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

Mireia Coma\textsuperscript{a}, Francesc X. Guix\textsuperscript{a}, Gerard Ill-Raga\textsuperscript{a}, Iris Uribesalgo\textsuperscript{a}, Francesc Alameda\textsuperscript{b}, Miguel A. Valverde\textsuperscript{a}, Francisco J. Muñoz\textsuperscript{a,*}

\textsuperscript{a} Laboratory of Molecular Physiology and Channelopathies, Universitat Pompeu Fabra, Parc de Recerca Biomédica de Barcelona, Barcelona, Spain
\textsuperscript{b} Servei d’Anatomia Patológica, Hospital del Mar, Universitat Autònoma de Barcelona, Barcelona 08003, Spain

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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 versus 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\textsubscript{1–40} and Aß\textsubscript{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\textsubscript{1–40} and Aß\textsubscript{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.

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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 (Ghiso and Frangione, 2001). 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 Attems, 2005).

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 (van Dorpe et al., 2000), the capability of vascular smooth muscle cells (VSMCs) to endocytose and accumulate Aß coming from the brain parenchyma (Wisniewski et al., 2000) and the link between reduced Aß-clearing capability and AD (Zlokovic, 2005).

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 Aβ was already hypothesized following the early observation that Aβ deposits are closely associated with cerebral VSMCs (Frackowiak et al., 1994) and the presence of APP and Aβ in VSMCs (Coria et al., 1992; Davis-Salinas and Van Nostrand, 1995; Frackowiak et al., 2004; Wisniewski et al., 1995). However, several facts have rested relevance to the contribution of VSMCs to CAA: (1) most reports just mention intracellular Aβ accumulation in VSMCs (Wisniewski et al., 1995), (2) absence of Aβ1–42 production by primary cultures of VSMCs (Frackowiak et al., 2005) and (3) the lack of a mechanism linking the production of Aβ 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) (Buxbaum et al., 1998; Lammich et al., 1999). Alternatively, the amyloidogenic pathway produces Aβ following the sequential cleavage of APP by a β-secretase identified as β-site APP cleaving enzyme 1 (BACE1) (Vassar et al., 1999) and a γ-secretase, identified as presenilin (PS) (Selkoe, 1998; Wolfe et al., 1999). 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 (Mattson and Magnus, 2006), has been implicated in the etiology of AD by increasing the expression of BACE1 in neuroblastoma cell lines (Misonou et al., 2000; Tamagno et al., 2002). Oxidative stress also contributes to the Aβ-induced pathophysiology seen in both brain parenchyma (Behl et al., 1994) and blood vessels (Coma et al., 2005; Munoz et al., 2002) of AD patients. Therefore, oxidative stress and Aβ generate an etiopathic loop that has been well studied in neurons but not in VSMCs.

In the present work we report the production of Aβ1–40 and Aβ1–42 by human cerebral VSMCs (HC-VSMCs). Moreover, oxidative stress activates the amyloidogenic processing of APP by increasing the expression of BACE1 and, subsequently, Aβ 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 Médica and the Universitat Pompeu Fabra (IMIM-UPF). Briefly, pieces of tunica media were incubated with 0.1% collagenase type IV for 35 min at 37°C 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 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, 5×10⁻⁷ g/l EGF, 1.5×10⁻⁶ 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 Neurologics (Serveis Científico-Tècnics, Hospital Clinic, 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 three control individuals and five AD patients.

2.3. Treatments

Determination of the optimal H₂O₂ sub-lethal concentrations related to the higher BACE1 expression (10 µM for HC-VSMCs) were obtained running dose-response (0–50 µM) 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 (10 µM; Calbiochem) were added to culture medium 1 h before H₂O₂ 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 was used in a reverse transcriptase-polymerase chain reaction (RT-PCR) using the OneStep RT-PCR Kit (Qiagen, Hamburg Germany). BACE1 specific primers (5′-CATTGGAGGTATCGACCCTCGCT-3′ and 5′-CCAGTCTCTTCCATGTCAGGTG-3′; Genbank accessions number: NM012104) were used for human BACE1 gene amplification and hypoxanthine phosphoribosyltransferase (hPRT) specific primers (5′-GGCCACAGTGTGTTGGATTGG-3′ and 5′-TGCGCTCATTTCAGGCTTTTG-3′. Genbank accessions 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$_2$O$_2$ 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 using 10% Tris-glycine gels and blotted onto nitrocellulose (Bio-Rad 680 and evaluated by microplate manager software. 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 (Fig. 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 β-
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 (Fig. 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 β- and γ-secretases.

3.2. Oxidative stress increases the expression of BACE1

Aging is associated to increased oxidative stress and lower antioxidant defenses (Mattson and Magnus, 2006). Moreover, oxidative stress has been associated to the origin and development of AD (Coma et al., 2005; Selkoe, 1998). Particularly interesting in the context of our work are the reports claiming the modulation of APP processing by oxidative stress (Tamagno et al., 2003a; Tong et al., 2004). The effect of oxidative stress on the APP and secretase expression was evaluated on HC-VSMCs (Fig. 1A). HC-VSMCs were exposed to sub-lethal concentrations of H2O2 (10 μM),...
previously adjusted carrying out dose-response cell viability experiments using MTT reduction assays (Fig. 1E). BACE1 expression was clearly increased in HC-VSMCs exposed to oxidative stress (Fig. 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$_2$O$_2$-induced BACE1 expression was examined at the transcriptional level by semiquantitative RT-PCR. Fig. 1B and C demonstrates an increase of BACE1 transcript that peaks 6 h after the addition of H$_2$O$_2$ 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$_2$O$_2$ was obtained using a SH-SY5Y human neuronal cell line (results not shown).

Oxidative stress conditions have been reported in AD brain blood vessels (Tong et al., 2004). 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 Aβ-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 (Fig. 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 (Fig. 1D, lower panel).

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

Several studies have demonstrated that both Aβ$_{1-40}$ and Aβ$_{1-42}$ species accumulated in CAA vessel walls (Shinkai et al., 1995) being Aβ$_{1-40}$ the most abundant one (Castano et al., 1996). Once we demonstrated an increase in the APP amyloidogenic pathway in HC-VSMCs exposed to oxidative stress, we set out to detect Aβ$_{1-40}$ and Aβ$_{1-42}$ under control conditions and following H$_2$O$_2$ treatment. We observed an increase in secreted (extracellular) Aβ$_{1-40}$ and Aβ$_{1-42}$ (Fig. 2A) without changes in the intracellular levels (Fig. 2B) detected in HC-VSMC cultures exposed to 10 μM H$_2$O$_2$. Secreted Aβ$_{1-40}$ reached a threefold increase ($p < 0.05$) and Aβ$_{1-42}$ a five-fold increase ($p < 0.05$) compared to control conditions. Increased secretion of Aβ$_{1-40}$ and Aβ$_{1-42}$ was reverted in HC-VSMCs pretreated with the antioxidant trolox (a vitamin E analogue) (Fig. 2A). The abolition of the Aβ increase by trolox can be explained by the inhibition of the oxidative-stress dependent up-regulation of BACE1 (Fig. 3, left panels), an effect already reported for neuronal BACE1 (Tamagno et al., 2002).

### 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 (Wang et al., 1998), and both kinases are up-regulated in AD (Zhu et al., 2001, 2002). 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 (Mielke and Herdegen, 2000). 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...
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 H2O2 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 H2O2 for 15 min. Pre-incubation with Trolox (500 μM) prevented BACE1 up-regulation and nuclear translocation of JNK and p38 MAPK induced by H2O2. Pictures are representative of three to five independent experiments under each condition.

in HC-VSMCs exposed to 10 μM H2O2. Immunofluorescence images of BACE1 up-regulation were run in parallel and shown in Fig. 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 (Fig. 3). Our results show that, similar to neuronal models (Tamagno et al., 2005), 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 (Badger et al., 1996; Bennett et al., 2001). 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 H2O2 showed increased expression of BACE1, determined both by western blot (Fig. 4A) and confocal immunofluorescence microscopy (Fig. 4E). Similarly to HC-VSMCs, the increase was specific for the β-secretase, without changes in α-secretases (ADAM10 or ADAM17) expression levels (Fig. 4A), and it was prevented by trolox. Quantitative analysis of the western blot experiments are shown for BACE1 (Fig. 4B), ADAM10 (Fig. 4C) and ADAM17 (Fig. 4D). The time course of BACE1 up-regulation in HA-VSMCs (Fig. 4F) was slightly faster than in HC-VSMCs (see Fig. 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). Fig. 4G shows a significant increase of C99 fragment after 3 h exposure of
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 H₂O₂ 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 H₂O₂ 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.

HA-VSMCs to H₂O₂ (p < 0.05), without changes in the p3CT fragment. Moreover, the increment in C99 (Fig. 4G) paralleled the time course of BACE1 up-regulation (Fig. 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 (Fig. 5A and B) and quantified by western blot (Fig. 5C–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 (Fig. 5A). After 3h of 25 μM H₂O₂ 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 signaling pathway (Tournier et al., 1997) (Fig. 5A, upper panel). SP600125 pre-treatment reduced c-jun protein expression induced by H₂O₂ 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 (Rouse et al., 1994) following 15 min of 25 μM H₂O₂ treatment (Fig. 5B, lower panel). Pre-treatment with SB203580 reduced the phosphorilation of MAPKAPK-2 induced by H₂O₂ but not the SP600125 pre-treatment. Inhibition of either JNK (Fig. 5A–C) or p38 MAPK (Fig. 5A–C) reverted the up-regulation of BACE1 in HA-VSMCs, an effect that reached statistical significance (p < 0.05) (Fig. 5E). The increased BACE1 protein expression with H₂O₂ 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 H₂O₂ 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 (Perry et al., 1999; Zhu et al., 2001). Using the ERK1-2 inhibitor,
PD98059, we demonstrated that, similar to neuronal models (Tamagno et al., 2005), 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 Aβ deposition (Tong et al., 2005). 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 Aβ deposits, although, some evidences support the possibility of a relevant contribution of brain vessels in the formation of vascular amyloid deposits (Frackowiak et al., 2005; Wisniewski et al., 1995).

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 (Grabowski et al., 2001; Levy et al., 1990; van Duinen et al., 1987), which was interpreted as the result of the more fibrillogenic nature of the mutants (van Dorpe et al., 2000; Wisniewski et al., 1991) and their aggregation in the proximity of the cells that produce them (i.e., vascular smooth muscle) (Van Nostrand et al., 1998). Although more recently is being favored the hypothesis involving the poor clearance of neuronal Aβ (Herzig et al., 2006). The identification of the Aβ species relevant to CAA is another interesting point for discussion. The generation of a murine model expressing either Aβ1–42 or Aβ1–40 clearly shows that both CAA and amyloid plaques require the presence of Aβ1–42 and that Aβ1–40 alone is not sufficient to generate these lesions (McGowan et al., 2005). A possible scenario based in existing evidence (Attems, 2005; Castano et al., 1996; McGowan et al., 2005; van Dorpe et al., 2000) might be as follows: the more fibrillogenic Aβ1–42 acts as a seed facilitating the accumulation of neuronal Aβ1–40 that has entered the perivascular drainage (McGowan et al., 2005). This raises the question of how the low soluble Aβ1–42 enters the perivascular drainage.

In the present work, we have addressed the study of Aβ production, secretion and its regulation by oxidative stress in human cerebral VSMCs (HC-VSMCs). We have demonstrated that HC-VSMCs produce and release Aβ1–40 and Aβ1–42. Our data also shows a higher secretion of Aβ1–40 than Aβ1–42 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 Aβ1–42. Taking into consideration that the highly fibrillogenic Aβ1–42 should aggregate in the proximity of the cells that produce it, we propose that the production of Aβ1–42 by VSMCs might act as a local seed (Harper and Lansbury, 1997; McGowan et al., 2005) to aggregate the less fibrillogenic Aβ1–40 produced by HC-VSMCs as well as the Aβ1–40 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 Aβ 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 Aβ 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 (Buxbaum et al., 1998; Lammich et al., 1999), 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) (Selkoe, 1998; Wolfe and Haass, 2001), 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 Aβ following the sequential cleavage of APP by α-secretase (BACE1) and a γ-secretase (Selkoe, 1998; Vassar et al., 1999). 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 (Smith et al., 2000). 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 (Tamagno et al., 2002; Tong et al., 2004). Oxidative stress, in addition to promote the expression of neuronal BACE1 may also contribute to the Aβ-induced pathophysiology seen in both brain parenchyma (Miranda et al., 2000) and blood vessels (Coma et al., 2005; Tong et al., 2005) of AD patients. Therefore, oxidative stress and Aβ generate an etiopathogenic loop that has been well studied in neurons but still not characterized in the second most important Aβ-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.
In accordance with previous studies carried out in neuronal cell lines demonstrating that hydrogen peroxide induces BACE1 promoter activity (Tong et al., 2004), Aβ production (Tamagno et al., 2003a) and the activation of JNK and p38 MAPK signaling 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. This process is independent of the proapoptotic signaling described for both JNK and p38 MAPK (Mielke and Herdegen, 2000) 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 Aβ secretion. In fact, several transcription factors induced by JNK and p38 MAPK can bind to the promoter of BACE1 gene as AP1, AP2, Sp1 and NFκB (Christensen et al., 2004; Sambamurti et al., 2004).

More importantly, oxidative stress increased markedly the secretion of Aβ1–40 and Aβ1–42 by HC-VSMCs. The antioxidant Trolox inhibited the oxidative stress-dependent BACE1 up-regulation and secretion of Aβ1–40 and Aβ1–42 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 (Tong et al., 2005). In this context, recent reports have pointed to the importance of neurovascular dysfunction in AD (Iadecola, 2004) and that vascular deposition of Aβ is determinant in the neuroinflammatory process and dementia in AD (Miao et al., 2005).

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 Aβ1–40 due to its poor drainage, may increase BACE1 activity and the release of Aβ, and hence, activating at the vascular level the etiopathogenic loop generated by Aβ and oxidative stress.

**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 3 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 Committe of the Institut Municipal de Investigació Mèdica and Universitat Pompeu Fabra (IMIM-UPF).

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