2002;10:279-288.

[36] Tamagno E, Guglielmotto M, Bardini P, Santoro G, Davit A, Di Simone D, Danni O, Tabaton M. Dehydroepiandrosterone reduces expression and activity of BACE in NT2 neurons exposed to oxidative stress. Neurobiol Dis 2003;14:291-301.

[37] Tamagno E, Parola M, Bardini P, Piccini A, Borghi R, Guglielmotto M, Santoro G, Davit A, Danni O, Smith MA, Perry G, Tabaton M. Beta-site APP cleaving enzyme up-regulation induced by 4-hydroxynonenal is mediated by stress-activated protein kinases pathways. J Neurochem 2005;92:628-636.

[38] Tamagno E, Robino G, Obbili A, Bardini P, Aragno M, Parola M, Danni O. H2O2 and 4-hydroxynonenal mediate amyloid beta-induced neuronal apoptosis by activating JNKs and p38MAPK. Exp Neurol 2003;180:144-155.

[39] Tong XK, Nicolakakis N, Kocharyan A, Hamel E. Vascular remodeling versus amyloid beta-induced oxidative stress in the cerebrovascular dysfunctions associated

with Alzheimer's disease. J Neurosci 2005;25:11165-11174.

[40] Tong Y, Zhou W, Fung V, Christensen MA, Qing H, Sun X, Song W. Oxidative stress potentiates BACE1 gene expression and Abeta generation. J Neural Transm 2004.

[41] Tournier C, Thomas G, Pierre J, Jacquemin C, Pierre M, Saunier B. Mediation by arachidonic acid metabolites of the H2O2-induced stimulation of mitogen-activated protein kinases (extracellular-signal-regulated kinase and c-Jun NH2-terminal kinase). Eur J Biochem 1997;244:587-595.

[42] van Dorpe J, Smeijers L, Dewachter I, Nuyens D, Spittaels K, Van Den HC, Mercken M, Moechars D, Laenen I, Kuiperi C, Bruynseels K, Tesseur I, Loos R, Vanderstichele H, Checler F, Sciot R, Van Leuven F. Prominent cerebral amyloid angiopathy in transgenic mice overexpressing the london mutant of human APP in neurons. Am J Pathol 2000;157:1283-1298.

[43] van Dorpe J, Smeijers L, Dewachter I, Nuyens D, Spittaels K, Van Den HC, Mercken M, Moechars D, Laenen I, Kuiperi C, Bruynseels K, Tesseur I, Loos R, Vanderstichele H, Checler F, Sciot R, Van Leuven F. Prominent cerebral amyloid angiopathy in transgenic mice overexpressing the london mutant of human APP in neurons. Am J Pathol 2000;157:1283-1298.

[44] van Duinen SG, Castano EM, Prelli F, Bots GT, Luyendijk W, Frangione B. Hereditary cerebral hemorrhage with amyloidosis in patients of Dutch origin is related to Alzheimer disease. Proc Natl Acad Sci U S A 1987;84:5991-5994.

[45] Van Nostrand WE, Melchor J, Wagner M, Davis J. Cerebrovascular smooth muscle cell surface fibrillar A beta. Alteration of the proteolytic environment in the cerebral vessel wall. Ann N Y Acad Sci 2000;903:89-96.

[46] Van Nostrand WE, Melchor JP, Ruffini L. Pathologic amyloid beta-protein cell surface fibril assembly on cultured human cerebrovascular smooth muscle cells. J Neurochem 1998;70:216-223.

[47] Vassar R, Bennett BD, Babu-Khan S, Kahn S, Mendiaz EA, Denis P, Teplow DB, Ross S, Amarante P, Loeloff R, Luo Y, Fisher S, Fuller J, Edenson S, Lile J, Jarosinski MA, Biere AL, Curran E, Burgess T, Louis JC, Collins F, Treanor J, Rogers G, Citron M. Beta-secretase cleavage of Alzheimer's amyloid precursor protein by the transmembrane aspartic protease BACE. Science 1999;286:735-741.

[48] Wang X, Martindale JL, Liu Y, Holbrook NJ. The cellular response to oxidative stress: influences of mitogen-activated protein kinase signalling pathways on cell survival. Biochem J 1998;333 (Pt 2):291-300.

[49] Wisniewski HM, Frackowiak J, Mazur-Kolecka B. In vitro production of beta-amyloid in smooth muscle cells isolated from amyloid angiopathy-affected vessels. Neurosci Lett 1995;183:120-123.

[50] Wisniewski HM, Wegiel J, Vorbrodt AW, Mazur-Kolecka B, Frackowiak J. Role of perivascular cells and myocytes in vascular amyloidosis. Ann N Y Acad Sci 2000;903:6-18.

[51] Wisniewski T, Ghiso J, Frangione B. Peptides homologous to the amyloid protein of

Alzheimer's disease containing a glutamine for glutamic acid substitution have accelerated amyloid fibril formation. Biochem Biophys Res Commun 1991;180:1528.

[52] Wolfe MS, Haass C. The Role of presenilins in gamma-secretase activity. J Biol Chem 2001;276:5413-5416.

[53] Wolfe MS, Xia W, Ostaszewski BL, Diehl TS, Kimberly WT, Selkoe DJ. Two transmembrane aspartates in presenilin-1 required for presenilin endoproteolysis and gamma-secretase activity. Nature 1999;398:513-517.

[54] Zhu X, Castellani RJ, Takeda A, Nunomura A, Atwood CS, Perry G, Smith MA. Differential activation of neuronal ERK, JNK/SAPK and p38 in Alzheimer disease: the 'two hit' hypothesis. Mech Ageing Dev 2001;123:39-46.

[55] Zhu X, Lee HG, Raina AK, Perry G, Smith MA. The role of mitogen-activated protein kinase pathways in Alzheimer's disease. Neurosignals 2002;11:270-281.

[56] Zlokovic BV. Neurovascular mechanisms of Alzheimer's neurodegeneration. Trends Neurosci 2005;28:202-208.

Figure 1

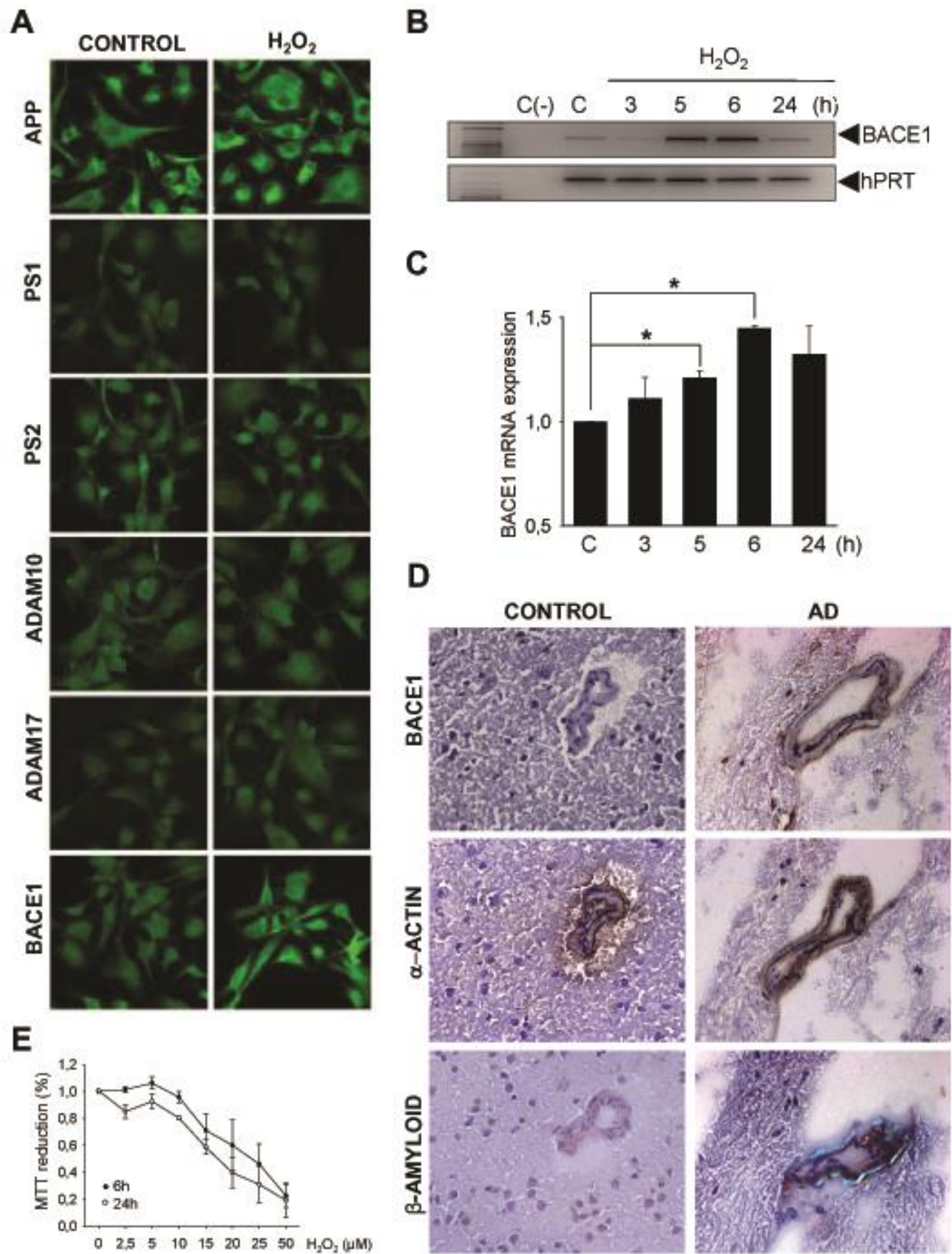

Figure 2

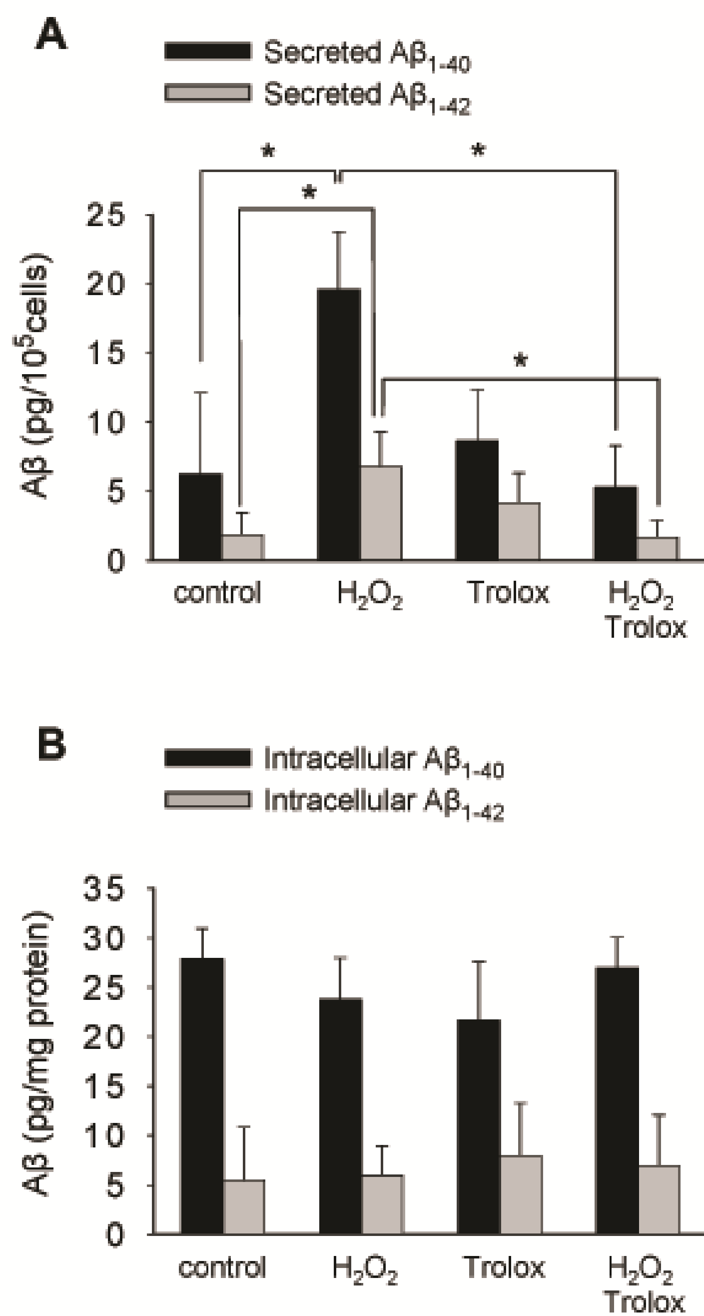

Figure 3

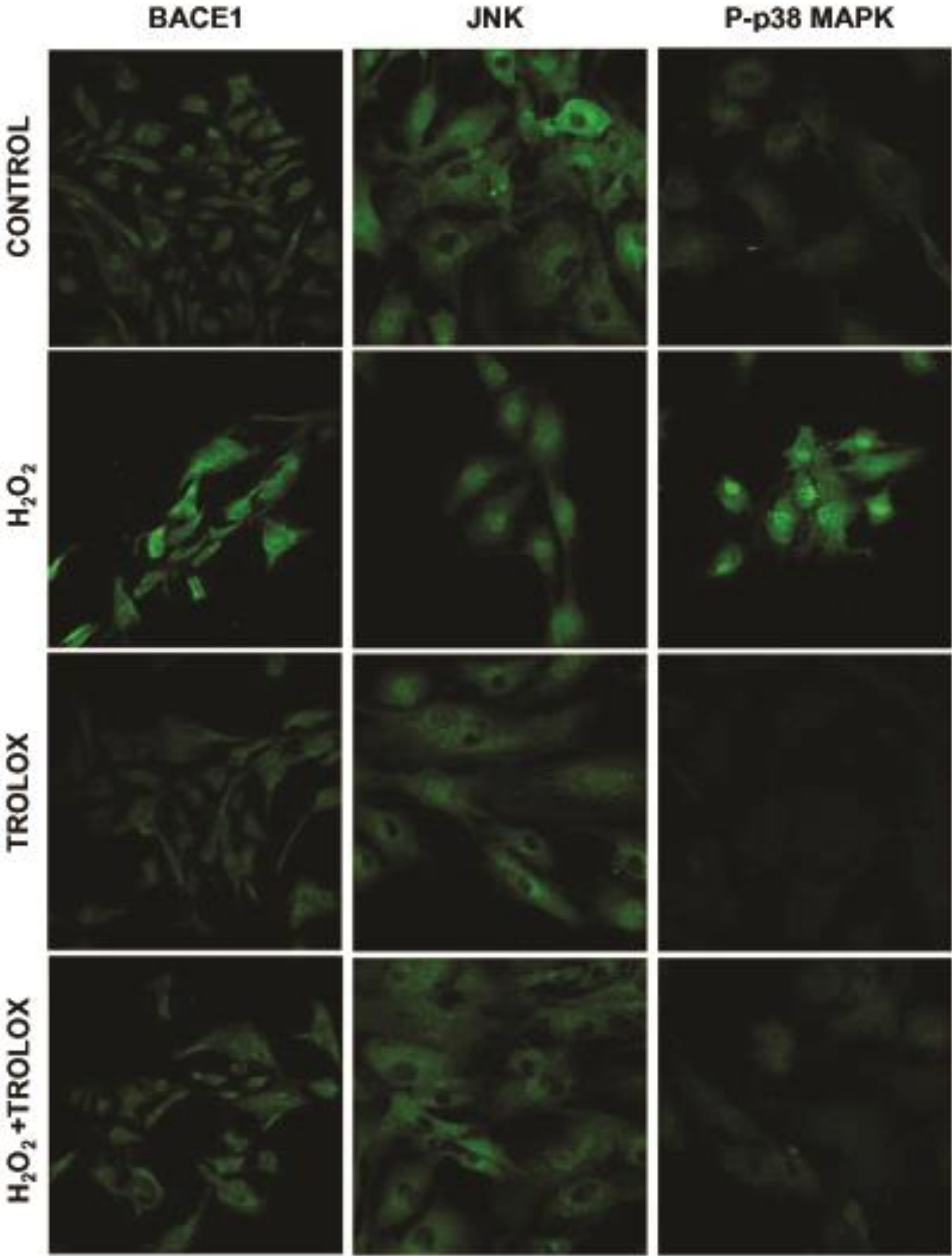

Figure 4

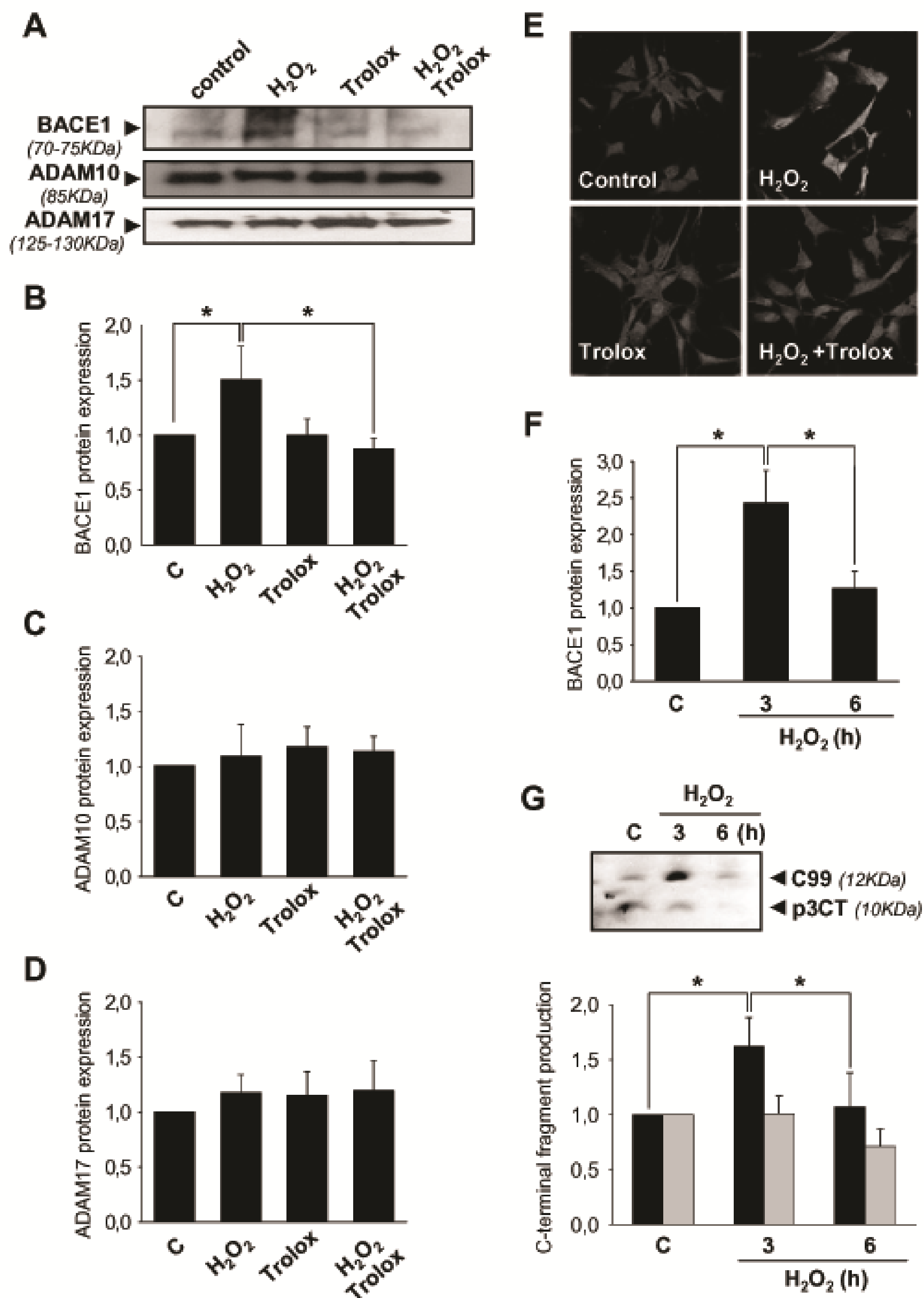

Figure 5